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COMPREHENSIVE REVIEW

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Critical features of an in vitro intestinal absorption model to study the first key aspects underlying food allergen sensitization

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Abstract

New types of protein sources will enter our diet in a near future, reinforcing the need for a straightforward in vitro (cell-based) screening model to test and predict the safety of these novel proteins, in particular their potential risk for de novo allergic sensitization. The Adverse Outcome Pathway (AOP) for allergen sensitization describes the current knowledge of key events underlying the

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complex cellular interactions that proceed allergic food sensitization. Currently, there is no consensus on the in vitro model to study the intestinal translocation of proteins as well as the epithelial activation, which comprise the first molecular initiation events (ME1-3) and the first key event of the AOP, respectively. As members of INFOGEST, we have highlighted several critical features that should be considered for any proposed in vitro model to study epithelial protein transport in the context of allergic sensitization. In addition, we defined which intestinal cell types are indispensable in a consensus model of the first steps of the AOP, and which cell types are optional or desired when there is the possibility to create a more complex cell model. A model of these first key aspects of the AOP can be used to study the gut epithelial translocation behavior of known hypo- and hyperallergens, juxtaposed to the transport behavior of novel proteins as a first screen for risk management of dietary proteins. Indeed, this disquisition forms a basis for the development of a future consensus model of the allergic sensitization cascade, comprising also the other key events (KE2-5).

KEYWORDS
allergen transport, cell culture, food allergy, intestine, novel proteins

1 | INTRODUCTION

By 2050, according to the United Nations Department of Economic and Social Affairs, there are expected to be 9.7 billion people on earth, which means a prospective rise of 2 billion people over the next 30 years. This relates to a 60% increase in food demand, as projected by the Food and Agriculture Organization (Alexandratos & Bruinsma, 2012). To sustain the ecological and societal footprint of this augmentation in the world's population, a rapid transition to a more sustainable food system and circular economy is crucial. Protein security rather than food security is important in this transition as malnutrition is often caused by a protein deficiency, rather than by a lack of calories (Aiking & de Boer, 2020).

With the growing consensus that animal protein has disproportionate environmental impacts (Aiking & de Boer, 2020), novel proteins from alternative sources (plant-derived, fungal-derived, or novel proteins from insects, algae, and seaweeds) are being rapidly introduced into our European diet. The most important health aspects to consider when introducing novel protein products onto the European market are acute toxicity, allergenicity, and de novo allergic sensitization potential, the latter presenting a critical issue that is often not adequately assessed and for which no clear guidelines are currently in place due to the lack of predictive models. Introducing novel protein sources in our diet, or increasing the intake of already consumed sources, will inevitably augment the risk of introducing new food allergies (see also Box 1 for a basic background on the loss of oral tolerance). For instance, before the introduction of kiwi fruit to European markets in 1962, practically no kiwi allergy was seen in Europe, while currently an estimated 1.8% of the general population in Spain is sensitized to kiwi fruit (Mills, 2007). More recently, there is an increased occurrence in sensitization and immunoglobulin (Ig)E-cross reactivity toward edible insects and novel protein products derived thereof (de Gier & Verhoeckx, 2018). Also, the increased use of concentrated pea protein as an alternative to meat protein has already slightly but significantly increased the incidence of pea-related allergic reactions, albeit its prevalence is still considered low (Lavine & Ben-Shoshan, 2019; Taylor et al., 2021).

In this context, any food that was not consumed "significantly" in Europe prior to May 1997 is considered to be a novel food, and thus before a new protein (as an isolate or food product) can be placed on the market in the European Union, its safety—including allergenicity—must be evaluated and addressed in a novel food application dossier for assessment by the European Food Safety Authority (EFSA), as laid down in the EU Regulation No. 258/97 (Regulation, 1997). Such a risk evaluation can be approached from two different angles: (1) risk assessment of the new protein becoming a primary sensitizer (de novo sensitization), resulting in a new type of food allergy, and (2) risk assessment of cross-reactivity toward existing allergens (allergenicity) (Remington et al., 2018). The last angle,

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Box 1

Loss of oral tolerance

The default, "healthy" reaction of the immune system to food proteins is called oral tolerance, which is the continuous unresponsiveness of the immune system to the 130–190 g of food proteins daily absorbed, a process that is largely mediated by the gut-associated lymphoid tissue (GALT) (Brandtzaeg, 2009; Yang et al., 2021). GALT is the largest lymphoid tissue in the body, comprising among others the Peyer's patches, the lymphoid follicles, and the mesenteric lymph node (MLN). During oral tolerance rupture, the GALT loses its ability to correctly discriminate self from external antigens and/or innocuous from dangerous/pathogenic antigens, which can result in allergic sensitization (Satitsuksanoa et al., 2018). In the GALT, gut-derived antigens are presented by professional antigen-presenting cells (APC), such as dendritic cells and macrophages, to T- and B-lymphocytes (Randall & Mebius, 2014). The broad antigenic sampling within the GALT facilitates the interaction between antigen-specific B- and T-lymphocytes leading to the initiation of an appropriate adaptive immune response. In a "healthy" environment, APC migrate to the MLN and present the sampled (food) antigen to the lymphocytes, which will be activated and will differentiate according to three signals: (1) interaction between the major histocompatibility complex class II (MHCII) of the APC, holding the epitope, and the T-cell receptor, (2) interaction between the CD80/CD86 surface molecules on the APC and the CD28 surface molecule on the T-lymphocytes, and (3) the environmental cytokines secreted by the APC and other surrounding cells. A "healthy," tolerogenic environment will promote the differentiation of naive T-lymphocytes to regulatory T-lymphocytes, which will then activate B-lymphocytes by surface marker interaction to start the production of antigen-specific tolerogenic antibodies (IgG). T-lymphocytes will migrate back to the intestinal epithelium to support the immune response. Oral tolerance is thus the default reaction to the oral exposure to food allergens. This default reaction of oral tolerance is disturbed when allergic sensitization occurs. One hypothesis, the "dual exposure hypothesis," postulates that oral tolerance might be lost due to a primary exposure to food allergens through nonoral routes (cutaneous or airway) (Kulis et al., 2021). Alternatively, the "epithelial barrier hypothesis" postulates that the "healthy," tolerogenic environment can be disturbed by different factors that might affect the integrity and the activation of the (intestinal) epithelial barrier, including microbiota dysbiosis, diet (saturated fatty acid consumption), and the environment (detergents, microplastics, nanoparticles, etc.), leading to the loss of oral tolerance (Celebi Sozener et al., 2022; Tokuhara et al., 2019). When oral tolerance is lost, APC will also present the antigen to T-lymphocytes in the MLN, but the naive T-lymphocytes will differentiate into a pro-inflammatory Th2 phenotype due to the presence of interleukin (IL)-4, IL-5, and IL-13, which are produced by innate lymphoid cells type 2 (ILC2) cells. When these T-lymphocytes activate B-lymphocytes, this will lead to the production of antigen-specific immunoglobulin (Ig)E. These IgE have the capacity to bind to FcεRI receptors on mast cells and basophils and the cross-linking of the IgE-FcεRI complex by repeated exposure to the antigen will trigger mast cell or basophil activation with subsequent release of substances that cause the allergic symptoms. The main pathophysiological processes leading to allergic sensitization are further described in Figure 1.

relative to sensitization, can in principle be tested quite easily since cross-reactivity is an elicitation event and thus part of the second stage of the allergic reaction cascade. However, in order to test new protein sources, the phylogenetic relationship with already known allergenic protein sources must be known to some extent, otherwise a targeted risk analysis will be difficult. In vivo, ex vivo, as well as in vitro immunoglobulin E (IgE) cross-linking models developed for existing food allergies can be applied, such as cellular basophil/mast cell mediator release assays (Bahri et al., 2018; Santos et al., 2021) or food allergy animal models (Ahrens et al., 2014; Ladics et al., 2010). Although such elicitation models have reliable predictive value, depending on the allergen source, they are not always perfect in their translational predictions for human clinical relevance and severity of symptoms (Eberlein, 2020), leaving oral food challenges the gold standard method of patient diagnosis (Foong et al., 2021). However, to assess if a novel protein can become a primary sensitizer, the first stage of the allergic reaction cascade needs to be evaluated, indicated as the sensitization stage, that is, the moment when a protein (peptide) is recognized by the immune system as potentially harmful. Limited options exist to predict de novo sensitization of novel proteins leading to food allergy and currently no suitable human or animal models are available that can make an accurate

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prediction or translation (Castan et al., 2020; Remington et al., 2018). In silico bioinformatics models predict many potential cross-reactions that are unlikely to have any clinical significance. Murine food allergy models are often unsuitable or time consuming because rodents, without the use of an inflammatory adjuvant, are generally not susceptible to sensitization (Bøgh et al., 2016; Castan et al., 2020), unless the exposure time is prolonged for up to 4–6 weeks (Smit et al., 2015). While the applicability of in vitro epithelial models in food allergy models has been recognized (Cubells-Baeza et al., 2015), their translational capacity to de novo food allergen sensitization has not been validated. Hence, considering the strongly growing societal demand to avoid the use of animal experimentation, there is a need for the development of in vitro-based screening models that have the capacity to distinguish between nonallergic and allergic proteins and can address the question of whether proteins from novel sources can lead to the development of novel food allergies. Within the COST Action ImpARAS (FA1402), an Adverse Outcome Pathway (AOP) for allergic sensitization has been proposed (Lozano-Ojalvo et al., 2019; Van Bilsen et al., 2017), describing the major molecular initiation events (MIE) and key events (KE) (Figure 1) underlying the de novo food sensitization response cascade. In this AOP, food protein uptake over the small intestinal mucosal barrier (via MIE1 to 3) and epithelial activation (KE1) are the prerequisite steps for the subsequent KE (KE2-5) and for allergic sensitization to occur (Figure 1).

2 | FOCUS OF THIS REVIEW

EFSA underlines the need for the integration and comparability between in vitro experiments to better understand the cellular and molecular mechanisms of sensitization (Mullins et al., 2022). Several in vitro intestinal epithelial barrier models employed in protein/allergen transport and absorption studies have been described, largely based on single-cell lines (Cubells-Baeza et al., 2015; Gavrovic-Jankulovic & Willemsen, 2015; Lozano-Ojalvo et al., 2019). However, these rather "simple" models are unable to accurately reflect the complexity of food allergen transport. There is a lack of agreement on a consensus model to investigate the first critical steps of the AOP (MIE1-3 and KE), and it is important to adopt a common scientific position on this, given their critical role in the allergic sensitization cascade. In this review, we aim at providing a critical assessment of what an in vitro gut mucosal model should ideally encompass, in order to study the potential effect of novel proteins on the AOP events MIE1-3 and KE1 (see Figure 1). To do this, this review will comprehensively describe key gastrointestinal physiology features, detail intestinal cell types (focused on the small intestine) that play a role in the epithelial transport of known allergens (MIE1-3), and review the epithelial biomarkers that are secreted following epithelial activation, as well as the factors that influence this secretion (KE1). This review will not detail KE2-5 (see Lamiable et al. [2020] for further reading) nor will it deal with how an in vitro gut mucosal model can be employed with food digesta in allergy sensitization research. We would like to emphasize that an intestinal epithelial model for AOP events MIE1-3 and KE1 does not allow one to perform a full AOP risk analysis and thus to draw conclusions about risks for immunological sensitization of the tested proteins.

3 | FEATURES THAT IMPACT ALLERGEN TRANSPORT (*MIE1-3*)

3.1 | Allergen transport

Transport of (partially) digested proteins/peptides, including allergens through the intestinal barrier, can occur by paracellular transport (through tight junctions) (MIE1) or by transcellular transport, involving endocytosis/exocytosis (transcytosis) mediated by membrane receptors (MIE2) or unspecific endocytosis (MIE3) (Figure 1). This transport relies on the intrinsic antigen properties (Perrier & Corthésy, 2011; Samadi et al., 2018), but also on the physiological features of the intestine (discussed in Section 3.3) and extrinsic, food-related factors (discussed in Section 3.4).

3.1.1 | Allergen transport by paracellular transport

Paracellular transport involves the passage of antigen through the intercellular space and is regulated by the integrity of tight junctions (Figure 2). This type of transport occurs principally for small hydrophilic compounds (≤3.5 kDa) and the antigens/peptides transported in this way are not degraded. Paracellular transport is considered a passive transport that does not implicate antigen processing, but that does require a previous stimulus for the disruption of the tight junctions—representing MIE1—allowing the antigen to pass through the epithelial layer and to be directly exposed to the cells of the immune system.

