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Applying the adverse outcome pathway (aop) for food sensitization to support in vitro testing strategies

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ABSTRACT

Background: Before introducing proteins from new or alternative dietary sources into the market, a compressive risk assessment including food allergic sensitization should be carried out in order to ensure their safety. We have recently proposed the adverse outcome pathway (AOP) concept to structure the current mechanistic understanding of the molecular and cellular pathways evidenced to drive IgE-mediated food allergies. This AOP framework offers the biological context to collect and structure existing *in vitro* methods and to identify missing assays to evaluate sensitizing potential of food proteins.

Scope and Approach: In this review, we provide a state-of-the-art overview of available *in vitro* approaches for assessing the sensitizing potential of food proteins, including their strengths and limitations. These approaches are structured by their potential to evaluate the molecular initiating and key events driving food sensitization.

Key Findings and Conclusions: The application of the AOP framework offers the opportunity to anchor existing testing methods to specific building blocks of the AOP for food sensitization. In general, *in vitro* methods evaluating mechanisms involved in the innate immune response are easier to address than assays addressing the adaptive immune response due to the low precursor frequency of allergen-specific T and B cells. Novel *ex vivo* culture strategies may have the potential to become useful tools for investigating the sensitizing potential of food proteins. When applied in the context of an integrated testing strategy, the described approaches may reduce, if not replace, current animal testing approaches.

APPLYING THE ADVERSE OUTCOME PATHWAY (AOP) FOR FOOD SENSITIZATION TO SUPPORT IN VITRO TESTING STRATEGIES

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Key words

IgE-mediated food Allergy; Adverse Outcome Pathway; In Vitro Models; Epithelial Cells; Dendritic Cells; T and B cells.

1. Introduction

Food allergy is one of the most common health disorders in the western world. The occurrence of food allergy drastically increased in the last decades with a current prevalence that reaches up to 10% of the population (Sicherer & Sampson, 2018). Food allergies are adverse reactions to an otherwise harmless food or food component that involves an abnormal response of the body's immune system to specific protein(s) in food. The clinical picture of food allergy is pleiomorphic and can range from gastrointestinal symptoms to severe anaphylaxis (Eigenmann et al., 2008). Most (approximately 90%) of food allergic reactions are caused by milk, egg, peanuts, tree nuts, fish, soya, wheat and shell fish (Boyce, Assa, Burks, Jones, & Hugh, 2010). Allergic disease develops in a two-step process comprising sensitization to the allergenic food and subsequent elicitation of the allergic reaction resulting in symptoms on re-exposure to the allergen.

The contribution of food proteins to healthy aging is increasingly documented and the role of these proteins in a healthy diet recognized. However, a growing global population places an increased pressure on the world's resources to provide not only more but also different types of food. The development and introduction of new dietary protein sources has the potential to improve food supply sustainability. This can be achieved via different ways, e.g. through development of new nutritional/protein sources, improvement of crops, by providing solutions to technical challenges during manufacturing, as well as by valorizing unused side products (Remington et al., 2018; Selb et al., 2017). These foods must not pose a risk to public health, thus a comprehensive risk assessment should be conducted in order to ensure their

safety. Risk assessment should cover different domains including kinetics, toxicology, nutritional effects and allergenicity (de Boer & Bast, 2018).

Studying the allergenicity of these foods is quite complex. Firstly, relevant allergenic testing material needs to be obtained. To this end, several factors (e.g. pH, buffer, fatty substances in matrix etc), should be taken into consideration to obtain an extract that provides a good picture of the novel food, which have to be optimized case-by-case (Mazzucchelli et al., 2018). In addition, the purification of proteins might also be also technically challenging and may result in chemical modifications, which needs to be assessed prior to testing for allergenicity (Mazzucchelli et al., 2018). Next to the difficulties to obtain relevant allergenic testing material, it is evident that properties of food matrix components are also relevant and important in the sensitization process. The allergen is never in initial contact with the immune system in a purified state; the matrix surrounds, interacts with, and can affect the physiochemical features of the allergens. Currently, the primary influences of the matrix are thought to be antigen bioavailability and release, digestibility and interactions with the immune system (McClain, Bowman, Fernández-Rivas, Ladics, & Van Ree, 2014). However there are no straightforward approaches to address the many variables represented by the matrix components in food (reviewed by McClain et al., 2014), so additional knowledge is required which will help to develop tools to incorporate their influence on sensitization into model systems (van Bilsen et al., 2017).

This manuscript focusses on the current available in vitro tools to study cellular and molecular mechanisms driving the non-symptomatic sensitization phase of food allergy resulting in the generation of food protein-specific IgE.

There are a considerable number of *in vivo* and *in vitro* data available describing molecular and cellular events potentially involved in food sensitization. Recently, these events have been organized in a sequence of related processes that is plausible to result in sensitization and useful to challenge current hypothesis by applying the concept of adverse outcome pathway (AOP) (van Bilsen et al., 2017). The proposed AOP framework provides a simplification of a complex biological process by collecting, organizing and evaluating data that describe the events of an adverse outcome at a biological level of organization with relevance for risk assessment. The application of the AOP concept allows to identify the major molecular initiating events (MIE) and key events (KE) underlying food sensitization (Figure 1). The AOP for food sensitization starts with a MIE involving the allergen uptake over the mucosal barrier

of the gut intestine. The food protein passage may induce the activation of intestinal epithelial cells (IECs), representing KE1, followed by the local activation of dendritic cells (DCs) and their migration to the mesenteric lymph nodes (KE2 and KE3). There, DCs present processed allergen to naive T cells priming them toward a T helper type 2 (Th2) response (KE4). Thus these events may cause the activation of B cells (KE5) and the production of specific IgE by plasma cells.

The events included in this AOP are still highly complex at molecular/cellular level, but the challenge is to integrate MIE and KE to better understand the mechanistic pathways of food sensitization induction. The AOP for food allergy offers the opportunity to anchor existing methods for the testing and assessment of sensitizing potential of food proteins. Moreover, it gives insight into which specific assays are suitable to evaluate the influence of novel food proteins and ingredients in the sensitization process.

It is still a matter of debate whether human, like mice, can be sensitized via other routes than the oral route such as via the skin or respiratory route. Epidemiological studies in human populations seem to confirm the skin as a relevant route for food sensitization induction and allergy in humans, however is mechanistically not sufficiently understood yet (van Bilsen et al., 2017). Evidence for the respiratory route is even more ambiguous (van Bilsen et al., 2017). Therefore, *in vitro* testing strategies focusing on the dermal or respiratory route are not explored in this review.

In this review, we aim to provide the state-of-the-art of existing *in vitro* approaches for assessing sensitizing potential of food proteins based on the identified MIE and KE proposed in the AOP for food sensitization, as described by van Bilsen et al. (van Bilsen et al., 2017). For this propose, we provide cell assays previously used for the study of food allergens focusing on major read-outs as well as strengths and limitations of these assays..

2. Antigen uptake over intestinal mucosal barrier and epithelium activation

2.1. *In vitro* models to assess tight junction disruption (MIE1)

During the gastrointestinal digestion, intact proteins and peptide fragments reach the intestinal lumen where they interact with the IECs resulting in antigen uptake over the gut barrier. Digestion products may be transferred across the intestinal epithelium by paracellular transport driven by disruption of tight junctions, adherent junctions and desmosomes, representing MIE1. Tight junctions are multiprotein

complexes composed by transmembrane proteins (occludin, claudin, junctional adhesion molecule A and tricellulin) that provide the integrity of the actomyosin ring, which controls inter-epithelial permeability.

