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The anti-inflammatory SSEDIKE peptide from Amaranth seeds modulates IgE-mediated food allergy

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ABSTRACT

The *in vivo* immunomodulatory effect of a bioactive peptide from *Amaranthus hypochondriacus* grains in an IgE-mediated food allergy mouse model was examined. The synthetic SSEDIKE peptide was orally administered to prevent food allergy. Upon oral challenge with milk allergens we observed an alleviation of hypersensitivity symptoms with negativization of skin test, a significant inhibition of specific IgE and IgG1 secretion, a substantial reduction in the secretion of IL-5 and IL-13 by antigen-stimulated spleen cells and mucosal reduction of transcripts coding for *ccl20*, *gata-3* and *nf-kb*. In addition *tgf-β* and *foxp3* mRNA levels were upregulated in jejunum. The charged SSEDIKE peptide from Amaranth inhibited the allergy reaction in a mouse model of food allergy, with suppression of IgE secretion and control of the intestinal inflammation preventing the activation of NF-κB, which might be attributed to the induction of local tolerance.

1. Introduction

The importance of amaranth as a functional food has resurged in the last years. *Amaranthus hypochondriacus* grain has an excellent nutrient composition with a high concentration of proteins (13%–19%) and bioactive peptides (Silva-Sanchez et al., 2008). These peptides are encrypted within the proteins and only after the enzymatic digestion or food processing are they released. Some studies using amaranth flour and protein isolates reported the occurrence of peptides with biological activities such as anti-hypertensive, anti-oxidant, anti-thrombotic, anti-proliferative among others (Barrio & Añón, 2010; de la Rosa et al., 2010; Quiroga, Barrio, & Añón, 2015; Sabbione, Scilingo, & Añón, 2015). Using an *in silico* approximation we identified two tetrapeptides, ALEP and VIKP, with proven anti-hypertensive activity (Vecchi & Añón, 2009). More recently, we also identified and characterized four peptides

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Abbreviations: CMP, cow's milk proteins; OD, optical density; CT, cholera toxin

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(AWEEREQGSR, YLAGKPQQEH, IYIEQGNGITGM, TEVWDSNEQ) with antioxidant activity, obtained by gastrointestinal digestion of amaranth proteins (Orsini Delgado et al., 2016).

However, there is limited evidence of immunomodulatory activity of these peptides.

Montoya-Rodríguez et al. showed that hydrolysates of *A. hypochondriacus* are capable of inhibiting the activation of NF-κB and the secretion of pro-inflammatory mediators on LPS-activated mouse and human macrophages (Montoya-Rodríguez, de Mejía, Dia, Reyes-Moreno, & Milán-Carrillo, 2014). In addition, our recent studies have described the biological activity of different peptides produced during the hydrolysis of Amaranth proteins, and the SSEDIKE peptide was targeted as a strong *in vitro* inhibitor of the NF-κB pathway on human intestinal epithelial cells (Moronta, Smaldini, Docena, & Añón, 2016). To gain a more complete characterization of the biological activity of this peptide, we investigated the *in vivo* anti-inflammatory properties in a mouse model of food allergy. As the gut mucosa is continuously exposed to a wide range of microbes and dietary components, it is armed with a highly organized immune system. Dysregulation of intestinal homeostasis promotes inflammatory disorders such as food allergy and inflammatory bowel diseases. The former is an immune-mediated adverse reaction against innocuous antigenic foods and constitutes a growing clinical problem worldwide (Sicherer & Sampson, 2014). The latter is a chronic inflammation of the gut, likely induced by the microbiota, resulting from a loss of immune tolerance that critically compromises the intestinal homeostasis.

It has been demonstrated that NF-κB pathway is one of the main factors critical for production of pro-inflammatory cytokines (via Toll-like receptors) to control infections. However, chronic activation of NF-κB has been linked to cancer, inflammatory and autoimmune diseases. An enhanced activation of NF-κB in epithelial cells of allergic mice (Ather, Foley, Suratt, Boyson, & Poynter, 2015; Ather, Hodgkins, Janssen-Heininger, & Poynter, 2011) and in asthmatic patients has been demonstrated (Pantano et al., 2008). Activation of NF-κB pathway through IL-4 triggering and STAT6 phosphorylation has been demonstrated to induce the pro-Th2 transcription factor gata-3 (Das et al., 2001). We therefore sought to determine whether controlling intestinal NF-κB activation would ameliorate allergic sensitization in mice allergic to milk proteins. If so, we could speculate to restore antigen-specific immunologic tolerance through the administration of this peptide. It is known that allergy is not spontaneously developed in mouse species and hence artificial procedures should be performed to trigger an allergic reaction. Using a well-defined murine model of milk-induced IgE-mediated food allergy (Smaldini, Ibañez, Fossati, Cassataro, & Docena, 2014), we triggered intestinal activation of the NF-κB pathway and mucosal allergic sensitization. Cholera toxin (CT) is a strong mucosal adjuvant for co-delivered antigens, which promotes NF-κB activation (Schnitzler, Burke, & Wetzler, 2007) and a predominant Th2-type immune response through the activation of human and murine dendritic cells (Eriksson, Fredriksson, Nordström, & Holmgren, 2003; Gagliardi, Sallusto, Marinaro, Lanzavecchia, & De Magistris, 2000). When studies in humans are limited for ethical reasons, mouse models hold great potential as biological tools for pre-clinical studies of novel immunotherapy procedures (Van Gramberg, De Veer, O'Hehir, Meeusen, & Bischof, 2013).

