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Article

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The major soybean allergen Gly m Bd 28K induces hypersensitivity reactions in mice sensitized to cow´s milk proteins.

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Abstract

Reactions to soy have been reported in a proportion of patients with IgE-mediated cow's milk allergy (CMA). In this work, we analyzed if Gly m Bd 28K /P28, one of the major soybean allergens, is a cross-reactive allergen with cow milk proteins (CMP). We showed that P28 was recognized by IgE sera from CMA patients and activated human peripheral basophils degranulation. Moreover, IgE sera of mice exclusively sensitized to CMP recognized P28. Splenocytes from sensitized animals secreted IL-5 and IL-13 when incubated with CMP or soy proteins, but only IL-13 when treated with P28. In addition, skin test was strongly positive for CMP and weakly positive for P28. Remarkably, milk-sensitized mice showed hypersensitivity symptoms following sublingual challenge with P28 or CMP. Using bioinformatics' tools seven putative cross-reactive epitopes were identified. In conclusion, using in vitro and in vivo test we demonstrated that P28 is a novel cross-reactive allergen with CMP.

Keywords: Food allergy; P28 soybean protein; Bovine caseins; Cross-reactivity.

Introduction

Food allergies are currently an important medical and social problem. Approximately 8% of children and 1-2% of adults have some type of food allergy[1]. Particularly, cow's milk allergy (CMA) constitutes the main food allergy observed in the pediatric population in Argentina and in many other countries[2–4]. Soy-based formulas are frequently used as a dairy substitute during the avoidance treatment. Although both milk and soy belong to the group of eight main allergenic foods, often referred as the Big-8, soy allergy (SA) is not as prevalent as CMA[5]. However, several cases of clinical soy-intolerance, at the beginning of the avoidance treatment in milk allergic patients, have been reported[6–9]. This situation introduces a further complexity in the management of patients. The identification and characterization of the components of soybean allergens responsible for allergy may be useful not only to understand the cross-reactivity observed, but also to raise a immunotherapy[10,11] using mutated and hypoallergenic soy allergens[12]. In this regard, we are focused on the investigation of cross-recognition between CMP and soy components, and we have found that bovine caseins (BC)[13,14] cross-react with three soy proteins (SP): Gly m 6.0401 sub G4, Gly m 5.0101 sub α and, more recently, Gly m Bd 30K)[15–19].

In this work, we focused in Gly m Bd 28K/P28, major soybean allergen[20–25]. P28 is a seed glycoprotein that was isolated from 7S globulin fraction and belongs to the cupin protein superfamily. Several major plant food allergens are included in this superfamily, such as the major storage globulins of legumes and nuts (Ara h 1, Ara h 3 from peanut, MP27/MP32 from pumpkin, Gea 8 from carrot, and Gly m 5 and Gly m 6 from soybean) [26–28]. The cupin superfamily includes a diverse group of proteins that share a beta-barrel core domain with very low level of sequence identity[29]. These structural domains are thought to be highly stable,

resisting thermal denaturation and certain types of proteolysis. Thus, the stability factor conferred by this fold may potentiate protein immunogenicity and allergenicity[30] .Gly m Bd 28 K cDNA encodes for a 476 amino acids residues protein a (52,9 kDa), which exhibits a high homology with the MP27/MP32 proteins in pumpkin seeds and carrot globulin-like protein[21,31]. In developing seeds, Gly m Bd 28 K preproprotein is processed into the mature 28kDa N-terminal and 23 kDa C-terminal polypeptides[20,21]. Both resulting polypeptides are recognized by IgE antibodies of patients sensitive to soybean[20,22,23,25], and based on these assays Gly m Bd 28 K is considered as a major soybean allergen. However, the *in vivo* ability of P28 to trigger hypersensitive symptoms has not been evaluated. Currently, animal models are a valuable biological tool to study the correlation of the immunochemical cross-reactivity assessed with the clinical outcome. In this study, we aimed to investigate the clinical relevance of cross-reactivity detected between Gly m Bd 28K /P28 and milk proteins.

Materials and Methods

Protein extracts and antibodies. Soybean protein extract was obtained from *Glycine max* L. Merr. seeds as described previously[16]. Cow's milk protein extract was prepared from commercial non-fat dry milk (Svelty, Nestle). Proteins were extracted with phosphate saline buffer pH 7.4 and filtered. The presence of soy components in the CMP extract was previously discarded by indirect ELISA using a (SP)-specific rabbit antiserum.

Sera of 10 pediatric patients (ranged 9 month to 7 years old, 5 male 5 female) and diagnosed as milk allergic according to history, skin Prick test, and serum specific IgE were used. Milk allergy was not diagnosed with the double-blind placebo-controlled food challenge because it is not currently performed in Argentina; instead milk elimination during two weeks and open challenge is done. Soy allergy was ruled out by history. In addition, sera from healthy individuals, with no allergy history and normal level of serum IgE according to age, or from patients allergic to aeroallergens, with no CMP-specific IgE antibodies and history of food allergy, were included as controls. Three monoclonal antibodies (mAb) with differential specificity for α-casein (1D5), ß-casein (4C3) and κ-casein (3B5), previously obtained and characterized [13], were used.

Cloning, expression and purification of Gly m Bd 28K/P28. The cDNA encoding sequence for P28 (GenBank: accession. AB046874.2) was obtained by PCR amplification of cDNA library[32]. Amplified PCR products were cloned directionally into pENTR/D TOPO (Life Technology, S.A. Argentina), and then transferred to pDEST-maltose-binding protein (MBP) destination vector for expression[33]. *E. coli* BL21 Codon Plus containing the constructs: pDEST His-MBP-P28 and pDEST His-MBP were induced and recombinant proteins were purified as described previously[16]. Depletion of lipopolysaccharide was carried out with a Sepharose-

polymyxin B resine (Sigma-Aldrich, St. Louis, MO, USA). Endotoxin determination was performed with Limulus amoebocyte chromogenic assay (LONZA, Buenos Aires, Argentina). Protein concentration was determined by the bicinchoninic acid assay with bovine seroalbumin as a standard (Pierce, Rockford, IL, USA).

Immunochemical assays. *i. Western blot.* Three μg of purified P28 and MBP were separated by SDS-PAGE, and transferred to nitrocellulose membranes. Blocked membranes (3% horse serum in phosphate buffer pH 7.4) were incubated with patient serum (1:5, overnight at 4 ºC), followed by biotinylated anti-human IgE monoclonal antibody (1:3000, 4 hs at 37 °C, Vector Laboratories Inc, CA, USA), and finally, horseradish peroxidase (HRP) -streptavidin conjugate (1:3000, 30 min at 37 °C, Sigma-Aldrich, MO, USA). Membrane were exposed to Luminol chemiluminescent substrate, revealed with X-ray film (Amersham Hyperfilm ECL, GE Healthcare Bio-Sciences Corp., USA) and scanned. Additionally, membranes were also revealed using the casein-specific mAbs (1 μg/ml) as primary antibody, followed by rat monoclonal anti-mouse immunoglobulin G antibodies conjugated with HRP (1:3000, 1 h at 37°C Bio-Rad Laboratories, CA, USA).