Typically, epithelial *in vitro* models employed to study the transport and absorption of food proteins or peptides along the intestinal epithelium are based on cell lines (including Caco-2, HT-29, T84, and IPEC-J2) grown in a transwell system (Cubells-Baeza et al., 2015). In this regard, Grozdanovic and co-workers demonstrated that the exposure of T84 cells to actinidin, a kiwifruit cysteine protease, resulted in the impairment of the epithelial barrier, due to the degradation of occludin promoted by the proteolytic action of actinidin (Grozdanovic et al., 2016). As a consequence, an increase of the intestinal permeability was recorded which could contribute to the process of sensitization in kiwifruit allergy. By employing the Caco-2 cell model, Price et al showed that peanut allergens Ara h 1 and Ara h 2 were able to alter the intestinal barrier permeability, modifying the co-localization of the transmembrane tight junction proteins occludin, JAM-A and claudin-1, with the intracellular adhesion protein ZO-1 (Price, Ackland, Burks, Knight, & Suphioglu, 2014). In another study, β-conglycinin from soy (Gly m 5) induced a downregulation of tight junction proteins by using a model of IPEC-J2 porcine cells (Zhao et al., 2015).

On the other hand, the breach of epithelial barrier may be a consequence of Th2 switching and may possibly reflect the abnormal responses and vicious cycle triggered by mast cell activation. It has been observed that mast cells releasing chymases and histamine have an impact on the physiology of the intestinal mucosa, promoting the degradation of the tight junction occludins (Bischoff & Kramer, 2007). Moreover, mast cells are involved in the release of Th2-realted cytokines, such as IL-4 and IL-13, that influence the modulation of IEC permeability in different human IEC cultures, decreasing the trans-epithelial electrical resistance (TEER) and selectively increasing the apical-to-basal movement of proteins (Ceponis, Botelho, Richards, & McKay, 2000). In order to address the integrity of the intestinal monolayer, the study of the TEER is an important issue that provides information about the inter-epithelial transport, generally associated with an alteration of the tight junctions and thus relates to paracellular transport. It has been reported that exposure of crude apple homogenate produces an increase of the paracellular resistance in Caco-2 cells, with an augmentation of the expression of several tight junction related genes, including claudin 4 (Vreeburg, Bastiaan-Net, & Mes, 2011). Zhao et al. have recently shown that peptides produced during simulated *in vitro* digestion of soybean β-conglycinin, determine an increase of alkaline phosphatase activity with a decrease of mitochondrial respiration (MTT

assay), TEER values and downregulation of claudin-3, claudin-4, occludin, and ZO-1 expression (Zhao, Liu, Zhang, Pan, & Qin, 2017).

However, not all food allergens affect TEER resistance. Moreno et al. reported that the transcellular transport of purified 2s albumins Ber e 1 (brazil nut) and Ses i 1 (sesame seed) across Caco-2 monolayer did not affect the permeability as assessed by the absence of any change of allergen absorption rate and TEER values (Moreno, Rubio, Olano, & Clemente, 2006). In other work, authors found that hydrolyzed ovalbumin did not affect MTT values or cell permeability indicating a protective effect exerted by the food matrix on the cell barrier (Grootaert et al., 2017). In addition, TEER values were restored to their original levels, demonstrating the absence of any permanent damage on the monolayer caused by egg digests tested (Grootaert et al., 2017). In line with this, previous studies also reported that purified proteins from wheat (ω5-gliadin and LTP 1) (Bodinier et al., 2007), peach (Pru p 3 and LTP 1) (Tordesillas et al., 2013), and peanut (Ara h 1 and Ara h 2) (Price, Ackland, & Suphioglu, 2017) were able to cross Caco-2 monolayers without compromising cell monolayer integrity.

2.2. *In vitro* models to assess receptor-mediated and unspecific endocytosis (MIE2 and MIE3)

Apart from paracellular transport, food proteins may also cross the intestinal epithelium via transcellular transport pathways, either receptor-mediated (e.g. CD23, the low affinity FcεRII-IgE receptor) (MIE2) or via unspecific endocytosis (MIE3).

CD23 is expressed by Caco-2, T84 and HT29 cells and their expression can be stimulated by IL-4 (Tu et al., 2005). Using Caco-2 cells, it has been demonstrated that IgE-antigen (nitrophenyl(NP)-BSA) complexes trigger the upregulation of IL-8 and CCL20. The supernatant of such triggered Caco-2 cells induced DC migration in a CCL20-dependent manner (Li et al., 2007). In HT29 cells, it has been shown that CD23 expression and transcellular transport of IgE-NP-ovalbumin complexes could be stimulated by factors present in supernatant of activated mast cell (HMC1) and monocyte (THP1) lines (Tu, Oluwole, Struiksma, Perdue, & Yang, 2009). The role of SIgA in transcytosis has been studied even less than that of CD23, and mainly focus on gliadin peptides. Caco-2 and HT29 cells express CD71 and transglutaminase 2 (TG2), both shown to co-precipitate with SIgA and important for endosomal transport routing (Lebreton et al., 2012). These reports on receptor-mediated endocytosis are from a few isolated studies, but they show that the role of CD23-mediated and of CD71/TG2 transcytosis could be examined using *in vitro* models, although more food proteins using these assays should be tested.

Unspecific transport of food proteins may occur via epithelial, goblet or M cells and may result not only in protein degradation within these cells and cellular activation, but also it can lead to process of proteins into peptides that can be expressed in the context of MHC-II. The route of uptake seems to have an important implication for the final outcome. Although insufficiently studied, it has been proposed that larger cross-linked proteins could be more sensitizing because they are preferentially taken up by M cells and directly interact with immune cells in Peyer's patches. Roth-Walter and co-workers described that the intestinal transport of soluble □-lactoglobulin occurs through enterocytes, while the uptake of its aggregates is redirected to Peyer's patches (Roth-Walter et al., 2008). On the other hand, smaller intact proteins are transferred via goblet-associated passage (also called GAP) and that may be linked to processing by CD103+ DCs, facilitating tolerance rather than to other DCs that are linked to sensitization (McDole et al., 2012). M cell transfer and GAP are not often studied using *in vitro* methods, but cell lines as well as organoids can be adapted to study the particular involvement of these transportation routes. For instance, cell lines and intestinal stem cell-derived organoids can be stimulated to form M cells by RANKL-RANK pathway activation (Kimura, 2018) and GAP could be studied in specialized cell lines (HT29-H cells) or in organoids (constitutive presence of goblet cells).

Endocytosis is a complex process that includes caveolea-mediated uptake mechanisms, clathrin-dependent and -independent uptake mechanisms, macro-pinocytosis and phagocytosis. These processes may operate alongside each other and can be studied by using specific inhibitors (Dutta & Donaldson, 2012). Important to mention here is that many of these inhibitors (e.g. filipin, cytochalasinD and monodansylcadaverine) also affect viability as well as TEER values (Price et al., 2017). Little information with regard to the importance of various mechanisms of endocytosis is yet available for food allergens. But studies with individual proteins show that different mechanisms of endocytosis may be involved for different proteins; for instance, Ara h 1 may be endocytosed via a combination of macro-pinocytosis and clathrin-dependent processes, whereas Ara h 2 may be endocytosed via a combination of macro-pinocytosis and caveolea-mediated uptake mechanisms (Price et al., 2017). Although authors speculate that the localization of Ara h 2 resulting from the specific way of endocytosis is linked to higher allergenicity of Ara h 2, other potential explanations such as their different molecular weight or physico-chemical properties should be also considered.

Altogether, *in vitro* models such as Caco-2 cells cultured onto transwell systems seem suitable to study the importance of MIE2 and MIE3 in sensitization to food proteins. However, no data is currently

available for ranges of allergens and clearly this information is needed to link endocytosis *per se* as well as the type of translocation to the sensitizing potential of a food protein.

2.3. *In vitro* models to assess epithelium activation (KE1)

Beyond being transported across the gut epithelial barrier, food proteins and their digestion products are able to interact with IECs in the intestinal lumen thereby triggering immune responses. It is widely accepted that food sensitization involves factors from gut epithelium, which are released after the activation of IECs (KE1). The presence of food allergens in the gut is mainly detected by pattern recognition receptors (PRRs), such as toll like receptors (TLRs), glycan binding receptors (galectins) or protease-activated receptors (PARs). This leads to the activation of inflammatory mechanism resulting in the activation of the NF-κB route and initiate the repair process for the damaged barrier with the production and secretion of chemokines, cytokines, reactive oxygen species (ROS), and lipid metabolites.