In the present work, we evaluated the anti-inflammatory capacity of the charged SSEDIKE bioactive peptide from *A. hypochondriacus* in an IgE-mediated mouse model of cow's milk allergy.

2. Materials and methods

2.1. Mice

Eight-week old male Balb/c mice free of specific pathogen were purchased from the School of Animal Science at the University of La Plata (La Plata, Argentina). Mice were housed in appropriate conventional animal care facilities and handled according to international guidelines required for animal experiments.

All the experimental protocol of this study was conducted in strict agreement with the international ethical standards for animal experimentation (Helsinki Declaration and its amendments, Amsterdam Protocol of welfare and animal protection and National Institutes of Health, USA NIH guidelines: Guide for the Care and Use of Laboratory Animals). All experimental procedures were approved by the local Institutional Animal Care and Use Committee (CICUAL) at the School of Sciences of University of La Plata (Protocol Number: 017-00-15). The number of animals per group (n = 5) was kept to a minimum and experimental procedures were adjusted to ensure minimal suffering.

2.2. Antigens

Cow's milk proteins (CMP) were obtained from commercial skimmed milk as described before (Smaldini et al., 2012). The peptide SSEDIKE was selected according to previous results (Moronta et al., 2016) and it was synthesized using the ABC technique Solid phase peptide synthesis (SPPS), by China Peptides Co. (Shangai, China). Peptide purity was >98%.

Endotoxin assessment was performed with Limulus amoebocyte chromogenic assay (LONZA, Buenos Aires, Argentina). Protein preparation contained less than 0.10 endotoxin U/mg proteins.

2.3. Sensitization and treatment of mice

Mice were sensitized as previously described (Smaldini et al., 2014). Briefly, mice received 6 weekly intragastric (i.g.) doses of 20 mg of CMP administered as homogenized commercial non-fat dry milk, plus 10 μg of cholera toxin (CT) (Sigma Aldrich, St. Louis, MO, USA) in a final volume of 200 μL of bicarbonate buffer (Sensitized mice). To prevent the allergic sensitization, during the sensitization, mice received 100 μg of peptide (P-treated mice) by gavage twice a week during 5 weeks. As control groups naïve mice received 6 weekly i.g. doses of 20 mg CMP with 100 μg peptide twice a week (P-Control) or without peptide (PBS) (Sham). Prior to oral administration mice were fasted (1 h) and administered 0,125 M ammonium bicarbonate solution to reduce gastric acidity (30 min). Ten days after the final boost

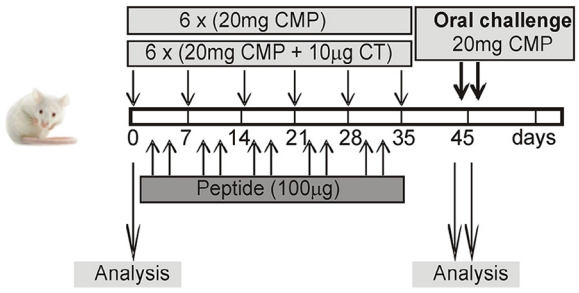

Fig. 1 – Schematic drawing of the experimental protocol in Balb/c mice (*n* = 5/group). CMP, cow's milk proteins; CT, cholera toxin.

mice were i.g. challenged with 20 mg CMP and symptoms were evaluated in a blinded fashion. Blood samples were collected during the whole protocol and sera were stored at –20 °C until use. The experimental design is shown in Fig. 1.

2.4. In vivo *evaluation of the allergic reaction*

2.4.1. *Assessment of clinical signs*

Symptoms were observed between 30 and 60 min after the oral challenge in a blinded fashion by 2 independent researchers. Clinical scores were assigned according to the following range: 0 = no symptoms; 1 = scratching and rubbing around the nose and head; 2 = puffiness around the eyes and mouth, diarrhoea, piloerection, reduced activity, and/or decreased activity with increase respiratory rate; 3 = wheezing, cyanosis around the mouth and the tail; 4 = no activity after prodding, or tremor and convulsion; and 5 = death.

