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Immunomodulatory Effects of Heated Ovomucoid-Depleted Egg White In a BALB/C Mouse Model of Egg Allergy

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Abstract

Oral immunotherapy (OIT) is a promising therapeutic approach for treating food allergy. The treatment with heated ovomucoid-depleted egg white (HOM`EW) in egg allergic patients is noteworthy, however, OIT protocols are still experimental and a better knowledge of the underlying mechanism is required. The objective of this work was to investigate the immunomodulatory effects of HOM'EW and characterize the underlying mechanism in a Balb/c mouse model of egg allergy. Mice were sensitized with EW and treated with HOM'EW. Post treatment, mice were challenged with EW and euthanized for collecting blood and spleen. Markers of allergic clinical outcomes were measured as histamine concentration, serum antibody activity and cytokine production from cultured splenocytes. Digestibility of HOM'EW was assessed mimicking physiological conditions *in vitro*. The HOM'EW demonstrated high digestibility. The treatment induced a marked increase of Th1/Th2 ratio in the high dose treatment group. Treated mice had significantly less histamine, EW-specific IgE and IL-4 and more of IFN-γ and IL-10. This study concludes mechanisms involved in successful tolerance induction with OIT using HOM'EW and allows understanding of the vital role of surrogate allergy markers involved in immune-modulation.

Keywords: food allergy, egg white, ovomucoid, Balb/c mice, oral immunotherapy.

Introduction

Food allergies are recognized as a global medical problem that affects more than 25% of the population in industrialized countries *(1)* and accounts for one third to one half of anaphylaxis cases worldwide *(2)*. Prevalence of food allergies is on the rise and a concerning increase of 18% has been reported in the USA from 1997 to 2007 *(3)*. Recently it has been reported that estimates are about 5% in children and 3% to 4% among adults *(4)*. Egg allergy is the second most common cause of food allergies in children *(5)* and four major allergens in the egg white [ovomucoid (OM or Gal d 1), ovalbumin (OVA or Gal d 2), ovotransferrin (OVT or Gal d 3) and lysozyme (LYS or Gal d 4)] contribute to induction of allergy *(6)*. Among the four, OM is considered immunodominant *(7)* due to its stability to heat treatment *(8)* and enzymatic digestion (9) and ability to retain IgE binding epitopes after *in vitro* digestion *(10)*.

At present, the main treatment for egg allergic patients is based on food avoidance; however, this poses a challenge due to the omnipresence of eggs in a wide range of food *(11)*. Furthermore, it has been shown that avoidance could lead to a lower reactivity threshold in human subjects *(12)*. For these reasons, a therapeutic approach seems more appropriate for the treatment of egg allergies. Oral immunotherapy (OIT) is one of the most studied therapeutic approaches and encouraging results have been recently reported both in mice *(13)* and human *(14)*. Nevertheless oral tolerance induction protocols to food allergens are still ongoing and are only experimental because there are remaining questions that need to be answered prior to exploring OIT as a treatment module such as to identify the severity and type of food allergy response to treatment, if OIT leads to desensitization or oral tolerance, if oral tolerance occurs naturally or is induced by treatment, the optimal

dose and duration, the degree of protection, and establishment of an adequate dose and if a maintenance dose is necessary, etc. Hence appropriate conditions for allergen preparation, treatment protocols and outcomes for OIT need to be standardized. Although few common facts related to successful OIT have been described such as reduction in specific IgE activity, induction of IgG4/IgG2a, altered T-cell cytokine balance (shift Th2 to Th1 type response) and T-cell anergy *(15)*; the overall mechanism is not completely understood, for example the role of specific IgA exerted at mucosal level *(16)*, or the controversial role of specific IgG and its respective isotype subclasses *(17)*.

With regards to EW OIT, the use of heated EW formulas has always been a common choice and is frequently reported *(18)*. The treatment with heated ovomucoid-depleted egg white (HOM'EW) is especially notable. The HOM'EW can be used in approximately 95% of egg allergic patients because of its hypoallergenic character and has been demonstrated to be safe *(19)*. Furthermore the effectiveness of HOM'EW in EW allergic patients has been proven recently *(18)* but the underlying mechanism still remains unclear. Reliable protocols for OIT are underway and more light is being shed on the mechanisms involved in OIT *(14, 20-22)*.

With this background, our objective was to study the immunomodulatory effect of HOM'EW in a Balb/c mouse model of egg allergy to reach a better understanding of the mechanisms involved by which HOM'EW desensitizes and may induce oral tolerance.

Materials and methods

Preparation of HOM'EW

HOM´EW was prepared as previously described *(19)* with slight variations. In brief, egg white was separated and diluted (1:10; v/v) with milli-Q water and sieved by a metallic strainer and the pH was adjusted to 5. The sample was heated at 95ºC for 30 min. After heat treatment, the sample was centrifuged at 7000 g at room temperature (RT) for 30 min. Since OM does not coagulate by heating, the OM was retained in the supernatant while the precipitate contained the HOM'EW. The precipitate was confirmed by western blotting (Figure 1) to ensure that the sample was composed of HOM'EW. The sample thus obtained was freeze dried and stored at -30ºC for further use.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE electrophoresis was performed according to the method of Laemmli *(23)*. Samples (40µg/well) were dissolved in sample buffer in the presence of 5% (v/v) β-mercaptoethanol, heated for 5 min at 95ºC and run on 4-12% Criterion XT gels (Bio-Rad Laboratories, Hercules, CA). Gels were stained using Coomassie G-250 (Bio-Rad) and destained using a 40% methanol, 7% acetic acid solution.

Reverse phase high-performance liquid chromatography (RP-HPLC)

The RP-HPLC analysis was performed using a Waters 600 HPLC instrument (Waters, Milford, MA) and a 250 mm X 4.6 mm Widepore C18 column (Bio-Rad, Richmond, CA). Operating conditions were as follows: column at RT; flow rate at 1 mL/min; injection volume at, 60 µL; solvent A (0.37 mL/L TFA in Milli-Q water); and solvent B (0.27 mL/L TFA in HPLC grade acetonitrile). A linear gradient of solvent B in A, from 0 to 60% in 60 min, followed by 60% B for 30 min, was used. Absorbance was recorded at 220 nm with a Waters 2487 λ dual detector. The software Empower 2000 system data (Waters) was used.

Western Blotting

Following SDS-PAGE electrophoresis, the proteins (20µg/well) were transferred onto a 0.45 µm nitrocellulose membrane (Bio-Rad) using a semi-dry transfer cell (Bio-Rad). The membrane was blocked at room temperature for 2 h with 1% casein dissolved in 1X TBS (25 mM Tris-HCl, 0.15 M NaCl, pH 7.4; blocking buffer) and then incubated at 4°C overnight with rabbit anti-OM IgG antibody with horseradish peroxidase (HPR) (Immune Systems Ltd., ISL, Paignton, U.K.) diluted 1:25000 in blocking buffer. Following overnight incubation, the membrane was washed (6 × 5 min) in 1 × TBS with 0.05% Tween-20. Eventually the blots were visualized using the ECL prime western blotting detection reagent (GE Heathcare, Buckinghamshire, UK).