In vitro studies have closely linked the production of ROS to the activation of epithelial cells in the case of aeroallergens. The presence of IL-13 in the environment and the activation of PAR-2 receptor have been implicated in the upregulation of ROS production by epithelial cells (Dickinson et al., 2016; Nadeem et al., 2015). However, the effect of food allergens on IECs have not been so furtherly studied and we can only report the case of peanut allergens, in which an increase in the nitric oxide synthase gene expression has been observed (Starkl et al., 2011).

In response to their activation, IECs are also able to release epithelial-specific cytokines (e.g. IL-1, IL-18, IL-25, IL-33, and the thymic stromal lymphopoietin –TSLP-) that are crucial for the initiation of food protein sensitization. This cytokine environment induces the activation of DCs, type 2 innate lymphoid cells (ILC2), basophils, eosinophils, and mast cells, skewing the intestinal immune system towards a Th2 response. IL-33, IL-25, and TSLP are constitutively expressed by epithelial cells being IL-33 a crucial regulator of mast cells. Besides that, IL-33 is able to enhance granulocyte, macrophage, and ILC2 responses (Saluja, Khan, Church, & Maurer, 2015). IL-25 elicits multipotent progenitor type 2 cells, a population of innate cells promoting type 2 cell immunity even in absence of ILC2 (Saenz et al., 2013). Regarding TSLP, its expression is increased in a NF-κB dependent-manner, being able to activate mast cells and influencing antibody production (Miron & Cristea, 2012). On the other hand, the production of IEC-derived TGF-β promotes DCs involved in tolerogenic signals, which is an important milestone for the control of allergic sensitization (Wang & Sampson, 2009). Tordesillas et al. examined the effect of the

major peach allergen Pru p 3, in comparison with that of the hypoallergenic peach LTP 1 using Caco-2 cells grown in a transwell system. Authors showed that in the presence of Pru p 3 an increased expression of Th2-driving cytokines (IL-25, IL-33, and TSLP) was observed. However, with the hypoallergenic protein LTP 1, the induction these cytokines was significantly lower (Tordesillas et al., 2013). In other study, the effect of the peanut allergen Ara h 2 on IECs showed upregulation of the inflammatory cytokines IL-1β and IL-8 (Starkl et al., 2011).

Concluding, the activation of epithelial cells is a heterogeneous process in which the cytokine environment is crucial, for this reason the effects of more food allergens on IECS should be described using other cell types than Caco-2 cells. Despite the fact that the majority of studies are focused on the analysis of the cytokines expression, other pathways should be explored, such as mucus secretion or Ca^{2+}-signaling pathway alterations.

2.4. *In vitro* models to assess antigen uptake combined with epithelium activation (MIE and KE1)

Approaches which combine the possibility to evaluate the contribution of allergens transport (MIE) to IEC activation and cytokine production (KE1) are important tools for studying the immunogenic properties of food proteins. The interaction of food allergens with the intestinal epithelium not only leads to the secretion of innate cytokines, but also the production of chemokines and other soluble factors such as alarmins (e.g. uric acid, ATP, HMGB1, and S100 proteins) that drive immune polarization by affecting DC function and the adaptive response (Gavrovic-Jankulovic & Willemsen, 2015; van Bilsen et al., 2017). Furthermore, there is evidence that mediators released by peripheral blood mononuclear cells (PBMCs) and mast cells contribute to increase intestinal permeability which means that immunologic status can, by itself, affect IEC activation and the access of the allergen through epithelium (Tordesillas et al., 2013).

In this sense, several strategies have already been attempted with relative success. IEC lines grown in transwell systems (e.g. Caco-2, T84, or HCT-8) have been used to assess allergen uptake and subsequent epithelium activation by measuring cytokine production or changes in gene expression induced by allergens (Starkl et al., 2011; Tordesillas et al., 2013). In order to study the influence of intestinal soluble factors from immune cells on IECs, some *in vitro* strategies have used separate monocultures of IECs and immune cells and then supernatant from immune cells has been applied to IECs (Martos, Lopez-Exposito, Bencharitiwong, Berin, & Nowak-Wegrzyn, 2011; Tordesillas et al., 2013; Yamashita, Yokoyama,

Hashimoto, & Mizuno, 2016) or *vice versa*. This strategy may contribute to a better comprehensive understanding of the *in vivo* cross-talk affecting KE1.

In addition, co-culture systems allowing cross-talk between structural cells (IECs) and effector immune cells (such as basophils) are being attempted to study whether a food allergen induces not only a direct epithelial activation, but also its consequences on the underlying immune cells, enabling to assess the immune cell responses through epithelial activation. An *in vitro* model based on the co-culture of Caco-2 cells and rat basophilic leukaemia cells (RBLs) has been used for the study of immune activation upon Gal d 2 challenge at the apical side of the IECs (Thierry, Bernasconi, Mercenier, & Corthesy, 2009). Although not performed, epithelial activation markers may be evaluated using this co-culture system.

More complex co-cultures combining mucoid IECs, non-mucoid IECs and B cell lines (Caco-2/HT29/Raji-B) have been suggested in the literature as systems to integrate allergen uptake with epithelial activation markers as well as with modulation by the immune cell signals (Araujo & Sarmento, 2013). However, such an integrative system has not yet been used for the study of food allergens.

3. Dendritic cells

3.1. *In vitro* models to assess dendritic cell activation and migration (KE2 and KE3)

As major antigen presenting cells, gut-associated DCs play a key role in immunological pathways associated to food protein sensitization process. Food proteins or their digestion products may induce activation of DCs (KE2) by C-type lectin receptor (CLRs) or other PRRs leading to a decrease in TLR-induced IL-12 production, as well as upregulation of OX40L, TIM-4, or both. In addition, DCs can be activated by IEC-derived IL-33 which also upregulates OX40L expression on DCs. After allergen uptake and activation, DCs migrate (KE3) to draining lymph nodes mediated by the expression of CCR2, CCR5 and CCR7 molecules. Other interesting surface markers to assess as indicators of antigen presentation are HLA-DR (MHC class II), CD86 (Katayama et al., 2013) and DC-SIGN binding by fluorescent-labeled food proteins (Kamalakannan, Chang, Grishina, Sampson, & Masilamani, 2016).

Besides DC assays analyzing the presence of (binding to) surface molecules and cytokine expression, other methods have been described evaluating the differences in DC endocytosis by incubating murine bone marrow-derived DCs (BM-DCs) with FITC-labeled β-lactoglobulin and by *in vitro* endolysosomal degradation of the native and cross-linked forms of this allergen (Stojadinovic, Pieters, Smit, &

Velickovic, 2014). These data indicated differences that correlated with the sensitizing potential *in vivo*, suggesting that these additional parameters may be useful *in vitro* parameters to evaluate the sensitizing potential of food proteins. On this regard, BM-DCs have been applied to estimate the effect of thermal process on ovalbumin and showed that a glycation product of this allergen, pyrraline, induced higher uptake by DCs associated with the scavenger receptor class A (Heilmann et al., 2014).

Transcriptomic profiling of allergen-activated DCs is another approach to compare the DCs activating potency of food proteins. Comparison of various cell lines with human monocyte-derived DCs (moDCs) by gene profiling suggested that the MUTZ-3 cell line resembles moDCs (Larsson, Lindstedt, & Borrebaeck, 2006). A clone of this cell line has been recently used to assess and predict the sensitizing potential of proteins in respiratory allergy in the Genomic Allergen Rapid Detection (GARD) assay. In this assay, cellular responses induced by eight selected proteins were assessed using transcriptional profiling, flow cytometry and multiplex cytokine analysis. A total of 391 potential biomarkers were identified as a predictive signature and series of cross-validations supported the effectiveness of this model. These results together with biological pathway analysis of the transcriptomic data indicate that the investigated cell system is able to capture relevant events linked to type I hypersensitization (Zeller et al., 2018). Although promising, the relevance of this model for food sensitization induction in general remains to be established.