2.4.2. *Cutaneous test*

Mice were injected intradermically with 20 µg of CMP in 20 µL of sterile saline in one footpad and saline in the contralateral footpad as negative control. Mice were also injected intravenously (tail vein) with 100 µL of 0.1% Evans blue dye (Anedra, Buenos Aires, Argentina). The presence of blue colour in the footpad minutes after the injection was considered positive. Footpad swelling was measured minutes after injection with a digital micrometer with a minimum increment of 0.01 mm.

2.5. In vitro *evaluation of the allergic reaction*

2.5.1. *Serum specific IgE detection*

For the evaluation of specific IgE antibodies against CMP serum samples were tested by the Enzyme Attached Sorbent Test (EAST) as described before (Smaldini et al., 2012). Briefly, cyanogen bromide-activated cellulose paper discs were coupled with CMP extracts containing 1.75 mg/mL of protein. Discs were blocked with ethanolamine, and incubated overnight at 4 °C with 50 µL of serum samples. IgE isotype was revealed using a biotinylated anti-mouse IgE monoclonal antibody (BD Pharmigen, San Diego, CA, USA) (1/1000, 5 h at 4 °C), followed with an alkaline phosphatase-streptavidin conjugate (Sigma-Aldrich Inc.) (1/3000, 30 min at 37°C). The enzymatic activity was revealed with p-nitrophenyl phosphate (Biochemika, Fluka, St. Louis, MO, USA) and stopped with 0.1 M EDTA. Optical density (OD) was measured at 405 nm and used to estimate the amount of specific IgE.

2.5.2. *Serum specific IgG1 and IgG2a detection*

CMP-specific IgG1 and IgG2a antibodies were measured by ELISA as previously described (Smaldini et al., 2012). Briefly, NUNC MaxiSorp ELISA plates (Thermo Fisher Scientific, Waltham, MA, USA) were coated overnight at 4 °C with 1 µg/100 µL CMP in carbonate–bicarbonate buffer (pH 9.6). Coated plates were blocked with 5% equine serum in saline for 2 h at 37 °C and then incubated with sera (1/200, 1 h at 37 °C). Bound antigen-specific immunoglobulins were detected using a sheep anti-mouse isotype-specific antibody (The Binding Site, Birmingham, UK) (1/1000, 1 h at 37 °C), followed by incubation with a horseradish peroxidase-conjugated goat anti-sheep antibody (Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA) (1/10.000, 1 h at 37 °C). The reaction was developed with o-phenylenediamine (Sigma-Aldrich Inc.), and stopped with 2 M H_2SO_4. OD was measured at 492 nm and used to estimate the amount of specific isotypes.

2.5.3. *Cytokine response of stimulated splenocytes analysis*

Twenty-four hours after the oral challenge mice were killed and spleens were aseptically removed. Single cell suspensions were prepared in complete medium. Splenocytes (4×10^6 cells/mL) were cultured for 72 h at 37 °C with complete medium alone or in complete medium containing CMP (0.35 mg/mL), previously optimized (Smaldini et al., 2012). Supernatants were harvested and assayed for IL-5, IL-13 and IFN-γ by ELISA using commercially available kits (eBioscience, San Diego, CA, USA) according to manufacturer's instructions.

2.5.4. *Mucosal gene expression analysis*

The jejunum was aseptically removed from mice killed by cervical dislocation 24 h after oral challenge and mRNA was isolated using illustra RNAspin mini isolation kit according the manufacturer's specifications (GE Healthcare, Freiburg, Germany). Peyer's patches were discarded prior to tissue processing. The amount of the extracted RNA was determined by UV absorption and the optical density ratio of OD_{280nm}/OD_{260nm} was used as a purity measure. Complementary DNA (cDNA) was obtained (Invitrogen, Carlsbad, CA, USA) and mRNA expression was determined by real-time quantitative PCR. The experimental procedure was performed on *ABI prims* sequence detection system using SYBRGreen fluorescence (BioRad, Hercules, CA, USA). β-Actin was used to standardize the total amount of cDNA and the fold change in mRNA expression was defined as the ratio of the normalized values corresponding to the sensitized mouse to that of control mouse (Sham mice). Genes of interest were *gata-3, t-bet, foxp3, nf-κb, ccl20, il10 and tgf-β.* Relative mRNA levels ($2^{\Delta Cts}$) were determined by comparing the PCR cycle threshold between cDNA of the gene of interest and that of β-actin (Cts), and comparing Cts values between the treated and untreated condition (ΔCts). (Smaldini, Stanford, Romanin, Fossati, & Docena, 2014).

