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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.

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

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8 **Key words**

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

11 **1. Introduction**

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

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

30 safety. Risk assessment should cover different domains including kinetics, toxicology, nutritional effects

31 and allergenicity (de Boer & Bast, 2018).

32 Studying the allergenicity of these foods is quite complex. Firstly, relevant allergenic testing material

33 needs to be obtained. To this end, several factors (e.g. pH, buffer, fatty substances in matrix etc), should

34 be taken into consideration to obtain an extract that provides a good picture of the novel food, which have

35 to be optimized case-by-case (Mazzucchelli et al., 2018). In addition, the purification of proteins might

36 also be also technically challenging and may result in chemical modifications, which needs to be assessed

37 prior to testing for allergenicity (Mazzucchelli et al., 2018). Next to the difficulties to obtain relevant

38 allergenic testing material, it is evident that properties of food matrix components are also relevant and

39 important in the sensitization process. The allergen is never in initial contact with the immune system in a

40 purified state; the matrix surrounds, interacts with, and can affect the physiochemical features of the

41 allergens. Currently, the primary influences of the matrix are thought to be antigen bioavailability and

42 release, digestibility and interactions with the immune system (McClain, Bowman, Fernández-Rivas,

43 Ladics, & Van Ree, 2014). However there are no straightforward approaches to address the many

44 variables represented by the matrix components in food (reviewed by McClain et al., 2014), so additional

45 knowledge is required which will help to develop tools to incorporate their influence on sensitization into

46 model systems (van Bilsen et al., 2017).

47 This manuscript focusses on the current available in vitro tools to study cellular and molecular

48 mechanisms driving the non-symptomatic sensitization phase of food allergy resulting in the generation

49 of food protein-specific IgE.

50 There are a considerable number of *in vivo* and *in vitro* data available describing molecular and cellular

51 events potentially involved in food sensitization. Recently, these events have been organized in a

52 sequence of related processes that is plausible to result in sensitization and useful to challenge current

53 hypothesis by applying the concept of adverse outcome pathway (AOP) (van Bilsen et al., 2017). The

54 proposed AOP framework provides a simplification of a complex biological process by collecting,

55 organizing and evaluating data that describe the events of an adverse outcome at a biological level of

56 organization with relevance for risk assessment. The application of the AOP concept allows to identify

57 the major molecular initiating events (MIE) and key events (KE) underlying food sensitization (Figure 1).

58 The AOP for food sensitization starts with a MIE involving the allergen uptake over the mucosal barrier

59   of the gut intestine. The food protein passage may induce the activation of intestinal epithelial cells

60   (IECs), representing KE1, followed by the local activation of dendritic cells (DCs) and their migration to

61   the mesenteric lymph nodes (KE2 and KE3). There, DCs present processed allergen to naive T cells

62   priming them toward a T helper type 2 (Th2) response (KE4). Thus these events may cause the activation

63   of B cells (KE5) and the production of specific IgE by plasma cells.

64   The events included in this AOP are still highly complex at molecular/cellular level, but the challenge is

65   to integrate MIE and KE to better understand the mechanistic pathways of food sensitization induction.

66   The AOP for food allergy offers the opportunity to anchor existing methods for the testing and

67   assessment of sensitizing potential of food proteins. Moreover, it gives insight into which specific assays

68   are suitable to evaluate the influence of novel food proteins and ingredients in the sensitization process.

69   It is still a matter of debate whether human, like mice, can be sensitized via other routes than the oral

70   route such as via the skin or respiratory route. Epidemiological studies in human populations seem to

71   confirm the skin as a relevant route for food sensitization induction and allergy in humans, however is

72   mechanistically not sufficiently understood yet (van Bilsen et al., 2017). Evidence for the respiratory

73   route is even more ambiguous (van Bilsen et al., 2017). Therefore, *in vitro* testing strategies focusing on

74   the dermal or respiratory route are not explored in this review.

75   In this review, we aim to provide the state-of-the-art of existing *in vitro* approaches for assessing

76   sensitizing potential of food proteins based on the identified MIE and KE proposed in the AOP for food

77   sensitization, as described by van Bilsen et al. (van Bilsen et al., 2017). For this propose, we provide cell

78   assays previously used for the study of food allergens focusing on major read-outs as well as strengths

79   and limitations of these assays..

80   **2. Antigen uptake over intestinal mucosal barrier and epithelium activation**

81   **2.1. *In vitro* models to assess tight junction disruption (MIE1)**

82   During the gastrointestinal digestion, intact proteins and peptide fragments reach the intestinal lumen

83   where they interact with the IECs resulting in antigen uptake over the gut barrier. Digestion products may

84   be transferred across the intestinal epithelium by paracellular transport driven by disruption of tight

85   junctions, adherent junctions and desmosomes, representing MIE1. Tight junctions are multiprotein

86 complexes composed by transmembrane proteins (occludin, claudin, junctional adhesion molecule A and

87 tricellulin) that provide the integrity of the actomyosin ring, which controls inter-epithelial permeability.

88 Typically, epithelial *in vitro* models employed to study the transport and absorption of food proteins or

89 peptides along the intestinal epithelium are based on cell lines (including Caco-2, HT-29, T84, and IPEC-

90 J2) grown in a transwell system (Cubells-Baeza et al., 2015). In this regard, Grozdanovic and co-workers

91 demonstrated that the exposure of T84 cells to actinidin, a kiwifruit cysteine protease, resulted in the

92 impairment of the epithelial barrier, due to the degradation of occludin promoted by the proteolytic action

93 of actinidin (Grozdanovic et al., 2016). As a consequence, an increase of the intestinal permeability was

94 recorded which could contribute to the process of sensitization in kiwifruit allergy. By employing the

95 Caco-2 cell model, Price et al showed that peanut allergens Ara h 1 and Ara h 2 were able to alter the

96 intestinal barrier permeability, modifying the co-localization of the transmembrane tight junction proteins

97 occludin, JAM-A and claudin-1, with the intracellular adhesion protein ZO-1 (Price, Ackland, Burks,

98 Knight, & Suphioglu, 2014). In another study, β-conglycinin from soy (Gly m 5) induced a

99 downregulation of tight junction proteins by using a model of IPEC-J2 porcine cells (Zhao et al., 2015).

100 On the other hand, the breach of epithelial barrier may be a consequence of Th2 switching and may

101 possibly reflect the abnormal responses and vicious cycle triggered by mast cell activation. It has been

102 observed that mast cells releasing chymases and histamine have an impact on the physiology of the

103 intestinal mucosa, promoting the degradation of the tight junction occludins (Bischoff & Kramer, 2007).

