Immunomodulating peptides for food allergy prevention and treatment

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Abstract

Among the most promising strategies currently assayed against IgE-mediated allergic diseases stands the possibility of using immunomodulating peptides to induce oral tolerance towards offending food allergens or even to prevent allergic sensitization. This review focuses on the beneficial effects of food derived immunomodulating peptides on food allergy, which can be directly exerted in the intestinal tract or once being absorbed through the intestinal epithelial barrier to interact with immune cells. Food peptides influence intestinal homeostasis by maintaining and reinforcing barrier function or affecting intestinal cell-signalling to nearby immune cells and mucus secretion. In addition, they can stimulate cells of the innate and adaptive immune system while supressing inflammatory responses. Peptides represent an attractive alternative to whole allergens to enhance the safety and efficacy of immunotherapy treatments. The conclusions drawn from curative and preventive experiments in murine models are promising, although there is a need for more pre-clinical studies to further explore the

immunomodulating strategy and its mechanisms and for a deeper knowledge of the peptide sequence and structural requirements that determine the immunoregulatory function.

Keywords

IgE-mediated allergy; intestinal barrier function; anti-inflammatory peptides; Th1 and Th2 modulators; immunotherapy

Introduction

IgE-mediated food allergy is a hypersensitivity reaction whose prevalence is rapidly increasing in the western world (Sicherer et al., 2011; Nwaru et al., 2014). Food allergy results from a failure in stablishing oral tolerance or a breakdown in existing tolerance (Burks et al., 2008). Allergic sensitization occurs when antigenic proteins enhance T lymphocyte differentiation into Th2 cells that synthetize cytokines such as IL-4, IL-5 and IL-13. This leads to the activation of B lymphocytes to IgE-producing plasma cells and the binding of the protein-specific IgE antibodies to the surface of tissue mast cells and blood basophils. Re-exposure to the allergen leads to cross-linking of the cell bound IgE, which triggers the release of mediators responsible for the allergic reaction (Berin and Sampson, 2013). In addition to an individual susceptibility, the ability of proteins or their accompanying food matrix components to promote Th2 effector pathways over Th1 immunity is considered to determine their capacity to induce an allergic response (Berin and Shreffler, 2008). In turn, activation of Th1 cells releases cytokines such as IL-2 and IFN-γ, which elicit the production of pro-inflammatory mediators, TNF-α and IL-8, and reactive oxygen species (ROS), boosting cellular anti-microbial and anti-tumoral defense mechanisms (Barnes, 2011). Both types of T cells, with the contribution of Th17, regulatory T (Treg) and regulatory B (Breg) cells, play crucial direct and indirect roles in the maintenance of immune tolerance and suppression of allergic inflammation (van de Veen et al., 2013; Palomares et al., 2014; Noval Rivas and Chatila, 2016).

Although allergen-specific immunotherapy (IT) has proved effective in allergic asthma and rhinitis, despite huge efforts to desensitize food allergic patients, there are still no validated

therapies to induce tolerance or provide effective protection from unintentional exposures, mainly because of the occurrence of severe adverse side effects, the varying efficiency of the traditional allergen extracts used for therapy and the excessive duration of the treatments (Muraro et al., 2014; Nowak-Wegrzyn and Albin, 2015). Thus, in most instances, the management of food allergy is limited to strict dietary avoidance and emergency treatment in case of adverse reactions.

Among the most promising strategies currently assayed against IgE-mediated allergic diseases stands the possibility of using immunomodulating peptides to stimulate oral tolerance towards offending food allergens or even to prevent allergic sensitization (Haney and Hancock, 2013; Berin, 2014). Antimicrobial peptides secreted by Paneth cells and peptide hormones secreted by enteroendocrine cells, both crucial for intestinal homeostasis, are good examples of endogenous immunomodulating peptides. Antimicrobial host defense peptides shape and preserve intestinal microbiota, but are also protective without a direct antimicrobial action because they combine anti-inflammatory and immunostimulatory properties that include effects on cell migration, survival and proliferation, and induction of antimicrobial and immune mediators (Easton et al., 2009). For their part, peptide hormones not only detect luminal nutrients aiding their absorption, but also respond to pathogens and microbial metabolites and exert direct immunomodulating effects on intestinal immune cells (Worthington, 2015).

Food proteins are a rich and varied source of peptides bearing valuable and diverse biological activities. Bioactive peptides can be released from food proteins *in vivo* by the action of enzymes from digestive fluids and intestinal epithelial cells (IECs) or through processing by antigen-

presenting cells. *In vitro*, they can be produced by enzymatic or chemical proteolysis or synthesis. *In vitro* generated peptides need to resist the influence of extreme pH, proteolytic enzymes and surfactants throughout digestion, to reach their sites of action and exert physiological functions. This review focuses on the beneficial effects of immunomodulating peptides of food origin on food allergy, which can be directly executed in the intestinal tract, by maintaining and reinforcing barrier function and immune homeostasis, or once being absorbed, through their action on cells of the innate and adaptive immune system. The most relevant IT experiments with food peptides in murine models are discussed, distinguishing the approaches targeted to achieve desensitization or tolerance and those aimed to prevent allergy progression or the development of allergic sensitization.

Peptides with local effects on the intestinal function

The intestinal epithelium includes 4 main cell types: absorptive enterocytes, goblet cells -responsible for mucus secretion-, enteroendocrine cells and Paneth cells -which secrete, respectively, hormones and antimicrobial peptides-. In addition to its double function to act as a physical barrier with the external environment and to allow the uptake of nutrients, it is now recognized that the cells that make up this protective interphase drive innate immune responses and ultimately act as modulators of adaptive immunity, therefore playing an essential role in the pathogenesis and development of food allergy. Food proteins and peptides influence the intestinal function by affecting barrier regulation, IEC-signalling to immune cells of the gut associated immune system (GALT) and mucus secretion (Martínez-Augustin et al., 2014). Moreover, the cross-talk of peptides with the microbiota, through prebiotic and antimicrobial

actions, although out of the scope of this review, is crucial in the regulation of intestinal homeostasis in view of the key role of commensal flora in the development and functioning of the GALT. Although allergic patients are not characterized by a consistent microbial pattern, it is well known that the healthy gut microbiome promotes oral tolerance and that its perturbations correlate with or predispose to food allergy (Chinthrajah et al., 2016). In this respect, administration of food protein hydrolysates tends to preserve the normal microbiota profile in rodents, reversing the intestinal dysbiosis that characterizes pathologies such as obesity or inflammation (Monteiro et al., 2016; Requena et al., 2016).

The barrier function of the intestine prevents the entrance of pathogens and antigens from the intestinal lumen to the mucosal tissue. In addition to commensal and probiotic bacteria, different components of the diet promote the maintenance of the barrier integrity and thus, contribute to regulate the passage of water and nutrients, and to protect against inflammatory, autoimmune and allergic diseases (Kotler et al., 2013; Ulluwishewa et al., 2011). It has been shown that fish protein hydrolysates can potentiate gut integrity and repair by virtue of both pro-migratory and proliferative activities, as demonstrated on rat (RIE-1) and human (HT29) IECs, and that they are also able to reduce sustained injury in an *in vivo* gastric damaging model (Fitzgerald et al., 2005). Similarly, bovine colostrum hydrolysed with pepsin and chymotrypsin contains protein fractions responsible for promoting the growth of the human intestinal epithelial cell line T84, with potential application in gastrointestinal restoration (Morgan et al., 2014).

On the other hand, milk proteins fed to rodents can strengthen the tight junctions -which seal the space between IECs and control the paracellular transport of water, ions and small molecules-

through changes in the expression of transmembrane and/or intracellular proteins that form the junctional complexes. However, while the beneficial effect of whey proteins on intestinal epithelial integrity, attributed to an increased the expression of claudin-4, was mediated by the presence of the anti-inflammatory cytokine TGF-β in the whey protein preparation (Hering et al., 2011; Ozawa et al., 2009), caseins may increase the expression of the pore-sealing protein claudin-1, and reduce that of the pore-forming claudin-2, through a TGF-β independent pathway (Visser et al. 2010). In fact, casein hydrolysates, unlike amino acid mixtures, were found to reinforce the intestinal barrier function *in vivo* through an effect on the tight junction proteins. This suggests the specific involvement of the peptides contained in the casein hydrolysates, although it remains to be stablished to what extent they modulate intestinal permeability by directly targeting signal transduction pathways, promoting the release of regulatory cytokines, such as IL-10 or TGF-β, stimulating mucin secretion, or inducing interactive support by the gut microbiota (Visser et al., 2012).

For instance, the peptide NPWDQ (α_{S2}-casein 107-111), the active form of GPIVLNPWDQ (α_{S2}-casein 102-111) identified in an enzymatic cheese hydrolysate, upregulates the expression of the occludin gene and the production of the protein (but not of claudin-1) in Caco-2 cells (Yasumatsu and Tanabe, 2010). NPWDQ inhibits the transport of ovalbumin *in vitro*, in Caco-2 cells cultured on filter supports (Tanabe et al., 2006), *ex vivo*, in jejunal and ileal loops, and *in vivo*, following oral feeding of ovalbumin to indomethacin-administered rats (Isobe et al., 2008). A similar activity was reported for DKIHPF (β-casein 47-52), which hinders intestinal permeation of β-lactoglobulin, suggesting a role in the prevention or treatment of food allergy by

avoiding allergen passage through the fortification of the intestinal barrier (Tanabe, 2012). Indeed, it has been suggested that augmented intestinal permeability is a factor promoting sensitization to food antigens in susceptible individuals (Heyman, 2005). Furthermore, in sensitized patients, the increase in permeability that is produced as a consequence of occasional food allergic reactions, and contributes to amplify the inflammatory response, does not return to normal values even long after the last allergen exposure (Perrier and Corthésy, 2010).

A contrasting example is that of gliadin, the 33-mer peptide that mediates T cell activation in celiac disease. Gliadin causes rearrangement of the cell cytoskeleton, loss of occludin-ZO1 protein-protein interaction and increased monolayer permeability in rat (IEC6) and human (Caco-2) IECs (Drago et al., 2006). Enhanced intestinal permeability upon gliadin exposure does not appear to be limited to celiac patients, although an exaggerated increase in gliadin-induced permeability is a characteristic of active celiac disease (Hollon et al., 2015).