2.5.5. *Cytokine quantification by ELISA in the jejunum*

Frozen sections of jejunum were minced and cells were treated with lysis buffer (10 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 10% Glycerol, 5 mM EDTA and a protease inhibitor

cocktail-Sigma-Aldrich Inc.). Homogenate was sonicated and the supernatant was collected. IL-5 and IFN-γ were determined by ELISA (eBioscience).

2.6. Statistical analysis

All statistical analyses and plotting were carried out using GraphPad Prism 5 software. A t test was conducted if 2 experimental groups were performed, whereas when more than 2 groups were conducted, the significance of the difference was determined using ANOVA test. Symptoms and skin test statistical analysis was done by Mann–Whitney test. A p value <0.05 was considered as statistically significant.

3. Results

3.1. In vivo responses to the oral administration of the Amaranth peptide in the food allergy mouse model

To study the anti-inflammatory property of the Amaranth peptide SSEDIKE *in vivo*, we administered an oral formulation containing the peptide during the allergic sensitization phase in Balb/c mice. An oral challenge following the sensitization step was performed to evidence the induction of hypersensitivity reactions immediately after the exposure to the allergen (Fig. 1). The clinical signs were scored (Fig. 2A) and we evidenced that treated animals (P-treated) showed significant lower clinical scores compared with sensitized animals exposed to CMP (Sensitized), suggesting that the allergic sensitization was ameliorated with the oral administration of the bioactive peptide. No symptoms were observed in control animals that received only CMP (Sham) or peptide (P-control).

To gain a more complete understanding of this inhibitory effect we next analysed the presence of sensitized mast cells in the skin (Fig. 2B). We found a lower extravasation of the blue dye in treated mice compared with sensitized mice, suggesting that the administered peptide reduced the presence or IgE sensitization of skin mast cells. Skin test was negative in the contralateral control footpad (Sensitized) and in sham and P-control mice. Fig. 2C shows the quantification of the footpad swelling of mice. As can be seen, P-treated mice showed a significant lower swelling compared with sensitized mice (0.28 ± 0.08 vs 0.61 ± 0.06, respectively), thus reflecting a reduced vascular permeability after the injection of the allergen. Control mice (Sham and P-control) showed a negative cutaneous test.

3.2. Serum specific isotypes and spleen cytokine secretion

To analyse the specific immune response induced following the allergic sensitization and peptide treatment, serum CMP-specific immunoglobulins and cytokines secreted by CMP-stimulated spleen cells were studied (Fig. 3). CT-driven sensitization induced a sustained and significant increase in serum milk-specific IgE ($p < 0.01$) and IgG1 antibodies ($p < 0.01$) with no induction of specific IgG2a, whereas significant low levels of these isotypes were detected in animals that received the peptide as treatment (P-treated) and in control mice (Sham and P-control) (Fig. 3A).

Cytokine analysis (Fig. 3B) showed that spleen cells stimulated with milk proteins secreted high amounts of IL-5 and IL-13 in sensitized mice ($p < 0.001$), whereas treated mice showed secreted Th2-cytokines comparable with sham and P-control animals, thus showing that production of Th2 cytokines was significantly decreased. Levels of IFN-γ remained low in all animals.

Collectively these findings reflect that the Th2-mediated immune response of sensitized mice was abrogated in P-treated animals and we found no sign of induction of a specific Th1 response.

3.3. Mucosal gene expression and production of cytokines

To address the effect of the administration of the peptide on the intestinal mucosa, we assessed the gene expression corresponding to the *ccl20* chemokine, regulatory cytokines (*il-10 and tgf-β*) and transcription factors (*gata-3, t-bet, foxp3, nf-κb*) in jejunum of individual animals. The data output shown in Fig. 4 are expressed as a fold-difference expressed as mean ± SEM of individual gene expression ratio of treated animals to sham group. As it can be seen in Fig. 4A, sensitized mice showed an increased expression of the *ccl20* transcript compared with P-treated and P-control mice. Analysis of intestinal samples from animals of the P-control group showed similar results to those from the Sham group. To further evaluate the potential mechanism that suppressed the inflammation, we analysed regulatory cytokines and we found a local significant induction of the *tgf-β* transcript, which was reduced in sensitized and P-control mice (ratio below 1). Consistent with this finding, we detected a significant up-regulated transcript level of *foxp3* in treated mice compared with the reduced mRNA level (ratio below 1) observed in sensitized mice. In addition, the pro-inflammatory transcription factors *gata-3* and *nf-κb*, which were up-regulated in allergic mice, were significantly reduced in treated and P-control mice. Overall, these findings provide a strong evidence of the anti-inflammatory properties of the peptide that was orally administered in IgE-mediated allergic mice. The low level of *t-bet* transcript suggests that Th1 cytokines are not induced either in sensitized or treated mice.

