

REVIEW ARTICLE

Immunomodulating properties of protein hydrolysates for application in cow's milk allergy

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Abstract

Cow's milk proteins cause allergic symptoms in 2–3% of all infants. In these individuals, the tolerogenic state of the intestinal immune system is broken, which can lead to sensitization against antigens and eventually to allergic responses. Although a true treatment for food allergy is not available, symptoms can be avoided by providing the infants with hydrolyzed proteins. Hydrolyzed proteins are proteins that are enzymatically degraded. They lack typical allergenic IgE-binding epitopes but are also thought to play a pertinent role in other mechanisms inducing hypoallergenic effects. This review discusses the mechanisms and evidence for immunomodulating properties of cow's milk hydrolysates. Hydrolysates are found to strengthen the epithelial barrier, modulate T-cell differentiation, and decrease inflammation. Some studies suggest a role for hydrolysates in manipulating pathogen recognition receptors signaling as underlying mechanism. Peptides from hydrolysates have been shown to bind to TLR2 and TLR4 and influence cytokine production in epithelial cells and macrophages. Current insight suggests that hydrolysates may actively participate in modulating the immune responses in subjects with cow's milk allergy and those at risk to develop cow's milk allergy. However, more research is required to design effective and reproducible means to develop targeting strategies to modulate the immune response.

Awareness of food allergy as a serious health issue is increasing in the Western world. Its burden on morbidity, quality of life, and healthcare costs is more and more recognized (1, 2). Despite this, there is a lack of treatment options. The only reliable therapy up to now is avoidance of allergens. To design more effective treatments, more insight is required in the mechanisms associated with food allergy.

A type of food allergy that has been subject of many studies is cow's milk allergy in infants. Infant formula, which is often cow's milk based, is the only approved alternative for breastfeeding. However, cow's milk proteins cause allergic symptoms in 2–3% of the infants (3). These allergic symptoms can be avoided by providing the infants with so-called hydrolysates of cow's milk protein instead of the intact proteins (4). Peptides in hydrolysates possess different immunological properties which prevent allergy (5).

The mechanisms by which hydrolysates modulate allergic responses are still subject of debate. One of the mechanisms is that after being taken up by intestinal epithelial cells and presentation of the peptides to the gastrointestinal immune

system, the response is different and more tolerogenic than the response against the larger proteins (6). The gastrointestinal immune system therefore seems to play an essential role. The barrier is composed of an epithelial layer and a variety of immune cells (7). It is covered with mucus. The proteins and peptides are taken up by the epithelial cells and presented to the mucosal immune system. Here, they interact with the gastrointestinal immune system (Fig. 1) to either induce tolerance or an immune response. In this review, we will give an overview on current knowledge on immunomodulatory properties of cow's milk hydrolysates and its interaction with the mucosal immune system. To do this, we will first discuss the immunological processes involved in maintaining oral tolerance and the onset of an allergic reaction.

Large proteins can cause allergy in infants

Newborns have specific dietary needs. To achieve optimal growth, development and health in the first months of life, breast milk is required. When breastfeeding is not possible, infant formula is the only approved infant nutrition. However,