Migration assays using BM-DCs (Rhee, Zhong, Reizis, Cheong, & Veillette, 2014) or MUTZ-3 cells (Rees et al., 2011) have been described in transwell systems in which activated DCs are applied in the upper chamber, whereas an appropriate chemokine is added to the lower chamber. After incubation, migrated cells are harvested from the lower chamber and quantified (Rhee et al., 2014). However, these migration assays have never been proven useful in a food allergen specific context.

3.2. *In vitro* models to assess interactions between epithelial and dendritic cells (approaches integrating KE1, KE2 and KE3)

The IECs- and DCs-derived signals constitute an allergen-induced inflammatory microenvironment that triggers DCs maturation and migration. Test methods incorporating IECs and DCs have been developed in a variety of formats: co-cultures of DCs/IECs, three-dimensional (3D) models reconciling the complex and dynamic interactions that exist *in vivo* between the intestinal epithelium and the luminal side, and between the epithelium and the underlying immune system on the basolateral side (Bermudez-Brito,

289 Plaza-Diaz, Fontana, Munoz-Quezada, & Gil, 2013). These methods have been applied to investigate
290 cellular and molecular mechanisms triggered by prebiotics and bacteria. However, the impact of allergens
291 on these mechanisms has not yet been investigated extensively with these test models. Cultures of DCs
292 supplemented with IEC-conditioned medium may also be considered as alternatives to co-cultures. This
293 method has been applied in the case of prebiotics using human moDCs cultured with HT-29 conditioned
294 medium (de Kivit et al., 2017).

295 Although several studies have used cultures of IECs or DCs to assess the sensitizing potential of food
296 allergens, only the previously mentioned study from Tordesillas *et al.* investigated the influence of a food
297 allergen on the function of co-cultured IECs/DCs. In that study, a transwell system with Caco-2 cells was
298 set up, including PBMCs from healthy donors in the basolateral compartment. The addition of the Pru p 3
299 onto the apical chamber induced increased expression of IL-1β, IL-6, IL-10 and TNF-α genes in PBMCs
300 which was related to the high transport rate of intact Pru p 3 over the Caco-2 barrier (Tordesillas et al.,
301 2013). These types of methods allow a further characterization of the sensitizing potential of allergens by
302 including the role of cell-cell contact as well as soluble molecules taking into account the cellular
303 interactions. However, several specific limitations may be encountered during the development of these
304 methods, such as compatibility of cell types or cell media, complexity of the systems, donor variability
305 when using primary human cells.

306 **4. T and B cell differentiation**

307 **4.1. *In vitro* models to assess murine Th2 cell priming (KE4)**

308 Allergens are thought to invoke an allergic response due to their ability to activate T cells through their
309 specific cell receptors (TCRs). Besides this primary pathway, the interaction of co-stimulatory and co-
310 inhibitory receptors of T cells (CD28, CTLA-4, OX40L) with ligands on antigen presenting cells
311 activates antigen unspecific signals that lead to the differentiation of T cells into a Th2 phenotype (van
312 Bilsen et al., 2017).

313 Due to the low allergen-specific T cell *in vivo* frequency, re-stimulation of pre-sensitized cells *ex vivo* is
314 the most widely used method to evaluate priming potential of common food allergens in mice. Most
315 studies use splenocytes, mesenteric lymph node cells, or a combination of both, although lamina propria
316 mononuclear cells (Sun et al., 2016) and isolated CD4+ T cells (Kanjarawi et al., 2011; Pochard et al.,

2010) have also been evaluated. Cell suspensions are prepared from immunized mice and cultured with the antigen for 3-6 days, depending on the subsequent analyses. T cell proliferation, expression of specific cellular surface markers and/or cytokine secretion are mostly determined in these studies. T cell proliferation is one of the most common ways to assess T cell activation upon *ex vivo* re-stimulation with the allergen. To this end, several methods such as labeling of cells with a fluorescent dye (Pochard et al., 2010; Sun et al., 2016), incorporate a radioactive nucleoside into new strands of chromosomal DNA during mitotic cell division (Freidl et al., 2017; Stojadinovic et al., 2014) or colorimetric assays for assessing cell metabolic activity (Sun, Liu, Wang, Liu, & Feng, 2013; Wai, Leung, Leung, & Chu, 2016) have been used.

Proliferation assays have been performed for □-lactoglobulin (Mizumachi, Tsuji, & Kurisaki, 2008; Stojadinovic et al., 2014), ovalbumin (Castro et al., 2012; Sun et al., 2016), tropomyosin (Capobianco et al., 2008; Wai et al., 2016), Cyp c 1 from carp (Freidl et al., 2017), peanut (Pochard et al., 2010), cashew and walnut extract (Kulis, Pons, & Burks, 2009), and soybean proteins (Sun et al., 2013), showing in all the cases a strong proliferative response of the cells. Expression of specific surface markers have been measured in sensitized mice after challenge with whey and ovalbumin, showing an increase in the percentage of both, activated Th1 and Th2 cells compare to control animals (Lozano-Ojalvo, Perez-Rodriguez, Pablos-Tanarro, Molina, & Lopez-Fandino, 2017; Vonk et al., 2017). However, when peanut extract was used there was no difference in the percentage of activated Th2 cells, and the percentage of activated Th1 cells was decreased (Vonk et al., 2017).

Levels of secreted cytokines are often evaluated by ELISA or flow cytometry in cell culture supernatants. This method has been used to evaluate several food allergens from cow's milk (Kanjarawi et al., 2011; Stojadinovic et al., 2014), peanut (Smit et al., 2015; Zhu et al., 2016), hen's egg (Pablos-Tanarro, Lopez-Exposito, Lozano-Ojalvo, Lopez-Fandino, & Molina, 2016; Sun et al., 2016), soybeans (Sun et al., 2013) and shrimp (Wai et al., 2016). Moreover, extracts from whey, hen's egg white, peanut, cashew, walnut and sesame seeds have also been evaluated using this method (Lozano-Ojalvo et al., 2017; Smit et al., 2015; Vonk et al., 2017). A high increased secretion of Th2 cytokines (IL-4, IL-5, IL-13) has been observed in all the studies, whereas some discrepancies were observed between allergenic proteins in their potency to induce IFN-γ (Stojadinovic et al., 2014; Vonk et al., 2017) and IL-10 (Vonk et al., 2017). A few studies have measured increased levels of IL-17 (Lozano-Ojalvo et al., 2017; Rupa, Nakamura,

Katayama, & Mine, 2014) , while in other limited number of studies IL-22 and TNF-α levels showed no differences compared to non-sensitized control cells (Pablos-Tanarro et al., 2016; Zhu et al., 2016).

The assays described above, have provided efficacy to identify known allergens that drive allergic reactions in individuals and allergen modifications that ameliorate their allergenic potential, as well as to identify T cell epitopes of cross-reactivity with other allergens. However, they are not effective to identify the allergenic potential of new proteins that are responsible for stimulating the underlying Th2 responses, mainly due to the need to use cells from previously sensitized mice against the same allergen to perform the assay. Moreover, although evaluation of T cell activation through T cell proliferation assays is widely describe in the literature using different strategies, the use of indirect methods such as those that measure mitochondrial activity (e.g. MTT or WST assays) should be avoided because they reflect viable cell metabolism and not specifically cell proliferation, requiring additional confirmations.

4.2. *In vitro* models to assess human Th2 cell priming (KE4)

The activation and priming of naive T cells (KE4) for allergic food proteins in the draining lymph nodes results from the recognition of an epitope presented by Th2 driving DCs in the context of HLA (MHC-II). Most of the approaches for investigating KE4 using human samples are based on the *ex vivo* re-stimulation of PBMCs from allergic patients and the study of the allergen-specific induced proliferation and cytokine production (Flinterman et al., 2010; Tao et al., 2016; Tiemessen et al., 2004; Vocca et al., 2011). Indeed, stimulation of PBMCs from cow's milk allergic children with β-lactoglobulin was found to induce cell proliferation and increased IL-13 over IFN-γ release compared to healthy or tolerant controls (Vocca et al., 2011). Also in PBMCs from peanut allergic patients, allergen-specific proliferation could be assessed by determining proliferation within the CD25+CD134+CD4+ T cell population after stimulation with raw peanut (Tao et al., 2016). However, in peanut allergic patients, *ex vivo* stimulation of PBMCs with peanut extract increased both IL-13 and IFN-γ as well as TNF-α levels compared to PBMCs from healthy controls (Flinterman et al., 2010).