ii. Flow cytometry-based modified basophil activation test (mBAT). Basophils were obtained from whole heparinized blood collected from non-allergic human donors using Ammonium-Chloride-Potassium (ACK) buffer pH 7.2 (0.15M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA) for erythrocyte lysis. Cells were re-suspended in ice-cold lactic acid buffer pH 3.9 (13.4 mM lactic acid, 140 mM NaCl and 5 mM KCl) during 5 min for stripping. Cells were washed and re-suspended in 2 ml of saline buffer containing 20 % of serum of milk allergic patients, 4 mM EDTA and 10 mg/ml heparin (90 min at 37ºC). Cells were washed with 20 nM HEPES buffer

pH 7.4, and incubated with HEPES buffer containing 1 mM $CaCl_2$ (30 min at 37 ºC)[34]. Then, cells were challenged as described previously[19] with whole milk proteins, soy proteins or the recombinant P28 soy allergen at different concentrations. Saline buffer was used to stop cell activation. Finally, washed cells were incubated with 7-Aminoactinomycin (7-AAD) (BD Pharmingen, USA), biotinylated anti-human IgE monoclonal antibody (Vector Laboratories Inc, CA, USA) and streptavidin-Allophycocyanin (APC) (eBioscience, USA), or Phycoerythrin (PE)-conjugated antibody specific to human CD63 (BD Pharmingen, USA), monoclonal antibodies specific to human CD203c (Macs, Miltenyi Biotec, Germany) followed by goat anti-mouse IgG fluorescein isothiocyanate (FITC) (Santa Cruz Biotechnology, USA). Fluorescence data were acquired with a Becton Dickinson FACSCalibur flow cytometer (Franklin Lakes, NJ, USA), and analyzed with the BD CellQuest Pro Software and FlowJo software (Tree Star Inc, Ashland, OR, USA). Controls with a non-related protein (OVA) or with sera from non allergic subjects were included.

ii. Competitive ELISA. Polystyrene microtitre plates were coated with 0,25 µg/well of α-casein or β-casein (Sigma-Aldrich, MO, USA) and blocked with 5% horse serum. Purified 1D5 or 4C3 mAbs were incubated with different quantities of soluble inhibitors: α-casein, β-casein, P28 or ovalbumin (OVA) as a non-related protein (2 hs at 37°C). Then, this pre-mixed dilution was added to the coated wells and incubated for 30 min at 37°C. Finally, rat monoclonal anti-mouse immunoglobulin G antibodies conjugated with HRP was added (1:3000, 1 h at 37°C) and color was developed with o-phenylenediamine. Optical density (OD) was measured at 492 nm.

Experimental mouse model of food allergy and immune response to antigens. *i. Mice sensitization and mucosal challenges.* Male 6- to 8-week old Balb/c mice were purchased from

the School of Animal Sciences, University of La Plata, and kept under pathogen-free conditions with water and commercial diet provided *ad libitum*. Mice were grouped into sensitization and control group (n=8 per group), and sensitized as described previously[15]. Briefly, mice received 6 weekly intragastric (*ig*) doses of CMP (20 mg/dose) plus cholera toxin (10 µg/dose) (Sigma Aldrich, St Louis, MO, USA) in a final volume of 200 ml of bicarbonate buffer (sensitization group), or CMP (20 mg/dose) without cholera toxin (control group). Mice were fasted for 2 h before sensitization, and 3% sodium bicarbonate solution was given 30 min before the immunization. Ten days after the final boost mice were *ig* challenged with 20 mg of CMP, 5 mg of SP. Additionally, some animals were sublingual (*sl*) challenged with 5 µg of P28, 5 µg of β-lactoglobulin (β-Lg) or 10 µg of OVA (Sigma-Aldrich, St Louis, MO, USA).

ii. In vitro evaluation of the allergic reaction. Serum specific IgE antibodies were assessed by western blot. Three µg of CMP, SP, purified P28 and MBP were separated by SDS-PAGE, and transferred to nitrocellulose membranes. Blocked membranes (3% horse serum) were incubated with sensitized mouse serum (1:2, overnight at 4 ºC), followed by biotinylated anti-mouse IgE monoclonal antibody (1:500, 3 hs at 37 °C, BD Pharmingen, USA), and finally, with horseradish peroxidase (HRP) -streptavidin conjugate (1:1000, 1 hs at 37 °C, Sigma-Aldrich, MO, USA). Membranes were incubated with luminol chemiluminescent substrate and exposed to X-ray film (Amersham Hyperfilm ECL, GE Healthcare Bio-Sciences Corp., USA). In addition, serum specific IgG1 and IgG2a were measured (ELISA) using CMP, SP, P28 or MBP as described previously[15]. Briefly, microtitre plates were coated with CMP, SP, P28 1 µg/100 µl, or MBP 0,5 µg/100 µl in carbonate/bicarbonate buffer, pH 9.6. The assay was developed as indicated[15].

To study T cell activation, 24 h following the oral challenge mice were killed, spleens were resected, and spleen cells were stimulated for 72 h with CMP (350 µg/ml), SP (200 µg/ml), P28

(15 μg/ml) or MBP (7,5 μg/ml). Concentration of IL-5, IFN-γ (Invitrogen Corporation, USA) and IL-13 (R&D Systems, UK) was assayed in the supernatants by ELISA following the manufacturer´s instructions.

iii. In vivo evaluation of the allergic reaction. Clinical symptoms were observed 30-60 min following the oral challenge in a blinded fashion by 2 independent investigators, and scored according to Table 1. Mice also underwent cutaneous tests: mice were injected into the pad of either rear foot with 20 μg of CMP, 10 μg of P28, or 10 ug of MBP in 20 μl of sterile saline, and saline in the contra-lateral footpad, as a negative control. Mice were also injected intravenously (tail vein) with 100 μl of 0.1% Evans blue dye (Anedra, 19 Buenos Aires, Argentina). The local presence of blue color minutes after the injection of proteins was considered a positive skin test, and footpad swelling was measured with a digital micrometer with a minimum increment of 0.01 mm.

Bioinformatics* analysis.** *i.* ***Sequence Alignment. The nucleotide sequences of Gly m Bd 28K [Glycine max] (Sequence ID: gi: 410067729 Length: 476 aas), αs1-casein [Bos Taurus] (Sequence ID: gi:162792 Length: 199 aas), β-casein [Bos Taurus] (Sequence ID: gi: 162931 Length: 210 aas) and κ-casein [Bos taurus] (Sequence ID: gi: 1228078 Length: 169 aas) were compared using BLAST (Basic Local Alignment Search Tool, http://blast.ncbi.nlm.nih.gov/Blast.cgi)[35] to identify putative cross- reactive epitopes.

ii. ***Property Distance index.*** to quantify the levels of similarity between P28 putative cross-reactive epitopes and caseins the Property Distance **(**PD) value was calculated using Property-Based Peptide Similarity Index PD for Two Sequences tool (http://fermi.utmb.edu/SDAP/sdap_pdi.html)[36].

192 *iii.* ***Peptide similarity of P28 with known allergen****:* The P28 putative cross-reactive epitopes
193 were assessed for similarity with known allergens in the Structural Database of Allergenic
194 Proteins (SDAP, http://fermi.utmb.edu/SDAP/) using the Peptide similarity tool
195 (http://fermi.utmb.edu/SDAP/sdap_pps.html) [36] .
196 *iv****. Prediction and analysis of the secondary structure:*** P28 secondary structure was predicted
197 with PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/) [37] server.
198
199 **Ethical Consideration.** All experimental protocols of this study were conducted in strict
200 agreement with international ethical standards for animal experimentation (Helsinki Declaration
201 and its amendments, Amsterdam Protocol of welfare and animal protection and National
202 Institutes of Health, USA NIH, guidelines: Guide for the Care and Use of Laboratory Animals)
203 and were approved by the local Institutional Animal Care and Use Committee at the School of
204 Animal Science (CICUAL, 700-003068 /14, University of La Plata). All efforts were made to
205 alleviate suffering during the whole experiment.
206 For the human sample analysis a written informed consent was obtained from parents of patients
207 and the project was approved by the Ethics Committee of the Argentinean Association of Allergy
208 and Clinical Immunology (#00589, February 2012).
209
210 **Statistical analysis.** The values are expressed as means ± standard error of the mean (SEM). All
211 statistical analyses were carried out using GraphPad Prism 5 software. The significance of the
212 difference was determined using an independent-sample t-test or ANOVA test. Statistically
213 significant differences were defined as $p < 0.05$.
214