104 Moreover, mast cells are involved in the release of Th2-realted cytokines, such as IL-4 and IL-13, that

105 influence the modulation of IEC permeability in different human IEC cultures, decreasing the trans-

106 epithelial electrical resistance (TEER) and selectively increasing the apical-to-basal movement of proteins

107 (Ceponis, Botelho, Richards, & McKay, 2000). In order to address the integrity of the intestinal

108 monolayer, the study of the TEER is an important issue that provides information about the inter-

109 epithelial transport, generally associated with an alteration of the tight junctions and thus relates to

110 paracellular transport. It has been reported that exposure of crude apple homogenate produces an increase

111 of the paracellular resistance in Caco-2 cells, with an augmentation of the expression of several tight

112 junction related genes, including claudin 4 (Vreeburg, Bastiaan-Net, & Mes, 2011). Zhao et al. have

113 recently shown that peptides produced during simulated *in vitro* digestion of soybean β-conglycinin,

114 determine an increase of alkaline phosphatase activity with a decrease of mitochondrial respiration (MTT

115 assay), TEER values and downregulation of claudin-3, claudin-4, occludin, and ZO-1 expression (Zhao,

116 Liu, Zhang, Pan, & Qin, 2017).

117 However, not all food allergens affect TEER resistance. Moreno et al. reported that the transcellular

118 transport of purified 2s albumins Ber e 1 (brazil nut) and Ses i 1 (sesame seed) across Caco-2 monolayer

119 did not affect the permeability as assessed by the absence of any change of allergen absorption rate and

120 TEER values (Moreno, Rubio, Olano, & Clemente, 2006). In other work, authors found that hydrolyzed

121 ovalbumin did not affect MTT values or cell permeability indicating a protective effect exerted by the

122 food matrix on the cell barrier (Grootaert et al., 2017). In addition, TEER values were restored to their

123 original levels, demonstrating the absence of any permanent damage on the monolayer caused by egg

124 digests tested (Grootaert et al., 2017). In line with this, previous studies also reported that purified

125 proteins from wheat (ω5-gliadin and LTP 1) (Bodinier et al., 2007), peach (Pru p 3 and LTP 1)

126 (Tordesillas et al., 2013), and peanut (Ara h 1 and Ara h 2) (Price, Ackland, & Suphioglu, 2017) were

127 able to cross Caco-2 monolayers without compromising cell monolayer integrity.

128 **2.2. *In vitro* models to assess receptor-mediated and unspecific endocytosis (MIE2 and MIE3)**

129 Apart from paracellular transport, food proteins may also cross the intestinal epithelium via transcellular

130 transport pathways, either receptor-mediated (e.g. CD23, the low affinity FcεRII-IgE receptor) (MIE2) or

131 via unspecific endocytosis (MIE3).

132 CD23 is expressed by Caco-2, T84 and HT29 cells and their expression can be stimulated by IL-4 (Tu et

133 al., 2005). Using Caco-2 cells, it has been demonstrated that IgE-antigen (nitrophenyl(NP)-BSA)

134 complexes trigger the upregulation of IL-8 and CCL20. The supernatant of such triggered Caco-2 cells

135 induced DC migration in a CCL20-dependent manner (Li et al., 2007). In HT29 cells, it has been shown

136 that CD23 expression and transcellular transport of IgE-NP-ovalbumin complexes could be stimulated by

137 factors present in supernatant of activated mast cell (HMC1) and monocyte (THP1) lines (Tu, Oluwole,

138 Struiksma, Perdue, & Yang, 2009). The role of SIgA in transcytosis has been studied even less than that

139 of CD23, and mainly focus on gliadin peptides. Caco-2 and HT29 cells express CD71 and

140 transglutaminase 2 (TG2), both shown to co-precipitate with SIgA and important for endosomal transport

141 routing (Lebreton et al., 2012). These reports on receptor-mediated endocytosis are from a few isolated

142 studies, but they show that the role of CD23-mediated and of CD71/TG2 transcytosis could be examined

143 using *in vitro* models, although more food proteins using these assays should be tested.

144 Unspecific transport of food proteins may occur via epithelial, goblet or M cells and may result not only

145 in protein degradation within these cells and cellular activation, but also it can lead to process of proteins

146 into peptides that can be expressed in the context of MHC-II. The route of uptake seems to have an

147 important implication for the final outcome. Although insufficiently studied, it has been proposed that

148 larger cross-linked proteins could be more sensitizing because they are preferentially taken up by M cells

149 and directly interact with immune cells in Peyer's patches. Roth-Walter and co-workers described that the

150 intestinal transport of soluble □-lactoglobulin occurs through enterocytes, while the uptake of its

151 aggregates is redirected to Peyer's patches (Roth-Walter et al., 2008). On the other hand, smaller intact

152 proteins are transferred via goblet-associated passage (also called GAP) and that may be linked to

153 processing by CD103+ DCs, facilitating tolerance rather than to other DCs that are linked to sensitization

154 (McDole et al., 2012). M cell transfer and GAP are not often studied using *in vitro* methods, but cell lines

155 as well as organoids can be adapted to study the particular involvement of these transportation routes. For

156 instance, cell lines and intestinal stem cell-derived organoids can be stimulated to form M cells by

157 RANKL-RANK pathway activation (Kimura, 2018) and GAP could be studied in specialized cell lines

158 (HT29-H cells) or in organoids (constitutive presence of goblet cells).

159 Endocytosis is a complex process that includes caveolea-mediated uptake mechanisms, clathrin-

160 dependent and -independent uptake mechanisms, macro-pinocytosis and phagocytosis. These processes

161 may operate alongside each other and can be studied by using specific inhibitors (Dutta & Donaldson,

162 2012). Important to mention here is that many of these inhibitors (e.g. filipin, cytochalasinD and

163 monodansylcadaverine) also affect viability as well as TEER values (Price et al., 2017). Little information

164 with regard to the importance of various mechanisms of endocytosis is yet available for food allergens.

165 But studies with individual proteins show that different mechanisms of endocytosis may be involved for

166 different proteins; for instance, Ara h 1 may be endocytosed via a combination of macro-pinocytosis and

167 clathrin-dependent processes, whereas Ara h 2 may be endocytosed via a combination of macro-

168 pinocytosis and caveolea-mediated uptake mechanisms (Price et al., 2017). Although authors speculate

169 that the localization of Ara h 2 resulting from the specific way of endocytosis is linked to higher

170 allergenicity of Ara h 2, other potential explanations such as their different molecular weight or physico-

171 chemical properties should be also considered.

172 Altogether, *in vitro* models such as Caco-2 cells cultured onto transwell systems seem suitable to study

173 the importance of MIE2 and MIE3 in sensitization to food proteins. However, no data is currently

174 available for ranges of allergens and clearly this information is needed to link endocytosis *per se* as well

175 as the type of translocation to the sensitizing potential of a food protein.