The control of epithelial permeability, innate immune recognition and adaptive immunity are all associated with gut inflammation. Perturbations in epithelial or immune homeostasis, either induced by infectious diseases or dietary antigens, can lead to inflammation -an excessive immune activation characterized by an influx of inflammatory cells from the blood- and to increased concentrations of cytokines, free radicals and lipid mediators (MacDonald and Monteleone, 2005). Inflammation represents a critical determinant in the development of food allergy, but also its consequence. In a situation of inflammation, intestinal permeability may be increased and the altered rate, route and mode of antigen presentation may lead to abrogation of oral tolerance (Bischoff et al., 2014). It has recently been demonstrated that the initiation of

peanut allergy in mice requires cellular damage or tissue injury that prevent the induction of tolerance through the overproduction of uric acid in the local microenvironment and the subsequent activation of dendritic cells (DCs) (Kong et al., 2015). On the other hand, as already mentioned, inflammation is the long term consequence of sporadic or repetitive exposures to allergens in sensitized patients (Galli et al., 2008).

Food derived peptides can prevent and repair the damage caused by oxidative stress and inflammatory reactions (Sarmadi and Ismail, 2010; Samaranayaka and Li-Chan, 2011; Power et al., 2013). Several publications report antioxidant and anti-inflammatory effects of protein hydrolysates and food peptides on animal models of inflammatory bowel disease (Daddaoua et al., 2005; Lee et al., 2009; López-Posadas et al., 2010; Mochizuki et al., 2010; Espeche Turbay et al., 2012; Wada et al., 2013; Zhang et al., 2015), as well as on different human cell lines (Phelan et al., 2009; Huang et al., 2010; McCarthy et al., 2013 and 2016; Fernández-Tomé et al., 2014;; Malinowski et al., 2014; Bamdad et al., 2015; Ko et al., 2016). In human IEC models, such as Caco-2 cells, subjected to oxidant stimuli, phosphopeptides from hen egg yolk phosvitin exhibit antioxidant activity, reducing IL-8 secretion and increasing intracellular glutathione levels and glutathione reductase activity (Katayama et al., 2006). These phosphopeptides also down-regulate the gene expression of IL-8 and IL-12 in lipopolysaccharide (LPS)- and TNF-α-stimulated HT29 cells (Xu et al., 2012). It was suggested that, in addition to the particular peptide structure, the phosphorus content plays a role in their activity and, in fact, casein phosphopeptides also enhance the antioxidant defense systems involved in the glutathione cycle in the Caco-2 cell line (Laparra et al., 2008). Similarly, whey protein hydrolysates inhibit IL-8 secretion and ROS generation, and lunasin (a 43-mer soybean peptide) reduces ROS levels,

induced by H_2O_2 and LPS, respectively, in Caco-2 cells (Piccolomini et al., 2012; García-Nebot et al., 2014).

The nuclear factor NF-κB pathway represents a prototypical pro-inflammatory signalling route. The NF-κB group of proteins -which consists of homo and heterodimeric subunits of the Rel family, including p50 and p65- is found in almost all animal cell types and controls the expression of pro-inflammatory genes involved in the production of cytokines, chemokines, and adhesion molecules. The activity of NF-κB is regulated by IκB kinase (IKK), which retains NF-κB in the cytoplasm complexed with the inhibitory protein IκBα. Upon stimulation, IKK is phosphorylated and, in turn, it phosphorylates the IκBα protein, leading to the dissociation of IκBα from NF-κB and to the activation and translocation of NF-κB to the nucleus, where it binds to its specific promoter elements and induces gene transcription. A variety of integral membrane receptors, such as Toll-like microbial pattern recognition receptors (TLR), switched by microbial products, or receptors which respond to pro-inflammatory cytokines -for instance, TNF-α and IL-1 that are rapidly released on tissue injury or infection- trigger the canonical NF-κB pathway (Lawrence, 2009). Although structurally different, these receptors use similar signal transduction mechanisms, that include phosphorylation of mitogen-activated protein kinases (MAPKs) -like JNK, ERK1/2 and p38, involved in directing cellular responses to a diverse array of stimuli, such as mitogens, ROS and pro-inflammatory cytokines- or activation of IRAK1 -responsible for IL-1-induced upregulation of NF-κB-.

The inhibition of the NF-κB route has long been considered a key goal for anti-inflammatory food components. A 10-mer peptide from durum wheat (QQPQDAVQPF), which reduces

cyclooxygenase-2 (COX-2) activity and production of pro-inflammatory cytokines, such as IL-6 and IL-8, triggered by gliadin peptides in the Caco-2 cell model, hampers IRAK1 activation and ERK1/2 and p38 MAPK phosphorylation, and thus, the NF-κB function (Capozzi et al., 2013). The flavour enhancing γ-glutamyl dipeptides, γ-glutamyl cysteine and γ-glutamyl valine, decrease TNF-α-stimulated pro-inflammatory cytokine expression and increase IL-10 expression in Caco-2 cells. It was demonstrated that these peptides inhibit JNK and IκBα phosphorylation through the activation of the calcium-sensing receptor (Zhang et al., 2015). A similar mechanism is followed by the dipeptide WH and by dietary poly-L-lysine, which attenuate the TNF-α-induced secretion of IL-8 from HT29 cells and reduce inflammation in dextran sodium sulphate-induced models of mouse colitis (Kobayashi et al., 2015; Mine and Zhang, 2015). Therefore, the calcium-sensing receptor, present on IECs among other cell types, could constitute a target for treating intestinal inflammation by virtue of its role in maintaining and restoring intestinal homeostasis.

The investigation of immunomodulating food peptides has not only focused on their ability to maintain the integrity of the intestinal epithelial barrier by hindering strong inflammatory responses, but also by inducing the secretion of IgA, which prevents bacterial and toxin translocation and subsequent breakdown of the barrier function (LeBlanc et al., 2002; Otani et al., 2003; Chalamaiah et al., 2014 and 2015). In mice, the oral administration of pasteurized kefir, fish and egg yolk protein hydrolysates increases the number of IgA^+ cells, along with the levels of IL-4, IL-6 and IL-10 in the lamina propria -which contribute to B cell activation and antibody production- and raises the IgA content of the intestinal lumen. The secretion of IL-10 is believed to counterbalance the potential actions of the pro-inflammatory cytokines INF-γ and

TNF-α, which are also enhanced as a result of these interventions, because peptide administration does not cause tissue damage (Vinderola et al., 2005; Duarte et al., 2006; Nelson et al., 2007; Ndiaye et al., 2012). It is likely that these outcomes are, at least partly, mediated by the interaction of the peptides with the IECs and the subsequent conditioning of the underlying immune cells (Mallet et al., 2014). Indeed, egg yolk and yellow pea protein derived peptides increase IL-6 secretion by mouse IECs (Nelson et al., 2007; Ndiaye et al., 2012), while casein phosphopeptides induce the expression of IL-6 in Caco-2 and its production in human Int-407 IECs (Kawahara and Otani, 2004; Kitts and Nakamura, 2006).Thus, Nelson et al. (2007) argued that IL-6 produced by IECs as a result of peptide stimulation is enough to trigger B cell differentiation into plasma cells for the production of IgA.

In fact, it is now broadly recognized that the IECs, which constitute the interface between the gastrointestinal contents and the GALT, most likely determine the immune response to food (Berin and Sampson, 2013). IECs release several factors involved in tolerance induction, such as TGF-β, IL-10 and retinoid acid, which drive the differentiation of non-inflammatory DCs and tolerogenic $CD103^+$ DCs that, in turn, induce antigen-specific T regs (Coombes et al., 2007; Iliev et al., 2009). Conversely, IECs can act on DCs promoting sensitization through the production of chemokines and cytokines such as thymic stromal lymphopoietin (TSLP), IL-25 and IL-33 (Chu et al., 2013). In particular, IL-33 regulation in the small intestine was found to be essential for gastrointestinal Th2 priming and subsequent humoral or cellular peanut-induced allergic responses in mice (Chu et al., 2013). On the other hand, TSLP acts directly on T lymphocytes amplifying the activation of Th2 cells and the production of IL-4, IL-5 and IL-13 (Ziegler and Artis, 2010). It has been shown that incubation of Caco-2 cells with the peach allergen Pru p 3

increases the expression of the epithelial cytokines TSLP, IL-33 e IL-25 (Tordesillas et al., 2013). Similarly, thermal processing of the major peanut allergen Ara h 2 results in structural changes which impact its interactions with IECs and the subsequent cellular signalling, enhancing its immunogenicity (Starkl et al., 2011).

It is speculated that, as opposed to allergenic proteins, certain factors derived from the diet and the microbiota, with direct access to the IECs, could indirectly prime DCs in the control of the immune response, stimulating tolerance rather than activating the development of an allergic phenotype (Berin and Shreffler, 2008). Leonard et al. (2012) described that, in mice, the protection against anaphylaxis that arises from oral IT is associated with significant changes in the intestinal expression of certain genes. This observation denotes the existence of a protective mechanism located in the intestinal mucosa, whose activation would depend on interaction and communication with IECs, and suggests that it may be switched by the stimulus of immunomodulating peptides. However, it remains to be elucidated to what extent food derived peptides exhibit intrinsic immunogenicity by interacting with IECs.

The mucus layer that covers the intestinal epithelium, in addition to offering physical protection against mechanical, chemical and microbial challenges, provides it with enzymatic activities and filter properties that affect the availability of antigens for entering the mucosa. In this respect, several food protein hydrolysates and peptides have shown stimulatory effects on mucin secretion in rat jejunum that are linked to a structure favourable to binding to opioid receptors (Claustre et al., 2002; Trompette et al., 2003). Further research demonstrated the induction of mucin production by peptides derived from caseins and whey proteins (such as YPFPGPI -β-

casein 60-66-, YGLF-NH_2 -β-lactoglobulin 102-105-, YLLF-NH2 -α-lactalbumin 50-53- and AYFYPEL -α_{S2}-casein 143-149-) through the modulation of the expression of mucin genes in human HT29-MTX goblet IECs (Zoghbi et al., 2006; Martínez-Maqueda et al., 2012 and 2013). In particular, peptide sequences with the ability to stimulate the production of Mucin 2 (Muc2), such as β-casein 94-123 (Plaisancié et al., 2015), could play a tolerogenic role. Muc2 -the major component of the mucus layer of the intestine- exerts anti-inflammatory properties on IECs and DCs (Shan et al., 2013). Furthermore, it has been shown that goblet cells of the small intestine can take up luminal material and deliver it to the tolerogenic $CD103^+$ lamina propria DCs, with recent observations pointing at a coupling between goblet cell antigen uptake and mucus secretion (McDole et al. 2012; Pelaseyed et al., 2014). Consequently, Muc 2 enhances gut homeostasis and tolerance by suppressing inflammatory responses in DCs while imprinting them with a tolerogenic function (Shan et al., 2013).