Results

Production of recombinant P28 in *Escherichia coli*. The 28kDa soybean protein is considered a major soybean allergen based on its interactions with IgE sera of soy sensitive patients [20,22,23,25,38]. To further analyze its involvement in hypersensitivity reactions, the cDNA encoding for the 52,9 kDa proprotein was cloned into pENTR/D-TOPO (Invitrogen) and transferred to the destination vector pDEST-HisMBP to fuse in frame with the sequences encoding a hexa-Histidine tag (His) and Maltose Binding Protein (MBP) at the N-terminal (Figure 1a). The obtained pDEST-HisMBP-P28 expressions vector (Figure 1b) was introduced into *E.coli* BL21 Codon Plus to produce the recombinant fusion protein MBP-P28 (Figure 1c). As a control, a pDEST-His-MBP, without the toxic ccdB gene, was also obtained and used to produce MBP. Both MBP-P28 and MBP fusion proteins were purified from the insoluble fraction under denaturing conditions by nickel affinity chromatography and purified proteins were analyzed by SDS-PAGE (Figure 1d). A 97kDa and 44kDa bands corresponding to the fusion MBP-P28 and MBP alone, respectively, were detected. These purified proteins were used in further analysis.

***In vitro* recognition of P28 by immunochemical assays with CMP-specific IgE.** *i. Immunoblotting with sera of CMA patients*. Human sera containing CMP-specific IgE antibodies were used to assess the recognition of the recombinant P28. Figure 2a shows that P28 was recognized as a coated antigen by ten IgE sera of milk allergic patients. No binding was detected for MBP with sera of allergic patients, and for P28 with sera of non allergic subjects and non-milk allergic patients (#11 and 12).

ii. mBAT with sera of CMA patients. To confirm the cross-reactivity a basophile activation test (BAT) was performed with CMP-specific human IgE sera. Stripped basophils from healthy

238 donors were passively sensitized with individual serum containing IgE and then challenged with
239 different concentrations of milk or soy proteins. A minimum of 100000 events per sample were
240 analyzed by flow cytometry and cells were gated based on physical properties (Figure 2biI), 7
241 AAD^- live cells (Figure 2biII), IgE^+ cells (Figure 2biIII) and further analyzed for membrane
242 CD63 and CD203c (Figure 2bii). Isotype controls were used to set the fluorescence thresholds.
243 Percentages of double positive cells were compared between basophils incubated with the
244 allergen or PBS. Figure 2biii showed that CMP and SP rendered a higher percentage of IgE^+
245 $CD63^+$ $CD203c^+$ cells compared with PBS (18.45±1.35% and 18.80±0.5% vs 9.16±1.26%,
246 respectively). When P28 was used for the IgE-dependent basophil activation we found
247 15.18±0.26% of double positive cells. The non-related protein OVA rendered 8.37±0.31% of
248 double-positive cells at different concentrations. In addition, there were no statistically
249 significant differences in cell activation using sera from non-allergic patients (data not shown).
250
251 *iii. Immunoblotting and competitive ELISA with the casein-specific monoclonal antibodies.* To
252 rule out co-sensitization in patients, immunoblotting was performed using three casein-specific
253 monoclonal antibodies, and we found that only for anti β-bovine casein (BC) mAbs revealed a
254 weak band corresponding to P28 (Figure 3a). MBP was not recognized. Then to rule out the
255 possibility of an artifactual reactivity due to new epitopes created during the coating of antigens,
256 P28 was used as soluble inhibitor in a competitive ELISA using the β-casein-specific 4C3 mAb
257 (Figure 3b) and α-casein-specific 1D5 mAb (Figure 3c). The sigmoid-shape of the dose-response
258 inhibition curve obtained with P28 as inhibitor demonstrated the specificity of the antigen-
259 antibody reaction. The β-casein-specific 4C3 mAb to the immobilized β-casein (0,25 μg/well)
260 was almost 100 % inhibited with 0,2 mg/ml of soluble β-casein, while 50% of inhibition (IC50)

was achieved with 0.06 mg/ml of β-casein, and 0.024 mg/ml of P28. In a separate assay antibody binding to the immobilized α -casein (0,25 µg/well) was 100 % inhibited with 0,002 mg/ml of soluble α -casein, while 50% of inhibition (IC50) was achieved with 0.0002 mg/ml of α-casein, however no inhibition was observed with α-casein-specific 1D5 mAb a wide range of concentration of P28. No inhibition was observed a wide range of concentration of OVA for both mAbs.

***In vivo* evaluation of P28 used a specific mouse model of CMP.** Mice exclusively allergic to milk were used to investigate the clinical relevance of the immunochemical co-recognition. The experimental protocol used to sensitized mice is depicted in Figure 4a. Intragastically sensitized mice developed hypersensitivity symptoms, scored according to Supporting Table S1, immediately after the oral challenge with the different allergens. CMP-sensitized animals showed higher scores upon the challenge with SP or CMP, compared to sham mice (Figure 4bi). When animals were sublingually challenged with individual proteins (β-lactoglobulin, P28 or the unrelated OVA) we observed high clinical scores with β-lactoglobulin, and intermediates scores with P28, while OVA was rendered no symptom (Figure 4bii). Finally, we examined the functionality of IgE-sensitized mast cells and basophils in the footpads of mice. The cutaneous injection of CMP or P28 led to a local and distant inflammation in minutes (Figure 4c). Footpad swelling was statistically lower when injected P28 compared CMP mice (0.27±0.05 and 0.49±0.06, respectively; and saline buffer 0.05±0.02 as control).

Humoral and cellular immune response to P28 fusion protein by sensitized mice of CMP. Afterwards, we questioned if the soy proteins are recognized by the secreted T-helper 2 (Th2) -

associated antibodies. We found that CMP and SP were highly and significantly recognized by mouse CMP-specific IgE and IgG1 antibodies. However, P28 was significantly recognized by CMP-specific IgG1 but weakly recognized by IgE antibodies (Figure 5ai and ii). No IgG2a specific antibodies were detected in sensitized or sham animals (Figure 5aiii). Also, to correlate humoral immune response with the cellular immunity we measured cytokine production in culture supernatants of splenocytes after being stimulated with different proteins. We found a significant increase of the secreted Th2 cell-associated cytokines (IL-5 and IL-13) in sensitized mice challenged with CMP or SP, while IFN-γ remained unchanged (Figure 5b). When the detoxified P28 protein was used to stimulate spleen cells, only IL-13 were secreted, while the addition of MBP induced no cell stimulation.

To further investigate immune response of milk sensitized mice a western blot was performed. Mouse Ig bound to CMP and SP (Figure 5ai). Interested, several components of the SP extract (i.e. α´- α- and β- β conglycinin, AB glicinin, 40kDa, P28) were specifically recognized by sera of mice exclusively sensitized to milk. Recombinant P28 was also recognized by IgE-containing serum antibodies.