176 **2.3. *In vitro* models to assess epithelium activation (KE1)**

177 Beyond being transported across the gut epithelial barrier, food proteins and their digestion products are

178 able to interact with IECs in the intestinal lumen thereby triggering immune responses. It is widely

179 accepted that food sensitization involves factors from gut epithelium, which are released after the

180 activation of IECs (KE1). The presence of food allergens in the gut is mainly detected by pattern

181 recognition receptors (PRRs), such as toll like receptors (TLRs), glycan binding receptors (galectins) or

182 protease-activated receptors (PARs). This leads to the activation of inflammatory mechanism resulting in

183 the activation of the NF-κB route and initiate the repair process for the damaged barrier with the

184 production and secretion of chemokines, cytokines, reactive oxygen species (ROS), and lipid metabolites.

185 *In vitro* studies have closely linked the production of ROS to the activation of epithelial cells in the case

186 of aeroallergens. The presence of IL-13 in the environment and the activation of PAR-2 receptor have

187 been implicated in the upregulation of ROS production by epithelial cells (Dickinson et al., 2016;

188 Nadeem et al., 2015). However, the effect of food allergens on IECs have not been so furtherly studied

189 and we can only report the case of peanut allergens, in which an increase in the nitric oxide synthase gene

190 expression has been observed (Starkl et al., 2011).

191 In response to their activation, IECs are also able to release epithelial-specific cytokines (e.g. IL-1, IL-18,

192 IL-25, IL-33, and the thymic stromal lymphopoietin –TSLP-) that are crucial for the initiation of food

193 protein sensitization. This cytokine environment induces the activation of DCs, type 2 innate lymphoid

194 cells (ILC2), basophils, eosinophils, and mast cells, skewing the intestinal immune system towards a Th2

195 response. IL-33, IL-25, and TSLP are constitutively expressed by epithelial cells being IL-33 a crucial

196 regulator of mast cells. Besides that, IL-33 is able to enhance granulocyte, macrophage, and ILC2

197 responses (Saluja, Khan, Church, & Maurer, 2015). IL-25 elicits multipotent progenitor type 2 cells, a

198 population of innate cells promoting type 2 cell immunity even in absence of ILC2 (Saenz et al., 2013).

199 Regarding TSLP, its expression is increased in a NF-κB dependent-manner, being able to activate mast

200 cells and influencing antibody production (Miron & Cristea, 2012). On the other hand, the production of

201 IEC-derived TGF-β promotes DCs involved in tolerogenic signals, which is an important milestone for

202 the control of allergic sensitization (Wang & Sampson, 2009). Tordesillas et al. examined the effect of the

203 major peach allergen Pru p 3, in comparison with that of the hypoallergenic peach LTP 1 using Caco-2

204 cells grown in a transwell system. Authors showed that in the presence of Pru p 3 an increased expression

205 of Th2-driving cytokines (IL-25, IL-33, and TSLP) was observed. However, with the hypoallergenic

206 protein LTP 1, the induction these cytokines was significantly lower (Tordesillas et al., 2013). In other

207 study, the effect of the peanut allergen Ara h 2 on IECs showed upregulation of the inflammatory

208 cytokines IL-1β and IL-8 (Starkl et al., 2011).

209 Concluding, the activation of epithelial cells is a heterogeneous process in which the cytokine

210 environment is crucial, for this reason the effects of more food allergens on IECS should be described

211 using other cell types than Caco-2 cells. Despite the fact that the majority of studies are focused on the

212 analysis of the cytokines expression, other pathways should be explored, such as mucus secretion or Ca^{2+}-

213 signaling pathway alterations.

214 **2.4. *In vitro* models to assess antigen uptake combined with epithelium activation (MIE and KE1)**

215 Approaches which combine the possibility to evaluate the contribution of allergens transport (MIE) to

216 IEC activation and cytokine production (KE1) are important tools for studying the immunogenic

217 properties of food proteins. The interaction of food allergens with the intestinal epithelium not only leads

218 to the secretion of innate cytokines, but also the production of chemokines and other soluble factors such

219 as alarmins (e.g. uric acid, ATP, HMGB1, and S100 proteins) that drive immune polarization by affecting

220 DC function and the adaptive response (Gavrovic-Jankulovic & Willemsen, 2015; van Bilsen et al.,

221 2017). Furthermore, there is evidence that mediators released by peripheral blood mononuclear cells

222 (PBMCs) and mast cells contribute to increase intestinal permeability which means that immunologic

223 status can, by itself, affect IEC activation and the access of the allergen through epithelium (Tordesillas et

224 al., 2013).

225 In this sense, several strategies have already been attempted with relative success. IEC lines grown in

226 transwell systems (e.g. Caco-2, T84, or HCT-8) have been used to assess allergen uptake and subsequent

227 epithelium activation by measuring cytokine production or changes in gene expression induced by

228 allergens (Starkl et al., 2011; Tordesillas et al., 2013). In order to study the influence of intestinal soluble

229 factors from immune cells on IECs, some *in vitro* strategies have used separate monocultures of IECs and

230 immune cells and then supernatant from immune cells has been applied to IECs (Martos, Lopez-Exposito,

231 Bencharitiwong, Berin, & Nowak-Wegrzyn, 2011; Tordesillas et al., 2013; Yamashita, Yokoyama,

232 Hashimoto, & Mizuno, 2016) or *vice versa*. This strategy may contribute to a better comprehensive

233 understanding of the *in vivo* cross-talk affecting KE1.

234 In addition, co-culture systems allowing cross-talk between structural cells (IECs) and effector immune

235 cells (such as basophils) are being attempted to study whether a food allergen induces not only a direct

236 epithelial activation, but also its consequences on the underlying immune cells, enabling to assess the

237 immune cell responses through epithelial activation. An *in vitro* model based on the co-culture of Caco-2

238 cells and rat basophilic leukaemia cells (RBLs) has been used for the study of immune activation upon

239 Gal d 2 challenge at the apical side of the IECs (Thierry, Bernasconi, Mercenier, & Corthesy, 2009).

240 Although not performed, epithelial activation markers may be evaluated using this co-culture system.

241 More complex co-cultures combining mucoid IECs, non-mucoid IECs and B cell lines (Caco-

242 2/HT29/Raji-B) have been suggested in the literature as systems to integrate allergen uptake with

243 epithelial activation markers as well as with modulation by the immune cell signals (Araujo & Sarmento,

244 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)

247 As major antigen presenting cells, gut-associated DCs play a key role in immunological pathways

248 associated to food protein sensitization process. Food proteins or their digestion products may induce

249 activation of DCs (KE2) by C-type lectin receptor (CLRs) or other PRRs leading to a decrease in TLR-

250 induced IL-12 production, as well as upregulation of OX40L, TIM-4, or both. In addition, DCs can be

251 activated by IEC-derived IL-33 which also upregulates OX40L expression on DCs. After allergen uptake

252 and activation, DCs migrate (KE3) to draining lymph nodes mediated by the expression of CCR2, CCR5

253 and CCR7 molecules. Other interesting surface markers to assess as indicators of antigen presentation are

254 HLA-DR (MHC class II), CD86 (Katayama et al., 2013) and DC-SIGN binding by fluorescent-labeled

255 food proteins (Kamalakannan, Chang, Grishina, Sampson, & Masilamani, 2016).