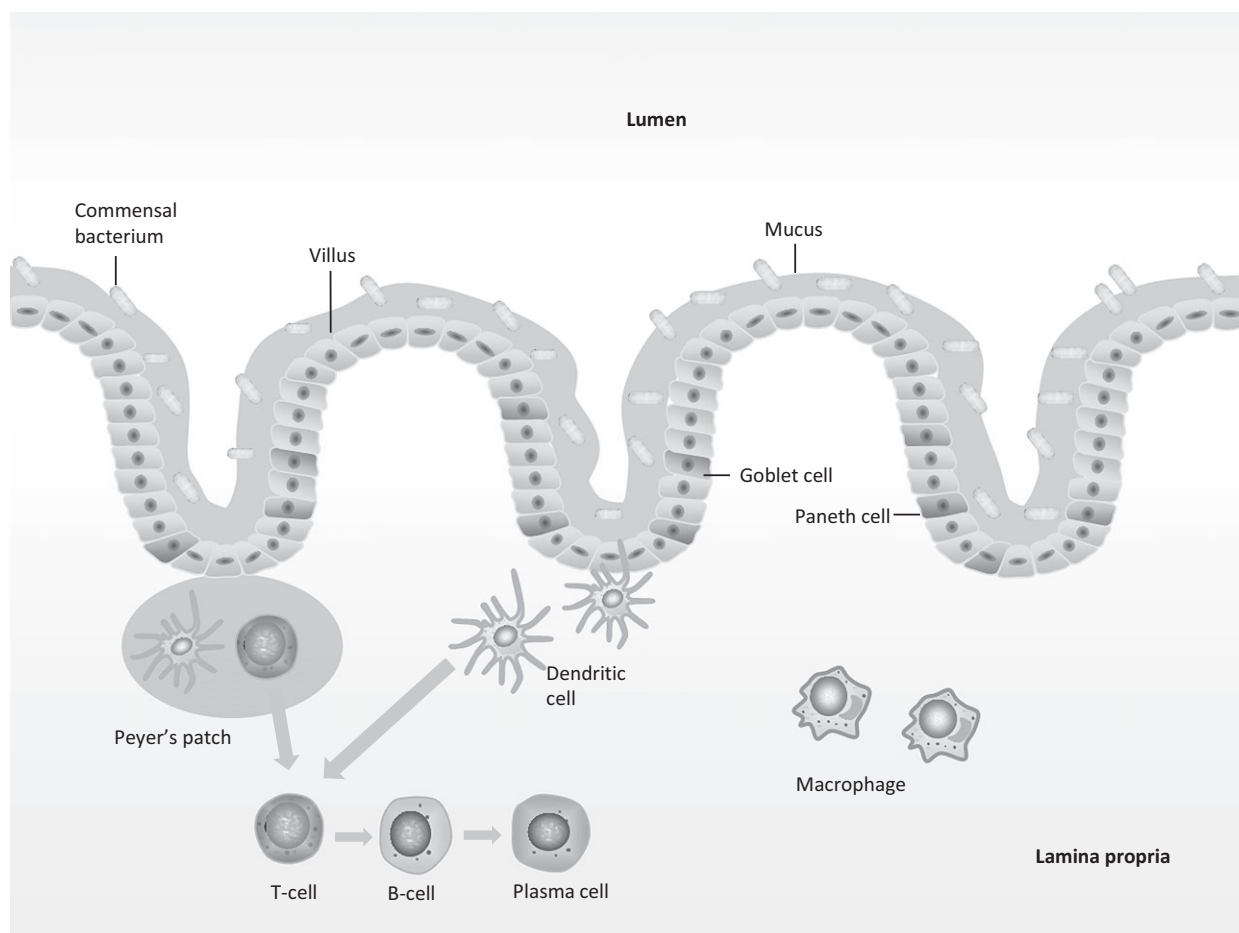


Figure 1 Layers of the intestinal immune barrier. The barrier consists of a protective layer of epithelial cells covered with mucus and a range of immune cells. The main cell type in the epithelial layer is gate-keeping epithelial cells, but other specialized cells are also present. The crypts of the villi contain both mucus-producing goblet cells and antibacterial compound secreting Paneth cells. The lamina propria under this epithelial layer contains many immune cells including dendritic cells, macrophages, and lymphocytes. After antigen passed the epithelial barrier, it is taken up by dendritic cells. The antigen-presenting dendritic cells migrate to the mesenteric lymph nodes and presented their MHC-antigen complex to T cells inducing T-cell activation. In the small intestine, Peyer's patches are present. In these organized lymphoid nodules, antigen-presenting dendritic cells also interact with T cells. T cells then regulate the immune response activating other immune cells, for example, B cells.

this is not without consequences. Infants are not yet able to digest all the novel enteral nutrients. The immature infant's digestive system is not producing enough enzymes essential for protein digestion, such as gastric pepsin (8). Also the relatively high pH in the infant's stomach does not accommodate optimal digestion of proteins (9). This results in partly or undigested proteins in the gastrointestinal tract of the infant during a vulnerable period for the development of food allergy. As a result, more and larger proteins may pass the gastrointestinal barrier and induce, in the presence or absence of a pathogen, an aberrant Th2-skewed immune response (10). This aberrant immune response is the first step toward allergic sensitization.

Furthermore, an important characteristic of the infant's intestine is the relatively high permeability of the epithelial layer separating the intestinal lumen from the mucosal immune

system (11), which is associated with allergic reactions (10). This can also contribute to a higher uptake of larger proteins, although it is more related to the later allergic reactions than to the sensitization phase.

To understand where and how food allergy against proteins can develop, we first need to discuss the comprehensive regulatory system that is involved in tolerance induction.

The gastrointestinal epithelial cells as gatekeeper

The first line of defense against intruders is, as outlined above, the epithelial cells separating the intestinal lumen from the mucosal immune system. The epithelial cells are highly organized and connected by tight junctions on both the apical and basolateral site. In adults, the connections make the barrier impermeable for molecules larger than

3.5 kDa (12). For infants, the exact permeability for macromolecules is not known and depends on birthweight, gestational and post-natal age (13). Epithelial cells carry the polymeric Ig receptors on the basolateral side that continuously transports neutralizing IgA antibodies into the lumen, which bind to harmful antigens that are then expelled by the peristaltic process (12).

The permeability of the epithelial layer is variable over time. Binding of food components to immune-related receptors can actively increase and decrease permeability to sample antigens from the lumen (14). Such receptors are for instance pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) (15). Binding of a luminal antigen to PRRs can lead to release of serine-proteases such as zonulin by epithelial cells (16). These serine-proteases digest the tight junctions between epithelial cells (17), which leads to an increase in permeability and consequently entry of luminal antigens in the lamina propria (Fig. 2). How often this occurs and how it

quantitatively contributes to tolerance for specific food antigens is unknown.

Another, more in detail-studied process, is antigen sampling by dendritic-like cells that are located in between the epithelial cells. These dendritic cells, often referred to as the CD11b+CX3CR1+ cells, create protrusions into the lumen of the gastrointestinal tract to sample its contents (18). The major part of luminal antigen sampling, however, is not occurring by the epithelial or dendritic cells but in the gut-associated lymphoid tissues (GALT). In the small intestine, these are called the Peyer's patches. Here, specialized epithelial cells called M-cells transport luminal antigens to the underlying dendritic cell-rich area (19). Here, the antigens are taken up by antigen-presenting cells (APC) (mainly dendritic cells) and processed for presentation to T cells. These dendritic cells are referred to as the CD103+CX3CR1-. They are found in high quantities in the lamina propria and take up food components, including proteins, that are sampled by the epithelial cells or

