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In vivo and in vitro models of food allergy

Epithelial models to study food allergen-induced barrier disruption and immune activation

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Changes in lifestyle, diet and environmental factors in westernized countries correspond with the rise in non-communicable diseases affecting metabolic and immune disorders, such as allergies. Therefore the mechanisms by which environmental factors and allergens are capable of elicitating allergic sensitization need to be further unraveled. *In vitro* models using human epithelial cells, with or without immune cells, are needed to achieve this purpose. Epithelial cells cover mucosal surfaces and provide a barrier between the external and internal environment. In mucosal tissues such as the respiratory and gastro-intestinal tract, epithelial cells not only contribute to barrier integrity but also actively regulate dendritic cell function and adaptive immune responses and can support tolerance induction or allergic sensitization. Certain allergens contain protease activity which may facilitate them to cross the barrier, others are transported via transcytosis. In addition, certain allergens may provoke epithelial activation resulting in production of T_H2 driving immune mediators. Preserving epithelial homeostasis is important to suppress allergic sensitization. This review describes *in vitro* models of human intestinal epithelial cells and co-culture models that are currently available to determine barrier disruption or immune activation induced by food allergens. These can be used for future development of *in vitro* models to study the contribution of intestinal epithelial cells in allergic sensitization and to identify sensitizing properties of novel proteins.

Introduction

Changing living conditions in industrialized countries, including dietary alterations, increased exposure to environmental pollutants, microbiome alterations and a sedentary lifestyle, have been linked to the increase in non-communicable diseases including allergies [1–3]. In the western world depending on the country 5–30% of young people are affected with asthma and/or rhinitis and 6% of children and 3–4% of adults with food allergy [4–6]. Allergic sensitization occurs

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for a large range of food allergens such as cow's milk, hen's egg and peanut proteins and/or inhalant allergens like house dust mite or pollen. Mucosal tissues covering the lung and gastro-intestinal tract provide a barrier against environmental antigens, and support immunological tolerance for harmless agents while immunity is raised against pathogenic intruders [7,8]. However in case of allergic sensitization a T helper cell 2 (T_H2) driven IgE mediated immune response is raised against relatively harmless proteins (allergens). Epithelial cells protect underlying mucosal lymphoid tissues from excessive exposure to allergenic proteins. They express pattern recognition receptors (e.g. Toll like receptors), glycan binding receptors (e.g. galectins), cytokine and chemokine receptors and produce cytokines, chemokines, galectins and growth factors that drive immune polarization by affecting dendritic cell (DC) function and the adaptive immune response [9,10]. This review describes the current knowledge on the contribution of intestinal epithelial cells (IEC) to allergic sensitization with regard to barrier properties and production of immune mediators and human *in vitro* models that can be used and/or further developed to study these processes.