However, as pointed out in the previous section for KE4, the precursor frequency of allergen-specific T cells is very low in the peripheral blood of allergic patients. In this regard, the generation of T cell lines and T cell clones is an interesting alternative that lead to analyze Th2 cell activation (Flinterman et al., 2010; Tiemessen et al., 2004). Assays to study the functionality of these allergen-specific T cells make the use of allergen induced selection and cloning to improve the sensitivity of the assay. A mixture of β-

lactoglobulin derived synthetic peptides were used to generate antigen-specific T cell lines and clones from PBMCs of cow's milk allergic patients (Sakaguchi et al., 2002). Limiting dilution clones were isolated and then used for epitope mapping. Results showed a sequence of 12 amino acids recognized by three out of six T cell clones from 5 different patients, which was associated with presentation via HLADRB1*0405 (Sakaguchi et al., 2002). Kondo et al. further studied intracellular cytokine expression in two of those clones, showing an increased production of IL-4 and IFN-γ both in combination with IL-10 (Kondo et al., 2005). In this sense, cytokine release measurement may provide additional information concerning the type of immune response that is raised against these epitopes. Tiemessen et al. generated T cell clones using whole cow's milk proteins and compared the cytokine response in a group of cow's milk allergic infants with non-symptomatic allergic patients and healthy donors (Tiemessen et al., 2004). Results showed that all different groups strikingly reacted to milk proteins, although cytokine production by allergic individuals was the highest for IL-4, IL-13, IFN-γ, and IL-10 (Tiemessen et al., 2004). However, only the symptomatic group revealed a negative or no correlation between IL-4/IFN-γ and IL-10, while in the other two groups there was a positive correlation, showing the importance of IL-10 as a regulatory cytokine involved in tolerance induction. In addition, T cells from the symptomatic group had a high expression of the activation marker CD69 (Tiemessen et al., 2004). Beyond studying differences in T cell phenotype between patients and controls, T cell epitope disruption can also be studied using T cell lines as showed by the fact that β-lactoglobulin was able to induce a higher proliferation and cytokine secretion than its products of hydrolysis (Knipping et al., 2012). In short-term peanut-specific T cell lines generated from PBMCs of peanut allergic patients, Ara h 1, Ara h 3, and Ara h 6 were identified to induce the highest proliferation and secretion of IL-13, showing that these cell lines created using crude peanut extract can be useful for allergen identification (Flinterman et al., 2010). Furthermore, allergen-specific T cell clones from HLA diverse donors can be generated in order to evaluate the epitopes involved in T cell activating capacity in a certain HLA context (Prickett et al., 2011, 2013). This method has been used to identify 10 core epitopes of Ara h 1 and 5 core epitopes of Ara h 2 that could effectively induce T cell proliferation in a HLA-DQ and/or HLA-DR and/or HLA-DP restricted manner (Prickett et al., 2011, 2013). In the latter study, it was also showed that Ara h 2 peptides were able to enhance IL-4 and IL-5 secretion (ELISPOT) in PBMCs from peanut allergic donors (Prickett et al., 2011).

Based on an *in silico* predictions, Ramesh et al. evaluated the immunogenic potential of 36 Ara h 1 derived peptides studying proliferation and cytokine production after *ex vivo* re-stimulation of PBMCs

from peanut allergic patients with those peptides (Ramesh et al., 2016). Almost all the *in silico* selected peptides induced proliferation and predominantly a high IL-13 release when compared to IFN-γ (Ramesh et al., 2016). This study indicates that PBMCs from allergic donors, combined with an appropriate *in silico* prediction, may yet be sufficient to identify allergenic epitopes of certain food proteins. In addition, MHC-peptide tetramers assays have been previously used to identify allergen-specific T cells in PBMCs. DeLong et al. cultured PBMCs from peanut allergic patients with Ara h 1 peptides loaded on biotinylated HLA-DR proteins and intracellular cytokine expression was identified in tetramer labeled cells (Delong et al., 2011). Results showed that CCR4 expressing Ara h 1 reactive T cells responded to different epitopes and produced mostly IL-4 beyond other cytokines (Delong et al., 2011).

In these types of assays, allergen-specific T cells can be identified by a highly upregulated Th2-prone culturing environment. They may not provide much information regarding the sensitizing potential of a given food protein, but merely indicate whether specific (or novel) food-derived proteins or peptides can be recognized by T cells and pose a potential risk. Moreover, these approaches are useful to identity novel food epitopes that are recognized by T cells.

4.3. *In vitro* models to assess DC activation/migration and T cell priming (approaches integrating KE2, KE3 and KE4)

After DC activation (KE2 and KE3), DCs migrate to the draining lymph nodes and drive T cell activation and differentiation (KE4). *In vitro* models to study antigen presentation and polarization of Th0 toward Th2 cells are usually based on co-culture approaches using DCs and primed T cells.

In methods based on murine cells, BM-DCs have been co-cultured with CD4+ T cells from mice sensitized to peanut (Pochard et al., 2010), whey (Stojadinovic et al., 2014) and a panel of purified food proteins (Smit, de Zeeuw-Brouwer, van Roest, de Jong, & van Bilsen, 2016). In addition, CD4+ T cells have also been obtained from TCR transgenic strains such as DO11.10 (Ilchmann et al., 2010) or OT-II mice (Rhee et al., 2014). These studies have showed that DCs increased the expression of activation/migration markers (MHC-II, CD80, and CD86) and the release of stimulatory cytokines such as IL-6 and IL-12 after re-stimulation with the allergen. Furthermore, activated DCs induce a high proliferation and a marked Th2 profile (enhanced production of IFN-γ, IL-4, IL-13, and IL-5 determined by ELISA) in primed CD4+ T cells. Similar results were obtained when DCs were directly isolated from

mesenteric lymph nodes and co-cultured with CD4+ T cells from DO11.10 mice (Blazquez & Berin, 2008).

Among human *in vitro* models, DCs used for co-culture with T cells have been obtained from differentiated THP-1 cell line (Katayama et al., 2013) and allergen-pulsed monocyte-derived DCs (Gomez et al., 2012; Scott-Taylor, Axinia, & Strobel, 2017). Allergens have been shown to activate DCs revealed by an enhanced expression of CD80, CD83, and CD86 (Gomez et al., 2012), production of inflammatory cytokines (IL-12p70, IL-1β, TNF-α, and IL-10) and up-regulated expression of MARCH genes. For the study of the cross-talk with T cells, PBMCs from healthy donors (Tordesillas et al., 2013) or cashew allergic patients (Archila et al., 2016) have been previously used. In addition, T cell lines generated from cow's milk allergic children (Meulenbroek et al., 2014) and CD4+ T cells directly isolated from patient allergic to peach (Gomez et al., 2012) and other food allergens (Scott-Taylor et al., 2017) have been previously used for co-cultures. Results have shown that, in presence of the food allergen, DCs induce T cell proliferation (observed by flow cytometry using carboxyfluorescein succinimidyl ester molecule -CFSE- or CD154 staining) in sensitized individuals compared to healthy donors. In addition, allergen-pulsed DCs trigger activation of T cells and increase the production of T cell-like cytokines such as IL-4, IL-13, and IFN-γ.