***In sílico* analysis of cross-reactive epitopes.** In order to understand the observed *in vitro* and *in vivo* cross-reactivity, P28 and αS1, β- and κ caseins sequence alignments were performed. Two small regions with discrete homology were detected when αS1 protein sequence was aligned with P28 (Table S1). The first homology region of αS1-casein with P28 had a length of 19 amino acids with 37% of identity and 57% of similarity, while the second homology region had only of 6 amino acids length, without gaps, with 83% of identity and similarity. Alignment of β-casein with P28 gave four homology regions of 8, 15, 17 and 23 amino acids length without

gaps, with identity in the range of 26% to 50% and similarity between 47-87% (Table 1). Only one discrete homology region of 12 amino acids was found in the alignment of κ-casein and P28 with 42% of identity and 75% of similarity. These putative cross-reactive epitopes were numbered according to their location along P28 sequence. The peptides 1 and 2 overlap in a sequence of 11 amino acids residues. Predictive cross-reactive epitopes were located in beta sheet and α helix structure as well disordered regions (Figure 6). To evaluate meaningful physical-chemical similarities, the Property Distance (PD) index between the bovine caseins and P28 peptides was calculated using Property-Based Peptide Similarity Index PD for Two Sequences (http://fermi.utmb.edu/SDAP/sdap_pdi.html)[36]. Peptide 5 and 6 has a PD value of 5.17 and 4.07, respectively while the other P28 peptides have PD values in the range of 9.06-12.19 (Table 1). Peptides with PD the value lower than 10 share high similarity[36], therefore P28 have several peptides with the potential cross-reactivity with caseins.

The seven putative cross-reactive epitopes were assessed for similarity with known allergens in the Structural Database of Allergenic Proteins (SDAP) using peptide similarity tool[36]. Cross reactivity with Bos d 8 allergen, which include αS1, β and κ caseins, was detected for all putative P28 cross-reactive epitopes except for peptide 2 and 4 (Supporting Table S2). In addition, P28 peptides showed significant similarity with allergens of peanut, sesame, soybean, carrot, pear, pistachio, etc, since the Property Distance (PD) score were lower than 10 (Supporting Table S2). Remarkably other IgE cross-reactive soybean allergens with bovine caseins described by our group [15,16,17,18], were retrieved by the SDAP peptide similarity tool. : Peptides 5 and 3 had PD value of 4.08, and 7.15 with alpha subunit beta conglycinin (Gly m 5.0101) [16,18], respectively. Peptides 5 had a PD value of 4.83 with glycinin G4 A4A5B3 (Gly m 6.0401) [15,17](Supporting Table S2).

Discussion

Gly m Bd 28K (P28) is a minor component of the 7S globulins[20,21,25] and it has been described, together with P34 and α subunit of β-conglycinin, as the third major soybean allergen[24,31,38,39]. We have shown that P28 was recognized by serum IgE from milk-allergic patients, CMP-specific IgE and IgG1 antibodies from milk-allergic mice and activated basophils passively sensitized with milk-allergic sera. Additionally, we showed that P28 was a good competitor of β-casein in an inhibition ELISA performed with a specific β-casein mAb in concordance with the recognition of P28 by this mAb in western blot. Furthermore, an immediate cutaneous reaction and hypersensitivity symptoms were observed in sensitized mice following oral exposure to P28, consistent with the Th2-biased immune response. These *in vivo* results are in good correlation with the *in vitro* cross-reactivity.

In previous studies, we demonstrated that bovine caseins and soy proteins: Gly m 6 G4 (glycinin $A_4A_5B_3$)[15,17], Gly m 5.0101(α subunit of β-conglycinin)[16,18] and Gly m Bd 30K (P34)[19], were recognized by IgE from milk allergic patients and they are able to trigger hypersensitive reaction in a mouse model to cow's milk allergy. These *in vivo* results partially explaining the clinical intolerance observed in a restricted population of IgE-mediated CMA patients, primarily not sensitized to SP[3]. However, and to rule out the possibility of co-sensitization of patients, casein-specific monoclonal antibodies were used to identify cross-reactive SP. Epitopes in P34 and α subunit of β-conglycinin were bound by α-, β- and κ-casein specific monoclonal antibodies[18,19], while P28 only reacted when confronted the β-casein specific mAb (4C3). Although α-casein 1D5 and κ-casein 3B5 monoclonal antibodies did not recognized P28, we cannot discard that P28 share epitopes with α- or κ-casein than were not recognized by these mAbs. This distinctive immunochemically cross-reactivity detected for P28, was also observed *in vivo* through IgE

mediated response of milk sensitized mice. CMA mice after sublingual challenge with P28, showed lower clinical hypersensitivity score compared with those confronted with P34 or α subunit of β-conglycinin [18,19]. Besides, P28 skin test was also weakly positive compared with CMP, P34 or α subunit of β-conglycinin that were strongly positive [18,19]. In addition, stimulation of sensitized mice spleen cells with P28 only induced IL-13 release, while the other cross-reactive soybean epitopes triggered both IL-5 and IL-13 discharge. Consequently, P28 T cell epitopes associated with Th2 responses are different from the ones present in bovine caseins, P34 and α subunit of β-conglycinin [18,19].

Based on our findings we cannot assure that cross-reactivity between P28 and caseins is based exclusively on sequential epitopes[40]. We know that the allergenicity of caseins is due to some small protein fragments containing IgE-binding epitopes, which are spread along the whole protein sequence, able to cross-link two IgE molecules, and thereby elicit an allergic reaction. In general, caseins appear to be the predominant allergen in patients with CMA[41]. The majority of milk allergenic patients showed a strong humoral and cellular response to caseins, with high titers of IgE specific for these proteins. Multiple B and T epitopes in caseins, ranging from 8 to 20 AA, have been identified by different immunoassays [42–50]. In agreement with this, when we aligned the sequences of αS1-, β- and κ- caseins with P28, seven peptides with discrete homology were distinguished (Table 1 and Figures 6). These peptides showed a length between 6 to 23 amino acids, degrees of identity from 26% to 83% and PD values from 4.07 to 12.19. Although, the threshold between cross-reactive and non-reactive peptides might vary among epitopes, PD values lower than 10 point out significant physical-chemical similarities[36]. Peptides 1 and 4-7 had PD values lower than 10, therefore are potentially cross-reactive epitopes with caseins. Thus, the *in silico* sequence analysis reveals that P28 had at least five cross-reactive

epitopes with bovine caseins, and only two are required for binding of IgE molecules and trigger hypersensitivity symptoms. These results contribute to validate the utility of the property distance (PD) scale for prediction of cross-reactivity.

Our group has described several soybean cross reactive allergens with caseins: P28 and α subunit of β-conglycinin (Gly m 5.01)n[16,18] that are 7S storage proteins and glycinin $A_4A_5B_3$ (Gly m 6 G4)[15,17] is an 11S storage proteins, P34 (Gly m Bd 30 K) a inactive cysteine protease of the papain-superfamily[19]. In this work, we showed that the putative cross-reactive P28 peptides with caseins, also share significant similarity with other member of the 7S and 11S soybean storage protein as well as cupins from legumes and legumes and tree nuts (Supporting Table S2). Although, it was thought that proteins with less of 50% sequence identity were rarely cross-reactive, recently it has been shown Ara h 1, Ara h 2, and Ara h 3 the three major allergen of peanut are cross-reactive[51]. This report is in coincidence with our results, that support cross-reactivity among non structural related proteins such as bovine caseins, soybean 7S and 11S proteins. It is important to remark that at difference of other reports that only analyzed in vitro Ig E recognition; we have demonstrated in vivo allergenicity among these proteins.

Experimental animal models are important to confirm the immunochemical data and to investigate the cross-allergenicity. Immunochemical cross-reactivity not always correlates with cross-allergenicity. In this work we probed that P28 triggered hypersensitivity symptoms in exclusively milk-sensitized mice, which was not observed with the un-related OVA antigen. Furthermore, the functionality of the IgE antibodies was also evidenced with the skin test, demonstrating that P28 contains at least two surface cross-reactive epitopes. This *in vivo* characterization that cannot be carried out in patients is critical to generate knowledge to be incorporated into the allergen databases.