The ability of food peptides to reinforce gut function through different mechanisms is of major importance in view of the increasing evidence that links the pathway of intestinal transport of food allergens and the allergen IEC-relationships with their intrinsic Th2-adjuvant activity (Roth-Walter et al., 2008; Price et al., 2014; Gavrovic-Jankulovic and Willensen, 2016). Whereas so far there is less evidence regarding food allergens, it is broadly recognized that house dust mite aeroalergens, such as Der p1, promote loss of tolerance and allergic sensitization due to their ability to activate innate pattern recognition receptors and protease-activated receptors and to cause direct injury to the bronchial epithelia (Lambrecht and Hammad, 2014). Furthermore, Der p1 has been reciently found in the gut of healthy humans, where, by virtue of its proteolytic activity, increases epithelial permeability, reduces the expression of tight-junction proteins and

weakens the mucus barrier, suggesting a role as an environmental trigger of food allergy (Tulik et al., 2016).

Intestinal transport of peptides

The passage of immunomodulating peptides across the intestinal barrier is a requirement for direct conditioning of the underlying immune cells of the GALT, as well as for systemic effects. As compared with intact proteins, hydrolysis products are endowed with distinct transport properties, both in terms of rate and pathway (Bernasconi et al., 2006). Food peptides can use the antigen sample pathway, that involves microfold cells (M cells) located above isolated lymphoid follicles and Peyer's patches (PP), which allows the trafficking of insoluble or particulate substances, although other transport mechanisms -such as the already mentioned paracellular passage regulated by tight junctions, passive diffusion through IECs, endocytosis and carrier-mediated transport systems- are also available to deliver soluble antigenic molecules to the lamina propria. Whereas there seems to be little unequivocal evidence that dietary bioactive peptides, other than di- and tri-peptides, are absorbed at physiologically relevant concentrations to produce systemic actions (Miner-Williams et al., 2014); the possibility exists that immunomodulating peptides can exert certain effects, at least those mediated by their interaction with cells of the adaptive immune system, at small concentrations, within the range of those required by large dietary antigens to induce tolerance. This is feasible by the specialized functions of lamina propria $CD103^+$ DC populations, that capture antigens and migrate to the

gut-draining mesenteric lymph nodes (MLN) where they instruct T cells promoting the development of gut-homing T regs by multiple mechanisms (Worbs et al., 2006).

Interestingly, it was found that IECs may constitute a powerful link between luminal antigens and local immune cells by mediating the transfer of only small amounts of soluble proteins or peptides. Most (90%) of the protein material that is taken by IECs is either totally (50%) or partially (40%) degraded in endosomes, yielding, in the latter case, peptides of around 1500 Da of molecular mass, a size compatible with binding to major histocompatibility complex class II (MHC-II) molecules. In IECs, as in professional antigen-presenting cells, endosomes containing partially degraded antigens may encounter MHC-II compartments, what allows peptides to be loaded on MHC-II molecules. This gives rise to exosome-like vesicles that very efficiently transfer MHC-II-peptide complexes to the lamina propria, where they can interact with local DCs and stimulate specific T cells at a concentration 100 times lower than free peptides (Ménard et al., 2010). Thus, the human IEC lines HT29 and T84 secrete exosomes bearing accessory molecules which strongly potentiate peptide presentation to T cells (Van Niel et al., 2001; Mallegol et al., 2007). However, it has been reported that, in mice, epithelial exosomes prime for immunogenic rather than tolerogenic responses (Van Niel et al., 2003), although the outcome would definitely depend on the phenotype of the local DCs and on the signals delivered by the IECs themselves and by exogenous factors, such as inflammatory stimuli. Indeed, exosomes derived from the serum of ovalbumin fed mice were found to prevent allergic sensitization in a model of allergic asthma, with the tolerant animals showing a significantly higher frequency of activated T regs (Almqvist et al., 2008).

On the other hand, in sensitized individuals, CD23 (FcεRII) -also called the low-affinity receptor for IgE- expressed by IECs and B cells, moves IgE and antigen-IgE complexes across the epithelium by transcytosis, exerting both negative and positive effects in the regulation of IgE production (Tu et al., 2005). This carrier-mediated transport enables, in cases of peptides with adequate length, activation of the high-affinity receptor of IgE, FcεRI, on mast cells and promotion of allergic inflammation, secretion of IL-4 and IL-13, local IgE production, facilitated antigen presentation by B cells and sensitization to additional antigens (Galli and Tsai, 2012). However, and as it will be explained in the following sections, since FcεRI is also expressed by human DCs, its activation by peptide-IgE complexes can influence, maturation, functional activation, migration of DCs and antigen presentation with a beneficial impact in tolerance induction (Platzer et al., 2015).

Effects on cells of the innate immune system: anti-inflammatory peptides

It has been reported that the oral administration of several food protein hydrolysates can activate the effector mechanisms of the innate immune response. For example, the phagocytic activity of peritoneal macrophages, and consequently their ability to respond to pathogens, increases after the administration to mice of hydrolysates of milk, fish and yellow field pea proteins (Biziulevicius et al., 2006; Duarte et al., 2006; Ndiaye et al., 2012; Chalamaiah et al., 2014).

Macrophage activation leads to an inflammatory response. Inflammation is part of the defense mechanism against infectious agents and injuries, but it is also associated to adverse chronic conditions such as cardiovascular diseases, diabetes and cancer. Macrophages are activated in response to oxidative stress or pro-inflammatory cytokines, including IFN-γ, and by bacterial

components, such as the TLR ligand LPS, resulting in the production of the pro-inflammatory substances: IL-1β, IL-6, TNF-α, NO and prostaglandin E2 (PGE2). NO and PGE2 are generated during inflammatory processes by inducible nitric oxide synthase (iNOS) and COX-2 -which catalyse, respectively, the reactions that convert L-arginine and oxygen to NO and citruline, and arachidonic acid to prostaglandins-. In this respect, and as shown in Table 1, a number of food-protein hydrolysates and derived peptides, particularly from soy, fish and shellfish, have shown anti-inflammatory properties on LPS-stimulated murine RAW 264.7 cells, as estimated by different markers associated with beneficial effects against inflammation and oxidative stress. The RAW 264.7 cell line is considered one of the most suitable *in vitro* model systems for inflammation-related investigations. In addition, other food protein hydrolysates have shown anti-inflammatory properties in human cell lines, such as monocyte (THP-1) derived macrophages (Millán-Linares et al., 2014; Montoya-Rodríguez et al., 2014).

The cascade of LPS-induced events in macrophages is initiated by its binding to the LPS-binding protein and transfer to CD14 on the macrophage surface for further interaction with TLRs. Through MAPKs, TLRs activate the already mentioned NF-κB pathway. A mechanism of action involving the inhibition of NF-κB activation, leading to the down-regulation of genes implicated in inflammatory processes in RAW 264.7 cells, such as COX-2, iNOS, IL-6, IL-1β or TNF-α, has been described for lunasin and lunasin derived peptides (de Mejía and Dia, 2009). A reduced transcriptional activity of NF-κB has also been deemed responsible for the antioxidant activity of hydrolysed common bean and soybean flours and amaranth hydrolysates (Oseguera-Toledo et al., 2011; Vernaza et al., 2012; Montoya-Rodríguez et al., 2014). Similarly, the anti-

inflammatory properties of pyroglutamyl-leucine in the RAW 264.7 cell line are probably due to the inhibition of NF-κB, via suppression of the phosphorylation of three types of MAPKs: JNK, ERK and p38, and of its dissociation from IκBα (Hirai et al., 2014).

In addition to interfering with TLR signal transduction pathways, there are also peptides which supress LPS-induced activation of macrophages through interaction with LPS itself and neutralization of its action. Antimicrobial host defense peptides, which are generally small (commonly 12-50 amino acids) cationic and, frequently, quite hydrophobic and amphipathic, interact with the negatively charged surface of LPS and suppress the LPS-triggered release of pro-inflammatory cytokines from macrophages (López-Abarrategui et al., 2013).This is, for instance, the case of antimicrobial peptides derived from shrimp, such as the shrimp anti-LPS cyclic peptide (ECKFTVKPYLKRFQVYYKGRMWCP) (Lin et al., 2010).

In contrast to the previously mentioned examples, wheat gliadin and its proteolytic fragments arising from pepsin digestion, such as VSFQQPQQQYPSSQ and FQQPQQQYPSSQ, have the ability to activate mouse peritoneal macrophages inducing the secretion of TNF-α and NO (Tuckova et al. 2002). Gliadin and its fragments also increase IL-8 and TNF-α production by human monocytes, what was suggested to be involved in the damage they cause to celiac intestinal mucosa. Unlike soy proteins, gliadin stimulates phosphorylation and subsequent degradation of the IκBα molecule and increases the DNA-binding activities of the NF-κB subunits p50 and p65, with an activation pathway independent of CD14 (Jelinkova et al., 2004).

It should be mentioned that it has been hypothesized that the suppression of Th1 responses by antioxidant compounds, while beneficial in reducing the side effects of inflammatory processes,

may provoke an up-regulation of Th2 type cytokines that promotes allergic sensitization and exacerbates allergic symptoms (Gostner et al., 2014). However, when the role of oxidative stress in allergic sensitization was investigated in humans, it was shown that it is the exposure to allergenic proteins combined with an inadequate antioxidant response what increases the likelihood for sensitization (Utsch et al., 2015). In fact, there is evidence that antioxidant and anti-inflammatory compounds that decrease the effects mediated by IFN-γ and slow down the intracellular pathways that lead to the generation of ROS, also reduce the expression of Th2 cytokines and IgE levels in response to food allergens in mice (Singh et al. 2011; Masilamani et al. 2012). For instance, dietary omega-3 fatty acids prevent allergic sensitization in mice via suppression of Th2 type antibody responses and enhancement of intestinal and systemic $Foxp3^+$ T regs (van den Elsen et al., 2013a). Noteworthy, the suppression of IL-4 and IL-13 secretion by mast cells, that contributes to decrease the susceptibility to develop allergy and the severity of the symptoms, is associated with the inhibition of the generation of ROS (van den Elsen et al., 2013b).

In line with those observations, it was reported that the fraction smaller than 3000 Da from an enzymatic hydrolysate of the edible microalgae *Spirulina maxima* and, particularly, the pentapeptides DAVNR and MMLDF, inhibit histamine release form RBL-2H3 mast cells sensitized with dinitrophenyl (DNP)-specific IgE and stimulated with DNP-BSA, in parallel with a suppressive effect on intracellular ROS production (Vo et al., 2013). Furthermore, the peptide PFNKGTFAS, derived from a gastrointestinal digest of abalone (*Haliotis discus hannai*), attenuates histamine release and production of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) from human HMC-1 cells. PFNKGTFAS hinders the phosphorylation of JNK and IκBα in

the stimulated mast cells, which points at the inhibition of the MAPK and NF-κB pathways as responsible for the restricted release of mediators (Ko et al., 2016). These results indicate that the ability of food peptides to favourably balance pro-inflammatory and anti-inflammatory/antioxidant responses could provide a tolerogenic milieu. In addition, it has been described that there are peptides able to interact with the ε-heavy chain of IgE and block its binding to the high-affinity receptor, FcεRI, on mast cells and basophils and protect mice from anaphylaxis (Zhou et al., 2013). These actions of peptides on mast cells and IgE signalling are highly interesting in view of the role of armed mast cells as effectors of anaphylaxis, but also as inducers of Th2 responses and suppressors of T regs (Burton et al., 2014).