Tight junctions form a continuous and tight network between the intestinal epithelial cells to seal the lumen and to protect the intestinal tissue from luminal

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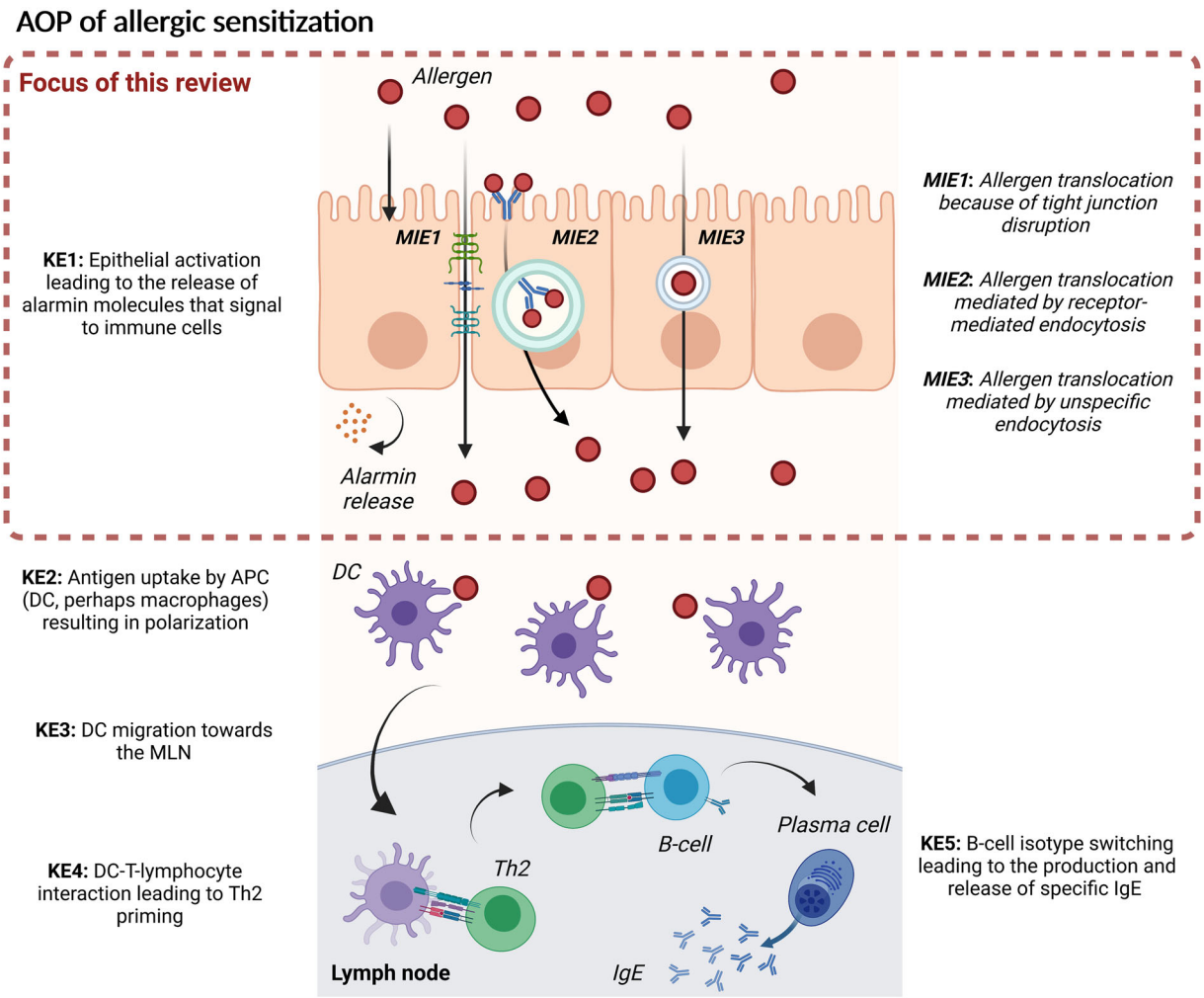

FIGURE 1 Schematic overview of the pathophysiological processes leading to allergic sensitization that have been described in the conceptual adverse outcome pathway (AOP), which summarizes the current knowledge of the cellular mechanisms and molecular pathways underlying allergic food sensitization (Lozano-Ojalvo et al., 2019; Van Bilsen et al., 2017). The focus of this review on MIE1-3 and KE1 is indicated by the red box. APC, antigen-presenting cells; DC, dendritic cells; KE, key events; MIE, molecular initiation events; MLN, mesenteric lymph node

contamination. Tight junction complexes are constituted by three families of transmembrane proteins, namely, the claudin family (that can be divided into pore-forming and barrier-forming claudins), the Marvel domain-containing family (e.g., occludins), and the immunoglobulin superfamily (e.g., junctional adhesion molecules). These transmembrane proteins are bound to the cytoskeleton by intracellular scaffold proteins, such as the zonula occludens (ZO) (Paradis et al., 2021) (Figure 2). Beyond tight junctions, adherens junctions (e.g., E-cadherin) and desmosomes also play a role in intestinal barrier integrity (Barbara et al., 2021). A link between food allergy and intestinal barrier permeability has been established. For example, food-allergic patients have a significantly increased intestinal permeability compared to control patients in the basal state and following allergen ingestion as demonstrated by different lactulose/mannitol urinary ratios (Andre et al., 1987; Reitsma et al., 2014; Van Bilsen et al., 2017; Ventura et al., 2006).

Some, but not all allergens can promote their paracellular passage by altering tight junction proteins and decreasing the barrier integrity, thus impacting allergic sensitization and the allergic response (Ali et al., 2020). This is the case of wheat gliadins, peanut extract, and kiwifruit Act d 1 (actinidin), which have been described to increase the monolayer permeability by modifying the interaction between occludins and ZO-1 (Drago et al., 2006; Grozdanovic et al., 2016; Price et al., 2014). Likewise, wheat α-gliadins seem to augment the paracellular permeability of the colonic epithelial cell line Caco-2 by binding to the chemokine receptor CXCR3 that is involved in tight junction damage (Lammers et al., 2008). Similarly, the oral administration of hen's egg Gal d 2 (ovalbumin) to rats downregulated the mRNA

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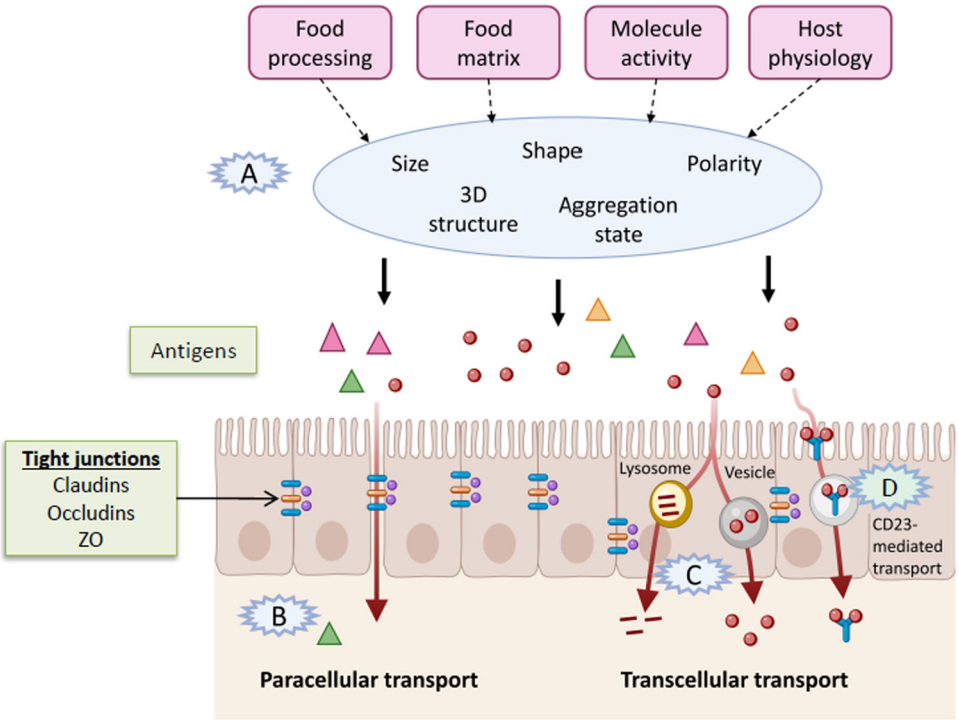

FIGURE 2 Allergen transport mechanisms across the intestinal epithelium. The paracellular and transcellular transport of allergens is modulated by the processing of the allergen-containing food, the food matrix, and intrinsic allergen activity, but also by host physiology. (a) Extrinsic and intrinsic factors that can affect allergen transport. (b) Paracellular transport regulated by the integrity of tight junctions that consist, among others, of claudins, occludins, and zonula occludens (ZO). (c) Vesicle-mediated transcellular transport in which proteins can either be endocytosed and directed to lysosomes for digestion, or transported intact to the basolateral side by transcytosis. (d) Receptor-mediated endocytosis, such as allergen transport mediated by IgE-CD23 complexes

expression of ZO-1 and claudin-2, claudin-8, and claudin-15 when compared to a phosphate-buffered saline (PBS) control, and increased tight junction permeability, as measured using lactulose/mannitol urinary ratios (Chen et al., 2014). The apical-to-basolateral translocation of shrimp Pen j 1 (tropomyosin from *Penaeus japonicas*) in Caco-2 cells has also been attributed to self-enhanced paracellular transport, since Pen j 1 increased intestinal permeability as measured using lucifer yellow translocation (Kunimoto et al., 2011).

More commonly, environmental factors seem to significantly influence the integrity of the epithelial barrier, including microbial factors (discussed in Section 3.3.3), a (low-grade) chronic intestinal inflammation, our diet, or other components such as nanoparticles or microplastics, as notably highlighted in the "epithelial barrier hypothesis" (Celebi Sozener et al., 2022; Tokuhara et al., 2019). Indeed, the expression of barrier-forming claudins and occludins is often downregulated in intestinal inflammatory, immune-mediated diseases in response to pro-inflammatory cytokines secreted by immune cells that populate the intestinal epithelium and the lamina propria, such as macrophages, T-lymphocytes, innate lymphoid cells (ILC), and intraepithelial lymphocytes (IEL). Notably, inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interferon gamma (IFN-γ), interleukin (IL)-1β, and IL-6 as well as allergy-related cytokines such as IL-13 have been shown to promote intestinal barrier permeability (Lee et al., 2018; Martini et al., 2017). For example, TNF-α promotes the delocalization of the claudins 5 and 8 from the tight junctions to the subtight junction compartments and the endosomes, whereas IL-1β increases the tight junction permeability through the redistribution of occludins (Al-Sadi et al., 2008; Zeissig et al., 2007). In contrast, the expression of claudin-2, which forms a pore channel promoting cation influx and water into the intestinal lumen, is generally increased in response to TNF-α, IL-6, and IL-13 in intestinal inflammatory diseases, including food allergy (Liu et al., 2013; Martini et al., 2017). Claudin-2 was reported to be present in the small intestine of nonallergic individuals, but its mRNA expression and protein levels were two- to threefold higher in patients with food allergies (Liu et al., 2013). Interestingly, claudin-2 was shown to bind protein antigen (horseradish peroxidase) and to promote its transport across the epithelial barrier (Liu et al., 2013).

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➢ When determining the allergic potential of proteins, their direct impact on tight junction integrity should be taken into consideration by, for example, the determination of transepithelial electrical resistance (TEER), the transport of paracellular-leaked molecule (lucifer yellow, AS-FITC, FITC-Dextran 4 kDa), or by determination of the mRNA or protein expression of some of the main tight junction proteins (occludins, claudins, ZOs). Similarly, the interplay between allergens, the epithelial barrier, and their environment should ideally be considered, possibly by testing the passage of an allergen under "healthy" conditions and under compromised, "pro-inflammatory" conditions.

3.1.2 | Allergen transport by transcellular transport

In transcellular transport, antigens are directly absorbed by intestinal epithelial cells through simple diffusion, carrier-mediated transport processes (passive/facilitated diffusion and active transport), endocytosis, and transcytosis (Figure 2). Carrier-mediated transport via facilitated diffusion regulates the transportation of the luminal content along the concentration gradient through membrane-bound carrier proteins. This mechanism is most commonly used by highly hydrophilic compounds (e.g., glucose) and is less relevant for larger peptides/proteins. Endocytosis and transcytosis are the most usual pathways for protein transport across the intestinal barrier and can occur through different types of cells (discussed in Section 3.2). Endocytosis is commonly used by large polar peptides, which can bind the cells and be incorporated into vesicles (Bouglé & Bouhallab, 2017). Upon endocytosis, proteins are either digested in lysosomes or transcytosed intact. Transcytosis is a process in which molecules are taken into cells by endocytosis, but then they are transported via transcytotic vesicles and exocytosed on the basolateral side (Tuma & Hubbard, 2003; Xu et al., 2019). Transcytosis tends to facilitate the transport of hydrophobic peptides, which due to their nonpolar character need to interact with the lipids in the membrane of the epithelial cells prior to their cell internalization (Shimizu et al., 1997; Xu et al., 2019). Positively charged proteins/peptides also seem to preferentially follow a transcytotic route (e.g., caseins), meaning that hydrophobicity and charge property affect peptide/protein transportation routes (Yang et al., 2019).

Receptor-mediated transcellular transport of food allergens—representing MIE2—have been described through the CD23 receptor, the low-affinity FcεRII-IgE receptor, which involves the delivery of immunologically intact IgE-bound allergen complexes across the epithelium through clathrin-mediated endocytosis (Engeroff & Vogel, 2021). However, the requirement for the presence of IgE suggests that this mechanism can only occur in already sensitized people (Bevilacqua et al., 2004; Yu et al., 2001). Similar to CD23, the neonatal Fc (FcRn) receptor IgG facilitates the uptake of IgG–antigen complexes and contributes to the development of oral tolerance in the neonate (Berin, 2012). Other receptor-mediated transcellular transport routes of food allergens have, to our knowledge, not been described.

Unspecific transcellular transport—representing MIE3—has been described for several allergens, including peach Pru p 3 (nonspecific lipid transfer protein [nsLTP]), wheat Tri a 19 (ω5-gliadin) and Tri a 14 (nsLTP), cow's milk Bos d 5 (β-lactoglobulin), peanut Ara h 1 (vicilin) and Ara h 2 (2S albumin), soybean P34 allergen, Brazil nut Ber e 1, and white sesame Ses i 1 (2S albumins) (Bernasconi et al., 2006; Bodinier et al., 2007; Moreno et al., 2006; Price et al., 2017; Sewekow et al., 2012; Tordesillas et al., 2013). The transcellular transport across the epithelial barrier of some of these allergens (Tri a 14, Pru p 3, Ses i 1, Ber e 1) seems to be facilitated by their high structural stability, allowing them to cross the epithelium in their native forms and making them probably more prone to function as primary sensitizers (Bodinier et al., 2007; Moreno et al., 2006; Sewekow et al., 2012; Tordesillas et al., 2013). A receptor-mediated transport (MIE2) is most likely to be involved in the transport of Pru p 3, although other transport mechanisms cannot be excluded (Tordesillas et al., 2013). Native and heat-denatured cow's milk Bos d 5 (β-lactoglobulin) can cross a Caco-2 cell monolayer by a transcellular pathway and is degraded by the epithelial cells during the transport (Bernasconi et al., 2006; Rytkönen et al., 2006). Overall, the physicochemical properties of allergens/proteins can thus be considered as key factors associated with unspecific transcellular transport, although other factors (e.g., cytokines) might also play important roles in this type of transport. Indeed, some ILs can potentiate the transcytosis of proteins across the intestinal epithelium (Berin et al., 1999; Moon, Vandussen, et al., 2014). IL-17 was reported to be the most efficient inducer of the expression of the polymeric Ig receptor, thus contributing to enhancing IgA-mediated antigen transcytosis across mouse intestinal epithelial cells (Moon, Vandussen, et al., 2014). Likewise, Berin et al. (1999) described that IL-4 affects epithelial barrier function by enhancing the transcellular uptake of antigenic proteins, thus suggesting that this IL might be crucial in the pathophysiology of food allergies (Berin et al., 1999).