256 Besides DC assays analyzing the presence of (binding to) surface molecules and cytokine expression,

257 other methods have been described evaluating the differences in DC endocytosis by incubating murine

258 bone marrow-derived DCs (BM-DCs) with FITC-labeled β-lactoglobulin and by *in vitro* endolysosomal

259 degradation of the native and cross-linked forms of this allergen (Stojadinovic, Pieters, Smit, &

260 Velickovic, 2014). These data indicated differences that correlated with the sensitizing potential *in vivo*,

261 suggesting that these additional parameters may be useful *in vitro* parameters to evaluate the sensitizing

262 potential of food proteins. On this regard, BM-DCs have been applied to estimate the effect of thermal

263 process on ovalbumin and showed that a glycation product of this allergen, pyrraline, induced higher

264 uptake by DCs associated with the scavenger receptor class A (Heilmann et al., 2014).

265 Transcriptomic profiling of allergen-activated DCs is another approach to compare the DCs activating

266 potency of food proteins. Comparison of various cell lines with human monocyte-derived DCs (moDCs)

267 by gene profiling suggested that the MUTZ-3 cell line resembles moDCs (Larsson, Lindstedt, &

268 Borrebaeck, 2006). A clone of this cell line has been recently used to assess and predict the sensitizing

269 potential of proteins in respiratory allergy in the Genomic Allergen Rapid Detection (GARD) assay. In

270 this assay, cellular responses induced by eight selected proteins were assessed using transcriptional

271 profiling, flow cytometry and multiplex cytokine analysis. A total of 391 potential biomarkers were

272 identified as a predictive signature and series of cross-validations supported the effectiveness of this

273 model. These results together with biological pathway analysis of the transcriptomic data indicate that the

274 investigated cell system is able to capture relevant events linked to type I hypersensitization (Zeller et al.,

275 2018). Although promising, the relevance of this model for food sensitization induction in general

276 remains to be established.

277 Migration assays using BM-DCs (Rhee, Zhong, Reizis, Cheong, & Veillette, 2014) or MUTZ-3 cells

278 (Rees et al., 2011) have been described in transwell systems in which activated DCs are applied in the

279 upper chamber, whereas an appropriate chemokine is added to the lower chamber. After incubation,

280 migrated cells are harvested from the lower chamber and quantified (Rhee et al., 2014). However, these

281 migration assays have never been proven useful in a food allergen specific context.

282 **3.2. *In vitro* models to assess interactions between epithelial and dendritic cells (approaches**

283 **integrating KE1, KE2 and KE3)**

284 The IECs- and DCs-derived signals constitute an allergen-induced inflammatory microenvironment that

285 triggers DCs maturation and migration. Test methods incorporating IECs and DCs have been developed

286 in a variety of formats: co-cultures of DCs/IECs, three-dimensional (3D) models reconciling the complex

287 and dynamic interactions that exist *in vivo* between the intestinal epithelium and the luminal side, and

288 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.,

317 2010) have also been evaluated. Cell suspensions are prepared from immunized mice and cultured with

318 the antigen for 3-6 days, depending on the subsequent analyses. T cell proliferation, expression of specific

319 cellular surface markers and/or cytokine secretion are mostly determined in these studies. T cell

320 proliferation is one of the most common ways to assess T cell activation upon *ex vivo* re-stimulation with

321 the allergen. To this end, several methods such as labeling of cells with a fluorescent dye (Pochard et al.,

322 2010; Sun et al., 2016), incorporate a radioactive nucleoside into new strands of chromosomal DNA

323 during mitotic cell division (Freidl et al., 2017; Stojadinovic et al., 2014) or colorimetric assays for

324 assessing cell metabolic activity (Sun, Liu, Wang, Liu, & Feng, 2013; Wai, Leung, Leung, & Chu, 2016)

325 have been used.

326 Proliferation assays have been performed for □-lactoglobulin (Mizumachi, Tsuji, & Kurisaki, 2008;

327 Stojadinovic et al., 2014), ovalbumin (Castro et al., 2012; Sun et al., 2016, tropomyosin (Capobianco et

328 al., 2008; Wai et al., 2016), Cyp c 1 from carp (Freidl et al., 2017), peanut (Pochard et al., 2010), cashew

329 and walnut extract (Kulis, Pons, & Burks, 2009), and soybean proteins (Sun et al., 2013), showing in all

330 the cases a strong proliferative response of the cells. Expression of specific surface markers have been

331 measured in sensitized mice after challenge with whey and ovalbumin, showing an increase in the

332 percentage of both, activated Th1 and Th2 cells compare to control animals (Lozano-Ojalvo, Perez-

333 Rodriguez, Pablos-Tanarro, Molina, & Lopez-Fandino, 2017; Vonk et al., 2017). However, when peanut

334 extract was used there was no difference in the percentage of activated Th2 cells, and the percentage of

335 activated Th1 cells was decreased (Vonk et al., 2017).

336 Levels of secreted cytokines are often evaluated by ELISA or flow cytometry in cell culture supernatants.

337 This method has been used to evaluate several food allergens from cow's milk (Kanjarawi et al., 2011;

338 Stojadinovic et al., 2014), peanut (Smit et al., 2015; Zhu et al., 2016), hen's egg (Pablos-Tanarro, Lopez-

339 Exposito, Lozano-Ojalvo, Lopez-Fandino, & Molina, 2016; Sun et al., 2016), soybeans (Sun et al., 2013)

340 and shrimp (Wai et al., 2016). Moreover, extracts from whey, hen's egg white, peanut, cashew, walnut

341 and sesame seeds have also been evaluated using this method (Lozano-Ojalvo et al., 2017; Smit et al.,

342 2015; Vonk et al., 2017). A high increased secretion of Th2 cytokines (IL-4, IL-5, IL-13) has been

343 observed in all the studies, whereas some discrepancies were observed between allergenic proteins in

344 their potency to induce IFN-γ (Stojadinovic et al., 2014; Vonk et al., 2017) and IL-10 (Vonk et al., 2017).

345 A few studies have measured increased levels of IL-17 (Lozano-Ojalvo et al., 2017; Rupa, Nakamura,

346 Katayama, & Mine, 2014) , while in other limited number of studies IL-22 and TNF-α levels showed no

347 differences compared to non-sensitized control cells (Pablos-Tanarro et al., 2016; Zhu et al., 2016).