399 In conclusion, P28 was recognized by IgE sera from CMA patients, activated human peripheral
400 basophils degranulation, produced a weakly positive skin test and elicited immediate
401 hypersensitivity symptoms in milk-sensitized mice. These in vitro and in vivo data confirmed
402 that P28 contains B and T cross–reactive epitopes with bovine caseins that were proposed using
403 different bioinformatics tools. Consequently, we demonstrated that P28 is a new cross-reactive
404 soybean allergen with bovine caseins. In vivo studies of cross-reactivity of allergens are scarcely
405 and this work is an important contribution for understanding physical-chemical characteristics of
406 allergens and for developing hypoallergenic soy formulations or news therapeutic intervention
407 protocols.
408

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570
571
572

573 **Figure legends**

574 **Figure 1. Construct and synthesis of recombinant *Gly m Bd 28K/* P28 protein.**

575 (a) pDEST-HisMBP expression vector. The vector contains the *cm* and toxic *ccd B* genes
576 between the recombination attR1 and attR2 sites.

577 (b) Schematic representation of MBP-P28 and MBP constructs. The hexahistidine (His) and
578 maltose binding protein (MBP) tags were fused to P28 proprotein (476 AA) in the N terminal to
579 produce MBP-P28. The vector pHisMBP is a modified version without the *cm* and toxic *ccd B*
580 genes, that was obtained to produce MBP protein.

581 (c) SDS-PAGE of total extracts: lane 1, *E. coli* wild type; lane 2, *E. coli* expressing MBP; lane
582 3: *E. coli* expressing MBP-P28.

583 (d) SDS-PAGE of the purified proteins: lane 1, MBP; lane 2: MBP-P28.

584 *MW, Molecular weight ranges; MBP, maltose-binding protein; His, hexahistidine tag; cm,*
585 *chloramphenicol resistance gen; ccd, toxin gene that targets DNA gyrase; P28, Gly m Bd 28K;*
586 *MW of MBP: 44 kDa; MW of MBP-P28: 97 kDa.*

587

588 **Figure 2. Immunochemical assays of soy allergen protein with sera of CMA patients.**

589 (a) ***P28 recognition by Ig E sera of CMA patients***: P28 recombinant protein was separated by
590 SDS-PAGE and membrane was revealed with individual sera of CMA patients (#1-10). In
591 addition, incubations with a representative serum of a non-CMA patient (#11) and of a non
592 allergic subject (#12) were used as negative controls. To discard recognition of IgE sera to the
593 His-MBP region of MBP-P28 fusion, the binding HisMBP protein was also analyzed. Ponceau S
594 staining of the blotted membrane was performed to check the efficiency of recombinant proteins
595 transfer.

(b) ***Basophil activation test by flow cytometry.*** Basophils from healthy donor subjects were stripped and passively sensitized with milk-specific IgE-containing sera from CMA patients (n=5). i. Cells were selected according to physical parameters I, viable cells; II, viable IgE-sensitized cells; III, Activated basophils: $CD63^+$ / $CD203c^+$ double positive cells.

ii. Activated basophils after stimulation with CMP, SP, and P28 at different concentrations were analyzed. OVA and PBS were used as negative controls. Results of representative experiments are showed.

iii. Percentage of activated basophils obtained with five CMA's IgE-sera. Data represent mean ± SEM % of double positive basophils. Statistical significant difference with t-test: ***$p<0.0005$, **$p<0.001$ vs PBS group.

Figure 3. Immunochemical assays with casein specific mAbs.

(a) Western blot of P28 and MBP revealed with different casein specific monoclonal antibodies. Ponceau S staining showed that proteins were efficiently transferred to the membrane.

.(b) Inhibition ELISA: wells were coated with β-casein (0,25 µg/well), and β-casein-specific monoclonal antibody 4C3 was pre-incubated with different concentrations of β-casein, P28 or OVA as soluble inhibitors.

(c) Inhibition ELISA: wells were coated with α-casein (0,25 µg/well), and α-casein-specific monoclonal antibody 1D5 was pre-incubated with different concentrations of α-casein, P28 or OVA as soluble inhibitors.

MBP, maltose-binding protein; P28, MBP-P28 recombinant fusion protein; 1D5, α-casein-specific monoclonal antibody; 4C3, β-casein-specific monoclonal antibody; 3B5, κ-casein specific monoclonal antibody. MW of MBP: 44 kDa; MW of MBP-P28: 97 kDa.

Figure 4. Allergic sensitization of mice to CMP and clinical symptoms .

(a) Schematic drawing of the experimental protocol: BALB/c mice (n=8 per group) were subjected to weekly intragastric sensitization with cholera toxin and CMP from day 0 through day 35. Challenge was performed at day 45 by intragastric (CMP or SP) (n=7 per group) or sublingual (β-Lg, P28 or OVA) (n=5 per group) administration of proteins. Control mice only received CMP and then they were orally challenged;

(b) Clinical symptoms were observed 30 min following the challenges with CMP or SP (i.) or with β-Lg, P28 or OVA (ii.) and then scored according to Table S1;

(c) Cutaneous test: sensitized control mice were injected into the pad of either rear foot with CMP, P28, MBP and PBS (n=4 per group).

i. Blue colour in the skin within minutes after injection, was considered a positive cutaneous test. The results are from a single experiment, which is representative of three independent experiments.

ii. Footpad swelling: the difference between the thickness of the footpad injected with allergens and negative control was calculated. Data are expressed as mean ± SEM. Statistical significant difference with ANOVA test: ***$p<0.005$, **$p<0.01$.

CMP, cow's milk proteins; SP, soy proteins; CT, cholera toxin; β-Lg, beta lactoglobulin; MBP, maltose-binding protein, P28, MBP-P28 recombinant fusion protein; OVA, ovalbumin.

Figure 5. Humoral and cellular immune response of milk sensitized mice. (a) i. Specific IgE (by WB): ii. Specific IgG1 (by ELISA) and iii. Specific IgG2a (by ELISA) in serum of milk-sensitized mice (mean values ± SEM);

642 (b) i. Levels of IL-5, ii. IL-13 and iii. IFN-γ (by ELISA) in supernatants of stimulated spleen
643 cells (mean values ± SEM). Results correspond to a single experiment with at least three mice
644 per condition; three independent experiments gave similar results. Statistically significant
645 differences with Student's *t* test: ***p<0.005, **p<0.01, *p<0.05.
646 *SP, soy proteins;, CS, Coomassie stained protein gel; MBP, maltose-binding protein, P28, MBP-*
647 *P28 recombinant fusion protein; CMP, cow's milk proteins, AB Gly: glycinin subunits, α′-, α-*
648 *and β- β conglycinin subunits.*
649

650 **Figure 6. *In silico* analysis of putative cross-reactive epitopes on P28.**
651 P28 protein sequence was alignment with bovine caseins. Regions of similarity to αS1-casein
652 (peptides 3 and 6) are shown with dashed lines, to β-casein (peptides 2, 4, 5, and 7) with solid
653 lines and to κ-casein (peptides 1) with dotted lines. Amino acid residue letter corresponds to its
654 degree of conservation: red for identical and purple for conserved. P28 secondary structure was
655 analyzed with PSIPRED prediction server (http://bioinf.cs.ucl.ac.uk/psipred/). The alpha-helix
656 regions are indicated with red cylinder, beta-sheets are presented in yellows arrows and
657 disordered regions with solid lines.
658

659 **Abbreviations: BAT**, basophil activation test; **β-Lg**, beta lactoglobulin; **CMA**, cow's milk
660 allergy; **CMP**, cow's milk proteins; **CR**, cross reactivity; **CT**, cholera toxin; **EAST**, Enzyme
661 Allergo Sorbent Test; **ELISA**, Enzyme-linked immunosorbent assay; **Gly m**, Glycine max (L.)
662 Merr. allergen; **HRP**, horseradish peroxidase; **IC50**, inhibitory concentration of 50%; **Ig**,
663 immunoglobulin; **IFN**, interferon; **IL**, interleukin; **IUIS**, International Union of Immunological
664 Societies; **i.v.**, intravenously; **i.g.**, intragástrica; **s.l.**, sublingual; **s.c.**, subcutaneous; **mAb**,
665 monoclonal antibody; **MBP**, maltose binding protein; **OD**, optical density; **OVA**, ovalbumin;
666 **PD**, property distance score; **P28**, 28 kDa soybean protein; **SDAP**, Structural Database of
667 Allergenic Proteins; **SDS-PAGE**, sodium dodecyl sulphate-polyacrylamide gel electrophoresis;
668 **SP**, soybean proteins; **Th**, T-helper cell; **WB**, Western Blot; **WHO**, World Health Organization.
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671

Table 1: Identification of putative cross reactive epitopes by alignment of amino acid sequences of P28 and bovine milk caseins.