➢ When assessing whether protein transport may pose a risk for allergic sensitization, factors such as the route of transport and whether the protein enters the

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lamina propria intact or as large peptides as well as their physicochemical properties should be considered. This could be determined by, for example, application of different types of endocytosis inhibitors and basolateral proteomics analyses (SDS-PAGE, LC–MS/MS). For example, using different endocytosis inhibitors (e.g., filipin III, monodansylcadaverine), Price et al. (2017) reported that peanut Ara h 1 and Ara h 2 were transported through the intestinal epithelial cells, starting in the endosomes (through multiple endocytotic mechanisms) and continuing in the lysosomes (Price et al., 2017).

3.2 | Intestinal cell types involved in allergen transport

Distinct types of intestinal epithelial cells have been implicated in the transport of allergens across the epithelial barriers and the cell type by which allergens are transported might be determinant for allergic sensitization. An overview of the relevant cell types and critical features that should be considered for an in vitro intestinal epithelial model to study the first key aspects of de novo allergic sensitization is presented in Figure 3. These cell types and critical features are more elaborately discussed in the sections below.

3.2.1 | Enterocytes

Enterocytes are highly abundant cells in the intestinal epithelium that are capable of transcellular uptake of proteins. Enterocytes are also involved in the transport of different immunoglobulins (IgA, IgG, and IgE) into the intestinal lumen. IgG and IgE promote allergen uptake (discussed in Section 3.1.2), whereas secreted IgA neutralizes allergens in the intestinal lumen and can thereby protect against allergic sensitization (Berin, 2012). Beyond their role in the transcellular uptake of allergens, enterocytes also express the major histocompatibility complex class II (MHC-II) and release exosomes containing MHC-II complexes loaded with endocytosed antigens both on the apical and basolateral sides (Heuberger et al., 2021). MHC-II expression is constitutive in the small but not in the large intestine, and it is upregulated by inflammatory stimuli such as IFN-γ (Heuberger et al., 2021). Exosomes released by enterocytes might initiate or shape the adaptive immune response by bringing antigen to antigen-presenting cells (APC), such as dendritic cells (DC), or by regulating T-lymphocyte activation, although it remains unclear whether these exosomes induce a tolerogenic or a pro-inflammatory response (Heuberger et al., 2021).

3.2.2 | Secretory epithelial cells

A subset of secretory epithelial cells implicated in protein/antigen transport are the mucus-producing goblet cells, which deliver low molecular weight (<10 kDa) soluble antigens to the lamina propria-located APC through the so-called goblet cell antigen passages (GAP) (McDole et al., 2012). These GAP are found in the small intestine, in line with the small intestine being considered the most common location for allergic sensitization (Newberry & Hogan, 2021). In the steady state, GAP appear to play a notable role in the induction of oral tolerance through the delivery of luminal antigens to APC, by imprinting tolerogenic properties on APC and by promoting the maintenance of T-regulatory lymphocytes (Kulkarni et al., 2020). Other secretory intestinal epithelial cell types, such as Paneth cells and enteroendocrine cells, have also been implicated in antigen passage (secretory antigen passages, or SAP), although their role is thought to be insignificant in the steady state (Kulkarni et al., 2020; Noah et al., 2019). However, an increased antigen sampling by Paneth cells, enteroendocrine cells, and goblet cells was observed upon the induction of food allergy, resulting in the direct delivery of allergens to the mucosal mast cells (Noah et al., 2019). Indeed, cell populations involved in allergen transport were significantly altered under the influence of IL-13, a cytokine secreted by the innate lymphoid type 2 (ILC2) cells in response to alarmins (KE1, discussed in Section 4.1) (Noah et al., 2019). It might be speculated that the patterning of epithelial antigen passage and the subsequent delivery to specific cell populations (APC, mast cells) is an important factor in the response of the immune system to dietary antigens (Newberry & Hogan, 2021; Noah et al., 2019).

3.2.3 | Microfold cells

Microfold cells (M-cells) are intestinal epithelial cells lacking microvilli that are predominantly localized at the epithelium overlying Peyer's patches, although they are also found interspersed among enterocytes (so-called villous M-cells) (Dillon & Lo, 2019). M-cells capture and actively transport particulate antigens (including large macromolecules, aggregated antigens, IgA-complexed antigens, bacteria, and viruses) by transcytosis across the epithelium to deliver these antigens to underlying immune cells. In support of this antigen-delivering

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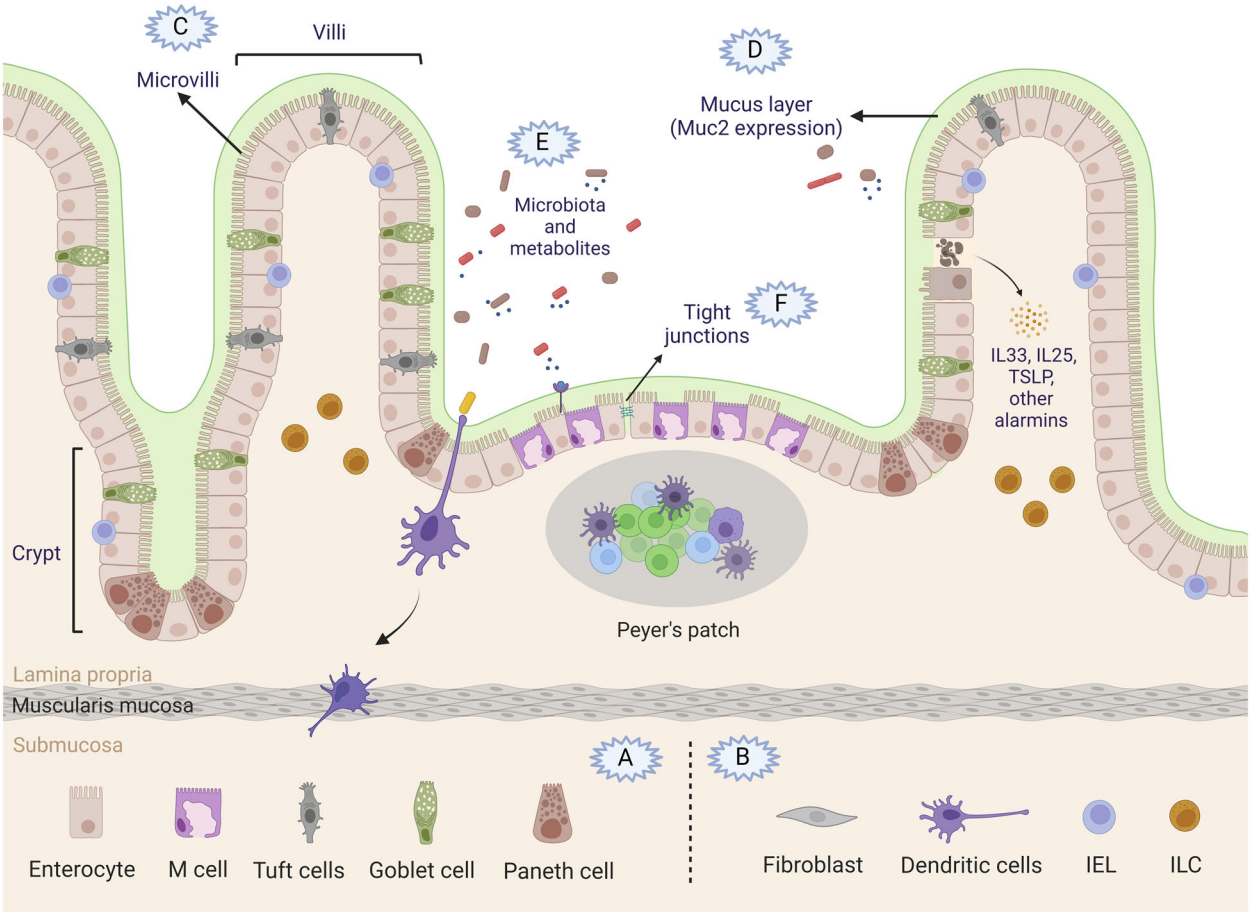

FIGURE 3 Critical features and cell types that should be considered for an in vitro intestinal epithelial model to study the first critical steps (MIE1-3, KE1) of de novo allergic sensitization. (a) A variety of intestinal epithelial cells that are implicated in allergen transport, including enterocytes (with MHC-II expression), goblet cells (but also other enteroendocrine cells), Paneth cells, and M cells (capable of transcytosing particulate antigens) that can secrete alarmins (enterocytes, tuft cells). (b) Submucosal cell populations that have the potential to modulate allergen transport and/or epithelial activation, such as DC, IEL, fibroblasts, and ILC. (c) Villi and microvilli. (d) A mucus layer from 50 to 450 μm comprising MUC2. (e) Microbiota (interacting with the host's immune system through various microbial factors). (f) Tight junctions that respond to external stimuli. DC, dendritic cells; IEL, intraepithelial lymphocytes; ILC, innate lymphoid cells; MHC, major histocompatibility complex

function, M-cells have a peculiar basolateral binding pocket that can accommodate B-, T-, or myeloid cells and have a low proportion of lysosomes compared to enterocytes, thus limiting antigen degradation (Berin, 2012; Dillon & Lo, 2019; Reitsma et al., 2014). M-cell-mediated transport can either induce an antigen-specific immune response or antigen tolerance (Dillon & Lo, 2019; Jang et al., 2004). The targeted delivery of ovalbumin to Peyer's patch M-cells by fusing ovalbumin to the recombinant reovirus protein sigma 1 protein prior to oral sensitization to native ovalbumin was associated with an improved oral tolerance compared to pretreatment with a PBS control, but it should be noted that pretreatment with a nonfused ovalbumin control was lacking (Suzuki et al., 2008). Other studies indicated that Peyer's patches are dispensable for oral tolerance induction, that M-cells are relatively rare in the steady state, and that mice deficient in M-cells do not show a loss of oral tolerance, suggesting that M-cell dietary antigen uptake is not essential for oral tolerance induction and is a minor route of uptake (Kulkarni et al., 2020; Newberry & Hogan, 2021; Pabst & Mowat, 2012).

A specific role for M-cells in sensitization toward aggregated allergens has been proposed, as aggregated allergens might be predominantly taken up by M-cells (Reitsma et al., 2014). The heat-induced aggregation of β-lactoglobulin and α-lactalbumin increased uptake by M-cells, whereas their soluble, native counterparts were predominantly transcytosed by enterocytes (Roth-Walter et al., 2008; Stojadinovic et al., 2014). Peanut protein was observed to cross the intestinal epithelium of BALB/c mice only in M-cells at intestinal Peyer's patches and was not observed in the paracellular spaces of normal intestinal epithelium (Chambers et al., 2004). M-cell-mediated

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uptake of aggregated proteins was associated with a significantly higher Th2 response and cytokine production during the sensitization phase, but with a reduced induction of symptoms during the challenge phase (Roth-Walter et al., 2008; Stojadinovic et al., 2014). Other studies using enzymatic protein cross-linking did not corroborate these findings and generally showed a lower allergic sensitization upon antigen aggregation (Radosavljevic et al., 2014; van Esch et al., 2013). However, these studies did not specifically investigate M-cell allergen transcytosis, indicating that the exact role of M-cells in oral tolerance and allergic sensitization remains to be further characterized. As allergen size might be a determinant factor in the localization of allergen uptake and the localization of allergen uptake might in turn impact the reaction of the immune system, M-cells are a requirement for an in vitro model to study the first key aspects of de novo allergic sensitization (Matsunaga et al., 2000).

3.2.4 | Submucosal cell populations

Submucosal cell populations that are close to the intestinal epithelial interface may cross talk directly with epithelial cells by establishing cell–cell interactions and cell–extracellular matrix interactions, which may significantly influence the structure and function of the epithelial barrier and subsequent intestinal protein translocation. Myeloid-derived submucosal DC, scattered along the entire small intestine, establish tight contact and associations with epithelial cells. The classical function of submucosal DC is to protect against luminal pathogens and to establish regulatory responses to antigens by continuously sampling the soluble and particle antigens transported by the various epithelial cells (as described in Section 3.2.1–3.2.3) or internalized by intraepithelial macrophages (KE2) (see also Box 1) (Liu et al., 2021; Schulz et al., 2009; Stagg, 2018). Different intestinal DC subtypes have been identified over the past years, which are called conventional DC (cDC) 1 or 2, or plasmacytoid DC based on their phenotype or function (see Liu et al. [2021] for a review). Besides this classical pathway, DC can also directly sample soluble antigens in the gut lumen by the extension of dendrites like periscopes between epithelial cells in the upper small intestine (Rescigno et al., 2001) or through M-cell-specific transcellular pores (Lelouard et al., 2012). During this close epithelial association, elongated dendrites maintain epithelial integrity by expressing tight junction proteins (Rescigno et al., 2001; Sebrell et al., 2019). Steady-state recruitment to and protrusion of the epithelium seem to be regulated by the chemoattractant C-X3-C motif chemokine ligand 1 (CX3CL1)/fractalkine and its receptor CX3CR1 (Niess et al., 2005). It has been shown that CX3CR1+-intraepithelial DC do not appear to be bona fide DC, as they do not migrate from the lamina propria to mesenteric lymph node and cannot present luminal antigen to naive T-lymphocytes (Butler et al., 2006; Chieppa et al., 2006). Nevertheless, they do seem to be loaded with antigen and, hence, they may play an accessory role, by passing it on to neighboring migratory CD103-expressing cDC for transport and presentation. Intraepithelial DC associated with Peyer's patches represent another DC subtype involved in antigen sampling, as they lack CX3CR1 expression and require C-C motif chemokine receptor (CCR) 6/C-C motif chemokine ligand (CCL) 20 attraction for epithelial association and luminal transepithelial-dendrite sampling (McDonald et al., 2017; Sebrell et al., 2019). It should be noted that phagocytic uptake of food allergens directly from the intestinal lumen by DC has not yet been visualized in vitro nor in vivo. In addition, epithelial association significantly reduced MHC-II expression on DC in an in vitro monocyte-derived DC/Caco-2 co-culture model (Butler et al., 2006). Thus, the functional significance of different modes of antigen sampling by DC on allergic sensitization remains poorly understood and requires further investigation.