Effects of peptides on cells of the adaptive immune system: Th1 and Th2 mediators

As already anticipated, there is evidence that immunomodulating peptides alter T lymphocyte functions and can contribute to a balanced Th1 and Th2 response, with potential implications in various clinical conditions, including allergy and oral tolerance to antigens (Kiewiet et al., 2015).

Antigen-presenting cells, such as DCs, are key initiators of innate immunity, although they also play a central role in shaping the properties of adaptive immunity by processing proteins or peptides and presenting them to T cells, together with co-stimulatory mediators that generate primary T cell responses. Food antigens can be directly sampled from the gut lumen by intestinal $CX3CR1^{+}$ $CD103^{-}$ fagocytes, which extend their dendrites between IECs. Alternatively, as mentioned, they can be transported through M cells into the lymphoid follicles, or by transcytosis to the lamina propria, and presented by DCs to naïve T cells in the PP or the MLNs (Ménard et al., 2010). As already indicated, intestinal DCs, with the help of other cells involved in innate

immunity, such as the nearby IECs, play a crucial role in the generation of tolerance to food proteins (Ruiter and Shreffler, 2012). Thus, while allergens, as well as certain food components, may exhibit intrinsic adjuvant capacity, stimulating DCs to enhance allergen-specific Th2 differentiation (Kean et al., 2006; Shreffler et al., 2006; Hsu et al., 2010; Gómez et al., 2012; Chu et al., 2013), it is also possible to prime DCs for allergy prevention or treatment. For instance, soybean isoflavones hinder allergic sensitization by inhibiting DC maturation and subsequent DC-mediated Th2 effector cell functions, which correlates *in vivo* with significantly reduced anaphylactic symptoms and mast cell degranulation upon allergen challenge (Masilamani et al., 2011). Accordingly, DCs are attractive targets for tolerance induction and the potential of food peptides to avoid Th2 type immunity by the enhancement of the tolerogenic properties of DCs warrants further investigation.

It is known for some time that FcεRI, in addition to mast cells and basophils, is also constitutively expressed on DCs and monocytes in humans, albeit not in mice (Shin and Greer, 2015). Experiments with transgenic mice led to postulate that FcεRI contributes to the rapid internalization of IgE-bound antigens by DCs, facilitating their presentation to specific T cells *in vivo* and promoting the development and activation of Th2 cells (Sallmann et al., 2011). However, other studies showed that, when monovalent antigens, unable to cross-link IgE-FcεRI complexes, are loaded by DCs, enhanced presentation results in a transient T cell proliferation, followed by systemic deletion of antigen-specific T cells (Baravalle et al., 2014). This mechanism, if confirmed in further studies, implies a role for peptides derived from allergenic proteins, focused by IgE-binding and transported by CD23, in the development of tolerance through the mediation of DCs. Furthermore, it was recently found that even antigen-specific IgE-

FcεRI cross-linking fails to induce maturation and production of inflammatory mediators by human and FcεRI-humanized DCs *in vitro*, which correlates with a reduced severity of food allergy *in vivo* (Platzer et al., 2015). These observations indicate that DC-intrinsic IgE signals function as feedback mechanisms to control allergic inflammation and suggest novel beneficial peptide-based approaches for allergy therapy.

As opposed to IgE-mediated food allergies, in the pathogenesis of celiac disease, and in addition to the inflammatory innate immune response that causes damage to the intestinal mucosa, the adaptive immune response to gliadin involves DCs that interact with gliadin-specific Th1 cells, producing inflammatory cytokines such as IFN-γ (Green et al., 2015). Stimulation of DCs with gliadin, unlike other food proteins, enhances the expression of maturation markers (CD80, CD83, CD86, and HLA-DR molecules) and the secretion of chemokines and cytokines (IL-6, IL-8, IL-10, TNF-α, growth-related oncogene, etc.), which results in more efficient processing and presentation of gliadin peptides to specific T cells (Palova-Jelinkova et al., 2005). However, two decapeptides naturally occurring in durum wheat, QQPQDAVQPF and its homologous QQPQRPQQPF, are able to prevent the gliadin-dependent functional DC maturation and T-cell stimulating capacity, raising the perspective of a potential therapeutic strategy alternative to a gluten free diet (Giordani et al., 2014).

Peptides from marine origin administered orally to mice enhance the proliferative response of spleen cells to concanavalin A (ConA) and the percentage of $CD4^+$ T cells (Yang et al., 2009a) or both $CD4^+$ and $CD8^+$ T cells (Chalamaiah et al., 2014). In addition, several studies describe that food peptides (Kawahara et al., 2004; Jiehui et al., 2014) and hydrolysates (Mercier et al., 2004;

Durrieu et al., 2006; Mao et al., 2007; Saint-Sauveur et al., 2008; Kong et al., 2008; Yang et al., 2009a; Hou et al., 2012; He et al., 2015) promote the proliferation of either resting or mitogen-treated murine spleen cells and human T cells *in vitro*, which was also interpreted as a stimulatory effect on cell-mediated immunity. However, in most cases, those works assessed cell viability -the number of healthy cells in a sample- rather than cell proliferation -the number of dividing cells-. Other reports described that protein hydrolysates do not change the number of viable cells, which allows discarding possible toxic effects, although they decrease ConA-induced proliferation as part of their immunoregulating role (Lozano-Ojalvo et al., 2016a).

The results available so far suggest that, beyond influencing proliferation, immunomodulating peptides can specifically drive lymphocyte responses in a certain direction. Milk protein hydrolysates have been reported to induce the secretion of Th1 cytokines (IL-2 and IFN-γ) by ConA-stimulated mice spleen cells and human Jurkat T cells, without an effect on the release of Th2 cytokines and thus, to play a role in fighting infections (Mao et al., 2007; Saint-Sauveur et al., 2008; Phelan et al., 2009). Conversely, the casein peptide QEPVL enhances Th2 type responses *in vivo*, as estimated by the concomitant up-regulation of IL-4 and down-regulation of IFN-γ and TNF-α in mice serum, and inhibits LPS-induced inflammation (Jiehui et al., 2014). These observations imply that food peptides may preferentially guide Th1 type immune responses or promote Th2 type responses, which are goals of recognized importance in infectious diseases, tumors and inflammatory processes.

In addition, other hydrolysates are able to combine Th1 and Th2 supresive properties and, therefore, they could be of interest as immunotherapeutic approaches against excessive

inflammatory conditions and Th2 responses, such as those typical of food allergies. Thus, hydrolysates of egg white proteins with alcalase reduce the ConA-stimulated release of TNF-α and IL-13 (Lozano-Ojalvo et al., 2016a). Moreover, these hydrolysates significantly neutralize oxidative stress in Th1-stimulated peripheral blood leukocytes, whereas they effectively down-regulate the production of Th2 cytokines and the secretion of IgE to the culture media of Th2-skewed human peripheral blood mononuclear cells (PBMCs) (Lozano-Ojalvo et al., 2016b). On the other hand, hydrolysates of egg proteins with pepsin help to re-establish the Th1/Th2 balance in Th2-skewed PBMCs by the simultaneous increase in IFN-γ and decrease in IL-5 and IL-13 (Lozano-Ojalvo et al., 2016b). In this respect, it should be noted that the therapeutic effect of peptides in mouse models of allergy has been associated both to the inhibition of allergen-induced Th1 and Th2 cytokine responses (Yang et al., 2009b), as well as to the promotion of Th1-biased responses to a detriment of the Th2 ones (Yang et al., 2010). Alternatively, other immunomodulating peptides could help to induce oral tolerance by stimulating T regs through the promotion of the secretion of regulatory cytokines, such as IL-10 (Prioult et al., 2004; Cian et al., 2012) or TGF-β (Rodriguez-Carrio et al., 2014).

Peptide IT: T and B cell epitopes

The production of allergen derivatives that are hypoallergenic and immunogenic is an attractive strategy for the development of IT agents for IgE-mediated allergies. Although its efficacy has not been formally demonstrated, oral IT -which consists in the gradual administration of increasing amounts of the allergen by the oral route- is one of the most promising treatment options. As compared with oral IT, which can produce severe systemic reactions, sublingual and

epicutaneous immunotherapy only lead to mild local reactions, but provide desensitization to substantially smaller amounts of food (Chinthrajah et al., 2016). However, the associated high frequency of allergic adverse events associated to oral IT has prompted the investigation of strategies aimed at reducing the allergenicity of the treatment preparations, while maintaining their immunogenicity, or even improving their immunomodulating potential, to achieve effective desensitization or sustained oral tolerance (Jutel et al., 2015; Vázquez-Ortiz and Turner, 2016; Wood, 2016).

Peptide IT focuses on the properties of fragmented allergens, which contain T cell-stimulating epitopes but are not capable of cross-linking IgE on basophils and mast cells. Peptides represent a safer alternative to full allergens, as they produce fewer side effects and increase adherence to therapy (Moldaver and Larché, 2011; Casale and Stokes, 2011). T cell epitope- mapping -that is the determination of the specific peptide sequences recognized by $CD4^+$ T cells- can be performed by different methods but, in all cases, it needs to be checked that as many as possible distinct MHC-II molecules, representative of the HLA genotypic frequencies of the patient population of interest, recognize and bind the selected epitopes (Malherbe, 2009).

T cell epitopes that can be potential candidates for peptide-based vaccines have been identified from many food allergens, but particularly from peanut, *in silico* -by the use of predictive algorithms- and *in vitro* -by screening the proliferative responses and cytokines released by PBMCs from allergic patients stimulated with consecutive overlapping peptides spanning the allergen sequence or by tetramer-guided epitope mapping- (DeLong et al., 2011; Prickett et al., 2011 and 2013; Pascal et al., 2013). Allergen-specific T cell lines or clones have been used to

overcome the limitation imposed by the low frequency or allergen-specific T cells (Prickett et al., 2011 and 2013; DeLong et al., 2011), although *in vitro* expansion can alter cell phenotypes or bias the results through the selection of the rapidly proliferating clones (Pascal et al., 2013). Specific T cell epitopes of food allergens, such as celery (Bohle et al., 2003), hazelnut (Bohle et al., 2005); shrimp-tropomyosin (Wang et al., 2012; Ravkov et al., 2013), β-lactoglobulin (Inoue et al., 2001, Kondo et al., 2008); α_{S1}-casein (Ruiter et al., 2006), ovomucoid (Holen et al., 2001), peach Pru P3 (Tordesillas et al., 2009; Pastorello et al., 2010), anisakis (Garcia Alonso et al., 2015) and walnut (Archila et al., 2015), among others, have also been described.