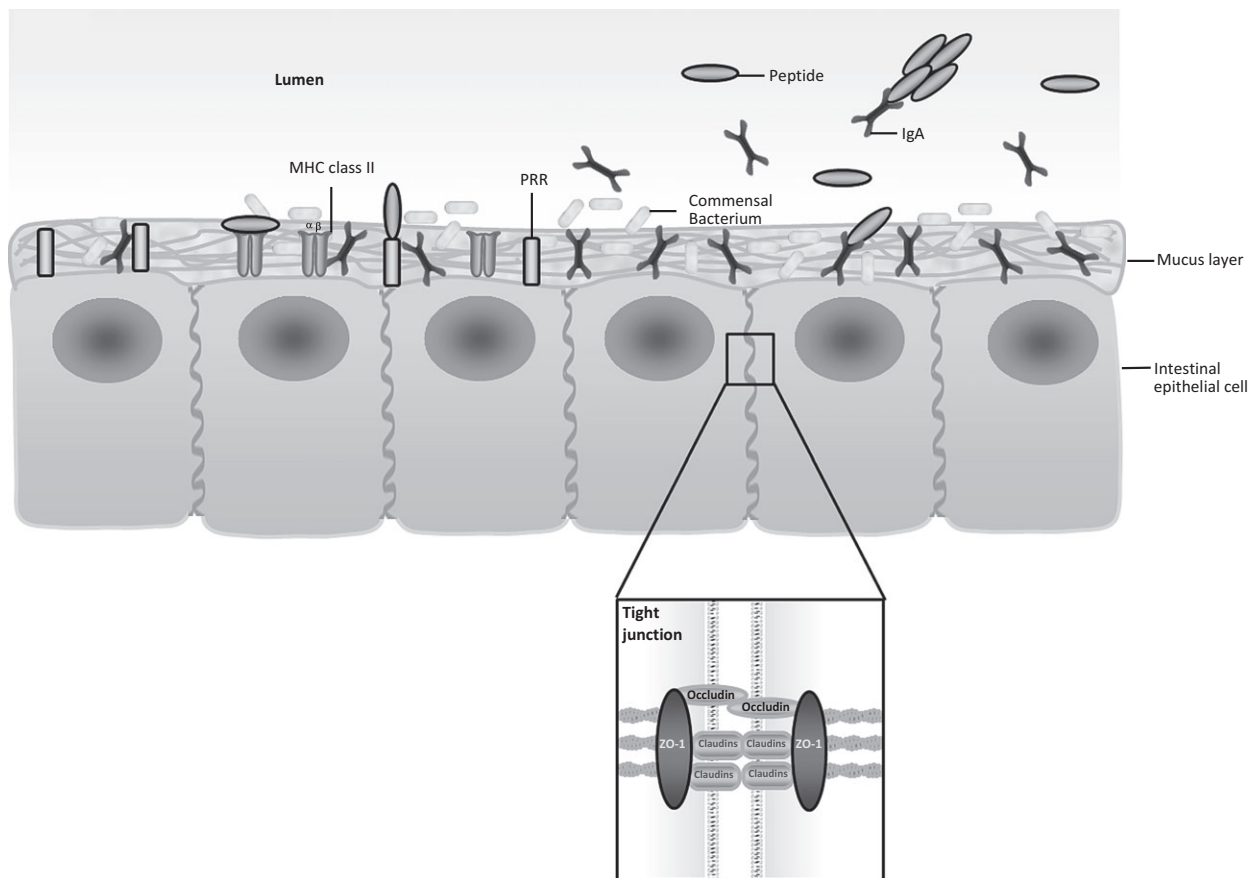


Figure 2 The intestinal epithelium separates the lumen of the intestine from the lamina propria and the immune system. It keeps pathogens and antigen from entering the body. This layer of epithelial cells is covered by a thick layer of mucus. The mucus layer is a reservoir for IgA, which agglutinates peptides. This stimulates expelling of the peptides. Epithelial cells are joined together by tight junctions. These structures, consisting mainly of the proteins ZO-1, occludin, and claudin, make the paracellular spaces impenetrable for most proteins. However, the permeability of the epithelial layer is not always that low. Epithelial cells express receptors including MHC class II and pattern recognition receptors. Binding of food-derived molecules to these receptors is able to regulate the permeability.

CD11b+CX3CR1+ dendritic cells. Currently, the CD103+CX3CR1- dendritic cells are considered to be the only dendritic cell population that is able to migrate to mesenteric lymph nodes (20), to induce tolerogenic responses. To do so, the cells need appropriate tolerogenic signals. An important source of tolerogenic factors is the epithelial cells (21). When the epithelial cells secrete factors such as IL-10, TGF- β , and TSLP (21), CD103+CX3CR1- dendritic cells differentiate into tolerogenic dendritic cells, before presenting their antigens to T cells in the mesenteric lymph nodes.

Adaptive tolerogenic T-cell responses to food antigens

Depending on the amount of antigen, three distinct immunological mechanisms can contribute to immunological tolerance to food antigens, that is, induction of regulatory T cells (Tregs), clonal anergy of T cells, and deletion of T cells (21). Tregs are mainly generated by CD103+CX3CR1- dendritic cells in the mesenteric lymph nodes. Here, the tolerogenic CD103+CX3CR1- dendritic cells present the food antigens to CD4+ T cells. Tolerogenic CD103+CX3CR1- dendritic cells, but also macrophages, mostly express IL-10, and TGF- β , which induce Treg formation (22). Upon interaction of dendritic cells with the Treg subtype Th3, a cascade of Treg subtype induction and maintenance is initiated, while the secreted TGF- β at the same time inhibits Th1 and Th2 subtype T cells (21). This type of tolerance induction is only initiated when the food antigen is present in lower doses. This mechanism is considered to be a central mechanism of tolerance induction for food antigens.

At higher doses tolerance is mainly caused by clonal deletion and clonal anergy. PRR-mediated activation, and especially TLR signaling, has also been found to be essential in these processes. TLRs have been shown to function as co-stimulatory molecule in the interaction between T cells and dendritic cells (23). By this, TLRs influence the T-cell response, as peptide-MHC-II-complex presentation together with a lack of co-stimulatory molecules on dendritic cells during high antigen exposure is thought to cause T-cell clonal anergy (24). Clonal deletion of T cells is the other process that occurs at high antigen exposure. This usually occurs in the Peyer's patches. It is thought to be caused by natural killer T cells, possibly via Fas-FasL interaction with T cells that leads to apoptosis of food antigen recognizing T cells (25) (Fig. 3).

Food allergy through broken tolerance

Sensitization to a food antigen may occur, when one of the above-mentioned processes fails. The antigen, such as a food protein, then causes an inappropriate Th2-skewed immune response. In the sensitization phase, antigens are processed by APCs and presented to T cells. These T cells become activated Th2 cells, which stimulate class switching to IgE and B cell differentiation into B plasma cells by secreting a mix of cytokines, including IL-4, IL-5, and IL-6 (26). The antigen-specific IgE antibodies bind to the high-affinity IgE receptor Fc ϵ RI on mast cells and basophils. Re-exposure to the same

antigen results in cross-linking of the IgE antibodies bound to Fc ϵ RI, inducing degranulation of the mast cells, which causes an immediate hypersensitivity reaction (27). Mast cells release several inflammatory mediators including histamine, which contributes to clinical symptoms such as nausea, vomiting, and abdominal pain (27) (Fig. 4). Upon activation, mast cells also secrete chymases, and various cytokines, such as TNF α , IL-13, and IL-8 (28), that can break open the tight junctions between epithelial cells, which allows more dietary antigens to cross the epithelial barrier. These changes in barrier function after antigen exposure lead to changes in osmotic water pressure over the epithelial barrier with diarrhea as a consequence (29). When the barrier cannot be closed the result will be severe inflammation which subsequently leads to more epithelial breakdown.