Gastric and duodenal digestion

Digestibility of EW and HOM'EW were demonstrated using an *in vitro* model system in two steps, which mimics gastric and duodenal digestion *in vivo (24)*. Both EW and HOM'EW were subjected to *in vitro* gastric digestion at 5.7 mg/mL final concentration. In brief, the digestions were performed in simulated gastric fluid (SGF, 35 mM NaCl) at pH 2.0, for 60 min at 37ºC, with porcine pepsin (EC 3.4.23.1**,** 3210 U/mg protein, Sigma-Aldrich) at an enzyme: substrate ratio (E:S) of 1:20, w/w (172 U/mg). Aliquots were taken at 0, 30 and 60 min of incubation and adjusting the pH to 7 with 1 M NaHCO3 stopped the reaction. Duodenal digestions were performed by using the 60 min gastric digests adjusted to pH 7, as described above, with the addition of: 1 M $CaCl_2$, 0.25 M Bis-Tris pH 6.5 and a 0.125 M bile salt mixture containing equimolar quantities of sodium cholate and sodium deoxycholate (Sigma-Aldrich). After incubation at 37 ºC for 15 min, pancreatin (Sigma-Aldrich) was added at an enzyme: substrate ratio of 1:25, w/w. The final composition of the mixture was: 4.27 mg/mL of EW or HOM'EW, 6.15 mM of each bile salt, 20.3 mM Bis-Tris, 7.6 mM $CaCl_2$; and pancreatin (enzyme:substrate ratio 1:25 w/w). Aliquots were taken

after 60 min of gastric digestion and 30 min of duodenal digestion. Duplicate digestions were conducted for each condition.

Animal Sensitization and Challenge

Female Balb/c mice (*n*=40) were purchased from Charles River Laboratories (Montreal, QC, Canada) at 6–8 weeks of age and randomly divided into four groups (*n*=10/group). All animals were housed in the campus animal facility at the University of Guelph under an egg-free diet [Teklad global diet, 14% protein (wheat and corn) and 3.5% fat] in a 12 h lighting cycle. Food and water were available ad libitum. All procedures were performed in accordance with the guidelines established by the Canadian Council of Animal Care (CCAC) and approved by the Animal Care Committee at the University of Guelph. As shown in Figure 2, following a 1-week acclimatization period, positive and treatment groups were sensitized with EW (5mg/mouse) and 10 µg of cholera toxin (CT) (List Biologicals, Denver, CO, USA) by oral gavage twice a week for a duration of 4 weeks. After the sensitization phase, EW-specific IgE activity was determined by ELISA to ensure that the positive and treatment groups were sensitized. Post the sensitization period HOM'EW was orally administered to the treatment groups thrice a week for six weeks in two different doses: 2.5 mg (high dose group) and 1.0 mg (low dose group). The positive and the negative groups received PBS. All mice were challenged on week 13 with 20 mg of EW diluted in PBS and euthanized for collection of blood and tissue samples.

Serum histamine release

Following the final oral challenge, whole blood was collected by cardiac puncture and two serum samples were pooled in equal volumes within each group (*n*=5) due to the limited volume in individual mouse serum for performing ELISA assays. Histamine concentrations

were assayed using a commercial ELISA kit (Labor Diagnostika Nord, Nordhon, Germany) following the manufacturer's instructions.

Total and specific IgE and IgG in serum

Concentrations of total IgE and IgG in mouse serum were determined by ELISA. In brief, flat-bottom 96-well microtitre plates (Corning, Costar Corp., MA, USA) were coated with 100 µl of anti-mouse IgE monoclonal antibody (BD Biosciences, San Diego, CA, USA) or goat anti-mouse IgG (Calbiochem, La Jolla, CA, USA) at 1 µg/ml final concentration. After overnight incubation at 4°C, the plates were washed with PBS with 0.05% (w/v) Tween-20 (PBST) three times and blocked with 200 µl of 1% (w/v) bovine serum albumin (BSA) in PBS for 2 hours at 37ºC. The plates were washed with PBST three times and dilutions of murine IgE (BD) (0.625-20 ng/mL) or IgG standards (AbD Serotec, Raleigh, NC, USA) (0.625–40 ng/ml) and serum samples (1:50 for IgE and 1:40,000 for IgG) in 1% BSA in PBST were added (100 µl) to the wells in duplicate and incubated for 2 hours at 37ºC. The plates were washed further with PBST four times and 100 µL of biotinylated anti-IgE monoclonal antibody (1:1000) (Caltag, CA, USA) or alkaline phosphate-conjugated goat anti-mouse IgG (1:2000) (Sigma-Aldrich) were added to the wells and incubated for 1 hour at 37ºC. After washing four times, 100 µL of avidin-horseradish peroxidise (BD) or extravidin conjugated to alkaline–phosphatase (1:3000) (Sigma-Aldrich) were added onto the plates and incubated for 30 min at 37ºC. The reaction product was visualized by adding 100 µL of 3,3′,5,5′-tetra methyl benzidine (TMB, Sigma-Aldrich) or *p*-nitrophenol phosphatase (1 mg/ml) (Sigma-Aldrich). The reaction was incubated for 30 min followed by the addition of 25µl of 0.5 M H_2SO_4 or 3 N NaOH (stop solution). The optical density absorbance readings (450 nm or 415 nm) were read using a microplate reader (iMark Microplate reader, Bio-Rad) and readings obtained from the individual serum samples were

converted to concentrations of IgE and IgG per millilitre for each assay from the values obtained from the standard curve.

Specific IgE and IgG levels were measured by coating the plates with 50 µg/ml of the intact EW, OM or OVA and a similar procedure to that described above was followed. Murine serum samples were diluted 1:5 for specific IgE and 1:1000 for specific IgG.

Measurement of specific IgG1 and IgG2a activity

Measurements of EW, OM and OVA specific IgG1 and IgG2a in mouse serum samples were performed by an indirect ELISA. In brief, 96 well microplates (Corning) were coated with 100 µl of EW or OM (50 µg/ml) and incubated overnight at 4ºC. Plates were washed three times using PBST and blocked with 200 µl of 1% (w/v) BSA for 1 h at 37ºC. Diluted serum samples (1:10,000 dilutions for specific IgG1 and 1:1000 for specific IgG2a) were added to each well and incubated for 1 h at 37ºC. After three washes with PBST, biotinylated monoclonal rat antibodies were added for IgG1 and IgG2a (100 µl/well; 1µg/ml) (BD) and the plates were incubated for 1 h at 37ºC. The plates were washed three times and incubated further with 100µl of avidin-HRP peroxidase-conjugated (BD) (1:2000) for 30 min at 37ºC before detection. The plate was washed six times with PBST and the reaction was visualized using 50 µl of TMB (Sigma-Aldrich) and incubated for 30 min. The reaction was terminated by adding 25µl of 0.5M H_2SO_4 and absorbance was read at 450 nm using a microplate reader (iMark Microplate reader, Bio-Rad).