Epithelial barrier and defects related to allergic sensitization

In the intestine a monolayer of epithelial cells exhibits numerous physical adaptations to separate the mucosal immune system from the external environment. A brush border on the apical surface of the epithelium produces digestive enzymes and allows uptake of nutrients, while intercellular tight junctions between neighboring epithelial cells prevent paracellular transport of immunogenic macromolecules. This physical barrier is reinforced by a glycocalyx formed by secretion and apical attachment of a heavily glycosylated mucin-rich layer further protecting the epithelial lining from microbial attachment and pathogen invasion [11]. In addition, IgA and digestive enzymes prevent the uptake of antigenic macromolecules into the body. The gut epithelium is created from a pool of pluripotent stem cells, which give rise to five types of IEC: absorptive columnar cells (enterocytes), goblet, endocrine, Paneth, and M (microfold) cells. Enterocytes form the vast majority and 10–25% of IEC consist of mucus producing goblet cells [8]. Cohesion and polarity of the epithelial layer are maintained by the apical tight and adherens junctions, and by the subjacent desmosomes [12]. Numerous aeroallergens (house dust mite (Der p1, Der p9), cockroach, pollen, *Penicillium* sp., *Aspergillus* sp.,) [11,13] and food derived allergens reveal protease activity (see Table 1). These allergens are involved in the pathogenesis of allergic diseases through (i) inducing the release of pro-inflammatory cytokines via activation of protease-activated receptors (PARs), which are widely expressed on leukocytes, endothelium, epithelium, and many airway cells; (ii) the cleavage of CD23 from activated B cells and CD25 from T cells to favor the development of T_H2-type responses [14,15]; (iii) the degradation of junctional proteins, thus increasing the permeability of the epithelium *in vitro*. Also non-proteolytic food allergens can cross the epithelial barrier for example via transcytosis (Table 2). Aeroallergens such as house dust mite allergen Der p2 or Timothy grass allergen Phl p1 [11,13,16–18], have recently been shown to induce airway epithelial activation resulting in the release of IL-1α, IL-33, IL-25, TSLP and/or GM-CSF which may contribute to recruitment and activation of DC and innate lymphoid group 2 cells (ILC2) and consequent T_H2 polarization. Similar aspects may apply for certain food derived allergens such as Peach LTP and peanut allergens (Table 2). In addition, IL-4 and IL-13 produced by T_H2 cells and/or ILC2, and tryptase secreted by mast cells, can enhance epithelial permeability via the IL-4/IL-13 receptor or PAR2 receptor respectively [19–22]. Beyond allergens increasing paracellular permeability and crossing the epithelial barrier via the transcellular route, IgE-allergen complexes can be transported over IEC via the low affinity IgE receptor CD23b [23].

Epithelial cells contribute to tolerance induction or allergic sensitization

The intestinal epithelium is in close contact with dendritic cells (DC) that sample luminal antigens. M-cells that cover Peyer's, caecal and colonic patches, are specialized in the uptake of particulate antigens and transfer these to DC in the subepithelial dome that can instruct naïve T-cells and B-cells [8]. The lamina propria is the effector site of the intestinal mucosa and contains DC, macrophages, ILC, T-cells, B-cells, intra epithelial T-cells, eosinophils and mast

Table 1. Examples of (food) allergens with proteolytic activity known to affect intestinal epithelial barrier integrity and/or activate mediator release *in vitro*

Allergen source	Enzyme	Mode of action	Effect
House dust mite [24]	Der p 1	Cleavage of tight-junction molecules (occludin, claudin) via cysteine protease activity	Increase in epithelial permeability of intestinal human biopsy
Kiwifruit [25,26]	Act d 1	Cleavage of tight-junction molecules (occludin)	Increase in epithelial permeability of Caco-2 and T84
Pineapple [27]	Ana c2	Widening intercellular junctions, strong mucolytic activity	Increase in epithelial permeability of Caco-2
Papaya [27]	Car p 1	Loosening of tight junctions	Increase in epithelial permeability of Caco-2

Table 2. Examples of food allergens with non-proteolytic activity that traffic the intestinal epithelial barrier and/or activate mediator release *in vitro*

Allergen source	Allergen	Mode of action	Effect
Peach LTP [28]	Pru p3	Lipid raft mediated uptake and increased epithelial TSLP, IL-33, IL-25 mRNA	Crosses epithelial barrier and activates Caco-2 epithelial cells
Cow's milk [29]	αLac βLac	Transcytosis	Crosses epithelial barrier of Caco-2
Peanut [30,31]	Ara h1/h2 Ara h2	Transcytosis Cellular activation	Crosses epithelial barrier of Caco-2 Stimulates a pro-inflammatory response in Caco-2/TC7 cells
Wheat [32]	ω5-gliadin LTP	Transcytosis	Crosses epithelial barrier of Caco-2
Egg white [33,34]	Gal d1 Gal d 2	Transcytosis Transcytosis	Crosses epithelial barrier of human breast Crosses epithelial barrier of human gastro-intestinal tract
Brazil nuts [35]	Ber e 1	Transcytosis	Crosses epithelial barrier of Caco-2
Sesame seeds [35]	Ses i 1	Transcytosis	Crosses epithelial barrier of Caco-2