To further confirm the mucosal cytokine regulation at the protein level, we quantified jejunum IL-5 and IFN-γ by ELISA. As depicted in Fig. 4C sensitized animals showed increased IL-5 compared to controls (Sham and P-control mice). Treatment of sensitized mice with the peptide significantly suppressed the production of IL-5, while IFN-γ remained unchanged in all animals.

4. Discussion

In search for a novel anti-inflammatory functional food containing Amaranth hydrolysates or supplemented with the bioactive peptide we here tested the *in vivo* anti-inflammatory and anti-allergic activity of a synthetic peptide from Amaranth. We showed that the oral administration of the peptide SSEDIKE alleviated hypersensitivity symptoms in a cholera toxin-driven IgE-mediated mouse model of food allergy. It has

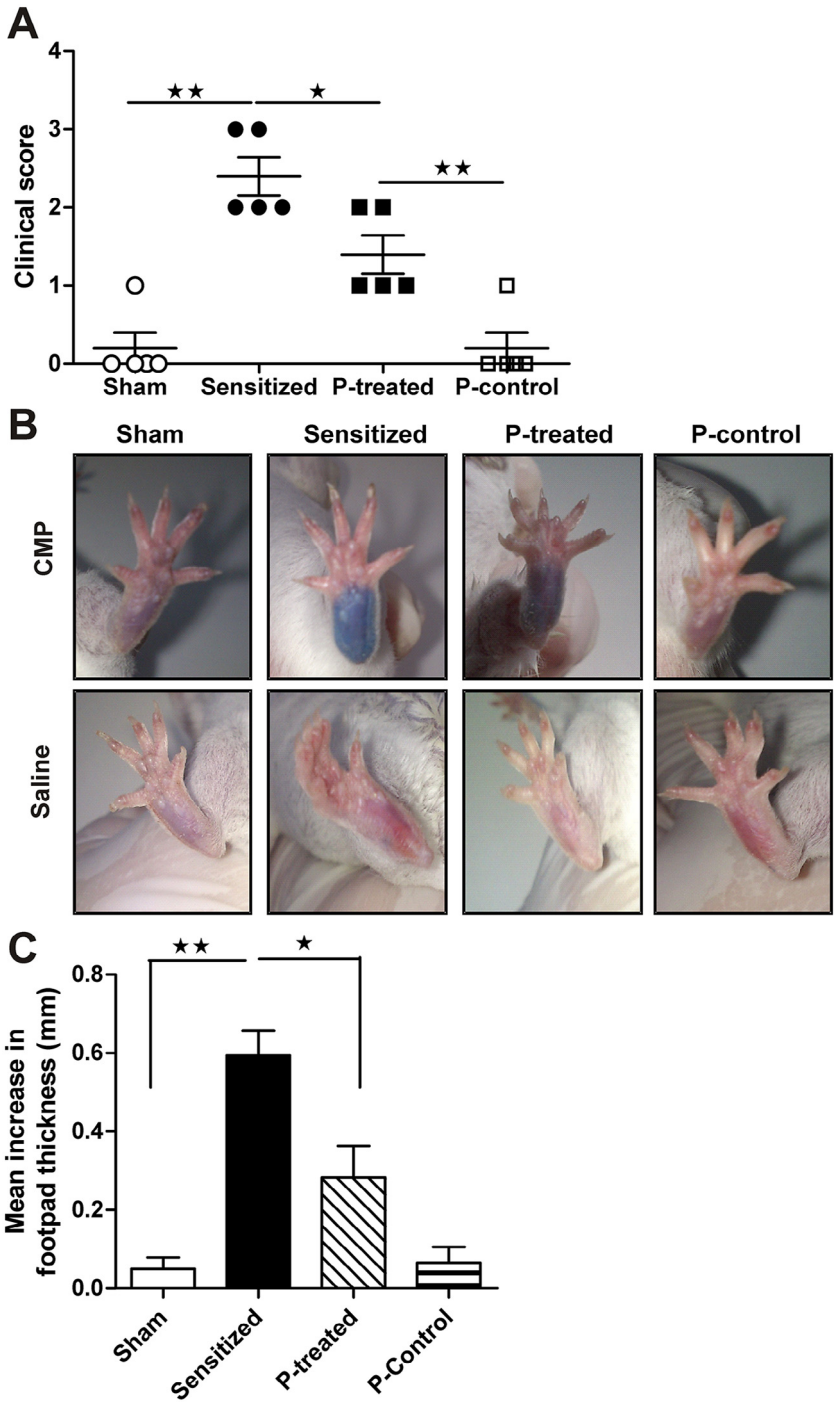

Fig. 2 – *In vivo* responses of the food allergy mouse model. (A) Clinical scores corresponding to symptoms elicited following the oral challenges with milk proteins. (B) Skin tests in sham, sensitized, P-treated and P-control mice. (C) Swelling of footpad was quantified and data are expressed as the mean values ± SEM. The results correspond to a single experiment, which is representative of two separate experiments that had similar results. Mann–Whitney test was used to determine statistical significance. $^{***}p < 0.005$, $^{**}p < 0.01$; $^{*}p < 0.05$. CMP, cow's milk proteins.