Measurement of EW-specific IgA in fecal samples

In an effort to further elucidate the underlying mechanism occurring locally at the intestinal level, mouse fecal pellets were freshly collected on a weekly basis from each mouse group cage, and were submitted to the following extraction procedure. In brief, fecal pellets were freeze-dried, diluted 1:7 (w/w) in PBS and homogenized by using a vortex. Samples were

subsequently centrifuged at 1600 g for 15 min at 4 ºC to remove large fibrous particles. Supernatants were carefully collected and centrifuged again at 9500 g for 10 min at 4ºC. Concentrations of EW-specific IgA were determined using a sandwich ELISA procedure. In brief, flat-bottom 96-well ELISA plates (Corning) were incubated with 100 µl/well of EW (50µg/well) in 100 mM $NaHCO_3$ (pH 9.6) and stored overnight at 4°C. Plates were subsequently washed four times PBST, and blocked with 200 µl/well of 1% BSA in PBS and incubated for 1 h at 37°C. An additional four washes were performed and 100 µl/well of fecal supernatants were added onto the plate in triplicate wells and incubated overnight at 4°C. The plates were washed four times with PBST and incubated with 100 µl/well of biotinylated-monoclonal anti-mouse IgA (1: 500; BD) diluted in 1% BSA in PBST for 1 h at 37°C. The wells were further washed four times with PBST, and 100 µl/well of avidin-HRP conjugate (1:2000; BD) were applied onto the plate for 30 min incubation at 37°C. After a final 4-wash cycle with PBST, EW-specific IgA binding activity was revealed by addition of 50µl/well of TMB (Sigma-Aldrich). The reaction was terminated after 30 min by addition of 25µl/well of 0.5 M H_2SO_4, and absorbance values were determined at 450 nm using an ELISA microplate reader (iMark Microplate reader, Bio-Rad).

Mouse spleen cell cultures and determination of cytokine secretion

At the experimental end-point, post oral challenge, spleen from individual mice was aseptically removed into ice-cold RPMI-1640 medium (Gibco Invitrogen, New York, NY, USA), containing $NaHCO_3$ (1.5 g/L), glucose (4.5 g/L), L-glutamine (2mM), sodium pyruvate (1mM), penicillin (50 U/mL) and streptomycin (50 mg/mL), and two whole spleens were pooled within each group (n=5/group). The cell suspensions were passed through a 100 µm nylon membrane cell strainer and transferred to 15 mL conical centrifuge tubes and centrifuged for 10 min at 500 g at 4ºC. Erythrocytes in spleen cell preparations

were lysed with 2 mL of red blood cell lysing buffer (Sigma-Aldrich) and 10 mL of RPMI media was added to stop the lysis. The splenocytes were washed twice with 10 mL RPMI by centrifugation. Splenocytes were resuspended in 10 mL of medium [RPMI 1640 supplemented with 8% fetal bovine serum (FBS)] (Hyclone, Fisher, Canada) and cell viability was assessed by trypan blue exclusion. Cells were cultured in 24-well plates (Corning) at a density of $2.5x10^6$/mL in the absence (negative control wells) or presence of purified EW (100 µg/mL) in triplicates. Supernatants were collected after 72 h of incubation in a 5% CO_2 humidified incubator and assayed for the presence of cytokines. Concentrations of IFN-γ, IL-4, TGF-β and IL-10 secreted in murine splenocyte culture supernatants were assayed by ELISA. Briefly, 96-well plates (Corning) were coated with 100 µL of the capture antibodies: rat anti-mouse IL-4 and INF-γ (BD) (1:250) or rat anti mouse IL-4 (BD) (1:250) and the plates were incubated at 4ºC overnight. The plates were washed three times with 200 µL of PBST and blocked with 200 µL of 1% BSA in PBS at 37ºC for 1 h. The plates were further washed three times with PBST and 100 µL of standard cytokines (BD) at concentrations of 31.250–1000 pg/mL (IFN-γ) or 15.625–5000 pg/mL (IL-4) diluted in 1% (w/v) BSA in PBST, and the culture supernatant samples were added at different dilutions: 1:10 (IFN-γ) or 1:2 (IL-4) and incubated at 37ºC for 2 h. After washing 4 times 100 µL of detection antibodies were added: biotinylated rat anti-mouse INF-γ (BD) (1:2000) or biotinylated rat anti-mouse IL-4 (BD) (1:2000) and incubated for 1 h at 37ºC. The plates were washed four times and bound antibodies were detected using 100 µL of HRP-conjugated avidin (BD) at 1:2000 dilution and plates were incubated for 30 min at 37ºC and washed six times with PBST. Then 50 µL of TMB (Sigma-Aldrich) was used as a substrate and the plate was incubated for 20 min in the dark at 37ºC and 25 µL of stop solution (0.5 M H_2SO_4) was added. The optical density was measured by an ELISA

reader (iMark Microplate reader, Bio-Rad) with a 450 nm filter. Production of TGF-β and IL-10 in the spleen cell culture supernatants was determined using the ready to use commercial kit "TGF-B Ready-Set-Go" and "IL-10 Ready-Set-Go" (eBiosciences Inc, San Diego, CA) following the manufacturer instructions. Standard curves for each cytokine (15.625–1000 pg/mL TGF-β; 15.625–1000 pg/mL IL-10) were used to quantify the levels of cytokines present in the culture supernatant samples.

Statistical analysis

Histamine, immunoglobulin and cytokine concentrations measured by ELISA assays were subjected to ANOVA analyses followed by post hoc multiple-comparison using Tukey's test. In all cases, p-values ≤ 0.05 were considered statistically significant. Statistical calculations were performed using the GraphPad Prism® package (Graphpad, San Diego, CA, USA).

Results and discussion

High in vitro digestibility of HOM'EW

Digestibility of EW and HOM'EW were performed to assess digestibility by using an *in vitro* system in two steps, which mimicked digestion in the stomach (gastric) and duodenum *(24)*. The HOM'EW was more susceptible to digestion than intact EW as shown in Figure 3 by SDS-PAGE electrophoresis. Looking at the 60 min gastric digests of EW and HOM´EW (lanes 3 and 6) the band of OVA (44 kDa), the most abundant allergen in the albumen, is mainly intact in the EW while is almost fully digested in the HOM´EW, which presented a wide band of digestion products with a molecular mass lower than 10 kDa. The OVT (76 kDa) was fully digested in the gastric phase in both EW and HOM´EW while the LYS (14.4 kDa) resisted the peptic action as part of the EW but not in the

HOM´EW. The higher digestibility of the HOM´EW was also keep along duodenal digestion (lanes 4 and 7) where the profile of HOM´EW is clearer than that of EW since most of the protein content was completely hydrolyzed. Non-digested EW and 60 min gastric digests of EW and HOM´EW were further analysed by RP-HPLC (Figure 4) and the profiles were in agreement with SDS-PAGE outcomes. In the RP-HPLC profile of non-digested EW the main EW allergens were identified such as: OVA that elutes at 55 minutes and OM after 33 minutes (25) OVT at 46 minutes and LYS at 42 minutes (26) (Figure 4A). Considerable amount of OVA was present in the EW (Figure 4B) while it was almost undetectable in the 60 min gastric digest of HOM´EW (Figure 4C). Upon peptic digestion OM profile displayed a wide non-resolved mix of peaks which were difficult to identify within the complex matrix of digested EW (Figure 4B). It is interesting to note in the profile of HOM´EW gastric digest (Figure 4C) the increased amount of peptides from minutes 15 to 32 compared to that of the EW gastric digest (Figure 4B), which indicates the higher degree of hydrolysis in the HOM´EW. This increase in susceptibility to digestion of HOM'EW can be attributed to the heat treatment because OVA is thermolabile and its digestibility increases by heat treatment *(25, 27)*. LYS and OVT have also been reported to be unstable to heat treatment *(18)*. Furthermore OM is reported to be digested into three fragments and two of them resist duodenal digestion and are able to retain IgE binding activity *(10, 25)*, hence low concentration of OM in HOM'EW made it more susceptible to digestion. A high stability through digestion is usually accepted as a characteristic nature of a food allergen which helps to keep the epitopes intact *(28)*, thus the high digestibility of HOM'EW may be related to the safety of OIT performed in this experiment. The gut associated lymphoid tissues are wide spread throughout the digestive tract in which the intestinal lamina propria contains a complex population of cells including activated CD4+