Beyond DC, also IEL impact the structure and function of the epithelial barrier and intestinal protein translocation. Interspersed into the basolateral side of the intestinal epithelium, IEL are highly abundant—with an estimated 1 IEL for every 10 intestinal epithelial cells—and directly interact with intestinal epithelial cells through the expression of the CD103 integrin and CCR9 (Van Kaer & Olivares-Villagómez, 2018). IEL directly promote the maintenance of epithelial cell barrier integrity by regulating the differentiation of intestinal epithelial crypt cells and by secreting keratinocyte growth factors and several other cytokines (Danese, 2008; James et al., 2021; Konjar et al., 2017; Qiu & Yang, 2013). IEL are of particular interest in the context of food allergy, as an important role has been described for the so-called $\gamma\delta$IEL in the development of oral tolerance. Indeed, the downregulation of the of the $\gamma\delta$-T-cell receptor using an anti-delta-chain antibody was linked to an impaired oral tolerance toward ovalbumin as demonstrated by antibody and T-lymphocyte cell responses (Ke et al., 1997). Also, it has been described that the phenotype of $\gamma\delta$IEL is modified toward a functional APC-like phenotype upon exposure to cholera toxin, which is classically used to induce allergic sensitization in mice (Frossard, Asigbetse, et al., 2015). If and how IEL directly impact allergen transport remains, however, to be further investigated.

➢ An in vitro intestinal epithelial model for the study of allergic sensitization would ideally include all cell types that are involved in antigen/allergen transport,

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including enterocytes, M-cells, secretory epithelial cells (such as goblet cells), and possibly DC and IEL. However, it will likely be challenging to create a model with the same physiological ratios and distributions of these cells as in vivo. To better characterize the respective function of the different cell types in allergen transport, cell type and morphology of the epithelial monolayer can be assessed by confocal microscopy of cell type marker proteins as it was previously done for the murine intestine (Knoop et al., 2020).

3.3 | Intrinsic factors that impact allergen transport

Physiological features of the gastrointestinal system also have the capacity to influence allergen transport and thus MIE1-3. Indeed, the gastrointestinal system has multiple functions and balances digestion, food absorption, oral tolerance, and immunity as well as hosting the intestinal microbiota.

3.3.1 | Villi and microvilli

To maximize the absorptive surface, the small intestinal epithelium consists of repetitive sequences of crypts and villi. To further increase the absorptive area for nutrients, vitamins, ions, and water, the enterocyte luminal surface is in turn covered by microvilli, which contain multiple transporter proteins, ion channels, and enzymes that together form the brush border (Dutton et al., 2019). To accurately determine allergen transport, the presence of villi and microvilli is thus needed. Ideally, these (micro)villi should be similar to those found in the normal human small intestinal epithelium, where villous height is 567–640 μm and villous diameter is 157–160 μm (Dutton et al., 2019). In the presence of bowel inflammation, villus area and height may decrease (Pereira e Silva et al., 2018), but whether this affects specific allergen translocation mechanisms or degree of alarmin release is not known.

3.3.2 | Mucus layer

On top of the apical intestinal epithelial surface, the mucus layer forms a chemical barrier consisting of high-molecular-weight glycoprotein complexes of gel-forming mucins secreted by the goblet cells, as well as water, lipids (1%–2%), electrolytes, secretory IgA, and antimicrobial peptides (Barbara et al., 2021). As the first layer of physical defense, the mucus plays an important role in reducing the adherence of pathogenic microbes and microorganism epithelial penetration and shielding the host from digestive enzymes. In the small intestine, the mucus consists of one layer of approximately 50–450 μm that is relatively loose and permeable, whereas the mucus layer in the large intestine is composed of two layers: an outer layer of 300–700 μm and an inner layer of 100–400 μm that is relatively impermeable (Dutton et al., 2019; Parrish et al., 2022). The gel-forming mucins have large numbers of O-linked oligosaccharide chains that give them a negative charge and which provide numerous binding sites for antigens (Li, Crouzier, et al., 2013). In the small and large intestines, Muc2 is the main secreted mucin, while Muc13 and Muc17 are the main transmembrane mucins that compose the carbohydrate-rich glycocalyx covering the intestinal epithelium (Schneider et al., 2018).

Although a direct link between allergen sensitization and the mucus layer has not been clearly established, it is reasonable to assume that the composition, porosity, or thickness of the mucus layer impacts antigen uptake. The mucus filters and limits the uptake of molecules, which means that only a relatively porous mucus layer, as found in the small intestine, allows for an effective antigen uptake (Johansson & Hansson, 2016; McDole et al., 2012). Interactions between the host immune system and the mucus layer also exist. On the one hand, the mucus layer might impact the host immune response to food antigen, as the mucin Muc2 was found to deliver tolerogenic signals to intestinal immune cells (Shan et al., 2013). Indeed, as opposed to wild-type mice, no oral tolerance was induced in Muc2-deficient mice that were gavaged with ovalbumin before an oral challenge with the same allergen. On the other hand, mucin production is regulated by host immune (e.g., cytokines) and microbial factors, and a depletion of the mucus layer has been observed in food-intolerant individuals and patients with inflammatory bowel disease (Parrish et al., 2022). Future studies are needed to better characterize the roles of the mucus layer in allergen transport (Parrish et al., 2022).

3.3.3 | Microbiota

The human gastrointestinal tract (GIT) harbors numerous species of commensal microorganisms—called the intestinal microbiota—that coexist in a symbiotic relationship with the host under normal circumstances. The microbiota has a key role in the physiological and immunological processes that take place in the intestine. In the context of food allergy, the intestinal microbiota can be considered an intrinsic environmental factor that is crucial not only for allergen sensitization but also for the maintenance of the

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integrity of the intestinal epithelial barrier, thus possibly influencing allergen transport (Renz & Skevaki, 2021). As such, when studying the impact of the microbiota on allergen transport, efforts must be made to mimic the microbial ecosystem of the upper part of the GIT rather than the colon. Nonetheless, it might be hypothesized that the current dietary shift from the consumption of easy-digestible proteins (milk, meat) to less digestible forms of proteins (e.g., legumes) might render the colon and its microbiota more important for allergic sensitization in the near future.

The resident microbiota interact with intestinal epithelial cells and the immune system by diverse mechanisms, such as proteinaceous molecules called microbe-associated molecular patterns or metabolism-derived byproducts (Delgado et al., 2020) that act as signaling molecules promoting regulatory responses in the host (Brown et al., 2013). For example, secretion of the epithelial cytokine APRIL (a proliferation-inducing ligand) in response to bacterial detection plays a critical role in T-lymphocyte-independent IgA class switching of B-lymphocytes (Wang et al., 2017). However, under certain circumstances or perturbations, the microbial equilibrium can be lost (dysbiosis) and some so-called pathobionts (likewise members of the microbiota) may overgrow, disrupting the microbial ecological balance and compromising the intestinal permeability and/or inducing inflammation (Chow et al., 2011). In a dysbiotic state, pathobionts and their metabolites and toxins can notably impact the intestinal epithelial barrier by impacting tight junction integrity. For example, tight junction integrity is reduced by the pathogenic species of *Escherichia coli* and *Salmonella typhimurium* (Lee et al., 2018). Similarly, lipopolysaccharide, which is an endotoxic component of the outer walls of gram-negative bacteria, might reduce epithelial barrier integrity by acting upon the Toll-like receptor 4 and by altering the expression and localization of ZO-1 and occludin tight junction proteins (Lee et al., 2018). Beyond this indirect interaction of the microbiota and the intestinal epithelial barrier, it remains unclear to what extent microbiota directly interact with protein allergens in a context of allergic sensitization.

An important aspect to consider for the design of in vitro intestinal absorption models regards the composition and diversity of the intestinal microbiota that is not the same throughout the small intestine (where protein absorption takes place, and the host immune system interacts with food antigens) and the large intestine. Another aspect to consider is the individual's age (Kondrashina et al., 2021) as most food allergies occur in infancy when the microbiota and the immune system are still under development. Colonization by diverse microbiota and microbiota–host cross talk is of key importance for the development of a functional immune system. After birth, the GIT of the newborn is colonized by different microbial communities that increase in number and diversity until reaching a more stable composition at approximately 2–3 years old (Yatsunenko et al., 2012), the age range that corresponds mostly to the acquisition of oral tolerance for the major food allergens (Rachid & Chatila, 2016). Indeed, a wide number of current reviews highlight the importance of the correct establishment of the gut microbiota for the prevention of food allergy (Lee et al., 2020; Nance et al., 2020; Rachid & Chatila, 2016; Stephen-Victor et al., 2020).

➢ Physiological features of a tissue or cell culture can be checked via histology and microscopy. For example, histochemical staining with Alcian blue/PAS can visualize the level of secreted mucus, while immunohistochemistry is a good method for visualizing cell type-specific marker proteins as well as villi formation. The use of enzyme-linked immunosorbent assays (ELISA) or the enzyme-linked lectin assay represents a simple alternative for the quantification of mucins, although this method will not provide insight into epithelial morphology and cell type distributions or cell–cell interactions (Plaisancié et al., 2013). The inclusion of the microbiota in a cell model of allergen transport remains a challenge.

3.4 | Extrinsic factors that can affect allergen transport

The transport of allergens can also be affected by extrinsic factors, which include structural modifications induced by digestion, food processing, or components of the food matrix. It is well-established that these factors are determinant in reducing or increasing the allergenic potential of different proteins, for example, by generating small peptides that cannot be recognized by the immune system or by the formation of protein aggregates that delay digestion (Costa, Bavaro, et al., 2022; Costa, Villa, et al., 2022).

3.4.1 | Allergen digestibility

The process of protein digestion starts in the stomach, where the main activators are hydrochloric acid and pepsin, and continues in the small intestine, where most of the protein digestion occurs through the action of the pancreatic enzymes. In the small intestine, peptidases tethered to the brush border membrane, such as aminopeptidases, carboxypeptidases, endopeptidases, and dipeptidases, complete peptide digestion by reducing them to di/tripeptides or free amino acids (Ozorio et al., 2020) (see Sun et al. [2022] for an excellent review on the

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gastrointestinal fate of food allergens and its relationship with allergenicity). The majority of dietary proteins are fully converted into di/tripeptides and amino acids and are absorbed by the intestinal epithelial cells in the form of nutrients. Still, large immunogenic peptides or even intact proteins can reach the epithelial barrier (Kohlmeier, 2015; Shen & Matsui, 2019). Although resistance to gastrointestinal digestion cannot be considered as a rule to identify allergenic proteins, many important allergens are (partly) resistant to gastrointestinal digestion and thus can reach the epithelial barrier immunologically intact (Akkerdaas et al., 2018; Verhoeckx et al., 2019). The extent of digestion determines the allergen transport mechanism, with intact allergens crossing the epithelial barrier by transcytosis/transcellular transport, while fragmented peptides can follow different routes, either by paracellular or transcellular pathways. These phenomena have already been described for cow's milk Bos d 5 (β-lactoglobulin) and hen's egg Gal d 4 (lysozyme) (Bernasconi et al., 2006; Yokooji et al., 2013). Data from literature suggest that when proteolytic activity during digestion is decreased, probably as a consequence of a lower enzyme (pepsin/trypsin)–substrate ratio, some allergens may be absorbed by paracellular rather than by transcellular transport, which could be linked to an increased potential for sensitization. Additionally, some pharmacological drugs (e.g., anti-inflammatory compounds such as aspirin and diclofenac) might also impact the mechanism of allergen transportation, thus favoring paracellular transport over the transcellular routes. When modeling allergen transport, the digestibility of food proteins should thus be considered, and special attention should be paid to allergen structure following digestion to understand the potential interaction of digested allergens with intestinal epithelial cells (Sun et al., 2022). In this context, the impact of brush border enzyme activity should also be taken into account (Di Stasio et al., 2020). Similarly, various kinds of host- and bacteria-derived proteases and peptidases might impact the digestibility of dietary proteins and their absorption.

3.4.2 | Food processing and food matrix

Food processing can increase the bioavailability of nutrients and enhance the safety of foods for human consumption. Food processing as well as the food matrix can also have a major impact on digestion and subsequent transport of the nutrients through the intestinal epithelial barrier. Different processing methods may induce several physicochemical alterations on food proteins, including hydrolysis of peptide bonds, denaturation, aggregation by disulfide and noncovalent bonds, as well as potential food component interactions of the matrix, namely, lipids, carbohydrates, or other micronutrients (e.g., vitamins, minerals, polyphenols), which may affect the allergenic potential of proteins (Costa, Bavaro, et al., 2022; Costa, Villa, et al., 2022; Sun et al., 2022) (see Sun et al. [2022] for an excellent review). For instance, a food matrix with a high content of proteins can delay gastrointestinal digestion and the epithelial transport of food allergens, shaping their sensitizing capacity (Schulten et al., 2011). Likewise, lipids have been demonstrated to have a protective effect on allergen stability during gastrointestinal digestion and allergen transport, also contributing to preserve the allergenic potential of different food allergens (Costa, Bavaro, et al., 2022; Costa, Villa, et al., 2022).

Heat-induced aggregation and glycation of allergens significantly lower allergen transport. Indeed, although soluble fractions of Bos d 5 (β-lactoglobulin) and Bos d 4 (α-lactalbumin) were rapidly transcytosed through enterocytes both in vitro and in vivo (mouse model), the formation of aggregated Bos d 5 and Bos d 4 structures induced by pasteurization reduced transcytosis across Caco-2 monolayers (Roth-Walter et al., 2008). Similarly, Bos d 5 glycation drastically reduced its transcytosis probably due to partial unfolding, and/or aggregate formation (Perusko et al., 2018). Likewise, the native form of the allergenic peptide derived from β-lactoglobulin, KIDALNENKVLVL, is more easily transported than their monolactosylated forms through the Caco-2 monolayer (Gasparini et al., 2022). Additionally, it was suggested that protein transport is glycosylation specific since the transport rate of bovine serum albumin glycosylated with α-Gal was different from an NA1 glycosylated form, a carbohydrate modification of similar size (Krstić Ristivojević et al., 2020).

In another study, heating hen's egg Gal d 2 (ovalbumin) and Gal d 1 (ovomucoid) prevented their transport across the human intestinal epithelial cells, probably by hampering the transcytosis process (Martos et al., 2011). An important role for the intestinal epithelium was proposed by Lupi et al. (2019), who demonstrated that heated and heated/digested wheat gliadins were not able to degranulate rat basophil leukemia cells before transport across a Caco-2 epithelial barrier, but that this capacity was partially recovered after transport (Lupi et al., 2019). The authors also concluded that especially the aggregated forms of α-gliadins were able to cross the Caco-2 epithelial monolayer, but that also the paracellular permeability of the Caco-2 monolayer was increased due to the α-gliadins exposure (Lupi et al., 2019).