cells [8]. Intestinal $CD103^+$ DC are crucial in determining the adaptive immune response to oral antigens, and they traffic to the mesenteric lymph nodes (MLN) in a CCR7 dependent manner where they promote tolerance or immunity [36,37]. The $CX3CR1^{hi}$ resident macrophages directly underlie the epithelium and under homeostatic conditions produce high amounts of IL-10. They can extend transepithelial dendrites through the epithelium via the paracellular space to sample luminal antigen and transfer this to $CD103^+$ DC via connexion 43. Similarly goblet cells transfer antigen via channels to $CD103^+$ DC [38]. Also $CD103^+$ DC themselves are in close contact with the epithelium and sample from the lumen. Oral tolerance is abolished in absence of MLN or CCR7 expressing DC, while the Peyer's patches are dispensable. This suggests that $CD103^+$ migratory DC from the LP are key in oral tolerance induction [39]. If these cells are instructed to produce retinoic acid (RA) (high expression of vitamin A converting enzyme aldehyde dehydrogenase) and TGFβ and/or indoleamine 2,3-dioxygenase (IDO) they can induce gut trophic $\alpha4\beta7^+CCR9^+FoxP3^+$regulatory T cells (Treg) that home back to the lamina propria where they are further differentiated and expanded by IL-10 producing $CX3CR1^+$ macrophages [8,36,37,40]. Local intestinal factors that generate these tolerogenic $CD103^+$ DC include the microbiome, dietary components, leukocytes, stromal cells and neuroendocrine mediators as well as IEC derived factors (including TGFβ, TSLP, RA and mucin MUC2) [8,37,40–44] (Fig. 1). Epithelial cells can instruct T_H2 driving OX40L expressing DC that secrete CCL17 and CCL22 and activate ILC2 [16,45,46]. This was convincingly shown for aero-allergens like HDM which contains specific allergens (Derp2) and LPS that activate NFkB signaling in airway epithelial cells. In response they release IL-1α which via a positive feedback loop induces IL-33, IL-25, TSLP and endogenous danger factors such uric acid and airway epithelial cells also can release DC chemo-attractants CCL2 and CCL20 upon allergen exposure [16,47]. TSLP, IL25, IL33 and uric acid are also increased in the intestine of mice affected with food allergy, and in particular IL-33 and uric acid contribute to allergic sensitization not only for inhalant allergen HDM but also for food allergen peanut in mice (Fig. 1) [46,48–50].

Human in vitro *models of intestinal epithelial cells*

The use of *in vitro* IEC models for transport studies and allergen uptake focusses on absorptive cells. Because of the difficulties in culturing isolated primary human IEC and limited viability, monolayers of human colorectal adenocarcinoma cell lines Caco-2, HT-29 and T84 are most often used. Caco-2 cells are the most popular for use and serve as model for human intestinal enterocytes. They differentiate spontaneously into polarized intestinal cells possessing an apical brush border and tight junctions between adjacent cells, and they express hydrolases and typical microvillar transporters [32]. In the context of food allergy the Caco-2 cell line is the most often used for allergen uptake [32,35]. However it remains to be revealed if the permeability data obtained from the Caco-2 model are predictive for human gastro-intestinal tract absorption since it is very difficult to measure absorption of proteins *in vivo*. HT-29 is another often used human cell line, and although essentially undifferentiated, HT29 cells in culture are heterogeneous and contain a small proportion (i.e. <5%) of mucus-secreting cells and columnar absorptive cells [51]. HT29-MTX, a stable homogenous subpopulation obtained from methotrexate treated HT29, exhibit an entirely differentiated goblet cell-like phenotype secreting low amounts of intestinal type MUC2 mucins [52]. The T84 cell line has been used as a model of intestinal cells which produces high molecular weight mucus [53,54]. A very high