T lymphocytes and B lymphocytes, macrophages, dendritic cells, eosinophils and mast cells. Also there are organized lymphoid tissues, Peyer´s patches being the most prominent, that likely have a pivotal role in triggering immune responses to digested antigens. Interestingly it was recently reported that heat treatment of OVA and OM prevented transport across human intestinal epithelial cells in a form capable of triggering basophil activation or T-cell activation *(29)* and together with the advanced degree of digestion of HOM'EW at intestinal level, compared to intact EW, might be critical in the use of HOM'EW for successful OIT. We recently reported oral administration of EW hydrolysates with peptic fragments of <1.4 kDa led to a specific immune hyporesponsiveness in EW-primed BALB/c mice (30). It was also shown that the higher the digestibility, the lower the antibody binding and heat treatment showed a significant influence on the potential allergenicity of the main egg white proteins that could be related to their resistance to denaturation and digestive enzymes (25). These data put together strongly supports and augments our study in which the HOM'EW was able to induce successful tolerance to EW sensitized mice, which may due to tolerogenic peptides present in HOM'EW.

Low histamine and EW-specific IgE activity post oral challenge.

Histamine concentration was checked in mice sera after oral challenge with EW (Figure 5A). Both treated mouse groups had significantly less histamine than the positive group and were similar to that of the negative group, which confirmed the success of OIT. Also EW-specific IgE antibody activity post oral challenge was less in both the treatment groups than the positive group but significantly higher than negative group. Interestingly, the high dose group showed higher EW- specific IgE activity than the low dose group (Fig 5B).

Total and allergen specific IgG and IgG subclasses

Attempting to find other mechanisms that may have contributed to a lower histamine concentration, we studied in depth sera IgG activity. Total IgG and EW-specific IgG levels were similar in sensitized mouse groups (data no shown). However, treated mice showed high OM specific-IgG levels and this increase was significant in the high dose group (Figure 6A). Antibody to specific IgG1 of EW, OVA and OM were also analyzed without significant differences between groups, however EW specific IgG2a was enhanced (data not shown) and OM and OVA specific IgG2a were significantly higher in the treated groups (Figure 6B and 6C). The functions of allergen specific IgG have been largely studied but the roles in immunotherapy are still being investigated and are a point of discussion. One of the mechanisms related to allergen specific immunotherapy is the induction of allergen-specific IgG antibodies and it is suggested that these antibodies may compete with specific IgE to bind the allergen at the mucosal surface and block the allergic response. On one hand this is questioned because serum concentrations of allergen-specific IgG are correlating with clinical improvement in some studies but not in others *(31)*, in addition many mast cells are on the mucosal surfaces and could meet allergens before antibodies can interpose themselves *(32).* On the other hand, it has been shown in a series of interesting experiments how allergen specific IgG can counteract allergen-specific IgE activity by direct competition by binding epitopes or via inhibitory FcRIIB-dependent signals *(17).* On this note in the current study the increase of OVA and OM specific IgG2a and the increase of OM specific IgG might be effective mechanisms induced during OIT that contribute to a lower histamine release.

Increased Th1/Th2 ratio

The role of Th1 and Th2 cell mediated type responses is well established in mouse models and recent research on T-regulatory cells sheds more light on balance of Th1 and Th2 cell mediated response. In the present study, analyses of culture supernatants stimulated *in vitro* with purified EW indicated that the levels of IFN-γ, the type 1 hallmark cytokine, was significantly higher in the high dose treated mice (Figure 7A) and no significant difference was observed in the low dose treatment group as compared to the positive control. With regards to IL-4 concentration, the Th2 hallmark cytokine, the treatment groups (both high and low) had significantly less concentration as compared to the positive control group (Figure 7B). Also concentration of TGF-β (Th3) and IL-10 (Tr1) were analyzed and no significant difference between groups was observed with TGF-β (Figure 7C), however a significant increase of IL-10 concentration was found in the high dose treatment group (Fig 7D) indicating a significant role of regulatory T cells in immune modulation caused by OIT with HOM´EW.

It was of interest to note that the treatment effects was dose dependent and the high dose group experienced a marked increase in Th1/Th2 ratio, as a result of a significant increase of IFN-γ and a significant decrease of IL-4. The switch from Th2 to Th1 response could be attributed to a significant role of the regulatory cytokine IL-10. It has been reported earlier in peanut allergic patients undergoing OIT, an increase in IL-10 secretion from peripheral mononuclear cells during the first months of OIT that eventually tends to go down and likely depends on the stage of OIT: escalation, build up and maintenance *(33)*. This finding could be attributed to the low dose group in which dose factor may play a significant role in OIT. In fact, it has been shown that IL-10 can modulate allergic reactions by different mechanisms: suppression of allergen-specific IgE, induction of allergen specific

IgG4/IgG2a, suppression both allergen-specific Th1 and Th2 cells, and reduction of the release of pro-inflammatory cytokines by mast cells *(34-36)*. The suppression of antigen-specific immune responses by IL-10 is essential in peripheral tolerance to allergens. In mice it was shown to be the pivotal role of IL-10 in the establishment of peripheral T-cell tolerance by administration of IL-10, which drove mice to antigen-specific T-cell unresponsiveness *(37, 38)*. Also Enrique et al., *(39)* reported high levels of IL-10 in sera after sublingual immunotherapy in hazelnut allergic patients. Based on our results it may be postulated that IL-10 has exerted a critical role in immune modulation of high dose treatment group that might be indicative of a role of T-regulatory cells for induction of oral tolerance.

EW-specific IgA in fecal samples

EW-specific IgA activity was determined in mouse fecal sample supernatants (Figure 8) in an effort to further elucidate the mechanisms occurring locally at the intestinal level. Both the allergic and treatment groups had an increase of EW-specific IgA along the sensitization phase. Once sensitization phase finished, the positive group experienced a decrease of EW-specific IgA while treatment groups had an increase of EW-specific IgA at the beginning of OIT. Then specific EW-IgA activity decreased slowly but was always higher than the positive group activity. At the end of OIT, treatment groups showed higher activity of EW-specific IgA than the positive control group. The negative group had low activity of EW-specific IgA throughout the study. Secretory IgA has an important role in the immune homeostasis of the gut *(16)* but its role in food allergy is still unclear. It has been earlier reported that low levels of allergen-specific IgA in the gut were associated with development of food allergies *(40)*. Although specific IgA was elevated during the

373 sensitization phase, at the end of OIT, just before the end point, we can clearly see how the
374 treated groups had a high activity of allergen specific IgA while the negative and positive
375 groups had a low activity of EW-specific IgA suggesting a susceptible state to develop
376 allergic disease, which supports previous studies *(13, 16, 40)*, and reinforces the fact that a
377 higher production of EW-specific IgA at the mucosal level may have contributed to the
378 allergy-suppressive effect of HOM'EW.
379 In conclusion, the present study established that the success of HOM'EW can be related to
380 its high digestibility. OIT with a high dose of HOM'EW induced desensitization through a
381 switch from Th2 to Th1 response marked by an increase in IL-10 concentration. Also less
382 histamine and EW specific IgE and more specific IgG and IgG2a could have contributed to
383 suppression of allergic response and high amounts of EW-specific IgA in fecal samples
384 suggest an important role at the mucosal level that might be contributing to the therapeutic
385 effect of HOM'EW.
386