In contrast to heat-induced aggregation and glycation, dietary lipids might facilitate the intestinal transport of proteins through lipid-mediated uptake or disruption of the intestinal barrier (Angelina et al., 2016; Mine &

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Zhang, 2003; Wang et al., 2009) and, in consequence, modulate the allergenic properties of proteins (López-Fandiño, 2020). Wang et al. (2009) found that the administration of allergens together with long-chain triacylglycerols to mice enhanced their absorption through intestinal epithelial cells and systemic dissemination via chylomicron formation, eventually promoting oral tolerance (Wang et al., 2009). Conversely, co-administration of shorter chain triacylglycerols or other lipids that are preferentially taken up through Peyer's patches favored subsequent transport of proteins to the draining mesenteric lymph nodes and increased their immunogenicity (Li, Wang, et al., 2013). Moreover, a recent study reported that egg phospholipids reduce the solubility of egg white proteins in simulated duodenal fluid, impairing epithelial absorption and promoting antigen delivery to Peyer's patches (Pérez-Rodríguez et al., 2021).

The interactions between food proteins and soluble carbohydrates used in the preparation of a wide range of foods, as stabilizers, thickeners, and emulsifiers, can exert a protective effect toward proteolysis and reduce protein digestibility, affecting protein transportation through the epithelial barrier. Jiménez-Saiz et al. (2013) reported higher IgE-binding activities of Gal d 1 (ovomucoid) and Gal d 2 (ovalbumin) in duodenal digests when those allergens were mixed with functional biopolymers commonly used in the food industry (pectin, gum Arabic, and xylan), which was attributed to a lower efficiency in the digestion of these proteins (Jiménez-Saiz et al., 2013). Similarly, a 0.2% concentration of the emulsifier polysorbate-80, but not the natural emulsifier lecithin, increased paracellular transport of different allergens by altering tight junction functionality through low cytotoxicity on Caco-2 monolayers (Khuda et al., 2021).

Other processing factors that modulate allergen transport include reduction or alkylation, or proteolysis following exposure to *Lactococcus lactis*, which were shown to result in a more efficient transepithelial transport of cow's milk Bos d 5 (β-lactoglobulin) (Bernasconi et al., 2006). Matrix factors such as the presence of aromatic amino acid ethyl esters also inhibited the transcellular transport of hen's egg Gal d 2 (ovalbumin) through Caco-2 monolayers, suggesting that food constituents, such as aromatic amino acids and their derivatives, can affect the permeability of the epithelial barrier and may in turn shape the immune responses to luminal allergens (Kobayashi & Watanabe, 2003).

➢ The digestibility and processing of the allergen, as well as the matrix in which it is ingested, heavily influence allergen transport. Globally, allergen digestibility impacts the transport of allergens and potentially allergic sensitization. The formation of aggregated structures during the application of heat treatments and glycation seems to hinder allergen transport by inhibiting their transcellular transportation, while lipids might facilitate allergen transport. It is thus advised to include an in vitro digestion procedure, with and without a food matrix, when applying protein samples within in vitro intestinal sensitization assays to mimic the in vivo-like consumption as much as possible, even when an allergen is studied that is known to be proteolytically resistant.

4 | FEATURES THAT IMPACT EPITHELIAL ACTIVATION (*KE1*)

Epithelial activation (KE1) is defined as a sensitizer-related inflammatory response at the epithelial level, most commonly characterized by the release of "alarmins." The release of these alarmins will shape the reaction of the underlying immune cells involved in the sensitization process (KE2-5) (see Figure 1). This section will be focused specifically on the activation of the gastrointestinal epithelium, although it should be noted that relatively little data are available on this type of epithelial activation, when compared to the skin or respiratory epithelium.

4.1 | Alarmin release

Alarmins are defined as "endogenous, constitutively expressed, chemotactic, and immune-activating proteins or peptides that are released following degranulation, cell injury or death, or in response to immune induction" (Yang et al., 2017). In the context of allergy, the alarmins IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) have been identified as drivers of the allergic response (Ali et al., 2020).

4.1.1 | Interleukin-33

IL-33 is an alarmin that is predominantly expressed by mucosal endothelial and epithelial cells, but also by immune cells and subendothelial myofibroblasts and that is responsive to tissue damage following injury or infection (Gupta et al., 2017; Pascual-Reguant et al., 2017). In healthy humans and mice, IL-33 is mainly detected in the endothelial cells and subendothelial myofibroblasts near the intestinal crypts, but its expression is induced in intestinal epithelial cells and infiltrating immune cells following inflammation, allergic sensitization, and celiac disease (Mahapatro et al., 2016; Pan et al., 2021; Pascual-Reguant et al., 2017; Perez et al., 2020). Secreted IL-33 acts

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upon the widely expressed ST2 receptor to regulate both inflammation and intestinal tissue homeostasis. For example, IL-33 can directly act upon intestinal immune cell populations, such as ILC2, DC, T-regulatory lymphocytes, cytotoxic T-lymphocytes, and natural killer cells (NK) (Perez et al., 2020). The importance of IL-33 in food allergy was shown using ST2 knockout mice, which showed an 80% reduction in peanut-specific IgE levels following a peanut sensitization regimen (Chu et al., 2013). Also, the injection of monoclonal antibodies against IL-33 prevented the development of food allergy upon oral sensitization with egg white and medium-chain fatty acids (Khodoun et al., 2018). The importance of IL-33 in food allergy is further underlined by the results of a phase 2 clinical trial using etokimab, an anti-IL-33 antibody, which shows to be promising for the desensitization of peanut-allergic individuals (Chinthrajah et al., 2019).

Despite the expression of IL-33 by cells of the intestinal mucosa, it is not yet clear whether (damaged) intestinal epithelial cells are the main source of IL-33 in food allergy (Ali et al., 2020; Hodzic et al., 2017). Indeed, treatment with recombinant IL-33 during skin sensitization with hen's egg Gal d 2 (ovalbumin) was also able to promote food-induced allergic symptoms (Han et al., 2018). Alternatively, the release of IL-33 from intestinal epithelial cells following inflammatory stimuli might (in part) be mediated by IEL (see Section 3.2.4) (Pascual-Reguant et al., 2017). Direct stimulation of IL-33 by allergens in intestinal epithelial cells has, however, been described. For example, the peach allergen Pru p 3 as well as the allergens tropomyosin and ovalbumin were able to upregulate IL-33 gene expression in Caco-2 cells or the mouse large intestinal model CMT93, respectively (Tordesillas et al., 2013; Wang, Lin, et al., 2021). Only few studies have, however, demonstrated the actual release of IL-33 from intestinal epithelial cell models. A recent study on milk formulas used to treat cow's milk allergy demonstrated a differential capacity of some of these formulas to induce IL-33 secretion from Caco-2 cells using a highly sensitive ELISA (Paparo et al., 2021). However, what cellular source of IL-33 is the main driver of food allergy remains to be further established and more studies on the role of intestinal epithelial cell-secreted IL-33 are needed.

4.1.2 | Thymic stromal lymphopoietin

TSLP is an alarmin with a structural analogy to IL-7 and is secreted by mucosal epithelial cells (skin, lung, intestine). Different signals and cytokines can promote TSLP release, including viruses, bacterial peptidoglycans, double-stranded RNA, cytokines (IL-1, IL-4, IL-13, TNF-α), and allergens (Cianferoni & Spergel, 2014). TSLP acts through a heterodimeric receptor complex consisting of an IL-7 receptor α-chain (IL-7Rα) and a TSLP receptor chain and that is expressed on a variety of immune cells, as well as epithelial cells (Ziegler, 2012). Secreted TSLP can promote lymphocyte proliferation and differentiation, and has been suggested to prime DC to promote the differentiation of T-lymphocytes toward the inflammatory Th2 phenotype (Ziegler, 2012). In mice intestine, steady-state mRNA levels of TSLP appear to be highest in the caecum and the large intestine, but the expression of TSLP is rapidly induced in mice duodenum following the oral sensitization to peanut using cholera toxin (Chu et al., 2013). A recent study also suggested a high expression of TSLP in a subset of Tuft cells, the so-called Tuft-2 cells (Haber et al., 2017). Despite the important role of TSLP as a promotor of atopic inflammation and Th2 inflammatory responses, the exact role of TSLP in the development of food allergy is not completely clear (Cianferoni & Spergel, 2014). Using TLSP receptor knockout mice, Chu et al. (2013) provided evidence that intact TSLP signaling was not required for the development of allergic symptoms upon peanut sensitization (Chu et al., 2013). In contrast, another study using oral sensitization to cow's milk Bos d 5 (β-lactoglobulin) using cholera toxin did find a role for TSLP, showing that the absence of the TSLP receptor partially prevented sensitization and significantly reduced anaphylaxis after an oral allergen challenge (Frossard, Zimmerli, et al., 2015). Also, monoclonal antibodies against TSLP prevented the development of food-induced allergic reactions upon oral sensitization with egg white proteins and medium-chain fatty acids (Khodoun et al., 2018). This specific study assessed the respective roles of IL-25, IL-33, and TSLP and found that the inhibition of the individual alarmins suppressed food allergy, but that optimal suppression was obtained when a cocktail of monoclonal antibodies against all three alarmins was used (Khodoun et al., 2018).

Allergens have the capacity to promote TSLP expression and secretion from intestinal epithelial cell models. Indeed, the roasted peanut allergen Ara h 3 (11S globulin) promoted the gene expression of TSLP in Caco-2 cells compared to raw peanut, while tropomyosin and ovalbumin increased TSLP gene expression in CMT93 cells, and the peach nsLTP allergen Pru p 3 induced TSLP expression in Caco-2 monolayers (Tordesillas et al., 2013; Wang, Lin, et al., 2021; Wang, Sun, et al., 2021). As for IL-33, only few studies have measured the actual release of TSLP from intestinal epithelial cell models. Using Caco-2 cells, an induction of TSLP release following IL-1, TNF-α, or butyrate treatment or specific milk formulas used to treat cow's milk allergy was observed (Cultrone et al., 2013; Paparo et al., 2021). Although TSLP release in response to allergens has not been studied in more complex models of the intestinal epithelium, it is thus possible that

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to determine the impact of allergens on TSLP release, a highly sensitive ELISA is needed (<5 pg/ml) and/or that co-treatment with TSLP-activating cytokines such as IL-1 or TNF-α is necessary.

4.1.3 | Interleukin-25

IL-25 is only secreted by the so-called tuft cells located at the intestinal epithelium. Tuft cells are relatively rare cholinergic chemosensory cells known to have an important role in the Th2-mediated immune reactions against parasitic helminth infections, protozoa, and likely food allergy (Harris, 2016). The ligands and receptors involved in the release of IL-25 from tuft cells are not yet fully understood, but known ligands for tuft cells include bacterial quorum-sensing molecules and the metabolite succinate (Billipp et al., 2021). Tuft cell-secreted IL-25 activates intestinal ILC2 (discussed in Section 4.2.2) to promote the secretion of IL-13, a cytokine that stimulates intestinal remodeling and that seems to impact aspects of innate tissue memory or "training" (von Moltke et al., 2016). Beyond IL-25, tuft cells also secrete cysteinyl leukotrienes, which are lipid signaling molecules that contribute to the activation of ILC2 (Billipp et al., 2021). In the context of food allergy, the role of IL-25 remains somewhat controversial. Using mice with impaired IL-25 signaling (IL-17RB knockout mice), no significant difference in peanut-specific IgE levels was observed following gastrointestinal sensitization to peanut (Chu et al., 2013). In contrast, a recent study using skin sensitization showed that IL-25 derived from intestinal tuft cells in combination with systemic IL-33 activated ILC2 and promoted IL-4 and IL-13 secretion, which in turn promoted anaphylaxis upon an oral food challenge (Leyva-Castillo et al., 2019). Although tuft cells do not directly transport allergens and it remains unclear how tuft cells respond to allergens to secrete IL-25, their exclusive secretion of IL-25 would warrant their inclusion in an intestinal barrier model for allergic sensitization.

4.1.4 | Other alarmins

Besides IL25, IL-33, and TSLP, other alarmins such as uric acid and high-mobility group Box 1 protein are also secreted by intestinal epithelial cells in response to stressors, such as cholera toxin and advanced glycation end-products, which can contribute to food allergy development (Kong et al., 2015; Smith et al., 2017; Wakabayashi et al., 2018). Other epithelial cell-derived cytokines and chemokines (soluble protein mediators critical for intercellular communication), such as IL-1α, IL-18, CCL20, CCL22, CX3CL1, granulocyte-macrophage colony-stimulating factor (GM-CSF), galectin-9, and transforming growth factor-β, also modulate the allergic response though their implication remains to be (further) validated in an intestinal context (Overbeek et al., 2019; Roan et al., 2019; Van Bilsen et al., 2017).

➢ No clear link has yet been described between the secretion of alarmins by the intestinal epithelium and the development of food allergic symptoms. Similarly, the impact of food allergens on alarmin section by the intestinal epithelium remains understudied and only few studies have managed to detect alarmin release from intestinal epithelial cell models (e.g., Caco-2). An in vitro model composed of a wide range of epithelial cell types and resembling the human intestinal epithelium is therefore needed to clarify whether and how the secretion of these alarmins is regulated in response to food allergens and innocuous proteins/peptides and to inform about their implication in allergic sensitization.

4.2 | Cell types that may affect and contribute to epithelial activation

Luminal cell populations that are close to the intestinal epithelial lining to establish cell–cell and cell–extracellular matrix interactions may significantly influence the structure and function of the epithelial barrier, possibly affecting allergen transport and intestinal epithelial activation. In this context, we here describe a selection of cells that could ameliorate an intestinal epithelial cell model for screening purposes.