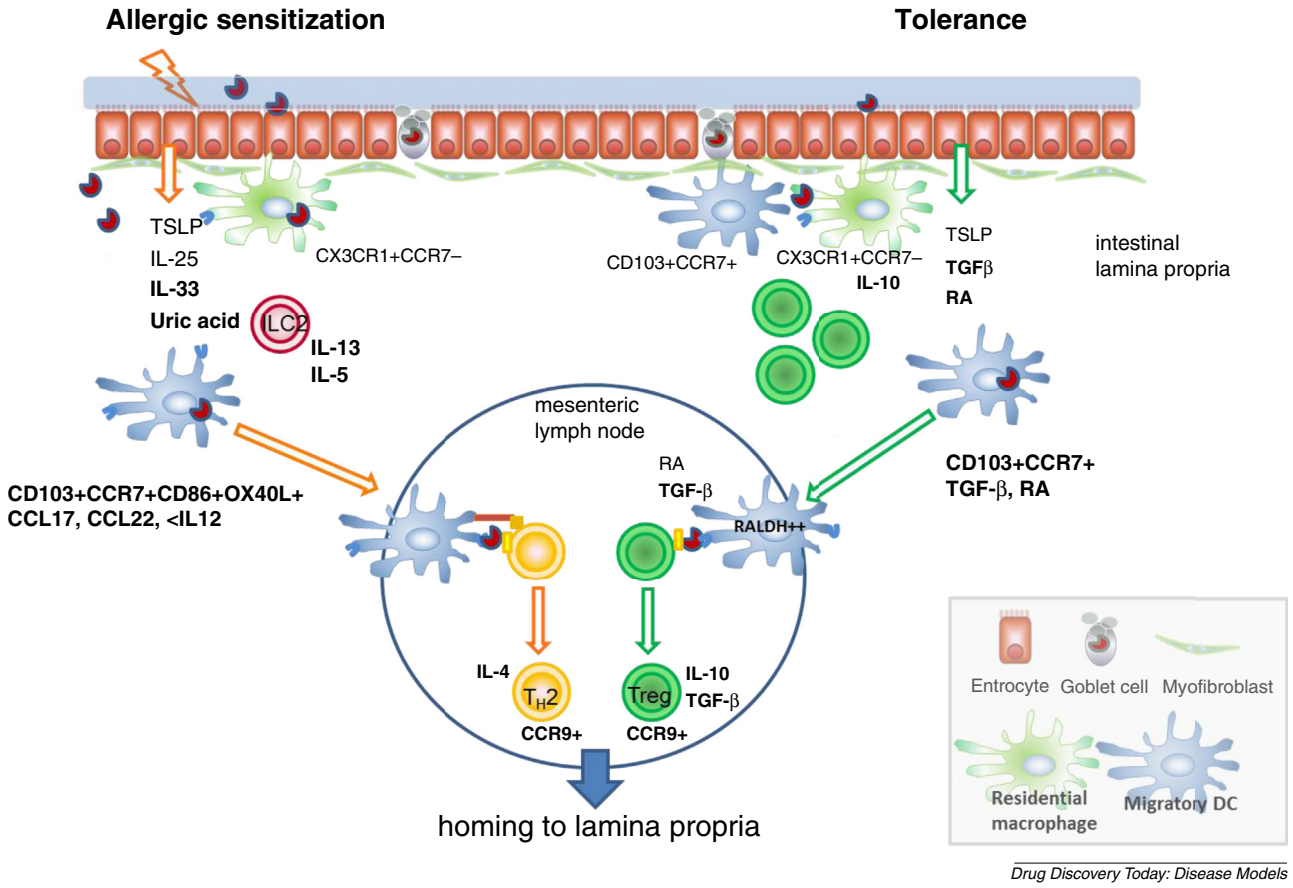

Figure 1. Antigen uptake in the intestine and contribution of IEC in tolerance induction or allergic sensitization. Allergen exposed IEC are in close contact with DC that sample luminal antigens. Allergens can enter the mucosa via the transcellular or paracellular route or be transferred via M-cells (not shown), goblet cells or sampled by resident macrophages and carried to migratory CD103^{+} DC, which can also directly sample from the lumen. Allergen loaded CD103^{+} DC traffic to the MLN in a CCR7 dependent manner where they instruct tolerance or allergic sensitization. If migratory DC are instructed by IEC derived factors (including TGFβ, TSLP, retinoic acid (RA) and mucin MUC2) to produce RA (via retinaldehyde dehydrogenase (RALDH)) and TGFβ they can generate CCR9^{+} regulatory T cells (Treg). The CX3CR1^{hi} resident macrophages directly underlie the epithelium and under homeostatic conditions produce high amounts of IL-10 to expand these gut homing Treg. On the other hand allergens and environmental triggers can induce IL-33, IL-25, TSLP and uric acid release by IEC which can activate ILC2 and instruct T_H2 driving CD86 and OX40L expressing migratory DC that secrete CCL17 and CCL22. In particular IL-33 and uric acid contribute to allergic sensitization for food allergen in mice (peanut allergy model).

trans-epithelial electrical resistance (TEER) is an indication of the enterocyte phenotype with well differentiated tight junctions. When grown on microporous filter supports coated with collagen cultures T84 cells maintain the polarity of goblet-like cells.

M-cells have a reduced glycocalix, irregular brush border with reduced microvilli and lack apical digestive enzymes. They are highly specialized for the phagocytosis and transcytosis of particulate antigens and pathogenic or commensal microorganisms [55]. An *in vitro* model system composed of a monolayer of Caco-2 cultivated with the human B-lymphoma cell line Raij has been widely used to study M cells [56]. Although these cells display efficient transcytosis activity, it is uncertain whether they accurately represent the characteristics of