There is a minimum core sequence of 9 to 15 amino acids required for recognition by reactive T cells. Consequently, -even if linear, soluble, 15-mer IgE-binding peptides are unlikely to act as full epitopes and cause allergic reactions (Albrecht et al., 2009)- the selection of peptides that combine the ability to stimulate T lymphocytes and modulate the immune response with a reduced capacity to trigger clinical symptoms implies a compromise, as longer peptides increase T cell epitope coverage but also the potential for IgE-cross-linking (Prickett et al., 2011 and 2013). The lack of overlapping between T- and IgE-binding epitopes has to be evaluated by searching B epitopes reported in the literature, and confirmed by serum IgE-binding and basophil activation assays (Wai et al., 2014). Peptide microarray technology allows the assessment of epitope diversity and affinity of the IgE response associated to clinical reactivity (Berin, 2015). Unfortunately, the evidence gathered so far highlights that B epitope multiplicity and variation in reactivity among allergic individuals is so large that the presence or absence of specific epitopes might not provide enough evidence for allergy prediction (Aalberse and Crameri, 2011; Ruiter and Shreffler, 2012; Scheurer et al., 2015).

Cytokine detection assays typically reveal a prevalent Th2 cytokine profile in PBMCs or specific T cells from allergic patients stimulated with the T cell epitopes regarded as most significant, while control donors show a trend towards a Th1 profile (Tordesillas et al., 2009; Pastorello et al., 2010; Prickett et al., 2011; DeLong et al., 2011; Pascal et al., 2013). Nonetheless, beyond trying to reduce IgE reactivity and retain T cell reactivity, the possibility arises to exploit the immunomodulating potential of peptides, in particular the ability to re-direct the immune response of specific cell types and the lack of inflammatory properties, to enhance the effectiveness and reduce the length of the IT treatments. In this respect, B cell epitope therapy, which uses non IgE-reactive peptides derived from the IgE-binding sites of allergens -with a diminished potential to release Th2 cytokines and inflammatory mediators, but able to induce allergen-specific blocking IgG antibodies when fused to the appropriate carrier protein- can make IT safer and more efficient (Valenta et al., 2016). Regarding food allergies, as it will be explained in the next section, mouse experiments have shown that peptides that do not induce Th2 cytokines and/or are able to increase allergen-specific IgG can play a valuable therapeutic role by providing and extra help to deviate predominantly Th2 to Th1 responses, by inducing T regs that downregulate both Th2 and Th1 responses, or by blocking allergen binding to IgE (Lozano-Ojalvo et al., 2016c; Prioult et al., 2005; Yang and Mine, 2009; Wai et al., 2014 and 2016).

Generally, many distinct fragments of food allergens are able to generate T and B cell responses (Prickett et al., 2011; Berin, 2015; Wai et al., 2016), although the final effect might depend on the occurrence of the right combination of peptides with synergistic or adjuvant actions, or on the peptides being in an aggregated state resembling the intact molecule (Yang et al., 2010; Bøgh et

al., 2012; Meulenbroek et al., 2013). On this basis, enzymatically hydrolysed allergens are a practical alternative to synthetic dominant T cell epitopes specific for each HLA type -as the former would contain enough peptides to address MHC-II diversity in patients- that could also provide non IgE-cross-linking B epitopes (Knipping et al., 2012; Kulis et al., 2012). Hydrolysis of food proteins emerges as an attractive and safe means for a reproducible and standardized production of immune-stimulating peptides at low cost, as compared with that associated with generating synthetic peptides on a large scale. Control of the enzymatic processes can be achieved through mass spectrometry methods for peptide identification and quantification, which provide great sensitivity in complex samples (Liebler and Zimmerman, 2013)

Immunodominant T cell epitopes with negligible IgE-binding and lacking inflammation stimulating capacity have been used to modify beneficially the immune reaction to allergens in clinical trials of IT by subcutaneous injection, demonstrating persistent efficacy with few adverse events for cat, house dust mite and pollen allergy, even though the precise mechanism of action is not yet clear (O'Hehir et al., 2016). T cell epitopes induce anergy or deletion of specific T cells, resulting in down-regulation of T cell proliferative and cytokine responses to the allergens, as in whole allergen IT, although the peptides may be too small to induce IgG-blocking antibodies (Prickett et al., 2015). Furthermore, also similarly to conventional IT, an important role of T regs, with an increased production of IL-10 -that further induces the expansion of T regs and the inhibition of Th1, Th2 and inflammatory cells- has been reported (O'Hehir et al., 2016). Interestingly, cross-reactive T cell epitopes have been identified and, in some cases, tolerance to one T cell epitope is found to protect against other epitopes of the same molecule, providing evidence for the induction of linked epitope suppression (Moldaver and Larché, 2011).

For its part, B cell epitope IT was proved to target different immune mechanisms aiding at pollen desensitization (Valenta et al., 2016). B cell epitopes prevent activation of mast cells and basophils, boost of memory IgE production and IgE-facilitated presentation to T cells, through the induction of IgG4 (Akdis and Akdis, 2014; Jongejan and van Ree, 2014). In the case of food allergies, IT with peptides has almost exclusively been assayed *in vivo* in murine models.

***In vivo* IT assays with food peptides**

The results of a double-blind placebo-controlled randomized IT study with a hypoallergenic hydrolysate of egg white in egg allergic children have been recently published by Giavi et al. (2016). A significant increase in egg-specific IgG4 and a decreased reactivity of basophils, usually associated with desensitization, was detected in the group of patients treated with the hydrolysate. The treatment, with few side effects, could be safely taken at home. However, the lack of statistically significant differences upon the final oral food challenge between the treatment and placebo groups suggests that further studies with a larger number of patients are needed to validate this therapeutic strategy and reveal the underlying mechanisms. To the best of our knowledge, this is the only published human trial with hydrolysates or peptides as IT for food allergy. For the meantime, promising findings have been obtained from studies using animal models of food allergy.

Mainly due to ethical reasons, animal models have become valuable tools to increase our understanding of the complex immunological and pathophysiological mechanisms involved in the development of food allergy (Finkelman, 2007; Van Gramberg et al., 2013). Such models allow the identification of factors responsible for the breakdown of oral tolerance and the

implementation of experimental studies to establish prevention strategies and curative approaches, as well as to investigate novel therapies (Oyoshi et al., 2014). Rodents are frequently used in the field of the food allergy because of their small size, short breeding cycle, well characterized immunology and ability to product protein-specific IgE; in particular, murine strains, with or without genetic modifications, such as BALB/c, C3H/HeJ, DBA/2, A/J, BDF-1 and C57B1/6 and Brown Norway rats (Fritsché, 2009; Ladics et al., 2010).

As already mentioned, a prerequisite of food peptides for being good candidates for IT is the lack of IgE reactivity and, consequently, of anaphylactic potential. In this respect, it should be noted that, in addition to increasing the safety of the IT treatments, the use of hypoallergenic preparations may also increase their efficacy and reduce their lengh. Activation of mast cells by IgE cross-linking releases Th2-driving cytokines, such as IL-25, and enhances IL-4, acting as an amplifier of Th2 responses (Burton et al., 2014). Thus, repeated intragastric challenge with intact allergens during IT may induce intestinal IL-33, IL-25 and TSLP signaling and enhance Th2 cells, promoting the expansion of mucosal group 2 innate lymphoid cells and the collaboration between these innate cells and allergen-specific Th2 cells, ultimately acting, through IL-4, as a Foxp3 inhibitor in Treg differentiation (Lee et al., 2016; Noval Rivas et al., 2016).

In vivo assessments of the allergenicity of food protein hydrolysates, mainly hydrolyzed milk proteins, have been conducted by passive cutaneous anaphylaxis tests, which measure the extravasation of a dye into the tissues after intravenous challenge of sensitized rodents or naïve rodents that previously received an intradermal injection of sera from sensitized ones (López-Expósito et al., 2012; Bøgh et al., 2015). Acute allergic skin responses -ear swelling- and local

release of mouse mast cell protease-1 (mMCP-1)- can also be measured in sensitized mice following intradermal injection of allergen hydrolysates (van Esch et al., 2010). Furthermore, graded oral administration of hydrolyzed and intact proteins to mice sensitized to the intact allergen allows comparing the doses that induce allergic symptoms and reduce body temperature (Kulis et al., 2012).

Table 2 summarizes the main outcomes of the studies evaluating food protein derived hydrolysates and synthetic peptides for the treatment of food allergy in mice sensitized to the intact food allergens. In general terms, 2-4 weekly doses were administered, either orally or intraperitoneally, for 1 to 4 weeks. Many of the listed IT treatments were found to decrease adverse reactions upon subsequent challenge with the allergen, as measured by symptom scores and/or body temperature drops, in parallel with reductions in serum histamine release (Yang et al., 2010; Rupa and Mine, 2012) or mMCP-1 (Wai et al., 2016), that denote reduced activation and degranulation of mast cells. However, none of these studies re-assessed protection from anaphylaxis after a sufficiently extended period of discontinuation of the treatment and, therefore, sustained unresponsiveness (Pesek and Jones, 2016) cannot be guaranteed.

As shown in Table 2, desensitization with immunomodulating hydrolysates and epitope peptides usually correlates with a significant reduction in allergen-specific IgE levels. However, the role of other allergen-specific antibodies, such as IgG and IgA, in mouse models, is under debate. Kulis et al. (2012) associated successful IT in the absence of changes in the IgE levels with significant increases in allergen-specific IgG1 and IgG2a, which would act as protective blocking antibodies. However, in other studies, no significant variations in allergen-specific total

IgG levels were detected in the effectively treated mouse groups (Yang et al., 2009b and 2010), although, on further examination, alleviation of allergy signs was found to correlate with reduced IgG1 and increased IgG2a (Rupa and Mine, 2012). Wai et al. (2016) referred reductions in specific IgE, no change in specific IgG1, and high levels of specific IgG2a whose blocking effect was verified *in vitro* and *in vivo*. In humans, many studies demonstrate that increases in specific IgG4 levels accompany clinical improvements (Akdis and Akdis, 2014). However, homology between mouse and human IgG subclasses is difficult to stablish, and there are differences in class switching: in mice, IL-4 induces IgG1 and IgE, whereas in humans, IL-4 induces switching to IgG4 and IgE (Mestas and Hughes, 2004; Finkelman, et al., 2016).