4.2.1 | Fibroblasts

Underlying the intestinal epithelium, fibroblasts and myofibroblasts are present as part of the mesenchymal cell population in the submucosa. Fibroblasts secrete extracellular matrix components that can ameliorate epithelial cell morphology and maintain the structural integrity of the intestinal mucosa (Dang et al., 2021; Darling et al., 2020). Fibroblasts also regulate the proliferation and differentiation of intestinal epithelial stem cells via paracrine action (Göke et al., 1998), as well as immune cell homeostasis, through the secretion of IL-6 and CCL2 and through the direct interaction with immune cells (Dang et al., 2021). A direct relationship between fibroblast function and the allergic sensitization pathway has not been described. However, it is worth mentioning the secretion of the KE1 alarmin IL-33 by colonic subepithelial fibroblasts upon microbial exposure as a result of a breached

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epithelial barrier (Mahapatro et al., 2016). Thus, in the context of food allergy, the role of fibroblasts remains ill defined, but given their role in intestinal barrier homeostasis and alarmin function, these cells should be considered.

4.2.2 | Cells of the innate immune system

The innate immune system is the first, nonspecific line of defense against pathogens, but it can also respond to allergens. Beyond its direct implication in the allergic sensitization cascade or allergic reactions (basophils, mast cells), the intestinal innate immune system might create an environment that supports epithelial cell activation. For example, IEL might promote IL-33 secretion from intestinal epithelial cells, as previously described in Section 4.1.1. Beyond IEL, ILC are a family of cytokine-activated, cytokine-secreting lymphocytes that reside in barrier tissues, including the intestinal epithelium, and that participate in maintaining mucosal homeostasis (Eberl et al., 2015). ILC contribute to maintain epithelial barrier integrity and thus potentially impact allergen transport, and also secrete cytokines that might affect epithelial cell activation (Fan et al., 2019; Jowett et al., 2021). ILC1 subsets produce IFN-γ, TNF-α, GM-CSF, granzyme, and perforin in response to viral- or pathogen-infected or injured tissues, whereas ILC3 produce IL-22 that promotes mucin production and epithelial cell proliferation (Fan et al., 2019). In the context of food allergy, ILC2 are important in shaping the immune response against allergens (Fan et al., 2019). ILC2 produce different cytokines (IL-4, IL-5, IL-9, IL-13, and amphiregulin) in response to the IL-25 alarmin secreted by tuft cells (discussed in Section 4.1.3), but also respond to other alarmins, such as IL-33 or TSLP. ILC2-secreted IL-13 has in turn been directly implicated in the regulation of cellular allergen transport routes (Noah et al., 2019). Although several of the cytokines produced by ILC2 have been implicated in the induction of food allergy, these cytokines also help to conserve and repair the epithelial barrier and might serve as a negative feedback to lower epithelial activation. ILC are thus an example of how cells of the innate immune system shape the intestinal environment. The impact of ILC-derived cytokines on epithelial cell activation has, to our knowledge, not been directly studied.

➢ ILC and IEL are key examples of cells of the innate immune system that create an environment that can influence epithelial barrier integrity and epithelial activation, whereas fibroblasts might directly contribute to IL-33 secretion. The targeted inclusion of these cell types in an intestinal barrier model for allergic sensitization might shed light on these interactions and the ramifications for de novo allergic sensitization.

5 | EXISTING COMPLEX IN VITRO MODELS THAT CAN BE USED TO MIMIC INTESTINAL ALLERGEN TRANSLOCATION AND EPITHELIAL ACTIVATION

To accurately model de novo allergic sensitization and to study the impact of novel food proteins on AOP events MIE1-3 (allergen transport) and KE1 (epithelial activation), we have highlighted the different features that impact and/or modulate these AOP events. In the past, tumor enterocyte cell lines of human or porcine origins have predominantly been used as models to study allergen transport, including Caco-2, HT29, T84, and IPECJ2 (see Cubells-Baeza et al. [2015] and Lozano-Ojalvo et al. [2019] for an extensive description). More recently, complexified variants of these models have been described in the literature, including a co-culture of Caco-2 with mucus-secreting HT29-MTX cells and triple co-cultures of Caco-2 with HT29-MTX and Raji-B lymphoma cells (to induce cells with an M-cell like morphology) (Lozano-Ojalvo et al., 2019). Although these co-culture models might represent a more physiologically relevant model than Caco-2 alone, they still lack a number of features described in the previous sections (see Table 1). Herein, we will focus on the characteristics of more complex models, either cell-line derived or based on primary cells, and indicate whether they could be relevant for the study of the first key aspects of de novo allergic sensitization.

5.1 | Complex cell line-based models

5.1.1 | Air–liquid interface models

Air–liquid interface cell culture models are two-dimensional (2D) culture models on transwells in which the basolateral side of cells is in contact with the culture medium, whereas the apical side is only covered by a thin film of liquid and is exposed to air. Through the reduction of the apical medium volume, the oxygen supply is enhanced to a level presumably more adequate for cultured cells (Nossol et al., 2011). Air–liquid interface models are commonly used to shape the airways and the skin, but several studies have suggested that the cultivation of intestinal epithelial cells at the air–liquid interface or a semi-wet interface (with a small amount of liquid on the apical side) might also improve intestinal epithelial cell physiology of commonly used intestinal epithelial cell

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TABLE 1 Overview of the advantages and disadvantages of different cell culture models that can be used to study the MIE1-3 and KE1 of the allergic sensitization AOP pathway

Cell model	Intestinal epithelial cells			Intestinal physiology					Immune cells	Human?	Through-put	Main advantages	Main disadvantages	Ref.
	Entero-cytes	*Secretory intestinal cells*	*M-cells*	*Mucus layer*	*Tight junctions*	*Villi*	*Microvilli*	*Alarmin release*						
Cell line-based models														
Caco-2	✓				(high)		✓	Very low		Yes	High	- Easy-to-use - Cheap - Reproducible	- Tumor cell line model with only one cell type - High TEER values	(Cubells-Baeza et al., 2015; Cultrone et al., 2013; Paparo et al., 2021)
Caco-2/HT29-MTX	✓			✓*	(high)		✓	Very low		Yes	High	- Presence of mucus layer (compared to Caco-2) - Easy-to-use - Cheap - Reproducible	- Tumor cell line model with only two cell types - High TEER values	(Cubells-Baeza et al., 2015; Cultrone et al., 2013; Paparo et al., 2021)
Caco-2/HT29-MTX/Raji-B	✓		✓	✓*	(high)		✓	Very low		Yes	High	- Presence of mucus layer and M-cell-like cells (compared to Caco-2) - Cheap	- Tumor cell line model with only three cell types - High TEER values	(Cubells-Baeza et al., 2015; Cultrone et al., 2013; Paparo et al., 2021)
Gut-on-chip (Caco-2 based)	✓			✓	?	✓ (in 3D scaffold)	✓	?	Can be added (PBMC)	Yes	Low	- Fluid flow which improves features of intestinal physiology (mucus production) - Peristalsis	- Low throughput - Technically challenging - Chip material has a big impact on the assay	(Donkers et al., 2021; Kasendra et al., 2018; H. J. Kim et al., 2012; Sontheimer-Phelps et al., 2020; Xiang et al., 2020)
**** thickness of mucus layer can be improved by using an air-liquid or semi-wet interface***														
Complex models														
Ussing Chambers	✓	✓	✓	✓	✓	✓	✓	likely	local	Yes	Low	- Physiologically relevant - Relatively easy to study transport	- Availability of material - Low throughput - Short viability - Labor intensive	(Clarke, 2009; Rozehnal et al., 2012; Sjöberg et al., 2013)
inTESTine	✓	✓	✓	✓	✓	✓	✓	likely	local	No	Medium	- Physiologically relevant - Relatively easy to study transport	- Availability of material - Not human; interspecies differences complicated extrapolation of data to humans - Short viability	(Smits et al., 2021)
3D enteroids	✓	✓	✓	✓	✓	✓	✓	likely	Can be added (T-lymphocytes, DC)	Yes	Medium	- Diverse intestinal epithelial cell types - Presence of specific cell types can be altered by using different cytokines - Villi and microvilli structures	- Availability of material - Technically challenging to study transport - Costly - No parenchymal cells	(Bellono et al., 2017; Howitt et al., 2016; Sato et al., 2009)
2D enteroids	✓	✓	✓	✓	✓		✓	likely	Can be added (PBMC)	Yes	Medium	- Diverse intestinal epithelial cell types - Presence of specific cell types can be altered by using different cytokines - Easy to study transport	- Availability of material - Technically challenging to obtain 2D monolayer - Costly - No parenchymal cells	(Moon, VanDussen, et al., 2014; van der Hee et al., 2018; VanDussen et al., 2015)
3D HIO	✓	✓	✓	✓	✓	✓	✓	?	Can be added	Yes	Medium	- Diverse intestinal epithelial cell types - Presence of specific cell types can be altered by using different cytokines - Villi and microvilli structures - Presence of parenchymal cells	- Necessity of in vivo transplantation to obtain a mature phenotype - Technically challenging to study transport - Costly	(Finkbeiner et al., 2015; Watson et al., 2014)
2D HIO (from 3D structures)	✓	✓	✓	✓	✓		✓	?	Can be added	Yes	Medium	- Diverse intestinal epithelial cell types - Presence of specific cell types can be altered by using different cytokines - Easy to study transport - Presence of parenchymal cells	- Not a fully mature phenotype - Technically challenging to culture and to obtain a 2D monolayer - Costly	(Kwon et al., 2021)
Gut-on-chip (organoid-based)	✓	✓	✓	✓	✓	✓	✓	likely	Can be added	Yes	Low	- Fluid flow which improves features of intestinal physiology - Peristalsis - Possibility to add immune cells & microbiota	- Low throughput - Technically challenging - Limited life span of primary cells in the devices - Chip material has a big impact on the assay	(Donkers et al., 2021; Kasendra et al., 2018; H. J. Kim et al., 2012; Sontheimer-Phelps et al., 2020; Xiang et al., 2020)
EpiIntestinal (MatTek Corp)	✓	✓	✓	✓	✓	✓	✓	likely		Yes	Medium	- Diverse intestinal epithelial cell types - Cultivation at air-liquid interface	- Costly - External supply so limited flexibility	(Markus et al., 2021)

Abbreviations: DC, dendritic cells; HIO; human-induced pluripotent stem cell (iPSC)-derived intestinal organoids; PBMC, peripheral blood mononuclear cells; TEER, transepithelial electrical resistance.

[a]Thickness of mucus layer can be improved by using an air–liquid or semi-wet interface.

models (IPECJ2, Caco-2, and HT29-MTX) (Elzinga et al., 2021; Navabi et al., 2013; Nossol et al., 2011). Most notably, air–liquid interface or semi-wet interface culture methods seem to increase cell numbers, improve cell polarization, and promote mucus accumulation on the apical cell surface, albeit in vivo different semi-anerobic conditions occur (Elzinga et al., 2021; Navabi et al., 2013; Nossol et al., 2011). Air–liquid interface models using primary cells have also been described in the literature and include the EpiIntestinal™ model (MatTek Corp.), which will be more elaborately discussed below, and 2D-cultured enteroid models (Wang et al., 2019). Air–liquid interface models have not yet been used in the context of food allergy, but these models might be of interest given the increased apical mucus layer that could be used to study the role of the mucus layer in allergen transport and/or to obtain a more physiological in vitro model.

5.1.2 | Co-culture systems of cell lines with immune cells

Different immune cell types were shown to have an impact on allergen transport and/or epithelial cell activation,

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including DC, IEL, and ILC. These immune cells can be co-cultured with intestinal epithelial cells, either by the direct addition of immune cells to the basolateral compartment or by the plating of immune cells onto the bottom of the transwell membrane on which intestinal epithelial cells are cultured to study the direct interaction between epithelial and immune cells (Ding et al., 2021). Different co-cultures with Caco-2 or HT29, among others, with monocyte-derived DC, peripheral blood mononuclear cells (PBMC), or THP-1 monocytes using these two techniques have already been published (Kleiveland, 2015) (see Table 1). Alternatively, the supernatant from cultured intestinal epithelial cells can be transferred to immune cell cultures or vice versa (Lozano-Ojalvo et al., 2019). In the context of food allergy, some of these co-culture models have been employed. Tordesillas et al. used a co-culture of Caco-2 with PBMC to demonstrate that the peach allergen Pru p 3 (nsLTP) increased the gene expression of the cytokines IL-1β, IL-6, IL-10, and TNF-α in PBMC after passage over the epithelial barrier, compared to cells not exposed to antigen (Tordesillas et al., 2013). More recently, Bogdanov et al. (2021) used a Caco-2 co-culture model with different THP-1-derived immune cells (mature DC, macrophages), PBMC or PBMC-derived monocytes, or NK/T/B-cell populations to analyze the changes in cytokine secretion following incubation with soybean Gly m 4 on the apical side (Bogdanov et al., 2021). More advanced co-culture systems, using, for example, a Caco-2/HT29-MTX immune cell co-culture, have not yet been described in the context of allergen transport or epithelial activation. The described models are clearly more sophisticated than Caco-2 culture alone and allow for the assessment of the impact of new food proteins on epithelial cell activation beyond the assessment of the transport of proteins or large peptides. It should be noted, however, that these models still lack the intestinal epithelial cell diversity that might be important for allergen transport and epithelial activation. Co-cultures of Caco-2 cultures with IEL or ILC have only rarely been described and might be technically challenging (Hu et al., 2018).

5.1.3 | Gut-on-a-chip models

Gut-on-a-chips are microfluidic devices that were designed to better mimic the dynamic mechanical environment of the gut, to support longer term cell culture in the presence of the gut microbiome, and to enable in vitro analysis of intestinal epithelial barrier functions (Kim et al., 2012). First designs of gut-on-a-chip microfluidic devices consisted of two chambers separated by a porous semipermeable membrane onto which epithelial cells were seeded, separating apical and basolateral compartments (Donkers et al., 2021). These first 2D models have rapidly evolved over the past 10 years into elaborated three-dimensional (3D) models that contain villi structures, peristalsis, and oxygen gradients (Xiang et al., 2020).

One of the most important additions of gut-on-chip models to classical models is the fluid flow, which is indispensable for villi formation, cell differentiation, barrier integrity, and proper tight junction functioning, as well as mucus production (Donkers et al., 2021; Kim et al., 2012; Sontheimer-Phelps et al., 2020). The most recent designs of gut-on-a-chip also incorporate additional cell types beyond Caco-2 cells, such as human intestinal microvascular endothelial cells, human PBMC, monocyte-derived macrophages, and the microbiota, to create an organ-level model (Beaurivage et al., 2020; Donkers et al., 2021; Xiang et al., 2020). Gut-on-a-chip models have been made using intestinal cell lines such as Caco-2 or HT29, but more recent studies have also used organoids or biopsies derived from the duodenum, the jejunum, or the colon to obtain a more diverse intestinal epithelium (discussed in Section 5.2) (Donkers et al., 2021; Kasendra et al., 2018; Xiang et al., 2020). For example, a gut-on-chip model seeded with Caco-2 and incubated with gut microbiota better resembled the human ileum based on hierarchical clustering analysis of genome-wide transcriptome profiles, and was successfully used to study intestinal bowel disease through the addition of PBMCs (Kim et al., 2016).