387 **Abbreviations used**

388 HOM`EW: heated ovomucoid-depleted egg white; OIT: oral immunotherapy; OVA:
389 ovalbumin; OM: ovomucoid; LYS: lysozyme; OVT: ovotransferin; SGF: simulated gastric
390 fluid; IL: interleukin; IFN-γ: interferon gamma; TGF-β: transforming growth factor beta;
391 Th1: T-helper cell response type 1; Th2: T-helper cell response type 2; T-reg: T regulatory
392 cell response.
393

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513

514 **Figure captions**

515

516 **Figure 1**. Immunoblot analysis of native OM, egg white (EW) and heated ovomucoid-
517 depleted egg white (HOM`EW) (20µg/well) by using rabbit anti-OM IgG-HRP. Lane 1:
518 molecular marker; Lane 2: pure OM; Lane 3: EW; Lane 4: HOM`EW.

519

520 **Figure 2**.Groups of Balb/c mice (n= 40) were orally sensitized twice/week for four weeks
521 with 5mg of egg white and 10 µg of cholera toxin and desensitized with two different doses
522 (1 and 2.5 mg) of heated-ovomucoid depleted egg white thrice/week for 6 weeks. Mice
523 were challenged with 20 mg of intact egg white at the end and blood and tissue samples
524 were collected for analysis of various parameters.

525

526 **Figure 3**. SDS-PAGE gel of *in vitro* gastric digestion at pH 2 (GD) and duodenal digestion
527 (DD) of egg white (EW) and heated ovomucoid-depleted egg white (HOM`EW)
528 (40µg/well). Lane 1: molecular marker. Lanes 2-3: GD of EW at 0 and 60 min. Lane 4: DD
529 of EW at 30 min. Lanes 5-6: GD of HOM`EW at 0 and 60 min. Lanes 7: DD of HOM`EW
530 at 30 min.

531

532 **Figure 4.** RP-HPLC analyses of non-digested egg white (EW) (a) and 60 min gastric
533 digests of EW (b) and heated ovomucoid-depleted egg white (HOM´EW) (c).

534

535 **Figure 5**. Serum histamine concentration and egg white-specific IgE activity in mice sera
536 after oral challenge are represented as mean standard deviation (n = 5) pooled sera.

Different letters indicate statistically significant differences (P<0.05), between groups of mice.

Figure 6. OM-specific IgG, IgG1 and IgG2a activity in mice sera post oral challenge. Data are represented as mean standard deviation (n = 5 pooled sera). Different letters indicate statistically significant differences (P<0.05), between groups of mice

Figure 7. Determination of cytokine concentrations of IFN-γ, IL-4, TGF-β and IL-10 following *in vitro* stimulation of spleen cell cultures with EW. Data are represented as mean±standard deviation (*n*=5 pooled spleens). Different letters indicate statistically significant differences (P<0.05), between groups of mice.

Figure 8. Time-course monitoring of egg white (EW)-specific IgA levels in mouse fecal extracts before and during oral immunotherapy with heated ovomucoid depleted egg white. Data are represented as mean±SD (*n* = 3).

1

Figure 1.

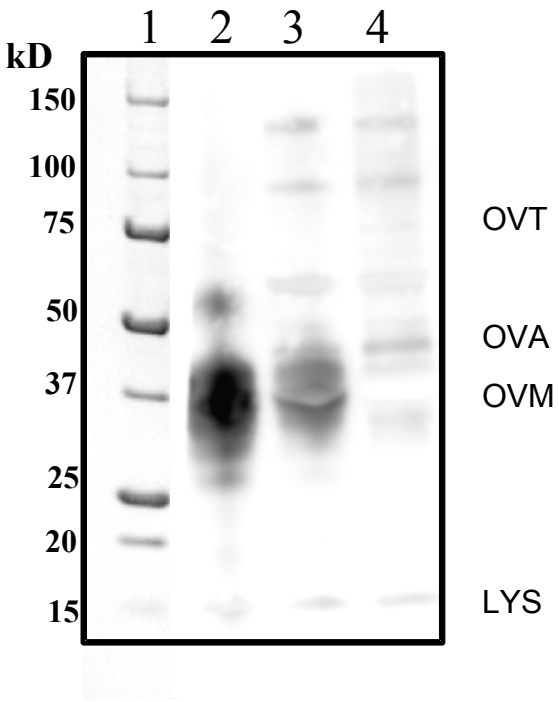


Figure 2.

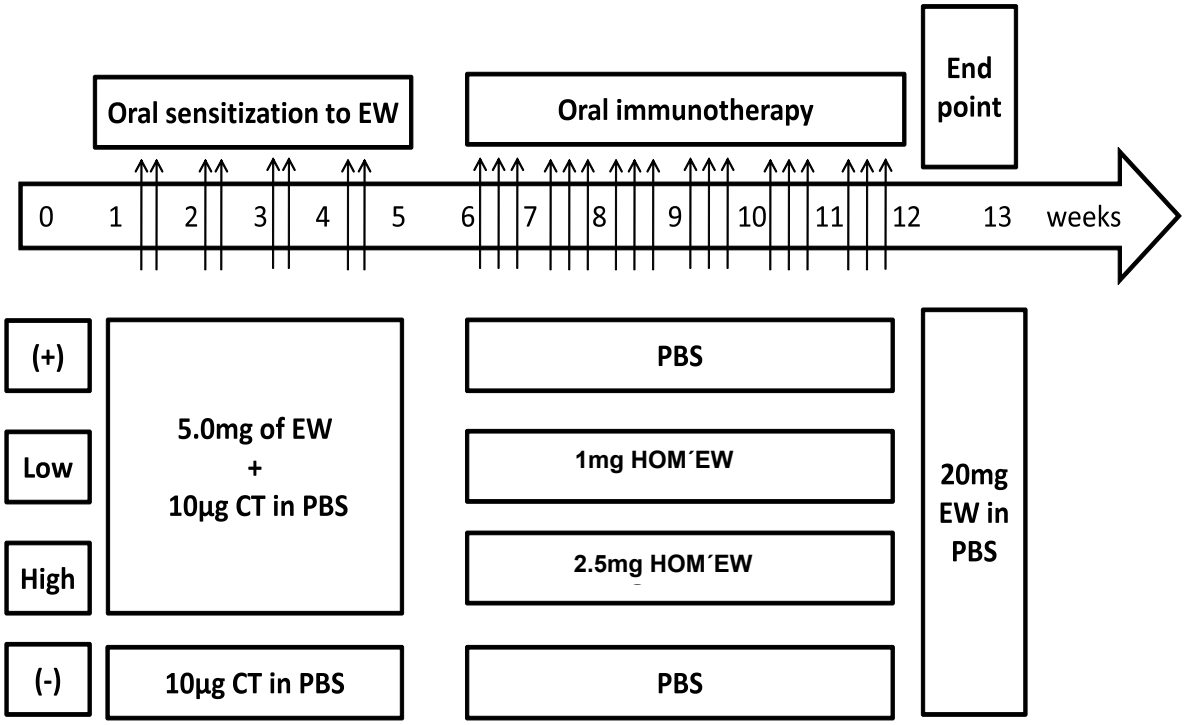

Oral sensitization to EW
Oral immunotherapy
End point
0 1 2 3 4 5 6 7 8 9 10 11 12 13 weeks
(+)
Low
High
(-)
5.0mg of EW + 10µg CT in PBS
10µg CT in PBS
PBS
1mg HOM´EW
2.5mg HOM´EW
PBS
20mg EW in PBS

Figure 3.