Cross-Reactive Epitopes	Casein (Bos d 8)	Matches regions in the alignment P28 vs caseins	AA length	Identities	Positives	Gaps	PD Value*
Peptide 1	κ casein	P28 70 HMHIGFISMEPK 81 H H+ F+++ PK K-cas 121 HPHLSFMAIPPK 132	12	5/12 (42%)	9/12 (75%)	0/12 (0%)	9.06
Peptide 2	β casein	P28 71 MHIGFISMEPKSLFVPQ 87 MH + P +F PQ b-cas 159 MHQPHQPLPPTVMFPPQ 175	17	6/17 (35%)	8/17 (47%)	0/17 (0%)	12.01
Peptide 3	α S1 casein	P28 115 RRLKTGDLYMIPSGSAFYL 133 R+ D Y PSG+ +Y+ a-cas 166 RQFYQLDAY--PSGAWYYV 182	19	7/19 (37%)	11/19 (57%)	2/19 (10%)	12.19
Peptide 4	β casein	P28 225 KDDKEQQLKKMMQDQ 239 + +++QQ + +QD+ b-cas 49 QSEEQQQTEDELQDK 63	15	4/15 (27%)	11/15 (73%)	0/15 (0%)	9.13
Peptide 5	β casein	P28 241 EDEEEKQT 248 + EE++QT b-cas 49 QSEEQQQT 56	8	4/8 (50%)	7/8 (87%)	0/8 (0%)	5.17
Peptide 6	αS1 casein	P28 309 EPDIGV 314 EP IGV a-cas 148 EPMIGV 153	6	5/6 (83%)	5/6 (83%)	0/6 (0%)	4.07
Peptide 7	β casein	P28 329 VNPISDEYTIVLSGYGELHIGYP 351 V P ++ ++ L+ LH+ P b-cas 131 VEPFTESQSLTLTDVENLHLPLP 153	23	6/23 (26%)	12/23 (52%)	0/23 (0%)	9.79

The nucleotide sequences of Gly m Bd 28K [Glycine max] (Sequence ID: gi: 410067729 Length: 476 aas), αs1-casein [Bos Taurus] (Sequence ID: gi:162792 Length: 199 aas), β-casein [Bos Taurus] (Sequence ID: gi: 162931 Length: 210 aas) and κ-casein [Bos taurus] (Sequence ID: gi: 1228078 Length: 169 aas) were compared using BLAST (Basic Local Alignment Search Tool, **http://blast.ncbi.nlm.nih.gov/Blast.cgi**)[35] to identify putative cross-reactive epitopes. Seven regions with high similarity were detected and these peptides were considered putative cross-reactive epitopes. The alignment of these peptides with caseins is shown: the identical amino acid residues are in red color, and the conserved residues are consigned with plus purple symbol. Statistics for each peptide is shown on the right columns. *The Property distance (PD) index between the bovine casein peptides and P28 epitopes identified in the alignment was calculated using Property-Based Peptide Similarity Index PD for Two Sequences (**http://fermi.utmb.edu/SDAP/sdap_pdi.html**)[36]. PD value detects meaningful physical-chemical similarities.

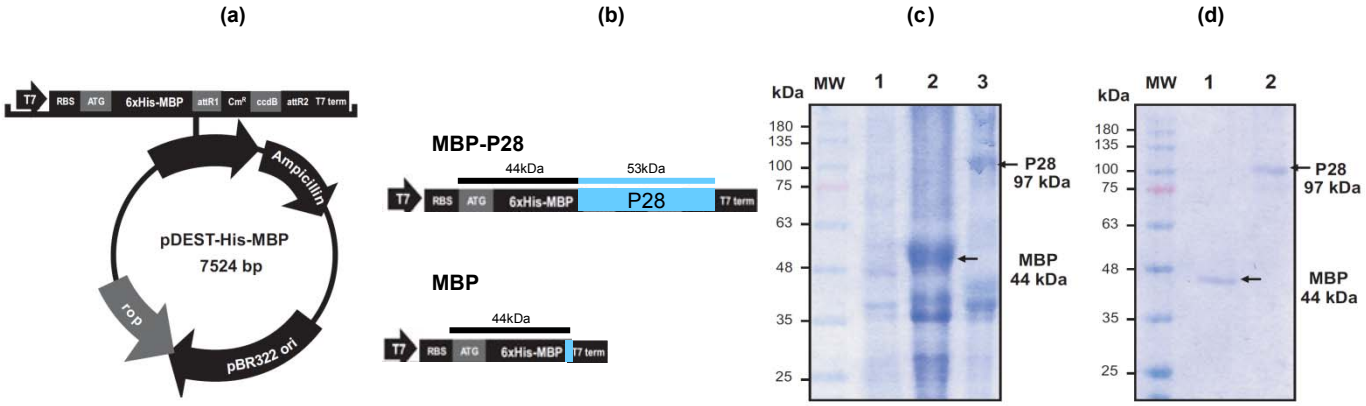

(a)
(b)
(c)
(d)
T7
RBS
ATG
6xHis-MBP
attR1
Cm^R
ccdB
attR2
T7 term
Ampicillin
pDEST-His-MBP
7524 bp
rop
pBR322 ori
MBP-P28
44kDa
53kDa
P28
MBP
kDa
MW
1
2
3
180
135
100
75
63
48
35
25
P28
97 kDa
MBP
44 kDa