The immediate hypersensitivity reaction can be followed by a T-cell-mediated late phase response, which occurs 12–48 h after antigen exposure (30) and is caused by the continuous presence of antigen (31). During this late phase reaction, Th2 cells and intestinal epithelial cells keep producing Th2-related cytokines such as IL-4, IL-5, IL-9, and IL-13 (28). This leads to maintenance of high IgE levels, elevated mucus production and the infiltration of basophils, eosinophils, and lymphocytes (32).

Beyond Th1 and Th2 cells

Although food allergy was classically thought to be a Th2-type response, recent studies have shown that the Th1/Th2 paradigm is an oversimplified view of the real situation. Besides Th1 and Th2 T cells, other T-cell subtypes, such as Th17 and Th22, have been identified and found to be important in gut homeostasis (33). In healthy individuals, low numbers of Th17 cells are present, mainly in the lamina propria (34). During infection, IL-17 induces the recruitment of neutrophils, and the epithelial increase of chemokine CCL20 attracts more Th17 cells (35). Current insight suggests Th17 cells also play a role in cow's milk allergy as will be outlined below.

Th22 cells produce IL-22. IL-22 has been found to be essential in the defense against Gram-negative bacteria in the intestine (36), showing its important role in the regulation of host defense and homeostasis. IL-22 elicits these protective effects by inducing for example the expression of antimicrobial peptides (36).

The above suggest that both Th17 and Th22 cells are important in maintaining homeostasis in the intestine and thus protect the state of tolerance. Although it is well established that these cells play a role in other allergic diseases, such as allergic airway inflammation (37), their exact role in cow's milk allergy needs more attention of researchers. Earlier, a trend toward a negative correlation between whole blood IL-17 levels and sensitization to some food antigens was described (38), which is in line with the study of Duhban and d'Hennezel who found a lower production of IL-17 in CD4+ T cells in children with food allergies compared to healthy controls. *In vitro*, CD4+ T cells from these allergic children showed impaired IL-17 production after antigen administration (39).