Since gut-on-a-chip models are more physiologically relevant than traditionally used 2D transwell systems using intestinal cell lines, these models could be of interest for the study of allergen transport or epithelial cell activation (Xiang et al., 2020). However, it should be noted that the throughput of gut microfluidic devices remains limited to few parallel chips and that significant training is required to operate these systems. Efforts toward more easy-to-use systems are being made, with the commercially available OrganoReady® Colon Caco-2 model from Mimetas and publicly available 3D printable chips as examples (Shin & Kim, 2022). To our knowledge, gut-on-chip models have not yet been used to study allergen transport or allergen-induced epithelial cell activation, but they have already been employed to study other intestinal inflammatory diseases (Beaurivage et al., 2020; Kim et al., 2016).

5.2 | Complex primary cell-based models

5.2.1 | Ussing chambers

Ussing chambers are physiological in vitro systems that have been used to measure the transport of ions, nutrients, and drugs across various epithelial tissues (Clarke, 2009).

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Ussing chambers consist of a fresh intestinal segment from mice, pigs, or humans that is mounted into an apparatus to measure protein transport and epithelial membrane properties. One advantage for food allergy research is the possibility of studying the effect of sensitization on intestinal protein absorption, using intestinal tissue from sensitized animals (Cubells-Baeza et al., 2015; Westerhout et al., 2015). Ussing chamber studies of intestinal mucosa have provided many of the key observations that improved our molecular understanding of transepithelial transport processes. An advantage of this system regards the use of different parts of the intestine (from the duodenum to the colon) (Westerhout et al., 2015). Although Ussing chambers have great advantages and are considered the "gold standard" for the study of the physiological complexities of the healthy and diseased intestinal mucosa (Clarke, 2009), it also has several limitations, namely, tissue availability; tissue viability is rapidly lost (2 h max); the tissue can be damaged during isolation, which may lead to overestimation of protein transport; and interspecies differences complicate extrapolation of data to humans (see Table 1) (Cubells-Baeza et al., 2015; Rozehnal et al., 2012; Sjöberg et al., 2013).

5.2.2 | InTESTine

A recently developed model called inTESTine circumvents one of the main shortcomings of human Ussing chamber experiments, tissue availability, by using intestinal waste from pigs in a medium-throughput Ussing chamber setting (see Table 1). This system has a good correlation with paracellular absorption (Westerhout et al., 2014) and drug absorption (Stevens et al., 2019), when compared to human Ussing chamber data. A recent study also successfully used this model to study the transport of peanut allergens (Ara h 1, 2, 3, and 6) across the intestinal epithelium (Smits et al., 2021).

5.2.3 | Intestinal organoids

A potential model to study allergen uptake that has gained substantial interest is the intestinal organoids. The term 'organoid' is rather broad and encompasses 3D cultures grown from stem cells, wherein one can distinguish intestinal tissue-derived organoids (enteroids, which consist only of epithelial cells and are grown from multipotent adult stem cells), or human-induced pluripotent stem cell [iPSC]-derived intestinal organoid [HIO], which consists of both epithelial and mesenchymal lineages. Organoids are generally cultured in a basement membrane matrix, such as Matrigel® and specific culture media containing the cytokines Wnt, R-spondin, Noggin, and epithelial growth factor to form a spherical monolayer with outward extending budding regions. In contrast to most cell line cultures, intestinal organoids can differentiate into a variety of epithelial cell types. Enteroid cell cultures have been described to comprise intestinal stem cells, Paneth cells, enteroendocrine cells, tuft cells, and M-cells (Bellono et al., 2017; Howitt et al., 2016; Sato et al., 2009). HIO cultures, in contrast, maintain a fetal phenotype in vitro, though they can mature following in vivo transplantation to yield all mature intestinal cell types: enterocytes, goblet cells, enteroendocrine, and Paneth cells (Finkbeiner et al., 2015; Watson et al., 2014). Additionally, significant efforts are undertaken to promote the maturation of HIO in vitro (Jung et al., 2018). With these different cell types, organoid cultures acquire several of the crucial features involved in MIE1-3 and KE1, which are lacking from more conventionally used cell lines (see Table 1).

The main challenge regarding the use of organoid cultures for allergen transport studies lies in their 3D nature and the presence of the luminal compartment at the interior of the organoid. One possibility is to administer antigens to the intestinal lumen of 3D organoids by microinjection (Noah et al., 2019), but this is a highly technical method. Another alternative is to alter the conformation of the organoid from basolateral-out to apical-out to allow for exogenous antigen administration (Co et al., 2019). Currently, the most commonly sought solution is to create an epithelial cell monolayer by temporarily culturing organoids on a porous membrane insert, providing access to the luminal/apical and basolateral compartments. These monolayers would ideally form an impermeable monolayer with a TEER that allows for physiological allergen transport. For enteroids, the creation of these monolayers has been reported for mice (Moon, VanDussen, et al., 2014), porcine (van der Hee et al., 2018), and humans (VanDussen et al., 2015). For HIO, 2D monolayers from 3D differentiated HIO or direct differentiation from iPSC have also been reported (Yamada & Kanda, 2019). As described above, to obtain an even more realistic intestinal model, duodenal organoids have already been used in gut-on-chip systems (Kasendra et al., 2018).

It should be noted that the cultivation of enteroids and HIO is technically complex and requires significant resources and might not be feasible for most research groups. The passage from 3D to 2D culture also remains technically challenging for routine lab implementation and the extent to which 2D monolayers accurately recapitulate features of the adult intestine and 3D organoid culture remains to be further established. Perhaps for these reasons, only few studies have so far employed organoid models in the context of food allergy. In a recent study, a monolayer (not grown on a porous transwell membrane insert) from mouse small intestinal organoids was used to

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show that the kiwi allergen Act d 1 (actinidin) increases pro-inflammatory cytokine secretion (IL-1β, TNF-α, and IL-33) and disrupts tight junction integrity (E-cadherin, claudin-3, and ZO-1) (Nešić et al., 2019). Also, in vivo matured HIO were used to show the presence of GAP and SAP in a human model system (Noah et al., 2019).

5.2.4 | Primary co-culture systems with immune cells

As for cell line co-culture systems, the cultivation of primary intestinal epithelial cells derived from enteroids or HIO might add information to the relationship between the intestinal barrier and the immune system. Different types of immune cell-enteroid co-cultures have already been described in the literature, using two techniques: the combination of murine or human enteroids with relevant immune cells (e.g., DC, ILC2, ILC3, among others) directly into the Matrigel® used for 3D cultivation, or the addition of immune cells to the basolateral side when enteroids are cultured in 2D on Transwells (see Han et al. [2021] for an overview). An example of the cross talk between intestinal epithelial cells and immune cells is given by a recent study that demonstrated the induction of goblet cell differentiation by the ILC2-produced cytokine IL-13 in mouse intestinal enteroids, whereas epithelial cell-secreted IL-33 promoted IL-13 production by ILC2 (Waddell et al., 2019). The impact of the direct interaction between the immune system and the intestinal epithelium was also shown using mice enteroids, where the proximity of T-lymphocytes to the intestinal epithelium resulted in the efficient expansion of $\gamma\delta$IEL (Nozaki et al., 2016).

In a future effort to include also the subsequent key events (KE2-5) of allergic sensitization into an in vitro model, these types of models might help to shed light, for example, on the tripartite interaction between epithelial cells, immune cells, and the microbiota by co-culture studies of organoids together with immune cells and microbiota components. A small number of studies have started to implement these triple co-cultures, by using 2D human enteroids in transwells cultured with PBMC or PBMC-derived macrophages in the basolateral compartment and *E. coli* in the apical compartment either submerged or at the air–liquid interface (Noel et al., 2017; Wang et al., 2019). It should be noted that the modeling of bacteria–host communication in vitro is not easy to implement. Researchers either produce, purify, and test microbiota-derived molecules obtained using bioreactors or directly co-culture bacteria with human cells in a system with an anoxic–oxic interface (Sardelli et al., 2021). For example, an oxygen-impermeable shell can be added to standard transwell chambers to create an anaerobic apical chamber or to more complex gut-on-chip models to create a more physiological anoxic–oxic interface (Sardelli et al., 2021).

5.2.5 | 3D organotypic small intestinal tissue models

In contrast to 3D growing organoids, organotypic small intestinal models have an open luminal surface onto which compounds such as allergens can be applied. Organotypic models aim to mimic the architecture and physiology of the human intestine and are thus more physiologically relevant than simple cell models (Markus et al., 2021). The main organotypic small intestinal model used is the MatTek EpiIntestinal™ model (EpiIntestinal™, MatTek Corporation, Ashland, MA), which is available with only small intestinal epithelial cells (from human donors) or with intestinal epithelial cells and intestinal fibroblasts (https://www.mattek.com/products/epiintestinal/). When seeded onto cell culture inserts, the small intestinal epithelial cells are cultured at an air–liquid interface for up to 14 days to allow for stratification, differentiation, the creation of apical–basolateral polarity, and the formation of villi-like structures (Markus et al., 2021). When intestinal epithelial cells and intestinal fibroblasts are seeded, the inserts are first cultured submerged and then at the air–liquid interface to create a self-assembled structure in which an apical epithelium is found on top of the fibroblasts (Markus et al., 2021). These models are now commonly used by pharmaceutical companies to test drug transport, but, probably due to their high costs, they have not yet been applied to allergen transport studies (see Table 1).

➢ Cell line-based models have predominantly been used in the past to study allergen transport, though these models generally lack one or several of the critical features important for modeling the MIE1-3 and KE1 steps (see Table 1). Recent technological advances have provided the possibility to use more complex models, such as gut-on-chip, organoids, and organotypic models, which could be used in the future to obtain a more physiologically relevant in vitro system to study allergic sensitization.

6 | OPINION

To be able to predict the sensitization capacity of novel protein products, there is a need for consensus models that are able to create insights into the fate of protein digesta along the GIT (Mullins et al., 2022). As members of INFOGEST,

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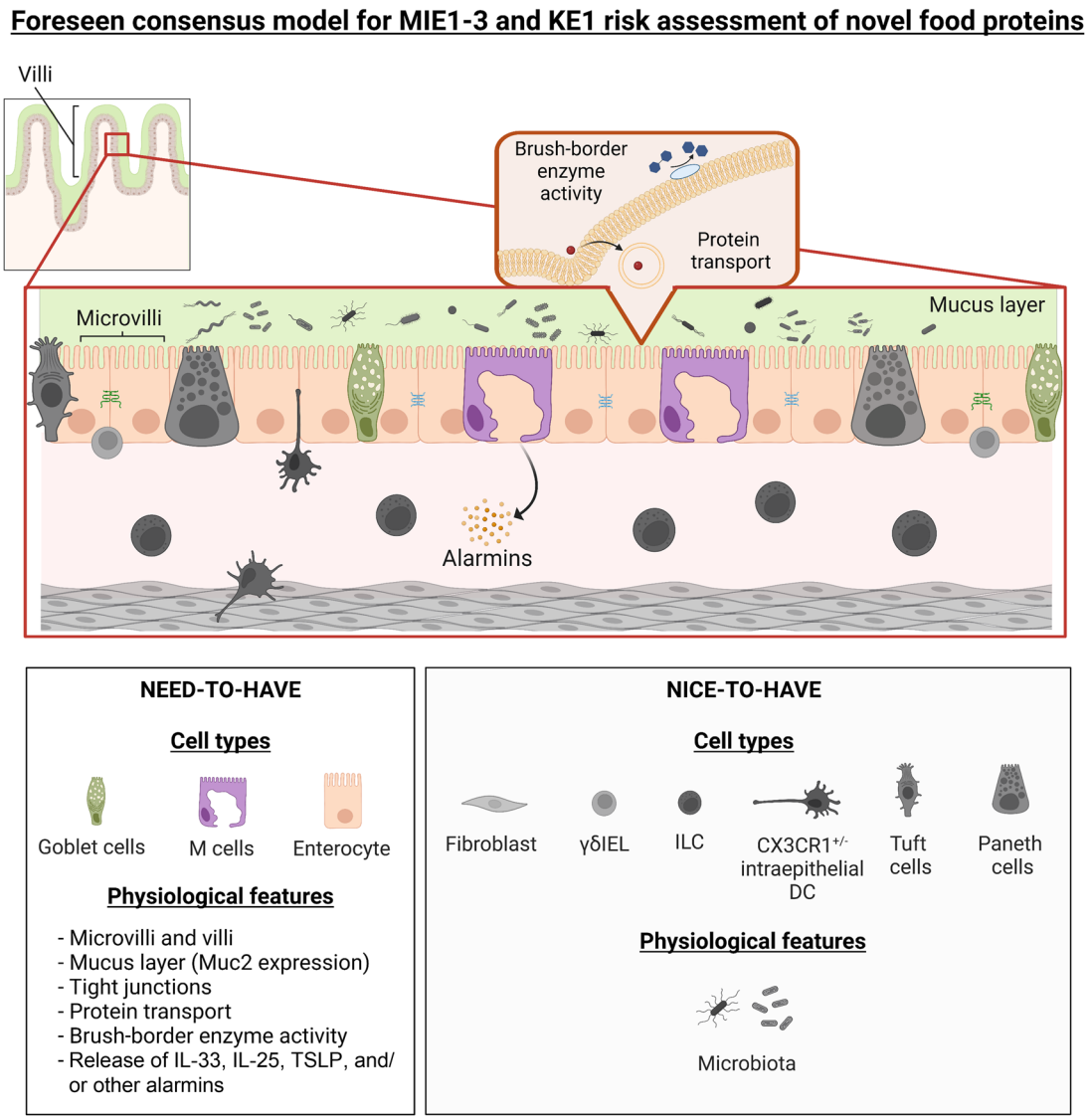

FIGURE 4 Need- and nice-to-haves in an in vitro intestinal epithelial cell model to study MIE1-3 and KE1 of the AOP of allergic sensitization. The need-to-haves are depicted in color, while features that are nice-to-haves are depicted in gray. Nice-to-haves notably include members of the innate immune cell complex, submucosal cell types, epithelial cell types, or the microbiota that are currently difficult to establish by using cell lines. ILC, innate lymphoid cells; DC, dendritic cells; IEL, intraepithelial lymphocytes; AOP, advanced outcome pathway; MIE, major initiating event; KE, key event

we have attempted to highlight several critical features that should be considered for implementation in an in vitro model for allergic sensitization that focalizes on the MIE1-3 and KE1 steps of the AOP (see Table 2 and Figure 4 for an overview). Such a model would be a first step toward a complete AOP screening model that must also contain KE2-5, in order to be able to make a total risk assessment of whether exposure with a novel protein may lead to tolerance or sensitization.