Figure 1

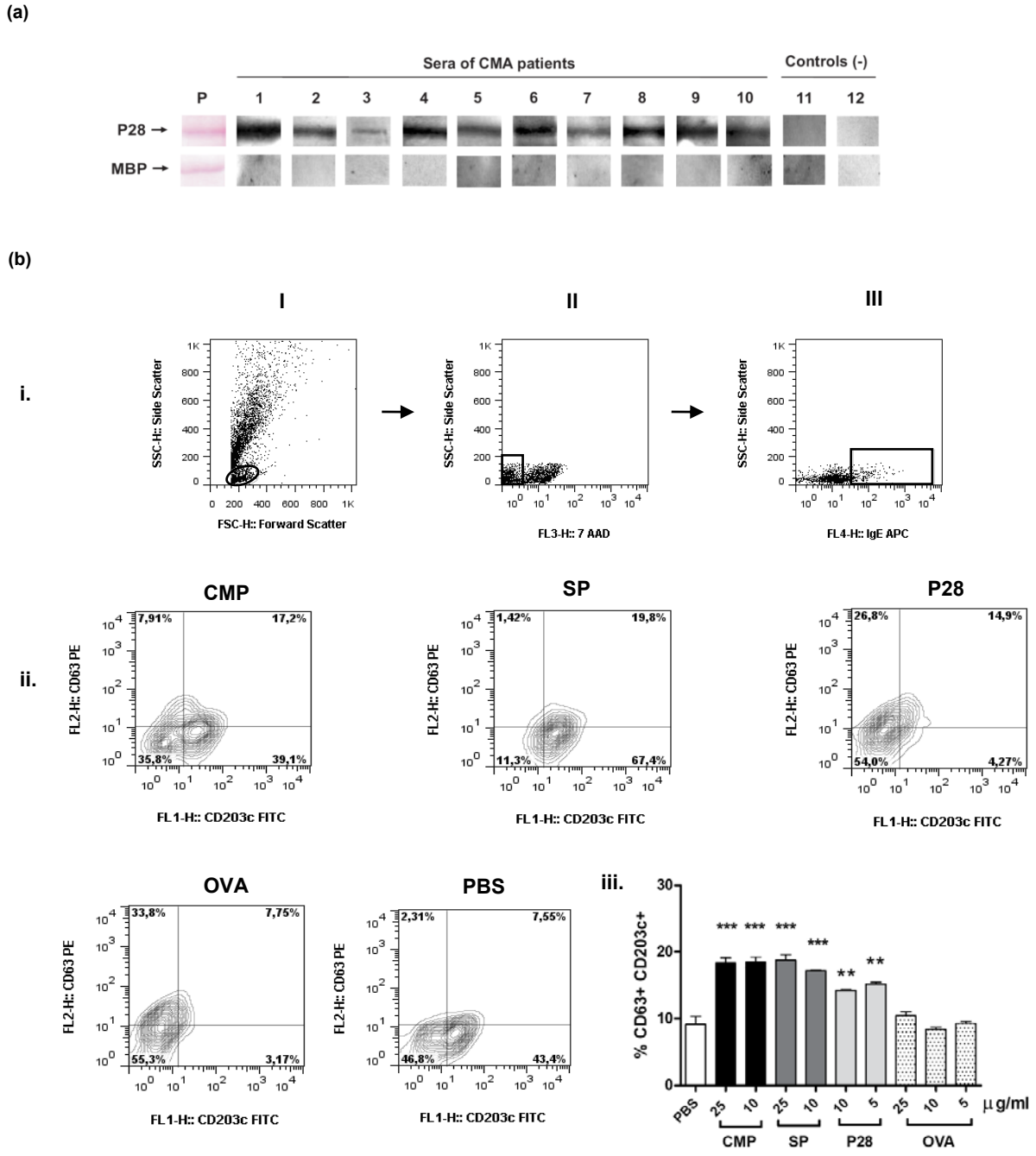
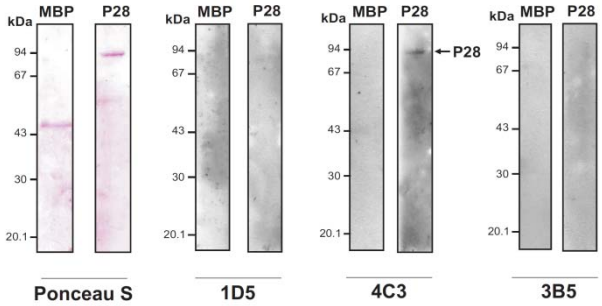
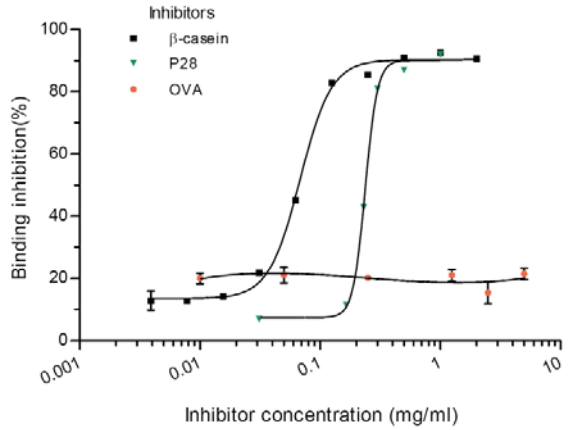

(a)
Sera of CMA patients
Controls (-)
P
1
2
3
4
5
6
7
8
9
10
11
12
P28 →
MBP →
(b)
I
II
III
i.
SSC-H:: Side Scatter
FSC-H:: Forward Scatter
FL3-H:: 7 AAD
FL4-H:: IgE APC
ii.
CMP
SP
P28
FL2-H:: CD63 PE
FL1-H:: CD203c FITC
7,91%
17,2%
35,8%
39,1%
1,42%
19,8%
11,3%
67,4%
26,8%
14,9%
54,0%
4,27%
OVA
PBS
33,8%
7,75%
55,3%
3,17%
2,31%
7,55%
46,8%
43,4%
iii.
% CD63+ CD203c+
PBS
25
10
CMP
SP
P28
OVA
µg/ml

Figure 2

(a)


kDa
MBP
P28
94
67
43
30
20.1
P28
Ponceau S
1D5
4C3
3B5

(b)


Inhibitors
β-casein
P28
OVA
Binding inhibition(%)
Inhibitor concentration (mg/ml)

(c)

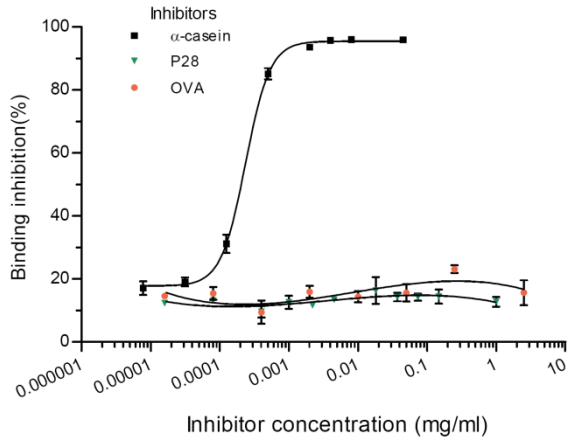

Inhibitors
α-casein
P28
OVA
Binding inhibition(%)
Inhibitor concentration (mg/ml)