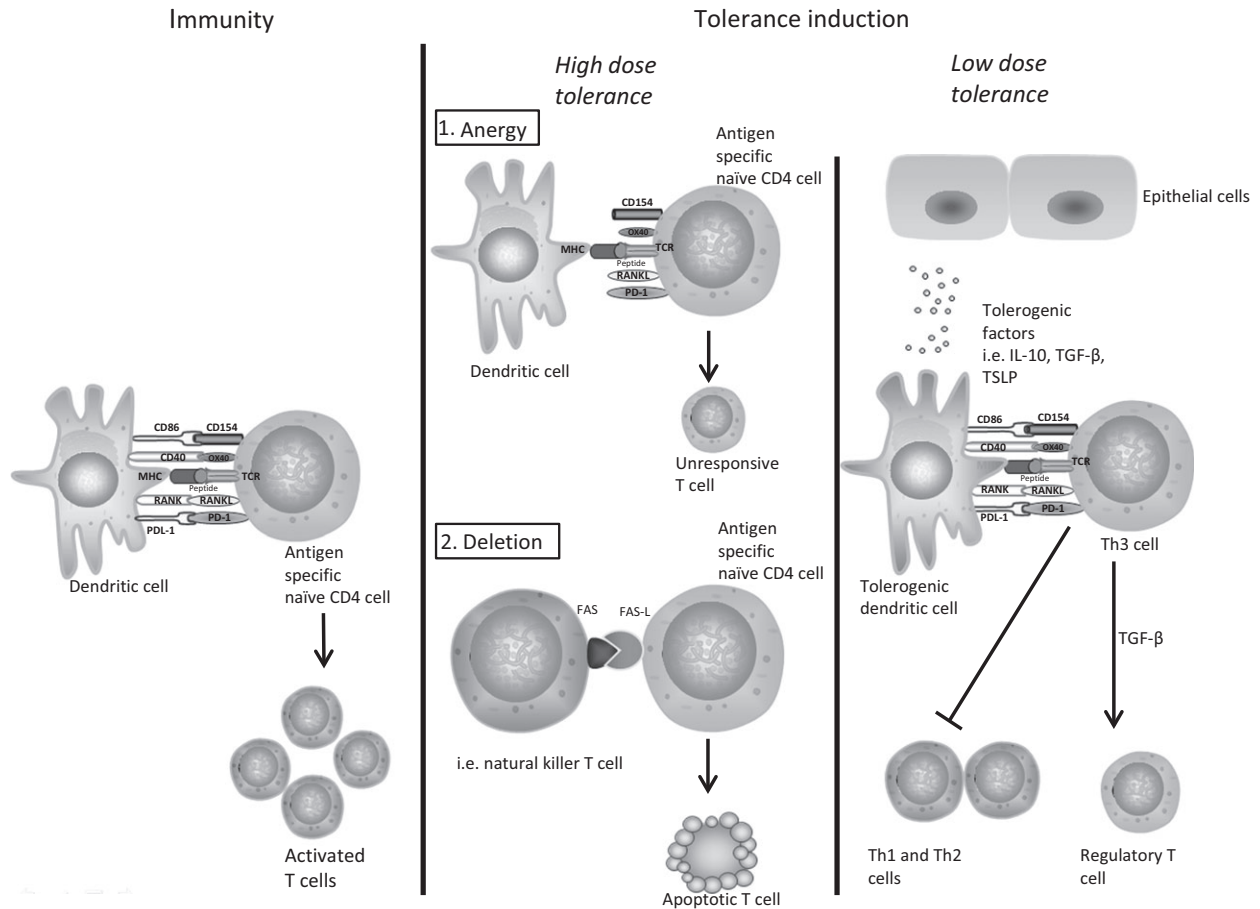


Figure 3 Mechanisms of oral tolerance induction. In a normal situation, immunity is induced by the interaction between an antigen-presenting dendritic cell and a naïve T cell. Due to the binding of the TCR (T-cell receptor) with the MHC-peptide complex and the co-stimulatory molecules the T cell gets activated. However, the default state in the gut is tolerance against dietary antigens. Different mechanisms are involved in inducing tolerance. When a high dose of antigen is present, the main mechanisms involved are anergy and deletion of T cells. Anergy occurs when co-stimulatory molecules are lacking during the interaction between dendritic cells and T cells. This leads to an unresponsive T cell. Deletion also occurs at high-antigen levels. Antigen-specific T cell go into Fas–Fasligand-induced apoptosis after interacting with, for example, a natural killer T cell. The main mechanism inducing tolerance at a low dose of antigen is the induction of regulatory T cells. Tolerogenic factors produced by epithelial cells result in a more tolerogenic dendritic cell. When this cell interacts with a T-helper 3 cell, the T cell produces TGF- β , which leads to differentiation towards a regulatory T cell and the inhibition of Th1 and Th2 cells.

Possibility to prevent/treat allergy with hydrolyzed proteins

As outlined before allergic reactions against cow's milk protein in cow's milk allergic infants can be prevented or avoided by administration of hydrolysates of cow's milk. During hydrolysis of the original protein, mixtures of smaller peptides with different properties can be produced. The formulas available differ by the degree of hydrolysis. There are extensively or partially hydrolyzed formulas available, made from whey or casein. Extensively hydrolyzed formulas are intended to avoid allergic reactions in already cow's milk allergic infants. This hypoallergenic effect has longer been known to be due to the destruction of the epitopes on the proteins, which are respon-

sible for IgE binding (40). Partially hydrolyzed formulas are used for infants at risk for developing food allergy (41) and are (together with extensively hydrolysates) now more and more considered to be hypoallergenic by modulating the immune response and therewith preventing sensitization. Some peptides in hydrolysates (but also proteins in cow's milk) have indeed been described to actively influence the immune system and modulate the allergic response (42). When encountering the epithelial cells and CD11b+CX3CR1+ dendritic cells in the intestine peptides can bind to specialized PRRs involved in inducing tolerogenic responses (43). By binding to the receptors, which are able to recognize a variety of molecules, hydrolysates may be able to affect the epithelial barrier or the dendritic cells.