348 The assays described above, have provided efficacy to identify known allergens that drive allergic

349 reactions in individuals and allergen modifications that ameliorate their allergenic potential, as well as to

350 identify T cell epitopes of cross-reactivity with other allergens. However, they are not effective to identify

351 the allergenic potential of new proteins that are responsible for stimulating the underlying Th2 responses,

352 mainly due to the need to use cells from previously sensitized mice against the same allergen to perform

353 the assay. Moreover, although evaluation of T cell activation through T cell proliferation assays is widely

354 describe in the literature using different strategies, the use of indirect methods such as those that measure

355 mitochondrial activity (e.g. MTT or WST assays) should be avoided because they reflect viable cell

356 metabolism and not specifically cell proliferation, requiring additional confirmations.

357 **4.2.** *In vitro* **models to assess human Th2 cell priming (KE4)**

358 The activation and priming of naive T cells (KE4) for allergic food proteins in the draining lymph nodes

359 results from the recognition of an epitope presented by Th2 driving DCs in the context of HLA (MHC-II).

360 Most of the approaches for investigating KE4 using human samples are based on the *ex vivo* re-

361 stimulation of PBMCs from allergic patients and the study of the allergen-specific induced proliferation

362 and cytokine production (Flinterman et al., 2010; Tao et al., 2016; Tiemessen et al., 2004; Vocca et al.,

363 2011). Indeed, stimulation of PBMCs from cow's milk allergic children with β-lactoglobulin was found

364 to induce cell proliferation and increased IL-13 over IFN-γ release compared to healthy or tolerant

365 controls (Vocca et al., 2011). Also in PBMCs from peanut allergic patients, allergen-specific proliferation

366 could be assessed by determining proliferation within the CD25+CD134+CD4+ T cell population after

367 stimulation with raw peanut (Tao et al., 2016). However, in peanut allergic patients, *ex vivo* stimulation of

368 PBMCs with peanut extract increased both IL-13 and IFN-γ as well as TNF-α levels compared to PBMCs

369 from healthy controls (Flinterman et al., 2010).

370 However, as pointed out in the previous section for KE4, the precursor frequency of allergen-specific T

371 cells is very low in the peripheral blood of allergic patients. In this regard, the generation of T cell lines

372 and T cell clones is an interesting alternative that lead to analyze Th2 cell activation (Flinterman et al.,

373 2010; Tiemessen et al., 2004). Assays to study the functionality of these allergen-specific T cells make

374 the use of allergen induced selection and cloning to improve the sensitivity of the assay. A mixture of β-

375   lactoglobulin derived synthetic peptides were used to generate antigen-specific T cell lines and clones

376   from PBMCs of cow's milk allergic patients (Sakaguchi et al., 2002). Limiting dilution clones were

377   isolated and then used for epitope mapping. Results showed a sequence of 12 amino acids recognized by

378   three out of six T cell clones from 5 different patients, which was associated with presentation via

379   HLADRB1*0405 (Sakaguchi et al., 2002). Kondo et al. further studied intracellular cytokine expression

380   in two of those clones, showing an increased production of IL-4 and IFN-γ both in combination with IL-

381   10 (Kondo et al., 2005). In this sense, cytokine release measurement may provide additional information

382   concerning the type of immune response that is raised against these epitopes. Tiemessen et al. generated T

383   cell clones using whole cow's milk proteins and compared the cytokine response in a group of cow's milk

384   allergic infants with non-symptomatic allergic patients and healthy donors (Tiemessen et al., 2004).

385   Results showed that all different groups strikingly reacted to milk proteins, although cytokine production

386   by allergic individuals was the highest for IL-4, IL-13, IFN-γ, and IL-10 (Tiemessen et al., 2004).

387   However, only the symptomatic group revealed a negative or no correlation between IL-4/IFN-γ and IL-

388   10, while in the other two groups there was a positive correlation, showing the importance of IL-10 as a

389   regulatory cytokine involved in tolerance induction. In addition, T cells from the symptomatic group had

390   a high expression of the activation marker CD69 (Tiemessen et al., 2004). Beyond studying differences in

391   T cell phenotype between patients and controls, T cell epitope disruption can also be studied using T cell

392   lines as showed by the fact that β-lactoglobulin was able to induce a higher proliferation and cytokine

393   secretion than its products of hydrolysis (Knipping et al., 2012). In short-term peanut-specific T cell lines

394   generated from PBMCs of peanut allergic patients, Ara h 1, Ara h 3, and Ara h 6 were identified to induce

395   the highest proliferation and secretion of IL-13, showing that these cell lines created using crude peanut

396   extract can be useful for allergen identification (Flinterman et al., 2010). Furthermore, allergen-specific T

397   cell clones from HLA diverse donors can be generated in order to evaluate the epitopes involved in T cell

398   activating capacity in a certain HLA context (Prickett et al., 2011, 2013). This method has been used to

399   identify 10 core epitopes of Ara h 1 and 5 core epitopes of Ara h 2 that could effectively induce T cell

400   proliferation in a HLA-DQ and/or HLA-DR and/or HLA-DP restricted manner (Prickett et al., 2011,

401   2013). In the latter study, it was also showed that Ara h 2 peptides were able to enhance IL-4 and IL-5

402   secretion (ELISPOT) in PBMCs from peanut allergic donors (Prickett et al., 2011).

403   Based on an *in silico* predictions, Ramesh et al. evaluated the immunogenic potential of 36 Ara h 1

404   derived peptides studying proliferation and cytokine production after *ex vivo* re-stimulation of PBMCs

405   from peanut allergic patients with those peptides (Ramesh et al., 2016). Almost all the *in silico* selected

406   peptides induced proliferation and predominantly a high IL-13 release when compared to IFN-γ (Ramesh

407   et al., 2016). This study indicates that PBMCs from allergic donors, combined with an appropriate *in*

408   *silico* prediction, may yet be sufficient to identify allergenic epitopes of certain food proteins. In addition,

409   MHC-peptide tetramers assays have been previously used to identify allergen-specific T cells in PBMCs.

410   DeLong et al. cultured PBMCs from peanut allergic patients with Ara h 1 peptides loaded on biotinylated

411   HLA-DR proteins and intracellular cytokine expression was identified in tetramer labeled cells (Delong et

412   al., 2011). Results showed that CCR4 expressing Ara h 1 reactive T cells responded to different epitopes

413   and produced mostly IL-4 beyond other cytokines (Delong et al., 2011).