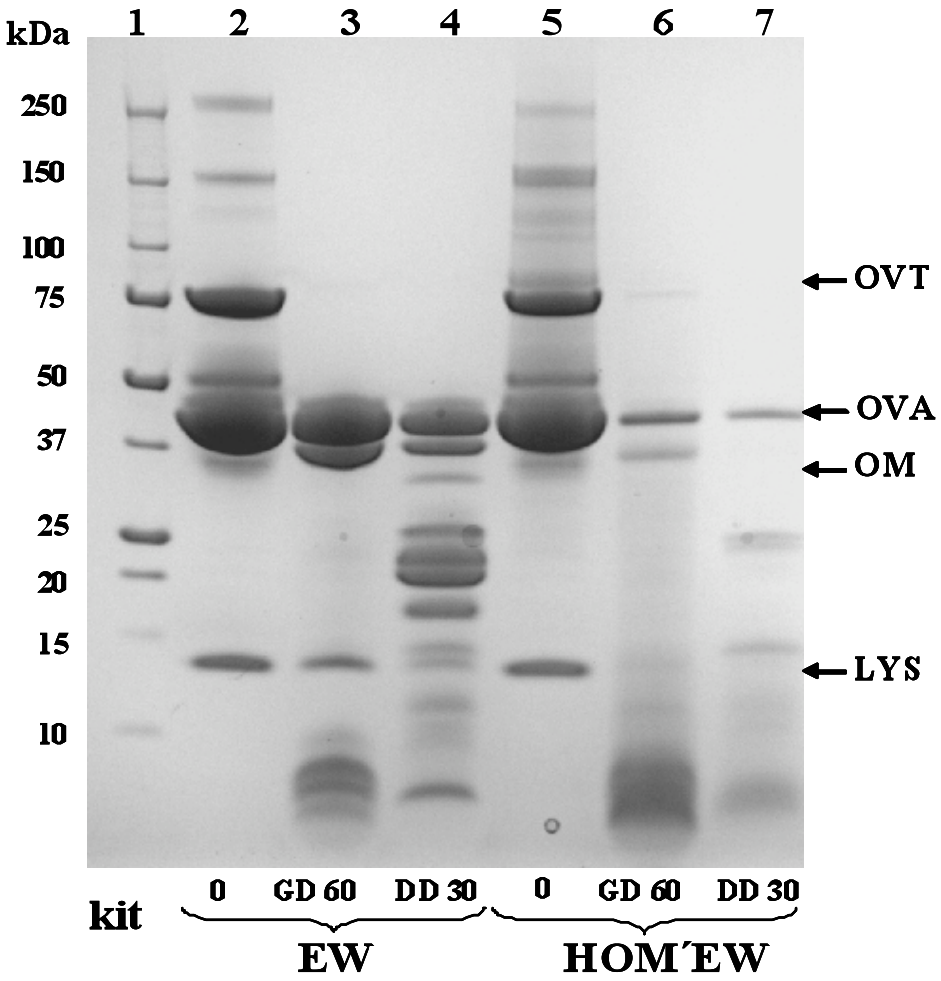

kDa
1
2
3
4
5
6
7
250
150
100
75
50
37
25
20
15
10
OVT
OVA
OM
LYS
kit
0
GD 60
DD 30
0
GD 60
DD 30
EW
HOM´EW

Figure 4.

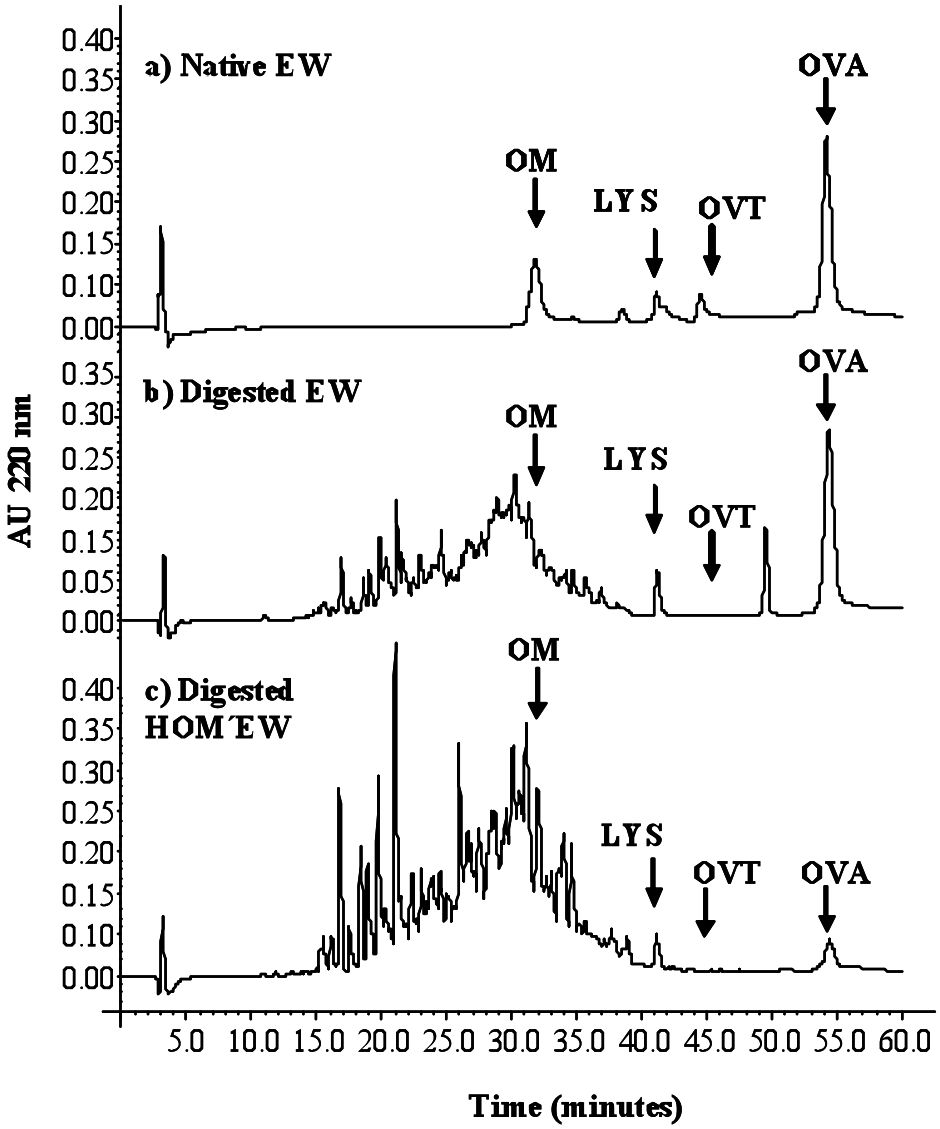

a) Native EW
OVA
OM
LYS
OVT
b) Digested EW
OVA
OM
LYS
OVT
c) Digested HOM EW
OM
LYS
OVT
OVA
AU 220 nm
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
5.0 10.0 15.0 20.0 25.0 30.0 35.0 40.0 45.0 50.0 55.0 60.0
Time (minutes)

Figure 5.