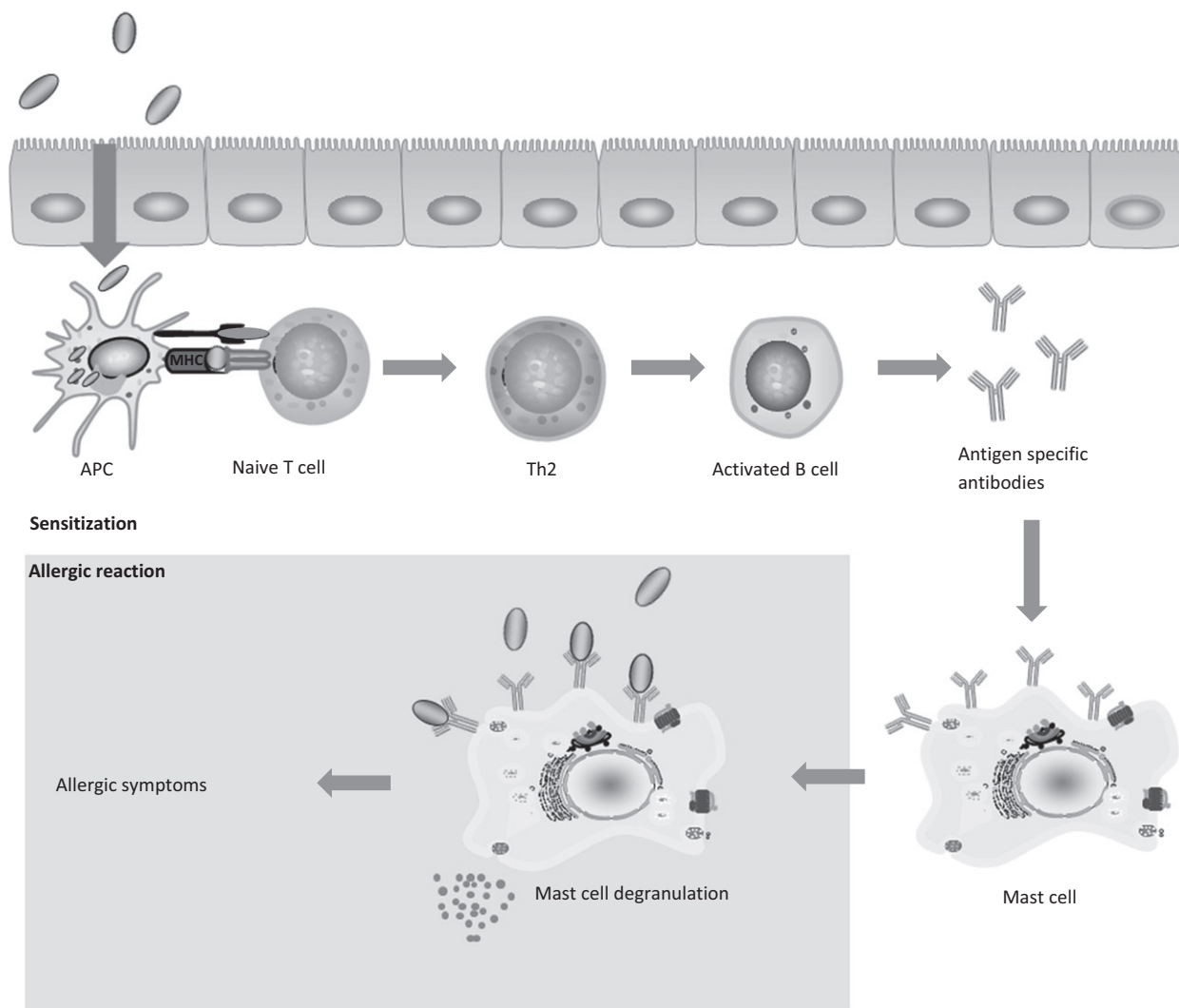


Figure 4 Overview of the events during sensitization and an allergic reaction. When antigen passed through the epithelial barrier, it is taken up and processed by antigen-presenting cells (APCs). These cells present the antigen in a MHC class II molecule together with co-stimulatory molecules to antigen-specific naive T cells, which get activated and develop a Th2 phenotype. Activated T cells stimulate B cells differentiation into plasma cells, which start to produce antigen-specific antibodies (IgE). These antibodies bind to the high-affinity receptor Fc3R1 on mast cells. Upon re-exposure of the same antigen the antibodies cross-link and degranulation of the mast cell is induced. The release of a range of compounds results in allergic symptoms.

Jaziri et al. (44) already proved in 1992 the concept of peptides binding to specific receptors. They showed that the immunostimulating peptides Gly-Leu-Phe (GLF) and Val-Glu-Pro-Ile-Pro-Tyr (VEPIP), which were isolated from casein, bound to specific sites on human phagocytic blood cells. Although the binding sites were not further characterized, it was thought that the two investigated peptides were recognized by two different receptors. GLF bound specifically to monocytes and polymorphonuclear leukocytes, while VEPIP only bound to monocytes and macrophages. Using different analogues, the great selectivity of the binding sites was demonstrated. Iskander et al. (45) recently observed a decline in the LPS-induced IL-8 production in respiratory epithelial cells after the administration of whey protein hydrolysates.

When they studied the mechanism involved, they found that the hydrolyzate did not suppress the IL-1 β and TNF α but induced IL-8 production. Therefore, TLR4, which binds LPS, was suggested to be involved. It was demonstrated that the hydrolyzate did neither neutralize LPS nor change the expression of the TLR4 receptor. Therefore, it was concluded that the effect of the hydrolyzate was probably due to a direct binding of the hydrolyzate to the TLR4 receptor, thereby preventing the binding of LPS. However, hydrolysates may not only hinder the binding of inflammatory stimuli to TLR receptors, they may also directly activate TLR signaling (46). Inhibition of either TLR2 or TLR4 was shown to abolish the increased production of IL-6 in intestinal epithelial cells treated with a yellow pea protein hydrolyzate (46).

Hydrolysates affect the intestinal epithelial barrier

Up to now, there are a few studies dedicated to modulation of the barrier function by hydrolysates. Visser et al. (47, 48) fed diabetes prone rats casein hydrolysates from the moment of weaning until an age of 140 and 150 days and compared the barrier function of these animals with animals fed regular chow with the same macronutrient composition (47, 48). The *in vivo* barrier function improved as demonstrated by a decrease in the lactulose:mannitol ratio in both studies (47, 48). Also *in vitro* the intestinal barrier was increased by hydrolysates, as demonstrated by measuring the transepithelial electrical resistance (TEER) of a sample from the ileum (48). Looking further into the mechanisms involved in the improvement of the epithelial barrier, it was found that the mRNA expression of genes encoding the tight junction proteins myosin IXb, claudin-1, and claudin-2 in diabetic rats on casein hydrolysates were normalized in the ileum after the casein hydrolyzate diet compared to healthy rats, together with an upregulation of the regulatory cytokine IL-10 (48). These studies demonstrate that hydrolysis not only deletes allergic epitopes but that hydrolysates also stimulate immune barrier function. Interestingly, one of these studies compared the hydrolyzate formulation with a formulation containing only single amino acids (47) and found that the hydrolyzate formulation was superior in epithelial barrier protection. This underscores the important role of peptides present in the hydrolyzate mixture.

Hydrolysates may also influence the 'inflammatory' status of the epithelial cells, and as inflammation is associated with cell damage and consequently damage to the epithelial barrier, hydrolysates can affect the epithelial barrier via this way. This was shown by Nielsen et al. (49) who studied the inflammatory state of intestinal epithelial cells *in vitro* using intestinal epithelial cells treated with the inflammation inducing drug indomethacin. Casein treated with the enzymes pepsin and corolase was observed to decrease the transcription of several inflammation markers, including cyclo-oxygenase 2 (COX-2) and NF- κ B, compared to casein treated with pepsin only, suggesting an anti-inflammatory effect of this hydrolyzate (49). Hydrolysates from other sources can also cause anti-inflammatory effects. For instance, a hydrolyzate obtained from pea protein was observed to decrease the production of IL-8, which is a proinflammatory cytokine, in CaCo2 cells compared to an unhydrolyzed pea protein extract (50). However, this hydrolyzate also inhibited epithelial cell division, and thereby hindered normal epithelial cell renewal and decreased epithelial integrity.

Hydrolysates thus appear to be able to stimulate the epithelial barrier. Because an increased permeability of the intestines is associated with intolerance and food allergy (51), an improved epithelial barrier is beneficial in at risk or already allergic infants. Therefore, peptides that improve the epithelial barrier are expected to have a hypoallergenic effect. It is therefore important that further (mechanistical) studies into the effect of hydrolysates on the intestinal epithelial barrier are performed.