414   In these types of assays, allergen-specific T cells can be identified by a highly upregulated Th2-prone

415   culturing environment. They may not provide much information regarding the sensitizing potential of a

416   given food protein, but merely indicate whether specific (or novel) food-derived proteins or peptides can

417   be recognized by T cells and pose a potential risk. Moreover, these approaches are useful to identity novel

418   food epitopes that are recognized by T cells.

419   **4.3. *In vitro* models to assess DC activation/migration and T cell priming (approaches integrating**

420   **KE2, KE3 and KE4)**

421   After DC activation (KE2 and KE3), DCs migrate to the draining lymph nodes and drive T cell activation

422   and differentiation (KE4). *In vitro* models to study antigen presentation and polarization of Th0 toward

423   Th2 cells are usually based on co-culture approaches using DCs and primed T cells.

424   In methods based on murine cells, BM-DCs have been co-cultured with CD4+ T cells from mice

425   sensitized to peanut (Pochard et al., 2010), whey (Stojadinovic et al., 2014) and a panel of purified food

426   proteins (Smit, de Zeeuw-Brouwer, van Roest, de Jong, & van Bilsen, 2016). In addition, CD4+ T cells

427   have also been obtained from TCR transgenic strains such as DO11.10 (Ilchmann et al., 2010) or OT-II

428   mice (Rhee et al., 2014). These studies have showed that DCs increased the expression of

429   activation/migration markers (MHC-II, CD80, and CD86) and the release of stimulatory cytokines such

430   as IL-6 and IL-12 after re-stimulation with the allergen. Furthermore, activated DCs induce a high

431   proliferation and a marked Th2 profile (enhanced production of IFN-γ, IL-4, IL-13, and IL-5 determined

432   by ELISA) in primed CD4+ T cells. Similar results were obtained when DCs were directly isolated from

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

434   2008).

435   Among human *in vitro* models, DCs used for co-culture with T cells have been obtained from

436   differentiated THP-1 cell line (Katayama et al., 2013) and allergen-pulsed monocyte-derived DCs

437   (Gomez et al., 2012; Scott-Taylor, Axinia, & Strobel, 2017). Allergens have been shown to activate DCs

438   revealed by an enhanced expression of CD80, CD83, and CD86 (Gomez et al., 2012), production of

439   inflammatory cytokines (IL-12p70, IL-1β, TNF-α, and IL-10) and up-regulated expression of MARCH

440   genes. For the study of the cross-talk with T cells, PBMCs from healthy donors (Tordesillas et al., 2013)

441   or cashew allergic patients (Archila et al., 2016) have been previously used. In addition, T cell lines

442   generated from cow's milk allergic children (Meulenbroek et al., 2014) and CD4+ T cells directly

443   isolated from patient allergic to peach (Gomez et al., 2012) and other food allergens (Scott-Taylor et al.,

444   2017) have been previously used for co-cultures. Results have shown that, in presence of the food

445   allergen, DCs induce T cell proliferation (observed by flow cytometry using carboxyfluorescein

446   succinimidyl ester molecule -CFSE- or CD154 staining) in sensitized individuals compared to healthy

447   donors. In addition, allergen-pulsed DCs trigger activation of T cells and increase the production of T

448   cell-like cytokines such as IL-4, IL-13, and IFN-γ.

449   One of the strengths of human *in vitro* models is the use of T cells from allergic patients in an autologous

450   setting, although it coincides with a downside, the limited number of cells that can be used (Lundberg et

451   al., 2008). In addition, there are variations in the experimental protocols used for pulsed-DCs alone with

452   the allergen of interest (Gomez et al., 2012; Scott-Taylor et al., 2017) or combined with maturation

453   factors, such as TNF-α, IL-1β, or LPS (Ashjaei et al., 2015). These differences during maturation can

454   affect expression of DC co-stimulatory and maturation markers and thus the subsequent proliferation of T

455   cells and cytokine production, although most of the studies were able to induce an allergen-specific

456   response. Despite differences between the *in vitro* models, co-cultures of DCs/T cells provide useful

457   information about antigen presentation and polarization ability of DCs in presence of primed CD4+ T

458   cells.

459   **4.4. *In vitro* models to assess B cell isotype switching (KE5)**

460   The mechanisms controlling the induction of class switch recombination and production of IgE by

461   switched B cells have been studied extensively. *In vitro*, the production of IgE by human B cells,

462 specifically induced by IL-4 or IL-13 and signalling via CD40 cell surface molecule, can be monitored

463 at various levels of the ε class switching process, including during the induction of the sterile ε

464 transcript, which precedes Ig heavy chain locus rearrangement by quantitative reverse transcription PCR

465 (RT-qPCR) or Northern blot assay, the detection of production of ε excision circles during the Ig heavy

466 chain locus rearrangement by PCR and the detection of production of IgE by ELISA as nicely reviewed

467 by Pène et al., 2005.

468 Even though several *in vitro* B cell activation protocols are available (Lin & Calame, 2004; Pène et al.,

469 2005), to the best of our knowledge no protocols have been established so far that investigate the

470 (various levels of) class switching in a food allergen-specific context.

471 **5. Future perspective**

472 **5.1. *In vitro* micro-fluidics systems**

473 Advances in micro-physiological systems are providing researchers alternative means to gain insights into

474 the molecular interactions of the gastrointestinal tract. These systems combine the benefits of micro-

475 engineering, micro-fluidics, and cell culture in a bid to recreate the environmental conditions prevalent in

476 the human gut. It becomes now possible to construct *in vitro* systems that more closely approximate those

477 conditions present within the gut on scales identical to those encountered *in vivo* (Kim, Huh, Hamilton, &

478 Ingber, 2012). These systems based on micro-fluidics offer numerous advantages over traditional cell-

479 culturing techniques, including a 3D culture environment, greater experimental flexibility, the ability to

480 precisely tune spatiotemporal oxygen and pH gradients, low shear environments, and the ability for high

481 throughput experimentation. Although currently available micro-fluidics systems provide promising

482 approaches to study local mechanisms that drive allergic responses, they have not yet been used to study

483 any of the MIE or KE including in the AOP for food sensitization. Some examples of available micro-

484 fluid devices are described below.

485 **5.1.1. Human gut-on-a-chip device**

486 A micro device "human gut-on-a-chip" has been developed by the Ingber group at Harvard University,

487 which is composed of two micro-fluidic channels, separated by a porous flexible membrane coated with

488 extracellular matrix and lined by Caco-2 cells to mimics the complex structure and physiology of living

489 intestine. The gut micro-environment is established by flowing fluid, which produces low shear stress

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

491   2012).

5.1.2. NutriChip

493   NutriChip is another integrated micro-fluidic platform developed by Swiss scientists for investigating

494   potential immunomodulatory function of dairy food and represents a miniaturized artificial human

495   gastrointestinal tract (Ramadan et al., 2013). NutriChip is a culture of a confluent layer of Caco-2 cells

496   separated from co-cultured immune cells by a permeable membrane, which allows studying processes

497   that characterize the passage of nutrients though the intestinal epithelium, including the activation of

498   immune cells. NutriChip allows application of *in vitro* digested food on its apical side and a basolateral

499   culture of a monocyte line (U937 cells) differentiated into macrophages (Ramadan et al., 2013).