Circulating IgG antibodies can neutralize allergens before they cross-link mast cell-bound IgE, although this ability to physically inhibit the binding of allergens to IgE is saturable and depends on the challenge dose (Finkelman, 2005). Furthermore, it has been described that anaphylaxis in the mouse can occur through two independent pathways, comprising either the classical mechanism associated with human allergy -that is the cross-linking of IgE bound to FcεRI on mast cells- or a second, alternative, pathway that requires IgG antibodies, FcɣRIII receptors and macrophages. Both share similar time courses and symptoms, but much higher antibody and antigen concentrations are required to induce anaphylaxis by the alternative IgG-mediated pathway, in line with the much higher affinity of FcεRI for IgE than FcɣRIII for IgG and with the need for antigen-IgG complexes to form in blood before they bind FcɣRIII on macrophages (Finkelman, 2007). IgE-mediated anaphylaxis predominates in the presence of low levels of IgG and allergen, even if the IgE levels are also low.

In some mouse models of allergy, sensitization stimulates high levels of specific IgG and mice are challenged with large quantities of allergen, which could inhibit IgE-mediated anaphylaxis, but also induce IgG-mediated anaphylaxis (Strait et al., 2006). This implies that the protective effect of blocking IgG antibodies could be restricted to a situation when the quantity of challenge allergen is less than that required to trigger IgG-mediated anaphylaxis; but when the dose increases, IgG or both IgG and IgE pathways could be triggered (Strait et al., 2006). Thus, the outcomes of different IT experiments with respect to changes in relative antibody levels may not be comparable, due to the use of different immunization protocols that produce different ratios of IgE to IgG and different amounts of allergen for challenge, particularly if antibody analyses are performed short after challenge. Furthermore, the IgE/IgG response may also vary depending on the administration route (oral, subcutaneous or intraperitoneal) of the sensitizing allergens and IT preparations (Finkelman et al., 2005; Kulis et al., 2012).

Secretory IgA has an important role in immune homeostasis in the gut, with low levels of intestinal allergen-specific IgA being associated with the development of food allergy, although its role is still unclear (Kukkonen et al., 2010). Following some effective IT studies with food peptides, increased levels of fecal allergen-specific IgA have been reported (Yang et al., 2010; Rupa and Mine, 2012). However, the hypothesis that IgA secreted at mucosal surfaces can bind allergens before they are absorbed, in a way similar to its neutralizing effect towards pathogens and toxins, could not be substantiated. Instead, the property of IgA to block systemic anaphylaxis by binding to allergens appears to be restricted to serum IgA and not to IgA contained in the gut lumen (Strait et al., 2011).

With respect to cellular responses, a consistent reduction in the production of Th2 cytokines (whether IL-4, IL-5 or IL-13) was observed when splenocytes from treated animals were stimulated with the whole allergens (Table 2). However, the reports refer either an associated increase in Th1 cytokines (IFN-γ or IL-12) and restoration of the immunological balance (Yang et al., 2010; Rupa and Mine, 2012; Wai et al., 2016), or a concomitant Th1 decrease (Yang et al., 2009b). Ileum expression levels of Th2 and Th1 cytokine genes parallel systemic changes in the Th2 and Th1 cytokine profiles (Yang et al., 2009b; Yang et al., 2010; Wai et al., 2016). Upregulation of TGF-β and IL-10 or of the transcription factor Foxp3 in intestinal tissues points at a role of T regs in tolerance induction by food peptides (Yang et al., 2009b and 2010; Wei et al., 2016). Increased percentages of T regs ($CD4^+$ $Foxp3^+$ and $CD4^+$ $CD25^+$ cells) were also detected in the blood of the desensitized animals (Rupa and Mine, 2012).

While the administration of multiple T cell epitopes appears more effective than that of the corresponding individual peptides (Yang et al., 2010), it is noteworthy that a single immunodominant T cell peptide can offer protection against challenge with the whole allergen, which reinforces the concept of linked epitope suppression (Table 2). In this respect, it is speculated that the only effective way to provide tolerance towards multiple epitopes within a protein -or even towards other allergens during co-exposure- by the use of single peptides is through the induction of a regulatory function versus a delectional function, which may depend on the choice of route, dose and timing of administration (Mackenzie et al., 2012). Indeed, there are examples of peptide IT in allergic airway diseases, where the regulatory environment generated extendes to T cells specific for other epitopes of the allergen (intramolecular tolerance)

or even to allergenic proteins not represented in the therapy (intermolecular tolerance) (Larché, 2014).

Decreased allergic symptoms following IT were induced by strong Th2-stimulating hydrolysates, which maintain the immunogenicity of the intact allergen and its sensitizing potential, but lack eliciting potential (Kulis et al., 2012). In any case, immunomodulating peptides, selected on the basis of their strong proliferative activity, IFN-γ stimulating properties and absence of IL-4 inducing capacity, stand as an interesting option (Yang et al., 2010; Wei et al., 2016). Nonetheless, the observation that different peptides that reduce allergen-induced clinical signs produce different immunological changes highlights the need for a deeper knowledge on the structure-function relationships to go deeper into their mechanism of action (Yang et al., 2010; Rupa and Mine, 2012).

***In vivo* allergy prevention assays with food peptides**

A recognized advantage of IT lays in its potential to restrain the progression of mild to severe forms of allergy. This suggests its use in sensitized individuals to avoid the development of allergic symptoms or, even as a prior strategy, to prevent sensitization (Valenta et al., 2016). In fact, it has been shown that, rather than their avoidance, the early introduction of eggs or peanuts in the diet significantly decreases the frequency of egg or peanut allergy among genetically predisposed children, which, in both cases, correlates with increased levels of peanut-specific IgG4 (Palmer et al., 2013; Du Toit et al., 2015). Furthermore, prophylactic house dust mite therapy in children at high heredity risk was demonstrated to reduce sensitization to common allergens (Zolkipli et al., 2015). However, as already indicated for curative treatments of food

allergy, the use of intact allergens for preventive treatments poses a risk of anaphylactic reactions and it does not fully exclude the possibility of a progress in the development of sensitization. Once more, peptides or hydrolysates appear as an attractive alternative, but it is necessary to assure that they lack sensitizing potential.

The sensitizing capacity of food hydrolysates can be evaluated in Brown Norway rats immunized by the intraperitoneal route without adjuvant, by analyses of specific antibody responses. Induction of specific IgE, IgG1 and IgG2a is taken as a measure of sensitization, together with the demonstration of the biological activity of the generated IgE in basophil activation assays (Bøgh et al. 2009 and 2013; Kroghsbo et al., 2014). Release of rat mast cell protease II (RMCPII) indicates IgE-mediated mucosal mast cell degranulation (Fritsché et al., 1997). The use of aluminum hydroxide as adjuvant enhances immunogenicity and narrows heterogeneity between experimental data, but may bias the results by changing antigen presentation to the immune system (Bøgh et al., 2015). On the other hand, the oral route, more physiologically relevant, gives comparable outcomes, but with different dose-response ratios, with respect to the intraperitoneal route (Kroghsbo et al., 2014).

Mice have also been used as models of food allergy for the evaluation of the sensitizing capacity of hydrolyzed proteins. Sensitization of mice to food proteins requires the use of Th2-polarizing adjuvants -such as staphylococcal enterotoxin B and, more commonly, cholera toxin (CT)- to overcome their strong tendency to develop oral tolerance, although this practice may conceal the intrinsic sensitizing properties of the co-administered proteins (Berin and Shreffler, 2008). In C3H/HeOuJ mice, comparison between the levels of allergen-specific IgE, IgG1 and IgG2a,

induced by hydrolyzed and intact allergens administered orally with CT is used for the assessment of lack of sensitizing potential of the formers, which can be confirmed by subsequent challenge with the whole allergen and measurement of anaphylactic symptoms, body temperature and mMCP-1 (van Esch et al. 2011a). An inter-laboratory evaluation of this model in four different research centers evidenced its transferability and adequacy to the prediction of the residual sensitization capacity of hydrolysed milk-based infant formulas (van Esch et al., 2013), which, according to European guidelines on hypoallergenic and follow-on infant formulas, should not be able to sensitize animals to the protein source they derive from (Commission Directive 96/4/EC).

Most interventions aimed at inducing protective responses against food allergy have been conducted with infant hypoallergenic formulas derived from enzymatic hydrolysis of cow's milk proteins. These have concluded that certain hydrolyzed infant formulas, in particular partially hydrolyzed whey formulas, have a role in the reduction of risk of atopic disease (Szajewska and Horvath, 2010; Vandenplas et al., 2014). Further evidence for the efficacy and mechanisms of action of protein hydrolysates in allergy prevention were obtained from mice trials, as shown in Table 3.

Administration of intact and hydrolyzed milk proteins avoid subsequent sensitization, as measured by inhibition of the generation of allergen-specific IgE, release of mast cell mediators and allergic symptoms (Table 3). However, in certain studies, only the intact proteins were able to significantly reduce serum levels of specific IgE and/or IgG1, indicating a more effective prevention of sensitization (Adel-Patient et al., 2011 and 2012; van Esch et al., 2011b). In any

case, protection against the development of an allergic response can also be induced by specific peptides present in partially hydrolyzed preparations but absent from extensively hydrolysed formulas (Fritsché et al., 1997; van Esch et al., 2011b), although more prolonged treatments may be necessary to achieve effects similar than those of the intact allergens (Peng et al., 2004). In addition, the effect is dose-dependent, with peptide enrichment decreasing the efficient oral dose, although correlation between peptide structure and tolerogenic properties has not been described (Pecquet et al., 2000; Hacini-Rachinel et al., 2014).

Simultaneous immunization of rats with intact β-lactoglobulin plus hydrolysed β-lactoglobulin reduces the sensitizing capacity of the former, which was attributed to a tolerogenic affect exerted by the digestion products (Bøgh et al. 2013). A single peptide from β-lactoglobulin (β-lactoglobulin 31-48) was reported to reduce sensitization and allergic response to the whole intact protein in CeH/HeOuJ mice, thus inducing linked epitope suppression, with stronger effects on its own than when present in a peptide mixture (Meulenbroek et al., 2013). However, the peptide β-lactoglobulin 25-107, which represents 50% of the protein sequence and comprises the former, was not found to decrease further sensitization in BALB/c, despite it exerts immunostimulating properties in splenocytes from β-lactoglobulin sensitized mice (Adel-Patient et al., 2012).

Preventive approaches using hydrolysates or peptides lead to reduced numbers of activated Th2 cells in the MLNs, following sensitization and allergen challenge of mice (van Esch et al., 2011b), as well as to decreased systemic Th2 responses, as shown by a diminished release of IL-4, IL-5 and IL-13- upon stimulation of spleen cells with the allergens used for sensitization

(Adel-Patient et al., 2012; Hacini-Rachinel et al., 2014). Changes in the Th1 response are, however, less consistent among the studies (Table 3). Combination of peptide treatment with a prebiotic diet that skews the immune response to a Th1 response may increase its tolerance-driving capacity (Meulenbroek et al., 2013). Conversely, co-administration of omega-6 polyunsaturated fatty acid-rich soya oil, which increases the Th2/Th1 ratio, abrogates peptide-induced tolerance (van den Elsen et al., 2015).