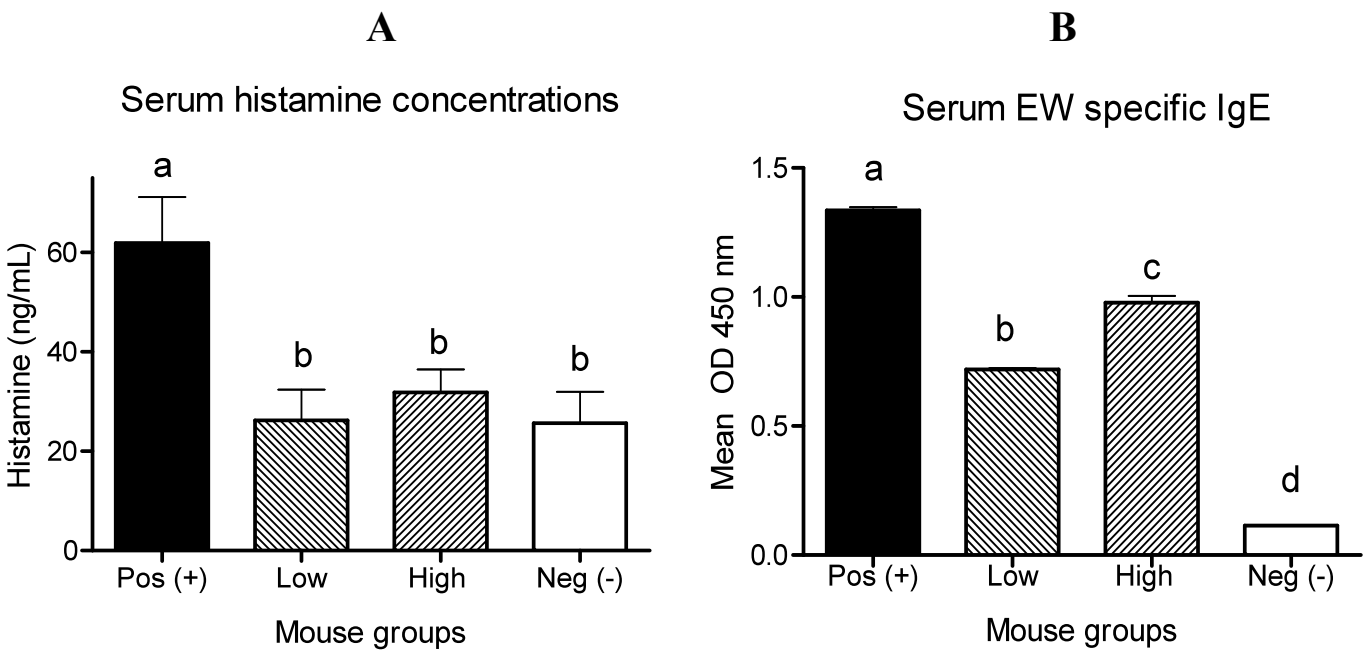

A
Serum histamine concentrations
Histamine (ng/mL)
0
20
40
60
a
b
b
b
Pos (+)
Low
High
Neg (-)
Mouse groups
B
Serum EW specific IgE
Mean OD 450 nm
0.0
0.5
1.0
1.5
a
b
c
d
Pos (+)
Low
High
Neg (-)
Mouse groups

Figure 6.

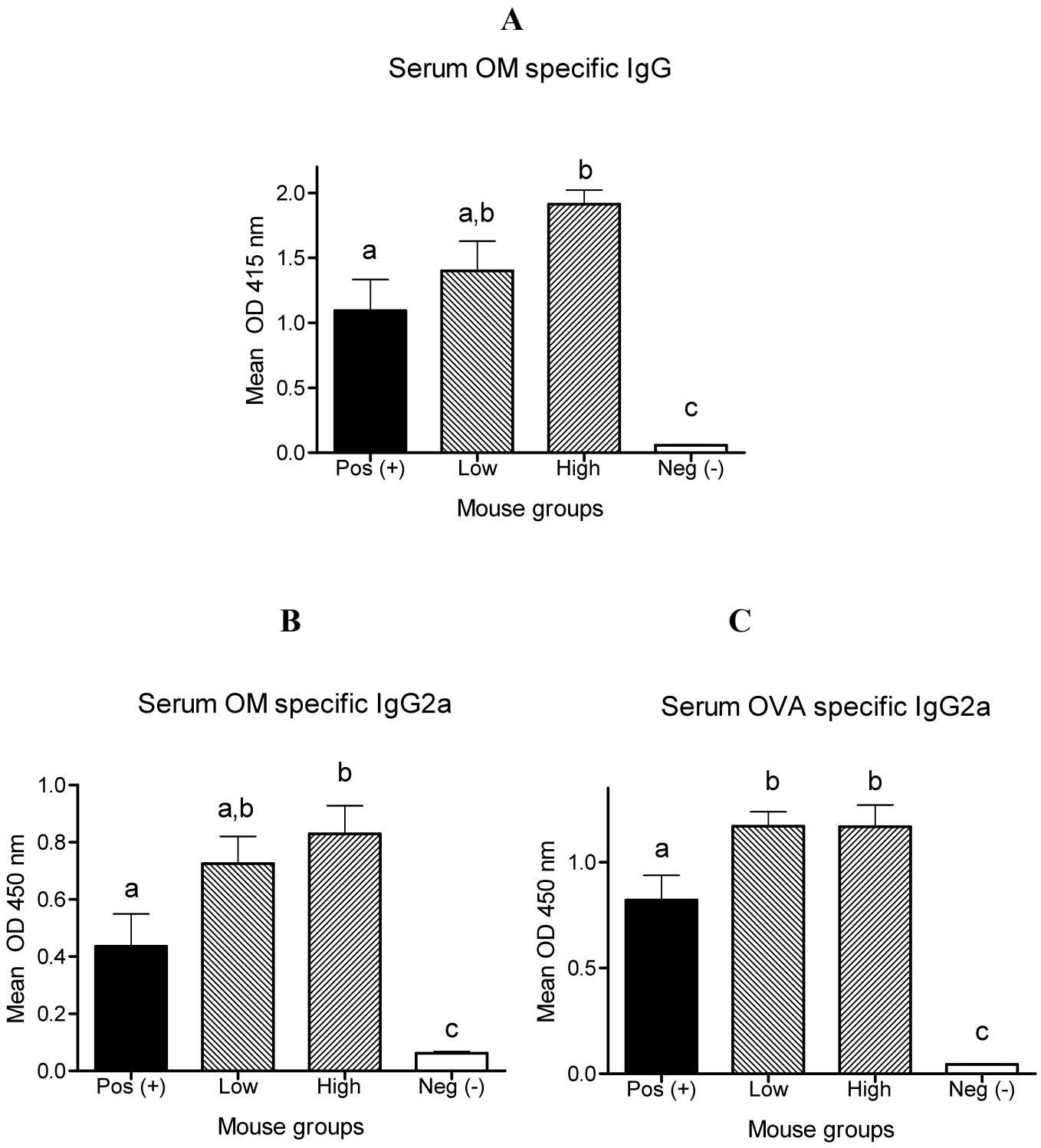

A
Serum OM specific IgG
Mean OD 415 nm
2.0
1.5
1.0
0.5
0.0
a
a,b
b
c
Pos (+)
Low
High
Neg (-)
Mouse groups
B
Serum OM specific IgG2a
Mean OD 450 nm
1.0
0.8
0.6
0.4
0.2
0.0
a
a,b
b
c
Pos (+)
Low
High
Neg (-)
Mouse groups
C
Serum OVA specific IgG2a
Mean OD 450 nm
1.0
0.5
0.0
a
b
b
c
Pos (+)
Low
High
Neg (-)
Mouse groups

Figure 7.