5.1.3. Immuno-HuMiX

501   Human-microbial cross-talk platform (HuMiX) is also a micro-fluid device that enables the study of

502   molecular interactions at the host-microbe interface (Eain et al., 2017). The features of HuMiX are similar

503   to those of the other fluidics systems in which also the microbiome component may be introduced.

504   Moreover, the system can be expanded to analyze the interactions between the immune system and the

505   intestinal microbiota in the human gut, the so called Immuno-HuMix model. First steps have been

506   undertaken to integrate human PBMCs in this system (Eain et al., 2017).

5.2. Organotypic *ex vivo* cultures

508   One of the major weaknesses of IEC lines is that they are different from the primary cells of the intestinal

509   epithelium, from which they had originally been isolated. The selection of cells that survive and expand

510   *in vitro* often leads to changes in gene expression profile and altered responses. In this respect, primary

511   isolated cells may therefore be a better model system to represent processes in the intestinal mucosa.

512   Promising *ex vivo* approaches are the generation of intestinal organoids and the culturing of *ex vivo*

513   intestine samples.

5.2.1. Intestinal organoids

515   Major advances have been made in establishing culture conditions that support the long-term propagation

516   and self-organisation of near-physiological tissue. In general, isolated somatic stem cells from various

517 organs are cultured in the presence of specific small molecules and growth factors, reflecting essential

518 niche components of the respective tissue of origin. A 3D scaffold mimicking the basal lamina has further

519 been shown to support *ex vivo* expansion of intestinal stem cells *ex vivo* and tissue formation that

520 resembles part of the cellular architecture, hierarchy and physiology of the *in vivo* counterpart (Sato &

521 Clevers, 2013). The culture of intestinal crypts and their growth and differentiation to organoids may be

522 an interesting and easy to handle tool to study allergen interaction and associated signal transduction

523 pathways in a complex intestinal system.

524 Despite the requirement for more expensive technology than IEC lines, intestinal organoids have been

525 shown to have multiple applications and with the recent development of efficient gene-editing tools, it is

526 now possible to generate highly physiological models of human gastrointestinal diseases (Leushacke &

527 Barker, 2014). However, the study of allergen transport across the epithelial barrier is limited by the

528 inaccessibility of food proteins to the apical side of IECs (directed to the lumen of the organoids).

529 **5.2.2. *Ex vivo* models**

530 Another approach to overcome the major drawbacks of the *in vitro* cell line-based models may be the use

531 of complete intestinal tissues. In these models, the asymmetrical distribution of proteins and lipids in the

532 two plasma membrane domains is facing the intestinal lumen. The internal milieu and the presence of

533 highly organized structures joining adjacent IECs, enable selective processes of absorption, transport, and

534 secretion to take place across the intestinal mucosa. The main e*x vivo* intestinal models for intestinal

535 protein transport studies include the everted sac technique and the Ussing chamber. These approaches

536 have been largely used to assess protein transport and, using intestinal tissue from sensitized animals, they

537 may lead to study the effect of sensitization on allergen uptake. However, both techniques have several

538 limitations including the rapid loss of the tissue viability and tissue damages during isolation, which may

539 lead to overestimation of protein transport. Furthermore, the presence of the muscle layer in the everted

540 sac method may lead to underestimation of protein transport. On the other hand, interspecies differences

541 complicate extrapolation of data to humans. In this respect, pigs share more physiological and

542 immunological similarities with humans than rodents and the recently developed InTESTine[TM] method

543 may be a medium-throughput alternative. InTESTine[TM] is based on intestinal tissues from pigs that are

544 incubated on a rocker platform in a high oxygen incubator (Westerhout et al., 2014).

545 **5.3. *In silico* approaches**

546   *In silico* methodologies and tools like databases and comparison software have been shown useful for the

547   assessment of potential allergenicity of food proteins based on their properties. These *in silico* methods

548   use a number of physico-chemical features (mainly amino acid searches) of proteins that can be predicted,

549   such as B cell epitopes, T cell epitopes and sequence homologies (as reviewed by Hayes, Rougé, Barre,

550   Herouet-Guicheney, & Roggen, 2015). They can identify whether a novel protein is an existing allergen

551   and/or has the potential to cross-react with an existing allergen. However, they cannot identify whether a

552   novel protein will 'become' an allergen, so therefore these approaches have limited value to identify truly

553   novel allergenic proteins (Hayes et al., 2015). The use of future innovative *in silico* approaches will be

554   largely influenced by the choice of databases and algorithms that will be developed, standardized, and

555   most importantly, empirically validated.

556   **6. Conclusion**

557   Although basic science studies have identified a lot of immune pathways behind the allergic response,

558   sensitization to food proteins is a complex process involving several molecular and cellular events

559   (Sicherer & Sampson, 2018; Tordesillas, Berin, & Sampson, 2017). The simplification of the biological

560   process of food allergy sensitization by applying the AOP concept is an effective strategy to identify *in*

561   *vitro* methods that lead to investigate the sensitizing potential of food proteins. Previously, such an

562   approach focusing on MIE and KE described in reported AOPs for skin and respiratory sensitization have

563   been used successfully to study the sensitizing potential chemicals in skin and respiratory allergy

564   (Ezendam, Braakhuis, & Vandebriel, 2016; Sullivan et al., 2017).

565   In this review, we have clustered, structured, and discussed the existing *in vitro* models that are suitable to

566   study the MIE and KE involved in the AOP for food allergy sensitization (van Bilsen et al., 2017). To the

567   best of our knowledge, this is the first time that the AOP concept is applied to structure all available *in*

568   *vitro* methods to identify the potential sensitizing capacity of food proteins. In order to recapitulate all the

569   reviewed assays, focusing on different (combinations of) MIE and KE reflecting the different building

570   blocks of the AOP, Table 1 summarized *in vitro* methods used to evaluate the three major food allergens:

571   chicken ovalbumin (Gal d 2), bovine β-lactoglobulin (Bos d 5) and peanut 2S albumin (Ara h 2).

572   In general, *in vitro* methods assessing mechanisms involved in the innate immune response are easier to

573   address than assays assessing the adaptive immune response of food sensitization: the recognition

574   molecules used by the innate system (here reflected in MIE, KE1, KE2, and KE3), are expressed broadly

575 on a large number of cells, which makes it easier to develop *in vitro* methodologies to study specific

576 building blocks of the AOP, as seen by the rich set of available tools. However, more *in vitro* studies of

577 the transcellular transport based on epithelial receptors (MIE2) or unspecific endocytosis (MIE3) should

578 be carried out to better understand the effect of this type of intestinal acquisition in the sensitizing

579 potential of food proteins.