M-cells *in vivo*. They highly express CCL20, but lack expression of mature M-cell marker genes, such as glycoprotein 2. A novel potentially physiologically relevant *in vitro* M-cell-model system was reported in which RANKL (Receptor Activator of Nuclear Factor-κB Ligand) stimulation induces M-cell differentiation in gut organoid cultures established from intestinal crypts or single LGR5^{+} (Leu-rich repeat-containing G protein-coupled receptor 5-expressing) crypt stem cells [57]. Besides exhibiting high transcytosis activity, the range of genes expressed by these organoid cultures closely resembles those of M-cells in *vivo*.

The studies on primary murine or human stem cell derived intestinal epithelium are expanding. Embryonic stem cells (ESCs) are grown under specific conditions to self-organize into organoids or 'mini guts' [58]. They form three-dimensional structures that incorporate many key features of the *in vivo* intestinal epithelium, including a crypt-villus structure that surrounds a functional central lumen. Intestinal organoids incorporate all of the known cell types found in the adult intestinal epithelium, and provide a physiologically

relevant model. Several methods have been used to grow 'organoids' from the small intestine [59], but the most successful method is a Matrigel-based three-dimensional culture system that supports the growth of self-renewing, near-native intestinal epithelia in the absence of stromal niche components [60].

The limit of the above *in vitro* intestinal models is that they do not recapitulate the mechanically active microenvironment of living intestine (peristaltic motions and intraluminal fluid flow) and cannot be colonized by microbes over a prolonged period [61,62]. Although not using primary cells a human gut-on-a-chip microfluidic device enables Caco-2 cells to be cultured in the presence of physiologically relevant luminal flow and peristalsis-like mechanical deformations, which promotes formation of intestinal villi lined by all epithelial cell types of the small intestine [63]. They could be co-cultured with a probiotic gut microbe (*Lactobacillus rhamnosus* GG) for more than two weeks.