One of the strengths of human *in vitro* models is the use of T cells from allergic patients in an autologous setting, although it coincides with a downside, the limited number of cells that can be used (Lundberg et al., 2008). In addition, there are variations in the experimental protocols used for pulsed-DCs alone with the allergen of interest (Gomez et al., 2012; Scott-Taylor et al., 2017) or combined with maturation factors, such as TNF-α, IL-1β, or LPS (Ashjaei et al., 2015). These differences during maturation can affect expression of DC co-stimulatory and maturation markers and thus the subsequent proliferation of T cells and cytokine production, although most of the studies were able to induce an allergen-specific response. Despite differences between the *in vitro* models, co-cultures of DCs/T cells provide useful information about antigen presentation and polarization ability of DCs in presence of primed CD4+ T cells.

4.4. *In vitro* models to assess B cell isotype switching (KE5)

The mechanisms controlling the induction of class switch recombination and production of IgE by switched B cells have been studied extensively. *In vitro*, the production of IgE by human B cells,

specifically induced by IL-4 or IL-13 and signalling via CD40 cell surface molecule, can be monitored at various levels of the ε class switching process, including during the induction of the sterile ε transcript, which precedes Ig heavy chain locus rearrangement by quantitative reverse transcription PCR (RT-qPCR) or Northern blot assay, the detection of production of ε excision circles during the Ig heavy chain locus rearrangement by PCR and the detection of production of IgE by ELISA as nicely reviewed by Pène et al., 2005.

Even though several *in vitro* B cell activation protocols are available (Lin & Calame, 2004; Pène et al., 2005), to the best of our knowledge no protocols have been established so far that investigate the (various levels of) class switching in a food allergen-specific context.

5. Future perspective

5.1. *In vitro* micro-fluidics systems

Advances in micro-physiological systems are providing researchers alternative means to gain insights into the molecular interactions of the gastrointestinal tract. These systems combine the benefits of micro-engineering, micro-fluidics, and cell culture in a bid to recreate the environmental conditions prevalent in the human gut. It becomes now possible to construct *in vitro* systems that more closely approximate those conditions present within the gut on scales identical to those encountered *in vivo* (Kim, Huh, Hamilton, & Ingber, 2012). These systems based on micro-fluidics offer numerous advantages over traditional cell-culturing techniques, including a 3D culture environment, greater experimental flexibility, the ability to precisely tune spatiotemporal oxygen and pH gradients, low shear environments, and the ability for high throughput experimentation. Although currently available micro-fluidics systems provide promising approaches to study local mechanisms that drive allergic responses, they have not yet been used to study any of the MIE or KE including in the AOP for food sensitization. Some examples of available micro-fluid devices are described below.

5.1.1. Human gut-on-a-chip device

A micro device “human gut-on-a-chip” has been developed by the Ingber group at Harvard University, which is composed of two micro-fluidic channels, separated by a porous flexible membrane coated with extracellular matrix and lined by Caco-2 cells to mimics the complex structure and physiology of living intestine. The gut micro-environment is established by flowing fluid, which produces low shear stress

over the micro-channels and exerts cyclic strain that mimics physiological peristaltic motions (Kim et al., 2012).

5.1.2. NutriChip

NutriChip is another integrated micro-fluidic platform developed by Swiss scientists for investigating potential immunomodulatory function of dairy food and represents a miniaturized artificial human gastrointestinal tract (Ramadan et al., 2013). NutriChip is a culture of a confluent layer of Caco-2 cells separated from co-cultured immune cells by a permeable membrane, which allows studying processes that characterize the passage of nutrients though the intestinal epithelium, including the activation of immune cells. NutriChip allows application of *in vitro* digested food on its apical side and a basolateral culture of a monocyte line (U937 cells) differentiated into macrophages (Ramadan et al., 2013).

5.1.3. Immuno-HuMiX

Human-microbial cross-talk platform (HuMiX) is also a micro-fluid device that enables the study of molecular interactions at the host-microbe interface (Eain et al., 2017). The features of HuMiX are similar to those of the other fluidics systems in which also the microbiome component may be introduced. Moreover, the system can be expanded to analyze the interactions between the immune system and the intestinal microbiota in the human gut, the so called Immuno-HuMix model. First steps have been undertaken to integrate human PBMCs in this system (Eain et al., 2017).

5.2. Organotypic *ex vivo* cultures

One of the major weaknesses of IEC lines is that they are different from the primary cells of the intestinal epithelium, from which they had originally been isolated. The selection of cells that survive and expand *in vitro* often leads to changes in gene expression profile and altered responses. In this respect, primary isolated cells may therefore be a better model system to represent processes in the intestinal mucosa. Promising *ex vivo* approaches are the generation of intestinal organoids and the culturing of *ex vivo* intestine samples.

5.2.1. Intestinal organoids

Major advances have been made in establishing culture conditions that support the long-term propagation and self-organisation of near-physiological tissue. In general, isolated somatic stem cells from various

organs are cultured in the presence of specific small molecules and growth factors, reflecting essential niche components of the respective tissue of origin. A 3D scaffold mimicking the basal lamina has further been shown to support *ex vivo* expansion of intestinal stem cells *ex vivo* and tissue formation that resembles part of the cellular architecture, hierarchy and physiology of the *in vivo* counterpart (Sato & Clevers, 2013). The culture of intestinal crypts and their growth and differentiation to organoids may be an interesting and easy to handle tool to study allergen interaction and associated signal transduction pathways in a complex intestinal system.

Despite the requirement for more expensive technology than IEC lines, intestinal organoids have been shown to have multiple applications and with the recent development of efficient gene-editing tools, it is now possible to generate highly physiological models of human gastrointestinal diseases (Leushacke & Barker, 2014). However, the study of allergen transport across the epithelial barrier is limited by the inaccessibility of food proteins to the apical side of IECs (directed to the lumen of the organoids).

5.2.2. *Ex vivo* models

Another approach to overcome the major drawbacks of the *in vitro* cell line-based models may be the use of complete intestinal tissues. In these models, the asymmetrical distribution of proteins and lipids in the two plasma membrane domains is facing the intestinal lumen. The internal milieu and the presence of highly organized structures joining adjacent IECs, enable selective processes of absorption, transport, and secretion to take place across the intestinal mucosa. The main e*x vivo* intestinal models for intestinal protein transport studies include the everted sac technique and the Ussing chamber. These approaches have been largely used to assess protein transport and, using intestinal tissue from sensitized animals, they may lead to study the effect of sensitization on allergen uptake. However, both techniques have several limitations including the rapid loss of the tissue viability and tissue damages during isolation, which may lead to overestimation of protein transport. Furthermore, the presence of the muscle layer in the everted sac method may lead to underestimation of protein transport. On the other hand, interspecies differences complicate extrapolation of data to humans. In this respect, pigs share more physiological and immunological similarities with humans than rodents and the recently developed InTESTine^{TM} method may be a medium-throughput alternative. InTESTine^{TM} is based on intestinal tissues from pigs that are incubated on a rocker platform in a high oxygen incubator (Westerhout et al., 2014).

5.3. *In silico* approaches

In silico methodologies and tools like databases and comparison software have been shown useful for the assessment of potential allergenicity of food proteins based on their properties. These *in silico* methods use a number of physico-chemical features (mainly amino acid searches) of proteins that can be predicted, such as B cell epitopes, T cell epitopes and sequence homologies (as reviewed by Hayes, Rougé, Barre, Herouet-Guicheney, & Roggen, 2015). They can identify whether a novel protein is an existing allergen and/or has the potential to cross-react with an existing allergen. However, they cannot identify whether a novel protein will 'become' an allergen, so therefore these approaches have limited value to identify truly novel allergenic proteins (Hayes et al., 2015). The use of future innovative *in silico* approaches will be largely influenced by the choice of databases and algorithms that will be developed, standardized, and most importantly, empirically validated.

6. Conclusion

Although basic science studies have identified a lot of immune pathways behind the allergic response, sensitization to food proteins is a complex process involving several molecular and cellular events (Sicherer & Sampson, 2018; Tordesillas, Berin, & Sampson, 2017). The simplification of the biological process of food allergy sensitization by applying the AOP concept is an effective strategy to identify *in vitro* methods that lead to investigate the sensitizing potential of food proteins. Previously, such an approach focusing on MIE and KE described in reported AOPs for skin and respiratory sensitization have been used successfully to study the sensitizing potential chemicals in skin and respiratory allergy (Ezendam, Braakhuis, & Vandebriel, 2016; Sullivan et al., 2017).