Figure 3

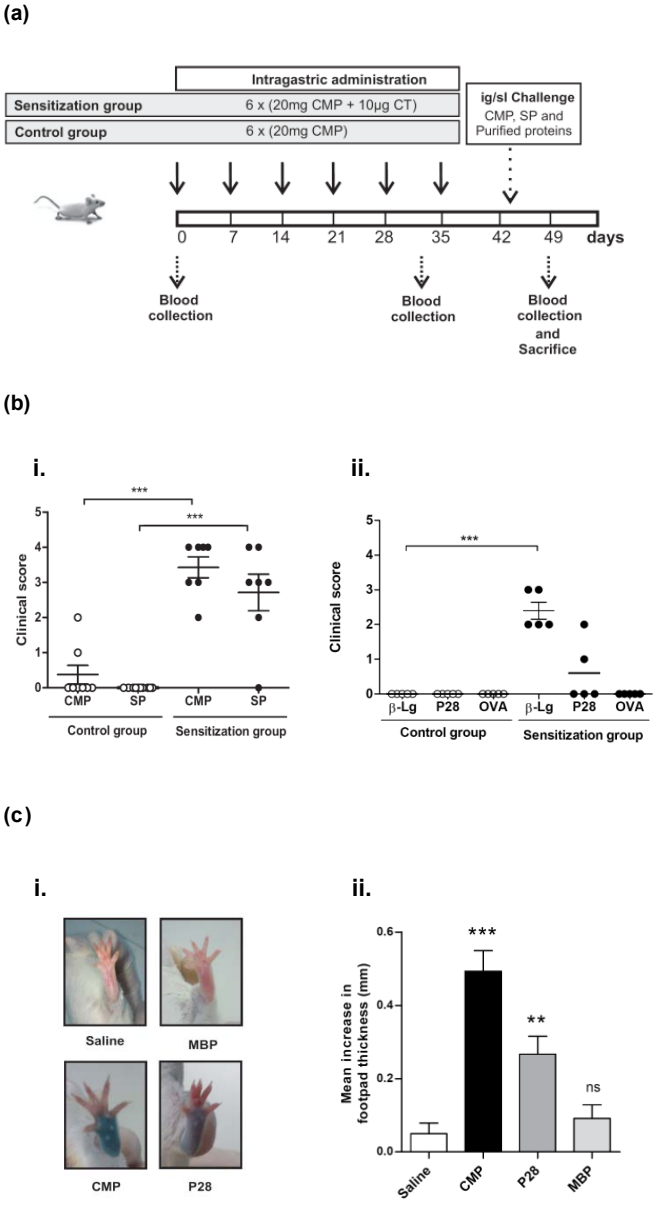

(a)
Intragastric administration
Sensitization group
6 x (20mg CMP + 10µg CT)
Control group
6 x (20mg CMP)
ig/sl Challenge
CMP, SP and
Purified proteins
0 7 14 21 28 35 42 49 days
Blood collection
Blood collection
Blood collection and Sacrifice
(b)
i.
Clinical score
CMP SP CMP SP
Control group
Sensitization group
ii.
Clinical score
β-Lg P28 OVA β-Lg P28 OVA
Control group
Sensitization group
(c)
i.
Saline
MBP
CMP
P28
ii.
Mean increase in footpad thickness (mm)
Saline CMP P28 MBP
ns

Figure 4

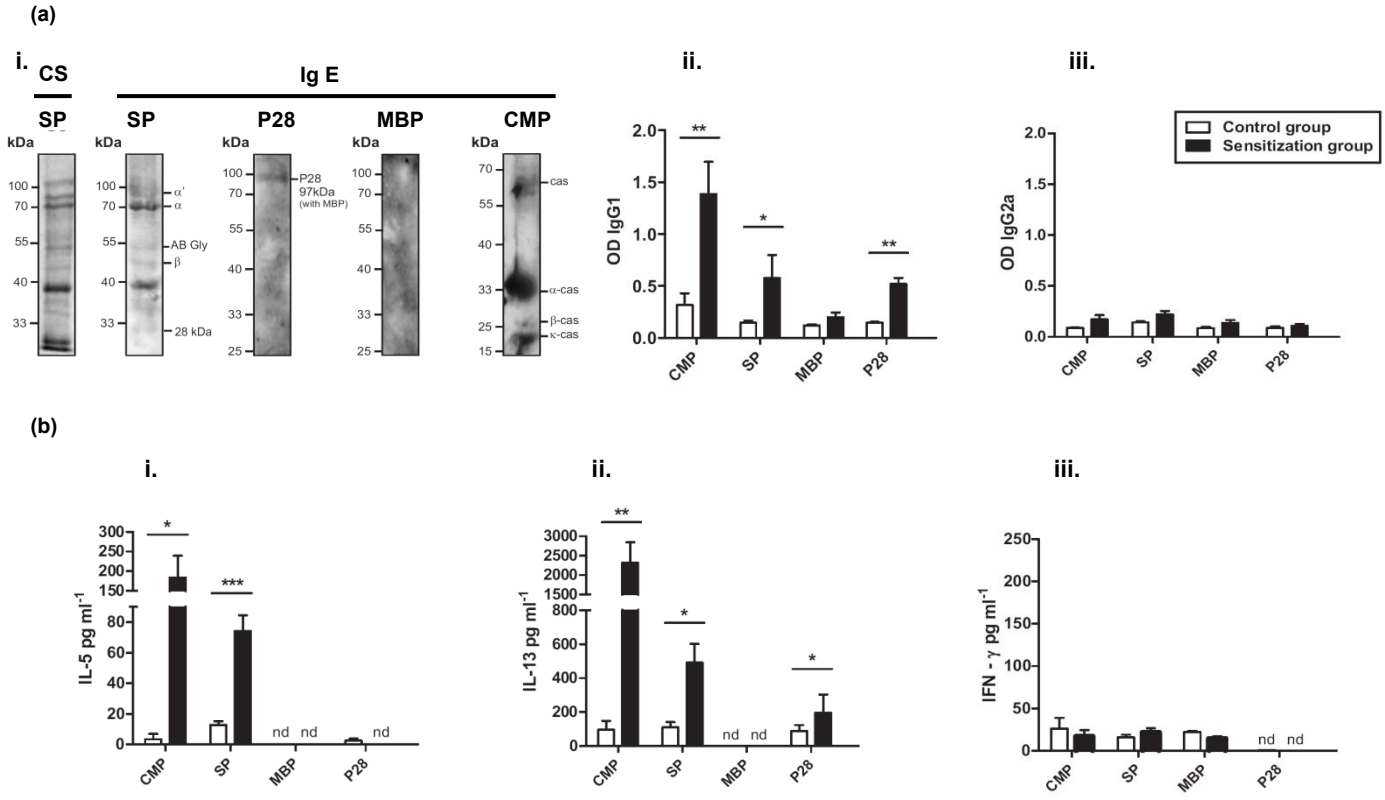
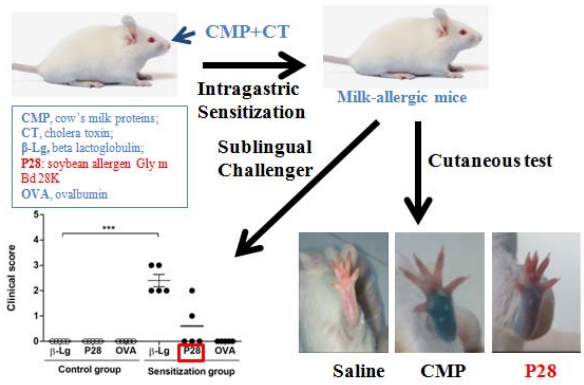

(a)
i.
CS
Ig E
SP
SP
P28
MBP
CMP
kDa
α'
α
AB Gly
β
28 kDa
P28
97kDa
(with MBP)
cas
α-cas
β-cas
κ-cas
ii.
OD IgG1
iii.
OD IgG2a
Control group
Sensitization group
CMP
SP
MBP
P28
(b)
i.
IL-5 pg ml-1
ii.
IL-13 pg ml-1
iii.
IFN - γ pg ml-1
nd

Figure 5


1 MGNKTTLLLLLFVLCHGVATTTMAFHDDEGGDKKSPKSLFLMSNSTRVFKTDAGEMRVLK 60
Peptide 1
Peptide 2
61 SHGGRIFYRHMHIGFISMEPKSLFVPQYLDSNLIIFIRRGEAKLGFIYDDELAERRLKTG 120
Peptide 3
121 DLYMIPSGSAFYLVNIGEGQRLHVICSIDPSTSLGLETFQSFYIGGGANSHSVLSGFEPA 180
Peptide 4
181 ILETAFNESRTVVEEIFSKELDGPIMFVDDSHAPSLWTKFLQLKKDDKEQQLKKMMQDQE 240
Peptide 5
241 EDEEEKQTSRSWRKLLETVFGKVNEKIENKDTAGSPASYNLYDDKKADFKNAYGWSKALH 300
Peptide 6
Peptide 7
301 GGEYPPLSEPDIGVLLVKLSAGSMLAPHVNPISDEYTIVLSGYGELHIGYPNGSRAMKTK 360
361 IKQGDVFVVPRYFPFCQVASRDGPLEFFGFSTSARKNKPQFLAGAASLLRTLMGPELSAA 420
421 FGVSEDTLRRAVDAQHEAVILPSAWAAPPENAGKLKMEEEPNAIRSFANDVVMDVF 476

Figure 6



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