580 On the other hand, the adaptive immune system (here reflected in KE4 and KE5) is composed of small

581 numbers of T and B cells with specificity of any individual allergen. Therefore the responding cells must

582 proliferate after encountering the allergen in order to attain sufficient numbers to mount an effective

583 response that can be detected. This feature of the adaptive system complicates the development of *in vitro*

584 approaches to assess KE4 and KE5. This is also reflected in Table 1 which depicts a limited number of *in*

585 *vitro* approaches to address KE4 and the absence of an available *in vitro* assay to assess B cell isotype

586 switching (KE5) in a food-allergen specific context.

587 It must be noted that the *in vitro* models discussed in this manuscript have been developed to obtain a

588 better understanding of the processes involved in food sensitization. Several of the described *in vitro*

589 models (summarized in Table 1) seem to be correlated with the sensitizing potential *in vivo* and some

590 show great promise to closely approximate *in vivo* conditions (e.g. *in vitro* micro-fluidics systems and

591 organotypic cultures), however as far as we know, none of the described models have been successfully

592 used to assess the sensitizing potential of a given food protein by comparing high and low/none

593 sensitizing food proteins in the assay. To this end, it would be an essential step forward to expand the

594 panel of tested food proteins by including also low/non-allergenic proteins. Most likely, none of the

595 assays will be able to distinguish high from low/non allergenic potency by itself; however the assays

596 combined can provide an important set of data which may be helpful to decide which of the assays are

597 essential to be part of the weight-of-evidence approach to determine the sensitizing potential of food

598 proteins. Unfortunately, to date it is not known whether the lack of a suitable *in vitro* model to assess KE5

599 (B cell isotype switching) will result in a crucial data gap to determine the sensitizing potential or whether

600 the KE5-model is redundant in the overall assessment.

601 *In vitro* models based on cell lines are very useful as research tools to investigate MIE and KE involved in

602 food allergy sensitization. However, the closer these cell-based systems are to the actual target tissue, the

603 better conclusions can be drawn. The main problem was that long-term propagation of native, non-

604 transformed single cells or cell clusters from the intestine was not feasible and it was generally assumed

605 that it would not be possible to establish long-term cultures of primary adult tissues without the

606 introduction of genetic transformations promoting cell proliferation and survival. In recent years,

607 significant progress has been made in this field and robust systems have been identified. A variety of *in*

608 *vitro* micro-fluidics systems and *ex vivo* culture strategies has been developed to investigate the function

609 of the intestinal mucosa, which will help to increase the knowledge of food sensitization process.

610 Concluding, the application of the AOP framework offers the opportunity to anchor existing testing

611 methods to specific building blocks of the AOP for food sensitization which provides insight which

612 specific methods are available and which still need to be developed. When applied in the context of an

613 integrated testing strategy, such an approach may reduce, if not replace, current animal testing

614 approaches.

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963   **9. Figure Caption**

964   Figure 1. A tentative Adverse Outcome Pathway describing the mechanistic events driving food

965   sensitization induction. Depicted are those events and relationships with substantial evidence for a role in

966   food sensitization induction in human. DC: dendritic cell; *Outside the scope of this manuscript. Adapted

967   from van Bilsen et al., 2017.

968   **10. Table Caption**

969   Table 1. Main *in vitro* approaches used to characterize major food allergen of bovine milk (Bos d 5),

970   hen's egg (Gal d 2) and peanut (Ara h 2), organized following the molecular initiation events and key

971   events described by the adverse outcome pathway for food allergic sensitization.

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.

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 ([3H]-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 ([3H]-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 ([3H]-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 ([3H]-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 ([3H]-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.
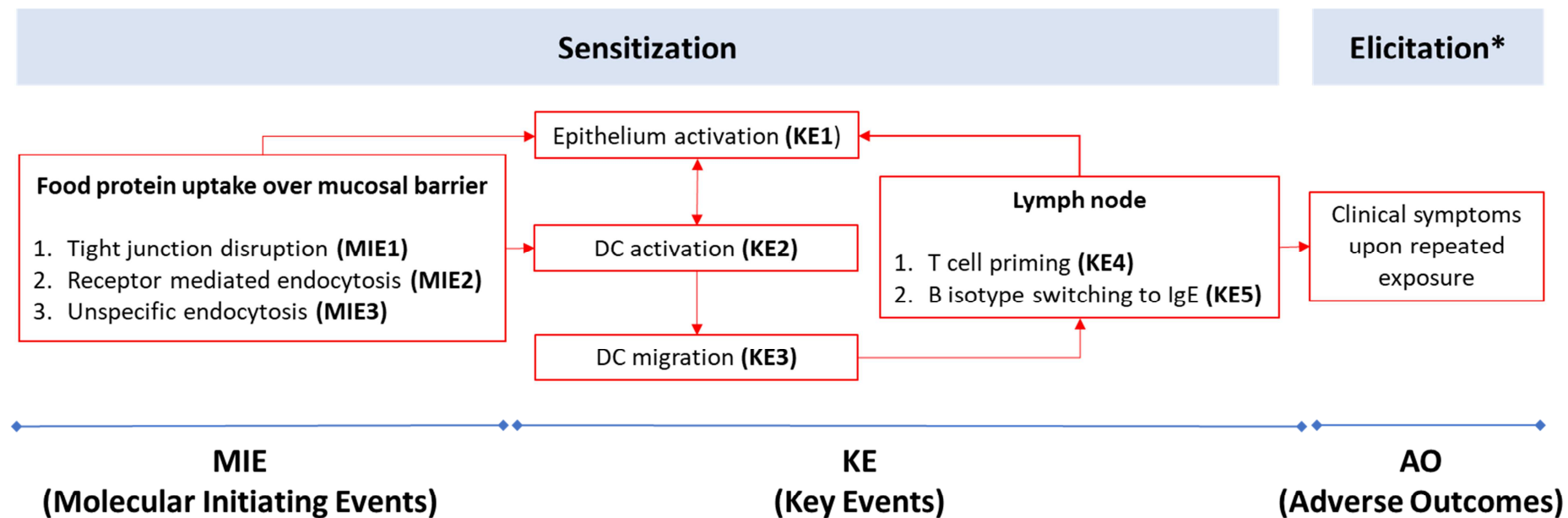
Figure 1. A tentative Adverse Outcome Pathway describing the mechanistic events driving food sensitization induction. Depicted are those events and relationships with substantial evidence for a role in food sensitization induction in human. DC: dendritic cell; *Outside the scope of this manuscript. Adapted from van Bilsen et al., 2017.

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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