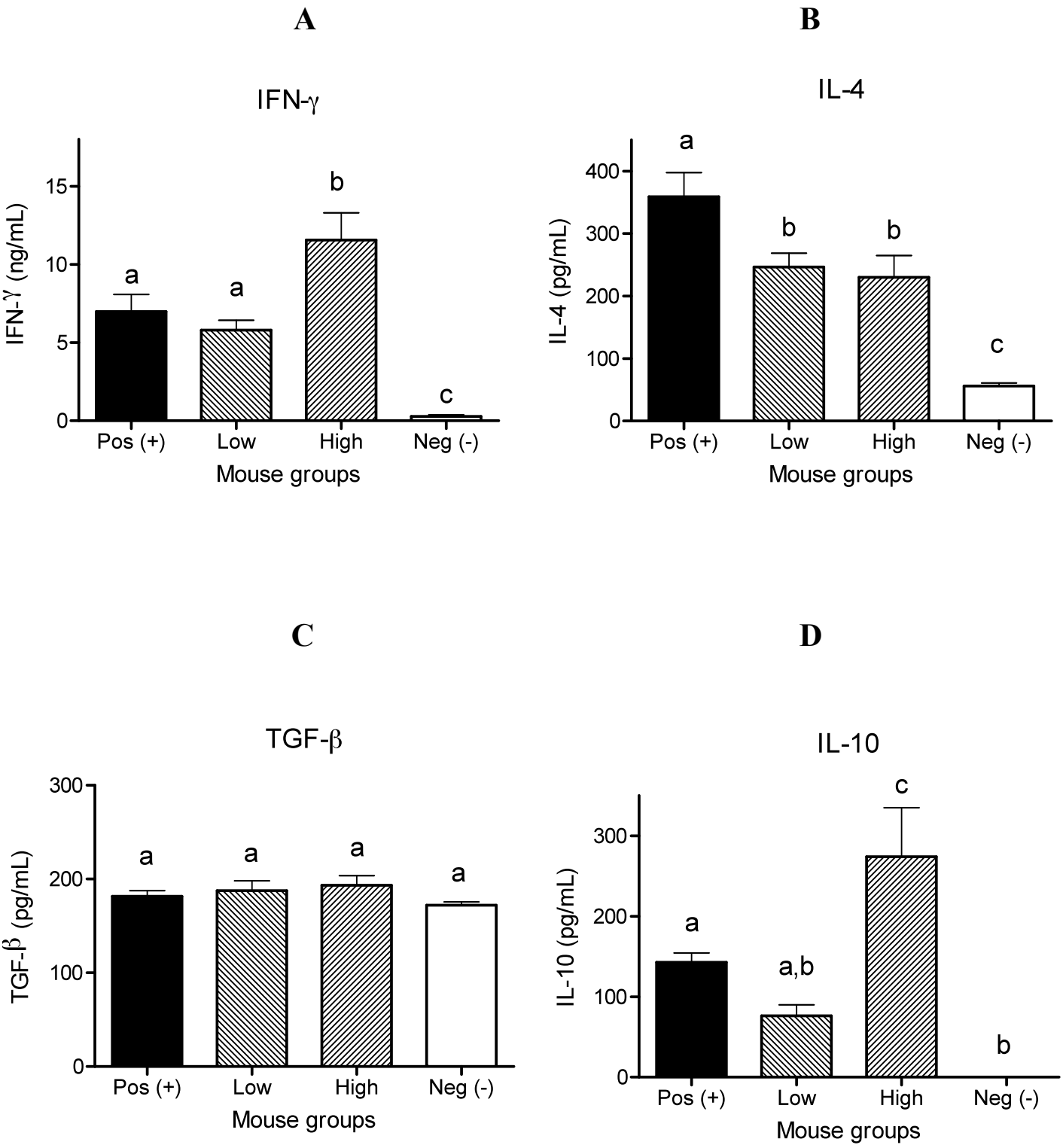
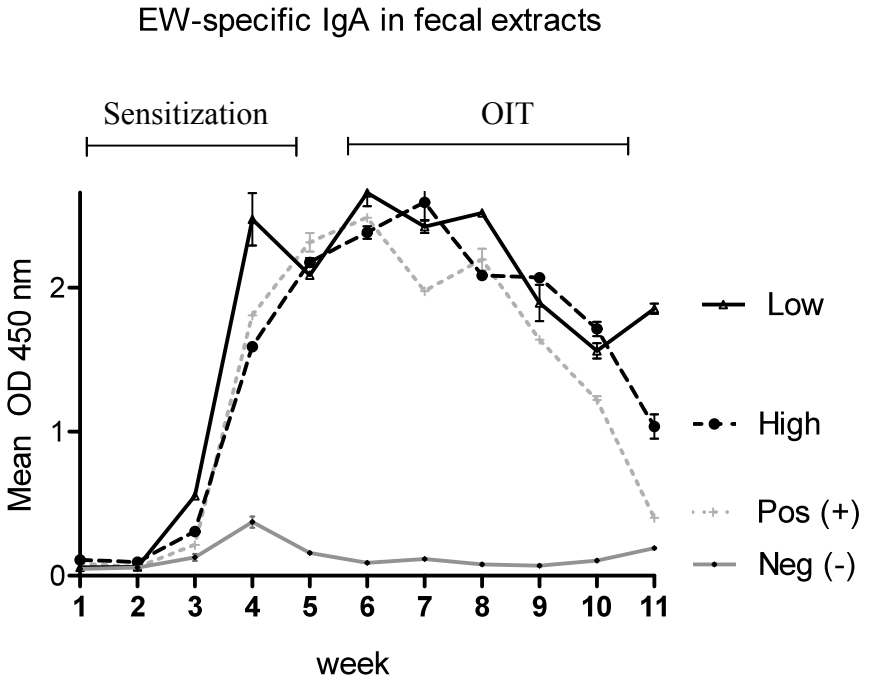

A
IFN-γ
IFN-γ (ng/mL)
15
10
5
0
a
a
b
c
Pos (+)
Low
High
Neg (-)
Mouse groups
B
IL-4
IL-4 (pg/mL)
400
300
200
100
0
a
b
b
c
Pos (+)
Low
High
Neg (-)
Mouse groups
C
TGF-β
TGF-β (pg/mL)
300
200
100
0
a
a
a
a
Pos (+)
Low
High
Neg (-)
Mouse groups
D
IL-10
IL-10 (pg/mL)
300
200
100
0
a
a,b
c
b
Pos (+)
Low
High
Neg (-)
Mouse groups

Figure 8.


EW-specific IgA in fecal extracts
Sensitization
OIT
Mean OD 450 nm
0
1
2
1
2
3
4
5
6
7
8
9
10
11
week
Low
High
Pos (+)
Neg (-)