been demonstrated by others using human and murine cell lines (Montoya-Rodríguez et al., 2014) that Amaranth hydrolysates reduced the cytosolic phosphorylation of Ikk-α and IkB-β thus preventing the translocation of p50 and p65 onto the nucleus, reducing PGE2 and COX-1, TNF-α secretion and cell activation. In another report they showed that this hydrolysate could exert anti-atherosclerotic and anti-inflammatory effects by controlling the LOX-1 signalling pathway that leads to the endothelial dysfunction and plaque formation (Montoya-Rodríguez, Milán-Carrillo, Dia, Reyes-Moreno, & González de Mejía, 2014). On the contrary, other authors have shown that protein extracts from Amaranth leaves exert a

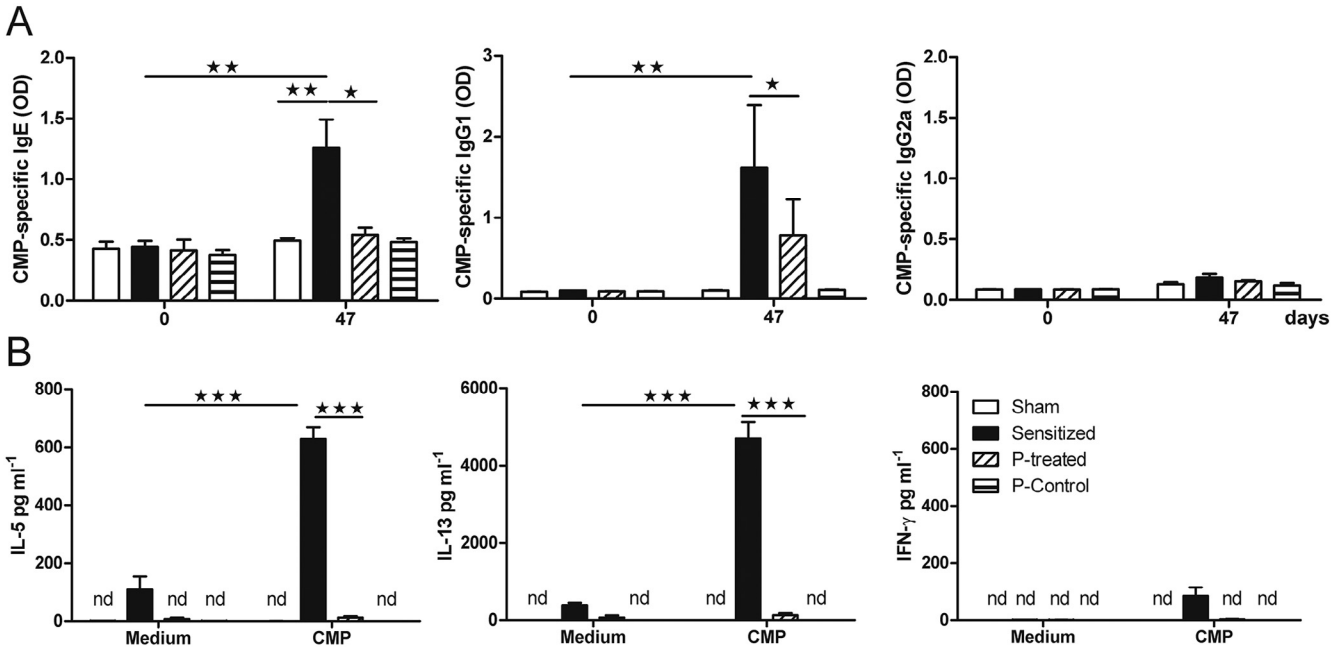

Fig. 3 – Serum specific isotypes and cytokines by ELISA. (A) Serum CMP-specific IgE, IgG1 and IgG2a. (B) Cytokines in the supernatants of spleen cells stimulated with CMP or medium for 72 h. These results are representative of two independent experiments with similar results. The data are expressed as the mean values ± SEM. Two-way ANOVA was used because all the data had normal distribution and equal variances. $^{***}p < 0.001$, $^{**}p < 0.01$. CMP, cow's milk proteins.