To come to a generally accepted and implementable consensus model for KE1/MIE1-3, several aspects have to be considered. A relevant in vitro model to study the translocation capacity of proteins or peptides as part of the AOP events MIE1-3/KE1 not only needs to represent the cellular complexity of the human small intestinal lining, but also to fulfill specific physiological features related to protein/peptide uptake (i.e., intestinal epithelial alarmin secretion, M-cell, goblet cell-mediated antigen translocation, etc.). On top of this, accuracy, reproducibility, and predictability of the readouts are important aspects to consider, but, above all, a consensus model should be readily applicable and reproducible in many labs. It also should have an accessible apical and basolateral compartment to allow for the addition of protein/allergen digesta and easy sampling. With these aspects in mind, although 2D-cultured intestinal organoid-based models and

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TABLE 2 Overview of critical features that should be considered for an in vitro model for the MIE1-3 and KE1 of the AOP pathway of allergic sensitization

Critical features to consider in an intestinal in vitro model for the MIE1-3 and KE1 of the AOP pathway of allergic sensitization					
MIE1-3		**KE1**		**Factors to take into consideration**	
What?	**Specifics**	**What?**	**Specifics**	**What?**	**Specifics**
Presence of tight junctions	• Responsive to external stimuli, such as pro-inflammatory cytokines secreted by immune cells or microbial factors	Presence of cell types with the potential to express and secrete alarmins	• Tuft cells (for IL-25, TSLP secretion) • Enterocytes (IL-33, TSLP secretion)	Physicochemical properties of allergens	• Enzymatic activity? Purity, endotoxin levels? Stability (Tm, pH)?
Capacity to transcytose antigens	• Responsive to external stimuli, such as pro-inflammatory cytokines	Presence of cell types that modulate epithelial activation	• Fibroblasts (might secrete IL-33) • Innate immune cells that might modulate epithelial activation (IEL, ILC)	In vitro allergen digestion	• Before transport studies and possibly including brush border enzyme-mediated digestion
A physiological resemblance to the human intestine	• Villi and micro-villi • A mucus layer of around 50–450 μm with MUC2 expression • Incorporation of duodenal microbiota?	Other factors that might modulate alarmin secretion	• Cytokines that can promote alarmin secretion (e.g., IL-1 and TNF-α)	Allergen processing and the food matrix	• Aggregate formation, allergen solubility, process-induced chemical modifications • Modulatory role of food matrix components (e.g., sugars, lipids, emulsifiers)
Presence of different intestinal cell types with functions in allergen transport	• Enterocytes with MHC-II expression • Goblet cells capable of antigen passaging • Other secretory epithelial cells • M-cells capable of transcytosing particulate antigens • Submucosal cell populations (DC, IEL)				

Abbreviations: AOP, adverse outcome pathwayKE1, key event, MIE, molecular initiating event.

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gut-on-chip models comprise many of the critical features that should be considered for an in vitro model for allergic sensitization, such as the cellular intestinal epithelial complexity (discussed in Section 5), they are also notoriously difficult to culture, are relatively expensive, and, for organoid-based models, have significant donor variability; however, the latter may actually be useful to enable studies to understand differences in sensitization affinity for different age and ethnic groups. Although developments in this research area are advancing fast, and it is likely that in the future most labs will have the necessary equipment at their disposal, at present combining well-defined human cell lines would be a well-considered choice. To capture the complexity of the intestinal mucosa, the development of a co-culture model representing the most relevant mucosal cell types is crucial since their interactions define, not only protein transport in general, but also maintain intestinal homeostasis. A disbalance due to, for example, inflammation or components in the food matrix can break this homeostasis, but this will go unnoticed if the relevant interacting cell types are not present in the model.

Considering missing knowledge and building on existing cellular models, we have pinpointed the so-called need-to-haves and nice-to-haves for an in vitro model to study allergic sensitization to allow for a gradual increase in complexity (Table 2 and Figure 4). The intestinal cell types that mediate antigen transport (i.e., enterocytes, M-cells, and goblet cells) and the intrinsic factors that exert impact on this (i.e., epithelial integrity, surface area, and mucus layer) are, for obvious reasons, need-to-haves for studying the AOP events MIE1-3. Complexity to cellular co-cultures can be added by applying fluid flow or air–liquid interface to increase villi-structuring and mucus secretion (discussed in Section 5). Another need-to-have is the capacity for alarmin release (KE1). However, this feature has been poorly studied in intestinal epithelial models, as compared to skin and lung models (discussed in Section 4), and we cannot yet state with certainty which intestinal cells should be included to physiologically reflect intestinal alarmin release. Further knowledge is needed concerning the full set of alarmin molecules that can be released by the epithelial lining upon allergen exposure and whether this release is variable and depends on the type of allergen and the barrier transport route followed. Currently, INFOGEST members are validating enterocyte-based cell lines to establish a consensus model of intestinal protein–peptide absorption, as well as a consensus protocol for their exposure with food digesta (Miralles et al., work in progress). Once established and thoroughly validated, such a model could form a basis to exploit and extend desired complexity that can be used for allergen translocation studies as part of the allergic sensitization risk assessment.

Colonization by diverse microbiota is a key issue in the development of a functional immune system, and a wide number of current reviews highlight the importance of the correct establishment of the intestine microbiota and its impact on food allergy (Lee et al., 2020; Nance et al., 2020; Rachid & Chatila, 2016). Being able to include such a microbiota–host cross talk in our proposed model would certainly add an extra dimension, though it holds certain difficulties. A tricky question to consider in the consensus design is the fact that protein absorption predominately takes place in the upper small intestine, while currently established models represent the microbiota from the colon that differs in composition and diversity. Efforts need to be made to mimic the microbial ecosystem of the upper part of the GIT and thus, there is a need to develop a synthetic microbiome (to meet the requirements reproducible and comparable) composed of microbiota representative of the small intestine. Ideally, it would be commercially available, or through a research group, which monitors composition and stable outgrowth in each batch. Since such a synthetic small intestinal microbiota blend is not yet available, we consider adding microbiota as a nice-to-have.

Since allergen sensitization mostly occurs in infancy (Dharma et al., 2018), we considered whether the intestinal epithelial model should be representative of an infant, toddler, or adult. After birth, the GIT of the newborn is colonized by different microbial communities that increase in number and diversity until reaching a more stable composition at approximately 2–3 years old (Yatsunenko et al., 2012). The establishment of this early microbiota provides a massive antigenic stimulus necessary for the adequate maturation of the gut and associated immune system. Infants have also an immature digestive system, low activity of some digestive enzymes, higher gastric pH, and an immature epithelium with high permeability. Although an intestinal barrier model that mimics infant permeability characteristics has been recently developed to study nutrient absorption (Kondrashina et al., 2021), capturing the complexity of an infant's immature intestinal epithelium for studying the AOP is currently considered difficult to perform in vitro. Thus, we consider that an adult epithelial consensus model is only feasible at this moment.

Lastly, we would like to highlight that the allergic sensitization assessment of novel protein isolates/concentrates should be evaluated, not only on its own, but also in the form and in the food product matrix in which it will be consumed. In this way, immunogenic adjuvants possibly present in the matrix are considered, as well as industrial processing aspects that may influence the rate of epithelial transport and thus sensitization capacity of the novel proteins. Thus, a consensus model should also include sample preparation steps, including an in vitro digestion protocol, and how digesta should be applied.

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Recommendations on how to best detoxify in vitro digesta samples for application in cellular intestinal absorption models are currently being drafted within INFOGEST (Kondrashina et al., to be submitted). Assessing a "healthy model," as well as an "inflamed or barrier damaged" model, is another aspect to consider. This comparison could indicate whether an atopic predisposed individual would have a larger risk to become sensitized than a nonatopic individual. In addition, the impact of drug use (antibiotics/antacids) or pathogenic colonization on allergen digestion and absorption is an additional assessment option. To study the full impact of matrix components, the addition of certain submucosal cell populations is important, so as not to miss feedback mechanisms toward the epithelium. Thus, co-cultures including $\gamma\delta$IEL and CX3CR1^{+}-intraepithelial DC are considered nice-to-haves.

7 | CONCLUSION

In this paper, we have evaluated the importance of certain parameters in allergen transport and provided a critical opinion on which cellular and physiological features should be included in an intestinal epithelial consensus model to create insights into the cellular and molecular mechanisms underlying the first critical steps in allergic sensitization: if a dietary protein or its derived peptides are unable to cross the intestinal epithelial barrier, interactions with the innate immune system are highly unlikely to occur, making additional screening using assays for AOP events KE2–5 largely unnecessary. The next step is the actual development of such a consensus model, with written protocols and clearly defined application guidelines, after which the model must be thoroughly validated by means of a ring trial using a panel of defined allergens and low/nonallergenic proteins. Currently, there are limited data available to clearly discriminate between allergenic and nonallergenic proteins, and this should be an important area of focus in the future. In addition, any guidelines should also include a downscaled INFOGEST digestion simulation protocol to deal with the availability of small quantities of pure allergens versus whole food product material. When this consensus for the first key event in the AOP is in place, complexity must be added by including guidelines for subsequent key events (KE2–5; see also Figure 1) using DC, macrophages, ILC, and T- and B-lymphocytes in the model, so that it is possible to carry out a full risk assessment of sensitization and to get an answer to the questions "which factor of a (novel) protein tilts the balance towards development of tolerance or allergic sensitization?"; "why are certain allergens more potent sensitizers than others?"; and "which protein properties determine the type and severity of clinical symptoms?." It is important to realize that with the development of the expected consensus model, only the first AOP steps in allergen sensitization (MIE1-3 and KE1) can be studied, and therefore no statements about sensitization risks can be made. We are still a long way from developing guidelines and filling knowledge gaps to ultimately apply a complex AOP consensus model for the risk assessments required by EFSA.

Nomenclature

2D	two-dimensional
3D	three-dimensional
AOP	Adverse Outcome Pathway
APC	antigen-presenting cells
APRIL	A proliferation-inducing ligand
CCL	C-C motif chemokine ligand
CCR	C-C motif chemokine receptor
CX3CL1	C-X3-C motif chemokine ligand 1
cDC	conventional dendritic cells
DC	dendritic cells
EFSA	European Food Safety Authority
ELISA	enzyme-linked immunosorbent assay
IEL	intraepithelial lymphocyte
IFN-γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
ILC	innate lymphoid cell
iPSC	induced pluripotent stem cell
GAP	goblet cell antigen passages
GALT	gut-associated lymphoid tissue
GIT	gastrointestinal tract
GM-CSF	granulocyte-macrophage colony-stimulating factor
HIO	human-induced pluripotent stem cell (iPSC)-derived intestinal organoid
KE	key events
M-cell	microfold cell
MHC	major histocompatibility complex
MIE	molecular initiation event
MLN	mesenteric lymph node
NK	natural killer cells
nsLTP	nonspecific lipid transfer protein
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
SAP	secretory antigen passage
TEER	transepithelial electrical resistance
TSLP	thymic stromal lymphopoietin
TNF-α	tumor necrosis factor alpha
ZO	zonula occludens

AUTHOR CONTRIBUTIONS

Wieneke Dijk, Caterina Villa, Sara Benedé, Isabel Mafra, Gregory Bouchaud Karen Knipping, Joana Costa, and

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Shanna Bastiaan-Net conceptualized the idea of the study. Wieneke Dijk, Caterina Villa, Sara Benedé, Emilia Vassilopoulou, Isabel Mafra, María Garrido-Arandia, Joana Costa, and Shanna Bastiaan-Net curated the data. Wieneke Dijk, Caterina Villa, Sara Benedé, Emilia Vassilopoulou, Isabel Mafra, María Garrido-Arandia, Mónica Martínez Blanco, Tamara Hoppenbrouwers, Susana Delgado, Joana Costa, and Shanna Bastiaan-Net performed investigation. Wieneke Dijk, Caterina Villa, Sara Benedé, Isabel Mafra, María Garrido-Arandia, Mónica Martínez Blanco, Joana Costa, and Shanna Bastiaan-Net designed the methodology. Wieneke Dijk, Caterina Villa, Isabel Mafra, María Garrido-Arandia, Simona Lucia Bavaro, Ana Maria Castro, Susana Delgado, Joana Costa, and Shanna Bastiaan-Net performed visualization. Wieneke Dijk, Caterina Villa, Sara Benedé, Emilia Vassilopoulou, Isabel Mafra, María Garrido-Arandia, Mónica Martínez Blanco, Gregory Bouchaud, Tamara Hoppenbrouwers, Susana Delgado, Joana Costa, and Shanna Bastiaan-Net wrote the original draft. Wieneke Dijk, Caterina Villa, Sara Benedé, Emilia Vassilopoulou, Isabel Mafra, Mónica Martínez Blanco, Gregory Bouchaud, Simona Lucia Bavaro, Linda Giblin, Karen Knipping, Ana Maria Castro, Susana Delgado, Joana Costa, and Shanna Bastiaan-Net reviewed and edited the manuscript. Gregory Bouchaud performed validation. Simona Lucia Bavaro, Susana Delgado, and Shanna Bastiaan-Net performed supervision. Linda Giblin and Shanna Bastiaan-Net administered the project. Ana Maria Castro provided resources and software.

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CONFLICTS OF INTEREST

WD, CMV, SB, EV, IMSGM, MGA, MMB GB, SLB, LG, AMC, SD, and JSBC declare no conflict of interest. KK is employee of Danone Nutricia Research. SBN and TH receive funding from the food industrial consortium partners within TKI-PPS LWV20.123. The TKI consortium did not influence the preparation of this manuscript and the views expressed are those of the authors and do not necessarily reflect the position or policy of the collaborators.

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