In this review, we have clustered, structured, and discussed the existing *in vitro* models that are suitable to study the MIE and KE involved in the AOP for food allergy sensitization (van Bilsen et al., 2017). To the best of our knowledge, this is the first time that the AOP concept is applied to structure all available *in vitro* methods to identify the potential sensitizing capacity of food proteins. In order to recapitulate all the reviewed assays, focusing on different (combinations of) MIE and KE reflecting the different building blocks of the AOP, Table 1 summarized *in vitro* methods used to evaluate the three major food allergens: chicken ovalbumin (Gal d 2), bovine β-lactoglobulin (Bos d 5) and peanut 2S albumin (Ara h 2).

In general, *in vitro* methods assessing mechanisms involved in the innate immune response are easier to address than assays assessing the adaptive immune response of food sensitization: the recognition molecules used by the innate system (here reflected in MIE, KE1, KE2, and KE3), are expressed broadly

on a large number of cells, which makes it easier to develop *in vitro* methodologies to study specific building blocks of the AOP, as seen by the rich set of available tools. However, more *in vitro* studies of the transcellular transport based on epithelial receptors (MIE2) or unspecific endocytosis (MIE3) should be carried out to better understand the effect of this type of intestinal acquisition in the sensitizing potential of food proteins.

On the other hand, the adaptive immune system (here reflected in KE4 and KE5) is composed of small numbers of T and B cells with specificity of any individual allergen. Therefore the responding cells must proliferate after encountering the allergen in order to attain sufficient numbers to mount an effective response that can be detected. This feature of the adaptive system complicates the development of *in vitro* approaches to assess KE4 and KE5. This is also reflected in Table 1 which depicts a limited number of *in vitro* approaches to address KE4 and the absence of an available *in vitro* assay to assess B cell isotype switching (KE5) in a food-allergen specific context.

It must be noted that the *in vitro* models discussed in this manuscript have been developed to obtain a better understanding of the processes involved in food sensitization. Several of the described *in vitro* models (summarized in Table 1) seem to be correlated with the sensitizing potential *in vivo* and some show great promise to closely approximate *in vivo* conditions (e.g. *in vitro* micro-fluidics systems and organotypic cultures), however as far as we know, none of the described models have been successfully used to assess the sensitizing potential of a given food protein by comparing high and low/none sensitizing food proteins in the assay. To this end, it would be an essential step forward to expand the panel of tested food proteins by including also low/non-allergenic proteins. Most likely, none of the assays will be able to distinguish high from low/non allergenic potency by itself; however the assays combined can provide an important set of data which may be helpful to decide which of the assays are essential to be part of the weight-of-evidence approach to determine the sensitizing potential of food proteins. Unfortunately, to date it is not known whether the lack of a suitable *in vitro* model to assess KE5 (B cell isotype switching) will result in a crucial data gap to determine the sensitizing potential or whether the KE5-model is redundant in the overall assessment.

In vitro models based on cell lines are very useful as research tools to investigate MIE and KE involved in food allergy sensitization. However, the closer these cell-based systems are to the actual target tissue, the better conclusions can be drawn. The main problem was that long-term propagation of native, non-

transformed single cells or cell clusters from the intestine was not feasible and it was generally assumed that it would not be possible to establish long-term cultures of primary adult tissues without the introduction of genetic transformations promoting cell proliferation and survival. In recent years, significant progress has been made in this field and robust systems have been identified. A variety of *in vitro* micro-fluidics systems and *ex vivo* culture strategies has been developed to investigate the function of the intestinal mucosa, which will help to increase the knowledge of food sensitization process.

Concluding, the application of the AOP framework offers the opportunity to anchor existing testing methods to specific building blocks of the AOP for food sensitization which provides insight which specific methods are available and which still need to be developed. When applied in the context of an integrated testing strategy, such an approach may reduce, if not replace, current animal testing approaches.

7. References

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9. Figure Caption

Figure 1. A tentative Adverse Outcome Pathway describing the mechanistic events driving food sensitization induction. Depicted are those events and relationships with substantial evidence for a role in food sensitization induction in human. DC: dendritic cell; *Outside the scope of this manuscript. Adapted from van Bilsen et al., 2017.

10. Table Caption

Table 1. Main *in vitro* approaches used to characterize major food allergen of bovine milk (Bos d 5), hen's egg (Gal d 2) and peanut (Ara h 2), organized following the molecular initiation events and key events described by the adverse outcome pathway for food allergic sensitization.