stimulating immunomodulatory effect through the activation of B cells (Lin, Chiang, & Lin, 2005). In this study we found that the oral administration of the charged SSEDIKE peptide abrogated the intestinal activation of NF-κB in a Th2-mediated allergic condition. In previous studies we provided evidence that NF-κB signalling pathway was inhibited in activated human colonic epithelial cells treated with Amaranth hydrolysates or purified synthetic peptides. The precise mechanism of inhibition behind this effect has not been elucidated yet. It has been demonstrated by Tan et al. that dietary antioxidants (Vitamins C and E) inhibited the NF-κB pathway through the suppression of reactive oxygen species in dendritic cells, and this promoted the induction of anergyzed allogeneic T cells with regulatory capacity (Tan et al., 2005). In addition, it has been demonstrated that antioxidants and NF-κB inhibitors induced tolerogenic dendritic cells, which then promoted Tregs (Mazzeo et al., 2002; Yang et al., 2003). In this study we evidenced an intestinal abrogation of *nf-κb* gene expression with the up-regulation of transcripts coding for *tgf-β* and *foxp3*, which are indicative of tolerance induction. We have recently demonstrated that Amaranth peptides exerted an antioxidant effect (Delgado, Galleano, Añón, & Tironi, 2015; Lado, Burini, Rinaldi, Añón, & Tironi, 2015; Orsini Delgado et al., 2016; Sabbione, Ibañez, Martínez, Añón, & Scilingo, 2016), and we propose that the induction of the *in vivo* anti-inflammatory effect of the SSEDIKE peptide might be promoted through the modulation of NF-κB on dendritic cells, which then locally generate regulatory T cells that suppress the response of other antigen-specific T cells (milk protein-specific T cells). We need further studies to confirm this hypothesis.

In the context of experimental allergy, Hibi et al. demonstrated in the OVA-TCR transgenic Balb/c mice fed with Amaranth-containing diet that the over-production of IgE and Th2 cytokines (IL-4 and IL-5) was likely suppressed through the induction of IFN-γ-producing OVA-specific T cells (Hibi, Hachimura, Hashizume, Obata, & Kaminogawa, 2004). Authors demonstrated that the addition of Amaranth grain powder-containing peptides to the T cell-spleen cell cultures deviate the OVA-specific T cell activation pattern from Th2 to Th1 dominant cells, with production of IL-4 and IFN- γ, respectively. These findings clearly indicate that Amaranth extract contained components with pro-Th1 adjuvant capacity. Adjuvant properties have been demonstrated in plant proteins, such as heat shock proteins (HSPs), which were proposed to be used as novel vaccine adjuvants. Corigliano et al. have reported that plant HSP90 induced an immune response through TLR-4 binding and subsequent activation of T (Th1 and cytotoxic cell responses) and B lymphocytes (IgG2a isotype) (Corigliano et al., 2011, 2013). In this regard, it has been reported that Amaranth species, including *A. hypochondriacus*, contain HSP proteins (Huerta-Ocampo et al., 2011). Here we used a purified synthetic peptide from *A. hypochondriacus* and we demonstrated that allergy can be modulated with the intragastric administration of the SSEDIKE peptide of Amaranth. The immunomodulatory effect observed was exerted by the peptide since no other grain component was administered. For this reason, we reasonably suggest that the antigen-specific Th1 activation in the study of Hibi et al. might be influenced by grain components with pro-Th1 adjuvant capacity. In contrast, the SSEDIKE peptide here employed did not promote any *in vivo* pro-Th1 effect (no induction of IgG2a, T-bet or IFN-γ). We found an increase in tolerance markers in the gut such as IL-10, TGF-β and FoxP3.