In addition, some studies showed a key role of T regs induced at the MLN level in the prevention of sensitization and development of food allergy (Adel-Patient et al., 2011; van Esch et al., 2011b; Meulenbroek et al., 2013; Hacini-Rachinel et al., 2014). MLNs connect the local and systemic immune system, making it possible to translate gut-induced regulatory responses to systemic tolerance. Indeed, adoptive transfer of MLN cells from tolerized donor mice inhibits sensitization and reduces consequent anaphylactic effects in recipient mice (van Esch et al., 2011b). The generation of a regulatory response by peptides is supported by a correlation between T regs and augmented tolerogenic $CD103^+$ DCs (Meulenbroek et al., 2013).

Conclusions

Progress in the understanding of the stimulating and anti-inflammatory properties of food peptides on cells of the innate and adaptive immune system has made apparent that they represent an attractive means to enhance safety and efficacy of allergen IT against food allergy. Moreover, the conclusions drawn from *in vivo* curative and preventive trials are promising, although there is a need for more pre-clinical studies to further explore the immunomodulating strategy and its mechanisms to maximice effective clinical translation. In particular, knowledge

of the peptide sequence and structural requirements which determine the immunomodulating action depending on the cell type, currently still poorly understood, will open new possibilities to impact the outcome of IT by differentially influencing the mechanisms by which oral tolerance to allergens is stablished.

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Table 1. Food protein hydrolysates and derived peptides with anti-inflammatory activity in the macrophage RAW 264.7 cell line

Peptide/hydrolysate	**RAW 264.7 stimulation**	**Main outcomes**	**Reference**
Lunasin	Lunasin pre-treatment for 6 h and LPS stimulation for 18 h	↓ROS, ↓TNF-α (23%) and ↓IL-6 (37%) production	Hernández-Ledesma et al., 2009
Lunasin-like peptide of 5000 Da	Simultaneous lunasin treatment and LPS stimulation for 24	↓NO (IC_{50}= 28 μM), ↓PGE2 (IC_{50}= 41 μM), ↓ IL-	de Mejía and Dia, 2009

	h	6 (IC_{50}= 2 μM) and ↓ IL-1β (IC_{50}= 13 μM) production COX-1 (no change), ↓iNOS (IC_{50}= 37 μM) and ↓COX-2 (IC_{50}= 25 μM) protein expression ↓NF-κB (IC_{50}= 21 μM) transactivation	

		↓P65 (IC_{50}= 48 μM) and ↓P50 (IC_{50}= 77 μM) nuclear translocation	
Soy flour hydrolysed with alcalase	Simultaneous hydrolysate treatment and LPS stimulation for 24 h	↓NO (18-35%) and ↓PGE2 (47-71%) production ↓iNOS (31-53%) and ↓COX-2 (30-52%) protein expression	Martínez-Villaluenga et al., 2009

Common bean flours hydrolysed with alcalase	Simultaneous hydrolysate treatment and LPS stimulation for 24 h	↓NO (IC_{50}= 4-48 μM) and ↓PGE2 (IC_{50}= 14-61 μM) production ↓iNOS (IC_{50}= 5-14 μM) and ↓COX-2 (IC_{50}= 35-44 μM) protein expression ↓NF-κB transactivation ↓P65 nuclear translocatio	Oseguera-Toledo et al., 2011

		n	
Germinated soybean flour hydrolysed with alcalase	Hydrolysate pre-treatment for 24 h and LPS stimulation	↓NO (21-69%), ↓PGE2 (64-88%) and ↓TNF-α (94-96%) production ↓iNOS (23-94%) and ↓COX-2 (36-77%) protein expression	Vernaza et al., 2012
Commercially available soy products hydrolysed with pepsin and pancreatin	Simultaneous hydrolysate treatment and LPS	↓NO (16-29%), ↓IL-1β (22-40%), ↓IL-6 (27-43%) and ↓TNF-	Dia et al., 2014

	stimulation for 24 h	α (48-333%) production ↓iNOS (65-88%) and ↓COX-2 (41-70%) protein expression	
Yellow pea proteins hydrolysed with thermolysin of less than 3000 Da	Hydrolysate pre-treatment for 12 h and LPS-IFN-γ stimulation for 24 h	↓NO (20%), ↓TNF-α (35%) and ↓IL-6 (80%) production	Ndiaye et al., 2012
Phosphopeptides derived from partial alkaline dephosphorylation and tryptic digestion of egg yolk	Peptide pre-	↓IL-6, ↓IL-1β,	Xu et al.,

phosvitin	treatment for 2 h followed by LPS stimulation for 6 h	↓TNF-α and ↓iNOS gene expression	2012
GCQQAVQSAV derived from clam (*Ruditapes philippinarum*) hydrolysed with alcalase	Peptide pre-treatment for 4 h followed by LPS stimulation for 18 h	IL6 (no change), PGE2 (no change) and ↓NO (48%) production ↓iNOS and ↓COX-2 protein expression	Lee et al., 2012
QCQCAVEGGL derived from Pacific oyster (*Crassostrea gigas*) hydrolysed with Protamex	Simultaneous peptide treatment	↓NO (72%) production	Hwang et al., 2012

	and LPS stimulation for 18 h		
GVSLLQQFFL derived from mussel (*Mytilus coruscus*) hydrolysed with Flavourzyme	Simultaneous peptide treatment and LPS stimulation for 18 h	↓NO (62%) production	Kim et al., 2013
Almond proteins hydrolysed with pepsin and pancreatin and the fraction higher than 5000 Da	Simultaneous peptide treatment and LPS stimulation	↓NO production ↓TNF-α, ↓IL-1β, ↓ IL6 production and gene expression ↓iNOS and	Udenigwe et al., 2013

		↓COX-2 protein and gene expression	
Gluten-derived pyroglutamyl-leucine	Simultaneous peptide treatment and LPS stimulation for various periods	↓NO, ↓TNF-α and ↓IL6 production ↓IκBα degradation ↓JNK, ↓ERK and ↓p38 phosphorylation	Hirai et al., 2014
Extruded amaranth hydrolysed with pepsin and pancreatin	Simultaneous peptide treatment and LPS	↓PGE2 (32%) and ↓TNF-α (34%) production	Montoya-Rodriguez et al., 2014

	stimulation for 24 h	↓COX-2 (67%) protein expression ↓IKK (88%) and ↓IκBα (66%) phosphorylation	
Tuna hydrolysed with alcalase and synthetic peptides PRRTRMMDGGR and MGPAMMRTMPG	Hydrolysate or peptide pre-treatment for 24 h and LPS stimulation for 24/28 h	↓IL-2, ↓TNF-α and ↓TNF-γ production	Cheng et al., 2015
Salmon myofibrillar protein conjugated with alginate	Simultan	↓NO	Saigusa

hydrolysed with pepsin and trypsin	eous hydrolysate treatment and LPS-IFN-γ stimulation for 24 h	(66%), ↓IL-6 (85%) and ↓TNF-α (67%) production COX-1 (no change), ↓iNOS, ↓IL-6, ↓COX-2 and ↓TNF-α gene expression	et al., 2015
Fermented (*Lactobacillus plantarum*) abalone (*Haliotis discus hannai Ino*)	Hydrolysate pre-treatment for 24 h and H_2O_2 stimulation	↓ROS (IC_{50}= 6.78 mg/mL), ↓NO and ↓PGE2 production	Hasnat et al., 2015

	Simultaneous or hydrolysate pre-treatment (18 h) and LPS stimulation for 24/28 h		
KAFAVIDQDKSGFIEEDELKLFLQNFSAGARAGDSDGDGKIGVDEFAALVK and AFAVIDQDKSGFIEEDELKLFLQNFSAGARAGDSDGDGKIGVDEFAALVK derived from tilapia (*Oreochromis niloticus*) hydrolysed with Flavourzyme	Peptide pre-treatment for 1 h and H_2O_2 stimulation on Simultaneous peptide treatment	↓ROS (83%) and ↓NO (40.9%) production	Kangsanant et al., 2015

	and LPS stimulation for 48 h		
PAY derived from salmon hydrolysed with pepsin	Simultaneous peptide treatment and LPS stimulation for 24 h	↓NO (64%), ↓PGE2 (45%), ↓IL-6 (44%), ↓TNF-α (58%) and ↓IL-1β (65%) production ↓iNOS (0.75-fold) and ↓COX-2 (0.48-fold) protein	Ahn et al., 2015

		expression	

Main outcomes: COX-1=cyclooxygenase-1; COX-2=cyclooxygenase-2; IκBα= nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha; IKK= IκB kinase; iNOS= inducible nitric oxide synthase; JNK, ERK and p38= mitogen-activated protein kinases; NF-κB= nuclear factor kappa-light-chain-enhancer of activated B cells; PGE2= prostaglandin E2; p50/p65= members of the Rel family of transcription factors; ROS= reactive oxygen species; ↑= increased or high levels; ↓= decreased or low levels

Table 2. Summary of successful immunotherapy trials in mouse models of food allergy with peptides and hydrolysates from allergenic proteins

Peptide/hydrolysate	**Experimental design**	**Main outcomes**	**Reference**
Egg white (EW) hydrolysed with a food grade aminopeptidase from *Aspergillus* sp. (EWH)	*Animal model:* BALB/c mice *Sensitization:* EW (5 mg) + CT (10 μg) i.g. (2 weekly doses for 4 weeks) *IT:* EWH (5mg) i.g. (3 weekly doses for 3 weeks) *Challenge:* EW (20 mg) i.g.	↓Histamine release ↓EW-specific IgE ↓IL-4 and ↓ IFN-γ released by EW stimulated splenocytes ↓IL-4, ↓IL-13, ↓IFN-γ, ↓IL-12p40, ↑IL-8, ↑IL-5,↑FoxP3 and ↑TGF-β intestinal (ileum) gene expression	Yang et al., 2009b
Mixture of ovalbumin (OVA) immunodominant T cell epitopes (15-mer): AMVYLGAKDSTRTQ SWVESQTNGIIRNVL	*Animal model:* BALB/c mice *Sensitization:* OVA (1 mg) + CT (10 μg) i.g. (2 weekly doses for 4 weeks)	↓Symptom score ↓Histamine release ↓OVA-specific IgE ↑OVA-specific faecal IgA ↓IL-4 and ↑IFN-γ	Yang et al., 2010