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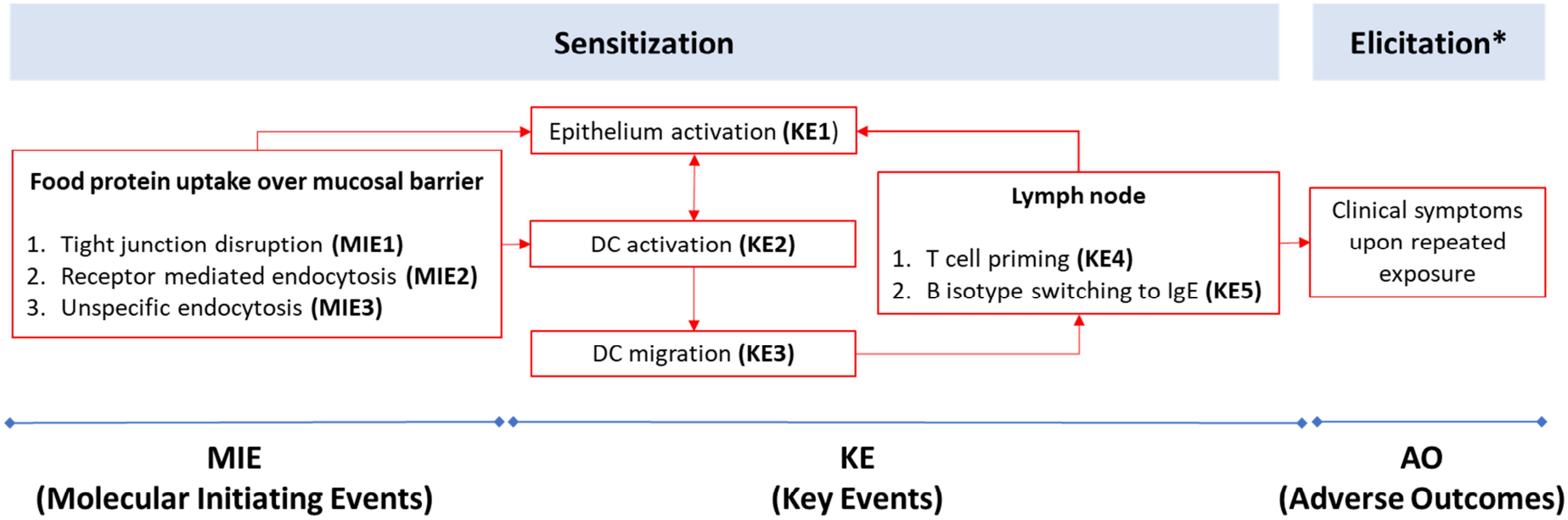
Event	Allergen	*In vitro* method	Read-outs	Main findings	Reference
MIE 1, 2, 3 & KE1	**Bos d 5**	Caco-2 and M cells	Allergen quantification (SDS-PAGE, Western blot and microscopy). Integrity of ZO-1 (microscopy).	Enhanced transcellular transport of intact Bos d 5 compared with heated Bos d 5.	(Rytkönen et al., 2006)
		Caco-2	Allergen quantification (ELISA and microscopy).	Bos d 5crossed epithelial barrier by endocytosis whereas tryptic peptides followed para- and transcellular transport.	(Bernasconi, Fritsché, & Corthésy, 2006)
		Caco-2	Allergen quantification (ELISA).	Enhanced transport of intact Bos d 5 compared with cross-linked Bos d 5.	(Stojadinovic et al., 2014)
		T84, Caco-2 and HCT-8	Monolayer integrity (TEER). Cytokine production (ELISA).	Bos d 5 maintained barrier integrity but increased production of IL-8 in HCT-8 cells.	(Yamashita et al., 2016)
	Gal d 2	Caco-2	Allergen quantification (ELISA).	Tryptophan residue without a free carboxyl group inhibited Gal d 2 transport.	(Tesaki & Watanabe, 2002)
		Caco-2	Monolayer integrity (TEER). Allergen quantification (ELISA and microscopy).	Gal d 2 crossed epithelial barrier by endocytosis and transcellular transport.	(Thierry et al., 2009)
		Caco-2	Allergen transport (RBL activation test)	Enhanced transport of intact Gal d 2 compared with heated Gal d 2.	(Martos et al., 2011)
		Caco-2	Monolayer integrity (TEER and Lucifer Yellow). Allergen quantification (ELISA and Western blot).	Gal d 2 showed higher epithelial passage than dephosphorylated Gal d 2.	(Matsubara et al., 2013)
	Ara h 2	HT-29	Monolayer integrity (TEER). Allergen quantification (ELISA). Integrity of A20 (Western blot and RT-qPCR).	Ara h 2 crossed epithelial barrier by endocytosis and reduced A20 expression.	(Song, Liu, Huang, Zheng, & Yang, 2012)
		Caco-2	Allergen quantification (ELISA and microscopy). Gene expression (RT-qPCR).	Ara h 2 induced inflammatory responses and showed a reduced binding ability.	(Starkl et al., 2011)
		Caco-2	Monolayer integrity (TEER). Allergen quantification (Western blot, microscopy and LC-MS/MS).	Ara h 2 induced disruption of tight junctions.	(Price et al., 2014)
KE 2 & 3	**Bos d 5**	Mouse BM-DCs	Allergen uptake (flow cytometry). Cytokine production (ELISA).	Intact Bos d 5 was internalized faster than cross-linked Bos d 5.	(Stojadinovic et al., 2014)
	Gal d 2	THP-1-derived DCs	Allergen uptake (flow cytometry). Gene expression (RT-qPCR). Cytokine production (ELISA).	Apple polyphenols suppressed Gal d 2 presentation via MHC-II degradation.	(Katayama et al., 2013)
		Mouse BM-DCs	Migration assay (flow cytometry). Cytokine production (ELISA).	PTPN12 regulated DC migration and antigen-induced T cell responses.	(Rhee et al., 2014)
	Ara h 2	Mouse BM-DCs	DC maturation (flow cytometry).	TLR signals modulated peanut-induce dendritic cells maturation.	(Pochard et al., 2010)
		Human Mo-DCs	Expression of DC markers (flow cytometry)	Peanut extract and agglutinin induced DC activation.	(Kamalakannan et al., 2016)
KE 4	**Bos d 5**	Human T cell clones	T cell proliferation ($[^3H]$-thymidine). Cytokine production (ELISA).	Bos d 5 induced Th2 cytokine production, but not enhanced proliferation in cow´s milk allergic patients.	(Schade et al., 2000)
		Human T cell clones	Cytokine production (ELISA). T cell activation (flow cytometry).	T cell activation status was associated with IL-4 and IL-13 production.	(Tiemessen et al., 2004)
		Human PBMCs	T cell proliferation (CFSE). Cytokine production (flow cytometry).	T cell response was associated with high proliferation and Th2 cytokine production.	(Tsuge et al., 2006)

		Human PBMCs	T cell activation (flow cytometry). Gene expression (RT-qPCR).	Enhanced expression of FoxP3, Nfat-C2, IL-16 and GATA-3 in patients with persisting cow's milk allergy.	(Savilahti et al., 2010)
		Human PBMCs	T cell proliferation ([^{3}H]-thymidine). Cytokine production (ELISA).	Bos d 5 induced T cell proliferation and production of Th2 cytokines.	(Vocca et al., 2011)
		Mouse MLN-isolated T cells	Cytokine production (ELISA).	Bos d 5 induced Th2 cytokine production, but low IFN-γ levels.	(Kanjarawi et al., 2011)
		Human T cell clones	T cell proliferation ([^{3}H]-thymidine). Cytokine production (ELISA).	Intact whey proteins induced higher T cell proliferation and production of cytokines than their hydrolysates.	(Knipping et al., 2012)
		Human T cell lines	T cell proliferation ([^{3}H]-thymidine). Cytokine production (ELISA).	Intact whey proteins induced T cell proliferation and production of IL-13, IL-10 and IFN-γ.	(Meulenbroek et al., 2014)
	Gal d 2	Human PBMCs	T cell proliferation ([^{3}H]-thymidine). Cytokine production (ELISA).	Gal d 2 induced T cell proliferation and high levels of IL-5.	(Ng, Holt, & Prescott, 2002)
		Mouse LP-isolated mononuclear cells	T cell proliferation (CFSE). Cytokine production (flow cytometry).	Gal d 2 induced T cell proliferation and increased IL-4+ and IFN-γ+ T cells.	(Sun et al., 2016)
	Ara h 2	Human PBMCs and T cell lines	T cell proliferation ([^{3}H]-thymidine). Cytokine production (ELISPOT).	Ara h 2 increased production of IL-4 and IL-5.	(Prickett et al., 2011)
		Human PBMCs	T cell proliferation and cytokine production (flow cytometry).	Ara h 2 induced T cell proliferation and production of Th2 cytokines.	(Vissers et al., 2011)
		Human PBMCs	T cell proliferation and expression of T cell markers (flow cytometry).	Peanut allergens increased proliferation of CD4+CD25+CD134+ T cells.	(Tao et al., 2016)
KE 2, 3 & 4	**Bos d 5**	Co-culture: BM-DCs/primed T cells	Allergen uptake (flow cytometry). T cell cytokine production (ELISA).	Intact and cross-linked Bos d 5 increased production of Th2 cytokines.	(Stojadinovic et al., 2014)
	Gal d 2	Co-culture: BM-DCs/CD4+ T cells	DC maturation (flow cytometry). T cell cytokine production (ELISA).	Glycation enhanced Gal d 2 uptake and CD4+ T cell activation compared with intact and heated Gal d 2.	(Ilchmann et al., 2010)
	Ara h 2	Co-culture: BM-DCs/primed T cells	DC maturation (flow cytometry). T cell cytokine production (ELISA).	Peanut pulsed-DCs induced Th2 response and production of IL-17 and IFN-γ.	(Pochard et al., 2010)

Ara h 2: peanut 2S albumin; BM-DCs: bone marrow-derived dendritic cells; Bos d 5: bovine β-lactoglobulin; CFSE: Carboxyfluorescein succinimidyl ester; DCs: dendritic cells;FoxP3: forkhead box P3; Gal d 2: chicken ovalbumin; GATA-3: GATA binding protein 3; KE: key event; LC-MS/MS: liquid chromatography tandem-mass spectrometry; LP: lamina propria; MIE: molecular initiating event; MLN: mesenteric lymph nodes; Mo-DCs: monocyte-derived dendritic cells; Nfat-C2: nuclear factor of activated T cells type 2; PBMCs: peripheral blood mononuclear cells; PTPN12: protein tyrosine phosphatase, non-receptor type 12; RBL: rat basophilic leukemia cells; RT-qPCR: quantitative reverse transcription PCR; TLR: toll-like receptor; TEER: trans-epithelial electrical resistance.



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HIGHLIGHTS

- The AOP for food sensitization helps to implement *in vitro* testing approaches.
- Innate immune mechanisms are easier to address than adaptive response.
- No *in vitro* protocols have been established for investigating IgE-class switching.
- *Ex vivo* strategies are promising to address multiple key events at the same assay.

APPLYING THE ADVERSE OUTCOME PATHWAY (AOP) FOR FOOD SENSITIZATION TO SUPPORT IN VITRO TESTING STRATEGIES

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