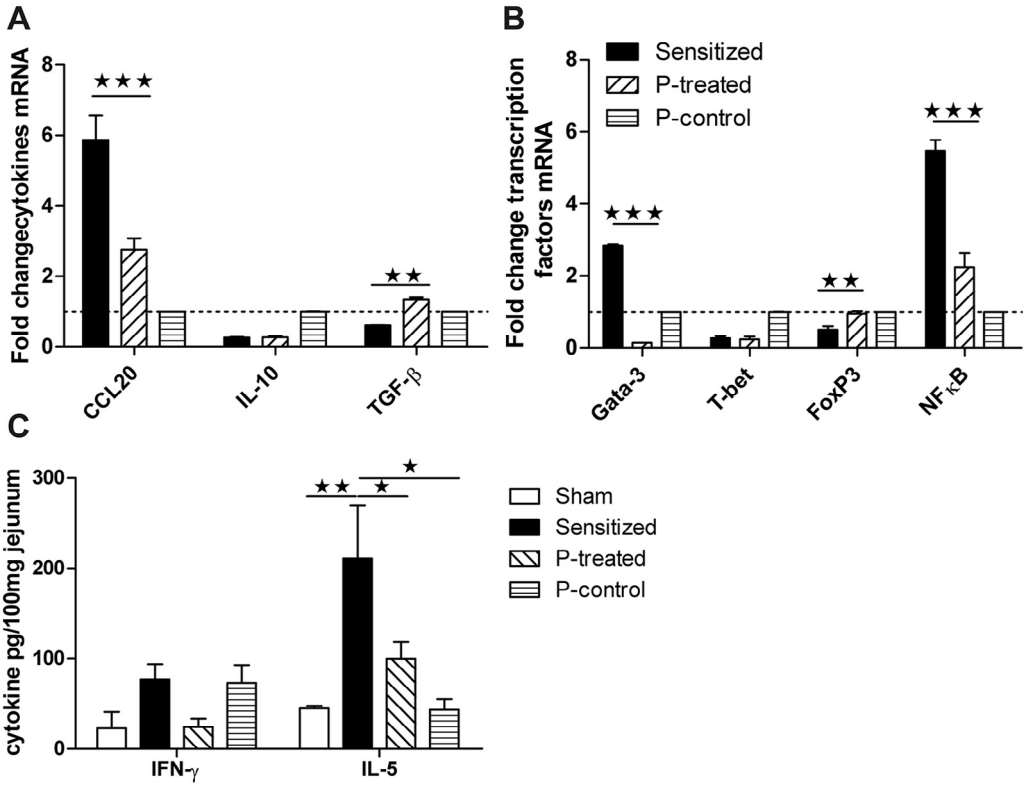

Fig. 4 – Intestinal expression of immune mediators. Transcript levels of chemokine and cytokines (A), and transcription factors (B) in the jejunum by quantitative PCR. β-Actin was used to standardize the total amount of cDNA and the fold change in mRNA expression was defined as the ratio of the normalized values corresponding to sensitized, P-treated or P-control mice to that of sham mouse. (C) Cytokine concentration in jejunum of animals. Data are expressed as the mean values ± SEM. These results are representative of two independent experiments with similar results. Two-way ANOVA was used because all the data had normal distribution and equal variances. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

Our findings also demonstrate that the oral delivery of the small and charged peptide resisted the acidity conditions of the stomach (the peptide was co-administered with bicarbonate), reached the mucosal surface of the small bowel, and probably modulated the local immune response via a yet unknown regulatory mechanism of enterocyte activation or any other molecular or cell target. The degradation and limited absorption of proteins and peptides in the gastrointestinal tract might be reduced with the use of different carrier systems that optimize stability and bio-availability of peptides as a therapeutic or supplement in a functional food. This approach will prompt us to reduce the amount of delivered peptide to assure a local immunomodulatory activity.

Although we could not demonstrate an increase of intestinal Tregs, the increased expression of *tgf-β* and *foxp3* prompts us to suspect that the treatment with the SEEDIKE peptide restores tolerance through the inhibition of NF-κB. Tan et al. demonstrated that the inhibition of IKK phosphorylation promoted the induction of Treg (Tan et al., 2005). This is the first report to demonstrate *in vivo* that hypersensitivity symptoms can be suppressed with an Amaranth grain-derived peptide. Our findings showed that IgE and Th2 cytokines were inhibited, thus reducing tissue and circulating sensitized cells responsible for clinical signs. These changes were accompanied by a significant suppression of intestinal gene expression of inflammatory markers such as *ccl20*, *gata-3* and *nf-kb*, and up-regulation of transcripts coding for *tgf-β* and *foxp3*. In addition, we ruled out the possibility of Th1 cell induction. On this basis, it is reasonable to suggest that the local immune tolerance induction might control the Th2-mediated allergic reaction. To confirm this hypothesis further studies are needed.

There is currently no commercial functional food with immunomodulatory properties available. Therefore, efforts should be focused on investigating the effects of Amaranth components on different human and animal health and mechanisms involved. It should be considered that depending on the Amaranth component that supplements the functional food the immunomodulatory effect achieved may differ substantially.

5. Conclusions

These findings led us to propose that the anti-inflammatory and anti-allergic SSEDIKE amaranth grain-derived peptide might be a useful supplement of functional foods for prevention of food allergy.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

Authors have contributed as follows: conceived and designed experiments: S.P.L. and D.G.H.; performed the experiments: M.J. and S.P.L.; analysed the data: M.J., S.P.L., F.C.A., A.M.C. and D.G.H.; contributed with reagents, materials, analysis tools: F.C.A., A.M.C., and D.G.H.; wrote the manuscript: S.P.L., A.M.C and D.G.H.

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