Hence several cell lines can be used to study epithelial function and gut-on-a-chip and primary epithelial cell cultures using organoids are being developed. Polarized Caco-2 cells are successfully used when studying barrier crossing properties of allergens via the paracellular or transcellular route *in vitro*. Alternatively T84 cells can be used since they also contain highly functional tight junction structures. In addition, these cells are sensitive for environmental triggers such as T_H2 driving IL-4 and IL-13 and PAR ligands [21,22]. Beyond studying the barrier crossing capacities of (potential) allergens, allergen induced epithelial activation may be indicative for its allergenicity. This phenomenon has only recently been revealed for airway sensitization and similar mechanisms may underlie food protein sensitization when occurring in the intestine [16,46,50]. Sensitive epithelial models enabling to measure this for food proteins are currently lacking and need to be developed. When developing these tools one should take into account that IEC are in close contact with the underlying mucosal cells such as DC (see Fig. 1) and effector immune cells which also may have impact on the epithelial interaction with allergens and environmental factors. Co-culture models combining IEC with mixed immune cells or DC may provide a better reflection of the mucosal tissue organization and allow cross talk between certain cell types in their reaction on allergens either or not in presence of other environmental factors. 2D and 3D co-culture models may be used to study these interactions.

Human 2D and 3D co-culture models of (intestinal) epithelial and immune cells

In a recent study colonic biopsies of healthy adults mounted in Ussing Chambers kept under high oxygen pressure were used to determine HDM induced intestinal barrier disruption and effects on IL-10 and TNF-α levels [24]. Hence it may be possible to maintain human intestinal biopsies for prolonged time. However the availability of fresh human intestinal biopsies for research purposes is limited and requires ethical approval. Co-culture models allowing cross-talk between structural cells and immune cells are being developed. Transwell 2D co-cultures in which T84 cells were grown on inserts and exposed to anti-CD2/CD28 activated lamina propria mononuclear cells (LPMC) in the basolateral compartment can be used to study the epithelial cell immune cell cross talk and barrier dysfunction [64]. Based on this model a 2D co-culture model using HT-29 and more easily accessible peripheral blood mononuclear cells (PBMC) instead of LPMC was developed. In this model the epithelial cells modified the cytokine secretion of underlying anti-CD3/CD28 activated PBMC when exposed to TLR ligands [65,66]. Epithelial derived galectin-9 (in HT-29 as well as T84) contributed to Treg and T_H1 polarization of PBMC and epithelial derived supernatant instructed Treg and T_H1 inducing monocyte derived DC (moDC). Epithelial galectin-9 expression was confirmed in the murine intestine and increased intestinal and systemic galectin-9 levels in association with enhanced intestinal Treg and T_H1 markers and suppression of food allergy symptoms, indicating the translational value of this 2D co-culture model [67,68]. Although allergens were not studied, in the co-culture LPS exposed HT-29 released TSLP and CCL22 (MDC) was increased [65]. Also Caco-2 may be able to produce T_H2 polarizing mediators. In a 2D Caco-2/PBMC co-culture Pru p3 transport and enhanced TSLP, IL-25 and IL-33 mRNA expression was measured while IL-1β, IL-6, IL-10 and TNFα mRNA in underlying PBMC was increased [28]. Hence, this type of model may not only indicate whether a food allergen induces epithelial activation, it may also determine the consequence of this effect on the underlying immune cells. In most cases human 2D co-cultures combine epithelial cells with DC. Caco-2 cells are grown on filters and moDC are seeded at the basolateral side and inflammatory mediator release and DC activation and migration is studied [69]. Supernatants of epithelial cells from healthy donors or Caco-2 enhanced CD103 expression on moDC or CD1c+ DC from human PBMC and instructed CD103+CCR7+ DC from human MLN to induce Treg [70]. RA, TGFβ and TSLP in the supernatant of Caco-2 cells were responsible for the induction of these Treg driving tolerogenic moDC [70]. When Caco-2 were cultured with moDC in the basolateral compartment and apically exposed to bacteria, epithelial derived TGFβ suppressed pro-inflammatory cytokine production by the moDC [71]. Caco-2 can also be grown inverted on the basolateral side of the filter while moDC are added to the apical compartment (contact model) [72]. In both models MHCII, CD86 and CD80 expression on moDC was reduced in the presence of IEC. However, only in the contact model also TGFβ concentrations increased while IL-8 decreased and moDC were less responsive to LPS maturation [72]. Future studies are warranted to determine whether this model would