AAHAEINEAGREVVG	*IT:* peptide mix (100 µg each) s.c. (3 weekly doses for 3 weeks) *Challenge:* OVA (20 mg) i.g.	released by OVA stimulated splenocytes ↓IL-5, ↓IL-13, ↑IFN-γ, ↑FoxP3 and ↑TGF-β intestinal (ileum) gene expression	
Ovomucoid (OM) immunodominant T cell epitope (15-mer): DNKTYGNKSNFSNAV	*Animal model:* BALB/c mice *Sensitization:* OM (1 mg) + CT (10 µg) i.g. (2 weekly doses for 4 weeks) *IT:* peptide (1 mg) i.g. (3 weekly doses for 4 weeks) *Challenge:* OM (20 mg) i.g.	↓Symptom score ↓Histamine release ↓OM-specific IgE, ↑IgG1,↓IgG2a, ↑faecal IgA ↓IL-4, ↑IL-12, ↑IL-10 released by OM stimulated splenocytes ↑circulating T regs (↑$CD4^+CD25^+$, ↑$CD4^+FoxP3^+$ cells)	Rupa and Mine, 2012
Native cashew protein (nCSH) hydrolysed with pepsin (pCSH)	*Animal model:* C3H/HeJ mice *Sensitization:* nCSH (2 mg) + CT (10	↓Symptom score and body temperature drop No change in nCSH specific IgE	Kulis et al., 2012

	μg) i.g. (4 weekly doses for 4 weeks) *IT:* pCSH (from 50 to 200 μg) i.p. (3 weekly doses for 4 weeks) *Challenge:* nCSH (0.25 mg) i.p. or pCSH (1 mg) i.p	↑nCSH-specific IgG1 ↑nCSH-specific IgG2a ↓IL-5 and ↓IL-13 released by nCSH stimulated splenocytes	
β-lactoglobulin (BLG) immunodominant T cell epitope AQKKIIAEKTKIPAVFKIDALN	*Animal model:* BALB/c mice *Sensitization:* BLG (50 μg) + alum i.p. (1 weekly dose for 3 weeks) *IT:* peptide (1 mg) i.p. (3 weekly doses for 4 weeks) *Challenge:* BLG (50 mg) i.g.	↓Symptom score and body temperature drop No change in BLG-specific IgE, IgG1, IgG2 or faecal IgA ↑IFN-γ, ↑IL-12, ↑IL-10 released by BLG stimulated splenocytes	Thang and Zhao, 2015

Mixture of 6 shrimp tropomyosin (Met e 1) immunodominant T cell epitopes	*Animal model:* BALB/c mice *Sensitization:* recombinant Met e 1 (0.1 mg) + CT (10 μg) i.g. (1 weekly dose for 5 weeks) *IT:* peptide mix (1.2 mg each) i.g. (2 weekly doses for 4 weeks) *Challenge:* recombinant Met e 1 (0.5 mg) i.g.	↓Symptom score, diarrhoea and intestinal inflammation ↓mMCP-1 ↓Met e 1-specific IgE ↑Met e 1-specific IgG2a ↓IL-4, ↓IL-5, ↑IFN-γ, ↑IL-12 released by Met e1 stimulated splenocytes ↑IL-10, ↑FoxP3, ↑CD25, ↑RUNX1, ↑RUNX3, ↓IL-5, ↓IL-13, ↑FN-γ intestinal (ileum) gene expression	Wai et al., 2016

Experimental design: i.g.= intragastric; i.p..= intraperitoneal; s.c.= subcutaneous; CT= cholera toxin. **Main outcomes:** mMCP-1= mouse mast cell protease 1; RUNX1= Runt-related transcription factor 1; RUNX3= Runt-related transcription factor 3;↑= increased or high levels; ↓= decreased or low levels

Table 3. Summary of successful preventive treatment trials in mouse models of food allergy with peptides and hydrolysates from allergenic proteins

Peptide/ hydrolysate	**Experimental design**	**Main outcomes**	**Reference**
Standard formula of intact cow's milk proteins Whey proteins (WP) partially hydrolysed with trypsin	*Animal model:* Sprague-Dawley rats *Preventive treatment:* Oral administration in the drinking water during 19 days (*ad libitum*) *Sensitization:* β-lactoglobulin (BLG) (100 µg) + AH i.p. (1 dose) *Challenge:* WP (1 g) i.g.	↓RMCPII ↓BLG-specific IgE ↓BLG-specific IgG ↓Lymphocyte proliferation by BLG-stimulated PLN cells	Fritsché et al., 1997
β-lactoglobulin (BLG) BLG hydrolysed with trypsin (TTH-BLG) Peptide fractions	*Animal model:* BALB/c mice *Preventive treatment:* BLG (5 mg/g body weight), TTH-BLG (2.5 mg/g) or peptide fractions (0.125	↓Footpad thickness ↓BLG-specific IgE ↓Lymphocyte proliferation by BLG-stimulated splenocytes	Pecquet et al., 2000

separated from TTH-BLG by ion exchange chromatography	mg/g) i.g. *Sensitization:* BLG (80 µg) + AH (1 dose) *Challenge:* BLG (100 µg) i.d. in the footpad		
Standard formula of intact cow's milk proteins (CM, Nan) CM partially hydrolysed (Nan-HA)	*Animal model:* C3H/HeN mice *Preventive treatment:* Oral administration at 2% (1 to 4 weeks) *Sensitization:* β-lactoglobulin (BLG), α-lactalbumin (ALA) or BSA (10 µg) + alum i.p. (1 dose)	↓BLG, ALA and BSA-specific IgE (1 week for Nan, 4 weeks for Nan-HA) ↓BLG, ALA and BSA - specific IgG (1 week for Nan, 4 weeks for Nan-HA) ↓BLG ALA and BSA, specific IgG1 (1 week for Nan, 4 weeks for Nan-HA)	Peng et al., 2004
Whey proteins (WP) Partially hydrolysed WP (pWH)	*Animal model:* C3H/HeOuJ mice *Preventive treatment:* WP and pWH (50 mg) i.g. (1 daily dose for 6 days) *Sensitization:* WP (20 mg) + CT (10 µg) i.g. (1	↓Acute skin response ↓mMCP-1 ↓WP-specific IgE and IgG1 (WP) ↑MLN T regs (pWH), ↑MLN Th1 (WP), ↓MLN Th2 (pWH and WP)	van Esch et al., 2011b

	weekly dose for 6 weeks) *Challenge:* WP (10 µg) i.d.		
β-lactoglobulin (BLG) BLG hydrolysed with trypsin (BLG-Try)	*Animal model:* BALB/c mice *Preventive treatment:* BLG (0.05-4 mg) and BLG-Try (2 mg) i.g. (6 doses in 10 days) *Sensitization:* BLG (5 µg) + alum i.p. (2 doses in 14-18 days) *Challenge:* BLG (10 mg) i.g.	↓mMCP-1 (BLG) ↓BLG-specific IgE ↓BLG-specific IgG1: (BLG) ↓IL-4, ↓IL-5 ↓IL-10 ↓IL-12 ↓IL-17 ↓IL-5 released by BLG stimulated splenocytes (BLG) ↑MLN T regs (↑ $CD4^+CD25^+$ $FoxP3^+$ cells) ↑PP and spleen T regs (↑ $CD4^+CD25^+FoxP3^+$ cells) (BLG)	Adel-Patient et al., 2011
Native β-lactoglobulin (nBLG) Denatured BLG (dBLG) nBLG hydrolysed with CNBr (nBLG-	*Animal model:* BALB/c mice *Preventive treatment:* nBLG, dBLG, nBLG-CNBr and dBLG-CNBr (2.4 mg) i.g. (6 doses in 10 days) *Sensitization:* nBLG (5 µg)	↓BLG-specific IgE ↓BLG-specific IgG1 ↓IL-4, ↓IL-5, ↓IL-13, ↓IL-10 released by BLG stimulated splenocytes	Adel-Patient et al., 2012

CBr) dBLG hydrolysed with CNBr (dBLG-CBr)	+ alum i.p. (1 dose)		
β-lactoglobulin (BLG) immunodominant T cell epitopes: BLG 31-48 Mix 1: 13-30, 19-36, 25-42, 31-48 Mix 3: 139-156, 145-162	*Animal model:* CeH/HeOuJ mice *Preventive treatment:* peptide mix or single peptide (4 mg each) i.g. (1 daily dose during 6 days) *Sensitization:* WP (20 mg) + CT (10 µg) i.g. (1 weekly dose for 5 weeks) *Challenge:* WP (10 µg) i.d. and WP (100 mg) i.g.	↓ Acute skin response ↓ WP-specific IgE ↓ WP-specific IgG2a ↑ MLN T regs (↑ $CD4^+CD25^+$ $FoxP3^+$ cells) (BLG 31-48) ↑ MLN DCs ($CD11b^+CD103^+$) (BLG 31-48)	Meulenbroek et al., 2013
Whole hen's egg (WE) WE hydrolysed with Protamex and Flavourzyme	*Animal model:* C57BL/6J mice *Preventive treatment:* Oral administration of OVA, WE, and HE (5 times more	↓Acute skin response ↓OVA-specific IgE ↓OVA-specific IgG1 ↓Proliferation of OVA	Hacini-Rachinel et al., 2014

1000L (HE)	concentrated) in the drinking water during 5 days (*ad libitum*) *Sensitization:* Ovalbumin (OVA) (100 µg) + alum (2 doses in 13 days) s.c. *Challenge:* OVA (20 µg) i.d.	stimulated splenocytes ↓IL-5, ↓IL-4, ↓IFN-γ, ↓IL-10, IL-12 released by OVA stimulated splenocytes ↑MLN T regs (↑ $CD4^+CD25^+$ $FoxP3^+ICOS^+$cells)	
Partially hydrolysed whey proteins (pWP)	*Animal model:* C3H/HeOuJ *Preventive treatment:* pWP (50 mg) i.g. (1 daily dose for 5 days) *Sensitization:* WP (20 mg) + CT (10 µg) i.g. (1 weekly dose for 6 weeks) *Challenge:* WP (10 µg) i.d. and WP (50 mg) i.g.	↓Acute skin response and symptoms score ↓WP-specific IgE	Van den Elsen et al. 2015

Peptide/hydrolysate: CNBr=cyanogen bromide. **Experimental design:** i.g.= intragastric; i.d.= intradermal; i.p.= intraperitoneal; s.c.= subcutaneous; CT= cholera toxin, AH= aluminium hydroxide. **Main outcomes:** mMCP-1= mouse mast cell protease 1; RMCPII= rat mast cell protease II; MLN= mesenteric lymph nodes; PLNs= peripheral lymph nodes; PPs = Peyer's patches; DCs= dendritic cells; ↑= increased or high levels; ↓= decreased or low levels