Hydrolysates show anti-inflammatory effects in innate immune cells, which can influence macrophage differentiation and the allergic reaction against dietary proteins

Hydrolysates have also been shown to inhibit inflammatory responses and even stimulate tolerogenic responses in antigen-presenting cells. Oseguera-Toledo (52) used a hydrolyzate mix from the common bean (*Phaseolus vulgaris* L.) obtained from hydrolysis using the enzymes alcalase and pepsin-pancreatin. After administrating the hydrolyzate, LPS-activated RAW 264.7 macrophages showed a decrease of inflammation markers, such as COX2 expression and related prostaglandin E2 production, inducible NO synthase (iNOS) expression and related NO production. The decrease of these inflammation markers was associated with a decrease of nuclear factor kappa beta (NF- κ B), due to a decreased translocation of its subunits p50 and p65 (53). However, these results were not compared to the intact protein extract from the common bean. Similar results for p50 and p65 were found when using a peptide called lunasin or a lunasin-like peptide, which was obtained from soy (54). Interestingly, this is one of the few peptides to be studied as individual protein giving unique insight in structure-function relationships. Lunasin was found to inhibit NO, prostaglandin E2, iNOS, and COX2 as well. Furthermore, the proinflammatory cytokines IL-6 and IL-1 β , which are also under the control of NF- κ B, were also found to be reduced.

Combined, the foregoing studies suggest that in addition to improving barrier function, peptides in hydrolysates may contribute to preventing allergy by inducing a more tolerogenic response in antigen-presenting cells. *In vitro* studies suggest that a decreased overall amount of NF- κ B and in particular the p50 subunit regulate the polarization of macrophages away from a pro-inflammatory (m1) toward an alternative (m2) state, which are directed toward a regulatory and tissue repair function (55) (Fig. 5). Therefore, the anti-inflammatory peptides could also help to control inflammation in the late phase of the allergic reaction (56).

Performing paw edema tests in mice fed with hydrolysates indeed showed that different whey hydrolysates have anti-inflammatory effects *in vivo*, which is promising in a setting of cow's milk allergy (57).

Specific hydrolysates skew the T-cell differentiation from a Th2 subtype toward Th1 or Treg, which is beneficial in food allergy

Peptides from hydrolysates may also encounter lymphocytes in the lamina propria when passing the barrier. In particular, in infants where the epithelial barrier is rather permeable (11) this is likely. Many studies have shown that hydrolysates from different sources can have direct stimulatory effect on the proliferation and activation of lymphocytes. For example, hydrolysates obtained from soy and whey (or more specifically the proteins β -lactoglobulin or lactoferrin) were shown to enhance proliferation in murine spleen lymphocytes (58). Also intact whey proteins stimulate proliferation in murine spleen

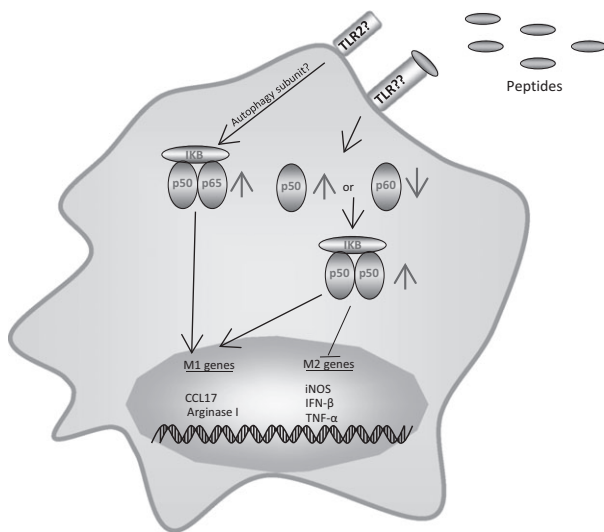


Figure 5 Immunomodulating peptides might be able to stimulate M2 differentiation in macrophages by regulating the expression of the NF-κB subunits p50 and p65. Specific peptides are able to decrease the overall NF-κB activity in the cell, which leads to a more M2-type macrophage. TLR2 activation, for example, results in autophagy of both p50 and p65. Administration of peptides that change the expression of a particular subunit can also change the phenotype of the macrophages. This results in a different amount of regulatory NF-κB complexes containing a p50 homodimer. This complex stimulates the expression of genes related to an M2 phenotype and inhibits the expression of multiple M1 genes.

lymphocytes; however, they were less potent than the whey hydrolysates (59).

Although the above-mentioned results show an effect of hydrolysates on number of lymphocytes, it is not the number of lymphocytes that may prevent or inhibit an allergic response, but the skewing of Th2 responses toward Th1 responses (60). These T-cell responses can be induced without causing an allergic response, depending on the size of the peptides in the hydrolyzate. For IgE cross-linking, the peptides need a minimal length of 30 amino acids. Smaller peptides are not able to induce mast cell degranulation, but can still be recognized by T cells and therefore skew T-cell differentiation (61).

Only a few studies have addressed this issue of T-cell differentiation skewed by peptides at the moment. One study investigated the effect of a yak milk casein hydrolyzate on the Th1/Th2 balance by measuring mRNA levels of Th1 cytokines (IL-2 and IFN- γ) and the Th2 cytokine IL-4 in murine spleen lymphocytes (62). They found that this hydrolyzate increased the Th1 cytokines, but it did not alter IL-4 levels. Therefore, this specific hydrolyzate skewed the differentiation of T cells toward a Th1 subtype. Furthermore, Wu et al. (63) mentioned that the IFN- γ /IL-4 ratio increased in spleen T cells from mice fed with chitosan hydrolyzate, suggesting a change toward a more Th1-like phenotype. On the contrary, specific

hydrolysates derived from whey did not show individual effects on specific cytokines (61).

Because IL-10 producing Treg cells can inhibit Th2 cells, another way to dampen the Th2 response is to promote the differentiation of Treg cells by administration of specific peptides (31). Various studies have shown an effect of hydrolysates on Treg formation by showing an IL-10 upregulation after treatment of lymphocytes with a hydrolyzate obtained from the seaweed *Porphyra columbina* (64) or from casein hydrolyzate (65). This upregulation of IL-10 production was also observed in splenocytes obtained from mice treated with β -lactoglobulin trypsin hydrolysates, while intact β -lactoglobulin on the contrary downregulated IL-10 (66). Ndiaye et al. (46) also found an increased amount of IL-10 producing cells in the small intestine lamina propria of mice after oral administration of yellow pea protein hydrolyzate.