be suitable to study intestinal epithelial cell/DC cross talk upon allergen exposure. Such an approach is already being developed using lung epithelial cells. For example, in a 2D bronchial epithelial cell (16HBe140) (inverted)/moDC contact model the effect of allergen exposure was studied. CD80 and PD-L1 expression on moDC was increased and the DC started to produce eotaxin and IL-10, which did not occur when DC were cultured with the epithelial cell supernatant. Furthermore, upon exposure to birch, grass or HDM extracts the DC from the co-culture model had reduced capacity to enhance autologous T-cell proliferation and T cell cytokine release [73]. In another 2D airway co-culture model BEAS-2B cells or primary bronchial epithelial cells from allergic donors that were cultured inverted on collagen coated transwell filters were basolaterally exposed to Der p1 and moDC precursors were added to the apical compartment. Der p1 increased the epithelial chemokine release and enhanced moDC migration [47]. Hence, in analogue to these 2D models studying the crosstalk between airway epithelial cells and DC upon aeroallergen exposure, this could be studied for food proteins using IEC. Beyond 2D also 3D co-cultures are being developed which include connective tissue cells that produce immune mediators as well as extracellular matrix components. In a 3D co-culture model T84 cells were grown on inserts on top of primary human CCD-18Co intestinal myofibroblasts and exposed to activated LPMC in the basolateral compartment. These studies revealed myofibroblasts to protect against inflammatory induced barrier disruption [64]. For lung disease such types of models have been further developed and combine epithelial cells, DC and fibroblasts. In a model in which human Calu-3 lung epithelial cells, moDC and human MRC-5 lung fibroblasts are grown on separate polyethylene terephthalate (PET) filters, papain induced barrier disruption was less pronounced when the fibroblasts were present. DC were found to migrate to the apical epithelial compartment upon exposure to HDM or LPS [74]. In an air exposed model in which MRC-5 cells, moDC and 16HBE bronchial epithelial cells were grown directly on top of each other on filters containing a collagen matrix, CCL17 and CCL22 release by DC was silenced, while CCL18 concentrations were high [75,76]. These 2D and 3D cultures show that several cell types present in mucosal tissues functionally interact and may impact on whether or not an allergen, in absence or presence of additional environmental triggers, can induce allergic sensitization. Hence, future development of *in vitro* IEC models that can identify the potential sensitizing capacity of allergens or novel proteins may not only make use of epithelial cells alone but also bring them in context with local tissue cells such as fibroblasts known to affect epithelial function and/or DC or mixed immune cells to reflect the impact on the immune response.

Conclusion

IEC models to study intestinal allergen uptake are widely used. Novel developments include the more physiological 'gut-on-a-chip' and stem cell derived primary organoids or 'mini guts' which in the future may be exploited for allergen testing as well. In addition, epithelial models suitable to measure T_H2 driving mediators such as IL-33, IL-25 and TSLP and relevant chemokines should be developed taking into account not only the exposure of the allergens but also environmental factors (such as inflammatory mediators, bacterial components or mycotoxins [77]) that can act as a secondary trigger to activate the sensitization cascade. Furthermore, taking into account the complexity of the mucosal tissue, *in vitro* models to study the sensitizing potency of allergens should also combine relevant mucosal cell types since their interaction may affect the functional response of IEC and therefore be more representative for the *in vivo* setting.

Conflict of interest

LW is employed at the Utrecht University and collaborates with Danone/Nutricia Research B.V. within a strategic alliance between the Utrecht Institute for Pharmaceutical Sciences of the Utrecht University and Danone/Nutricia Research B.V, Utrecht, The Netherlands.

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