Hydrolysates are not only thought to elicit effects on the epithelial barrier by binding to TLRs, but the observed effects on lymphocytes described above could also be induced via TLRs expressed on dendritic cells and T cells. TLR activation not only induces tolerogenic dendritic cells, but in general, activation of TLRs on antigen-presenting cells also prevents a Th2 driven allergic response by skewing a more Th1-like response (67). Multiple animal studies have indeed shown that stimulation of a range of TLRs ameliorates the allergic response, including TLR2, TLR3, TLR4, TLR5, TLR7, and TLR9 (68). Some clinical studies using non-pathogenic probiotics as TLR ligand showed a decrease in the incidence of atopic dermatitis, although the number of sensitized individuals was the same in the placebo and the experimental group (69). However, although effects of hydrolysates on TLR signaling in epithelial cells (45) and effects of hydrolysates on dendritic cell and T-cell activation and proliferation have been shown, more research is needed to show the direct interaction of hydrolyzate peptides and TLRs on dendritic cells and T cells.

Conclusion and future perspectives

To prevent immune reactions against the numerous antigens present in the lumen of the gut, the different arms of the intestinal immune system contribute to maintaining a tolerogenic state. When this state of tolerance is broken, this can lead to the onset of an allergic reaction. The present review discusses the evidence that although bioactive proteins may serve as allergic-antigens, some bioactive peptides from hydrolysates have the ability to modulate the immune response in a hypoallergenic or other beneficial way (70).

An important part of these beneficial effects is attributed to the immunomodulatory properties of the hydrolysates (71). There is increasing *in vitro* evidence that hydrolysates contain specific immunomodulating peptides, which, possibly by binding to TLR, have been found to improve the epithelial barrier, modulate the Th1/Th2 balance and the amount of Tregs toward a less Th2-skewed response and decrease inflammation. The limited *in vivo* studies confirm these findings (Table 1).

Table 1 Overview recent studies investigating immunomodulating effects of hydrolysates

References	Author(s)	Year of publication	Described effects	Type of studies	Species in vivo studies
45	Iskander et al.	2013	Decline in the LPS-induced IL-8 production in respiratory epithelial cells after the administration of whey protein hydrolysates	<i>In vitro</i>	–
49	Nielsen et al.	2012	Decrease in expression of inflammation markers in casein hydrolysate treated epithelial cells	<i>In vitro</i>	–
50	Swiatecka et al.	2012	Hydrolyzed pea protein decreased IL-8 production in CaCo2 cells	<i>In vitro</i>	–
52	Oseguera-Toledo et al.	2011	LPS-activated macrophages showed a decrease of inflammation markers after administration of hydrolyzed common bean protein	<i>In vitro</i>	–
54	de Meija	2009	Lunasin inhibited inflammatory markers and reduced the production of IL-6 and IL-1 β in macrophages	<i>In vitro</i>	–
58	Wong et al.	1989	Whey hydrolysates enhanced proliferation in murine spleen lymphocytes	<i>In vitro</i>	–
61	Knipping et al.	2012	Whey hydrolysates did not show individual effects on specific cytokines	<i>In vitro</i>	–
62	Mao et al.	2007	Yak milk hydrolysate increased Th1 cytokine expression, but did not affect Th2 cytokines	<i>In vitro</i>	–
64	Cian et al.	2012	A seaweed hydrolysate increased IL-10 production in splenocytes, macrophages and T cells.	<i>In vitro</i>	–
65	Lahart et al.	2011	A casein hydrolysate increased IL-10 production in T cells.	<i>In vitro</i>	–
66	Duan et al.	2012	β -lactoglobulin trypsin hydrolysates increased IL-10 production in splenocytes from sensitized mice	<i>In vitro</i>	–
46	Ndiaye et al.	2012	Increased amount of IL-10+ cells in the small intestine lamina propria after oral administration of yellow pea protein hydrolysate	<i>In vivo</i>	Mice
47	Visser et al.	2012	Decreased in lactulose:mannitol ratio (also compared to AA diet) after casein hydrolysate diet	<i>In vivo</i>	Rats
48	Visser et al.	2010	After casein hydrolysate diet <ul style="list-style-type: none"> ● decreased lactulose:mannitol ratio ● increased transepithelial electrical resistance in an ileum sample (<i>ex vivo</i>) ● mRNA expression tight junction genes were normalized ● IL-10 upregulation 	<i>In vivo</i>	Rats
57	Tavares et al.	2013	Anti-inflammatory effects from a whey hydrolysate were observed using a paw edema test	<i>In vivo</i>	Mice
63	Wu et al.	2006	IFN- γ /IL-4 ratio increased in spleen T cells from mice fed with chitosan	<i>In vivo</i>	Mice

However, to obtain a better understanding of the immunomodulatory effects of hydrolysates especially human studies are needed, as many different effects of peptides, of both a pro- or anti-inflammatory nature, were found, which makes it hard to predict the final outcome in humans.

Another complicating factor is the diversity and a lack of documentation of the chemical properties of the hydrolysates tested, making side-by-side comparison of studies complicated. Every hydrolyzate composition, and therefore its effects, is unique due to the use of different raw materials and hydrolysis production methods. Therefore, general statements about the hypoallergenic and immunomodulating effects of hydrolysates are not possible. Our recommendation is to document as many details as possible about the hydrolysates, including their degree of hydrolyzation, the source, lot-number and way of processing. It is also important to isolate individual peptides from hydrolysates and study characteristics of these individual peptides separately, to

elucidate the exact interaction between a specific peptide and, for example, a receptor.

Hydrolyzed proteins are already used in infant formula for infants with cow's milk allergy or infants at high risk to become allergic. However, up to now, the suitability of these peptides in cow's milk allergy is based on observations of absence of symptoms in the infants treated, while the underlying mechanisms are not well understood. Elucidating the exact effects and working mechanisms of specific peptides together with a better understanding of tolerance induction could provide mandatory information for a more efficient application of specific immunomodulating peptides to induce a hypoallergenic effect in infants. Therefore, more knowledge about the hypoallergenic effects of these hydrolysates will contribute to a more efficient treatment of this vulnerable group of newborns and could ultimately also be beneficial for other individuals with a higher risk of allergy.

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