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Ovalbumin-Induced Epithelial Activation Directs Monocyte-Derived Dendritic Cells to Instruct Type 2 Inflammation in T Cells Which Is Differentially Modulated by 2′-Fucosyllactose and 3-Fucosyllactose

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Abstract
Allergic sensitization starts with epithelial cell activation driving dendritic cells (DCs) to instruct T helper 2 (Th2) cell polarization. Food allergens trigger intestinal epithelial cell (IEC) activation. Human milk oligosaccharides may temper the allergic phenotype by shaping mucosal immune responses.We investigated in vitro mucosal immune development after allergen exposure by combining ovalbumin (OVA)-preexposed IEC with monocyte-derived DCs (OVA-IEC-DCs) and subsequent coculture of OVA-IEC-DCs with Th cells. IECs were additionally preincubated with 2′FL or 3FL. OVA activation increased IEC cytokine secretion. OVA-IEC-DCs instructed both IL13 ($p < 0.05$) and IFNγ ($p < 0.05$) secretion from Th cells. 2′FL and 3FL permitted OVA-induced epithelial activation, but 2′FL-OVA-IEC-DCs boosted inflammatory and regulatory T-cell development. 3FL-OVA-IEC lowered IL12p70 and IL23 in DCs and suppressed IL13 ($p < 0.005$) in T cells, while enhancing IL17 ($p < 0.001$) and IL10 ($p < 0.005$).These results show that OVA drives Th2- and Th1-type immune responses via activation of IECs in this model. 2′FL and 3FL differentially affect OVA-IEC-driven immune effects. 2′FL boosted overall T-cell OVA-IEC immunity via DC enhancing inflammatory and regulatory responses. 3FL-OVA-IEC-DCs silenced IL13, shifting the balance towards IL17 and IL10.This model demonstrates the contribution of IEC to OVA Th2-type immunity. 2′FL and 3FL modulate the OVA-induced activation in this novel model to study allergic sensitization.

Introduction

Allergic disease, which currently affects over 150 million Europeans, has become the most common chronic disease in Europe. It is estimated that by 2025, more than 50% of the European population will suffer from at least one type of allergy [1]. Up to 10% of infants are affected by food allergies in Western countries, mainly caused by allergens in hen's eggs, cow's milk, and peanuts [2]. Previously the increasing prevalence had been observed in Westernized countries only; however, similar trends are now shown globally [3–5]. Although key components of

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the underlying immunological mechanisms of food allergies have been extensively studied, a deeper understanding of the onset and regulation of these mechanisms is lacking [6]. In vitro models to study the development of allergic sensitization at mucosal and other epithelial sites need to be developed to further investigate the intrinsic capacity of potentially allergenic proteins to drive sensitization and develop strategies to prevent sensitization [7]. Key events in the mechanisms of allergic sensitization involve in particular the direct crosstalk between epithelial cells and dendritic cells (DCs) and the consequent effect on T-cell development, which can be studied using human in vitro coculture assays, although these models have not made use of allergens to provoke epithelial activation [8–10].

The mucosal immune system in the gastrointestinal (GI) tract defends the host against pathogenic intruders, while it is also essential for the establishment of oral tolerance to harmless food proteins. In the case of food allergy, oral tolerance is hampered by specific food allergens such as the major hen's egg allergen ovalbumin (OVA). OVA is an important food allergen as it can escape GI digestion and has an intrinsic capacity to induce immune activation [11]. Allergic sensitization to a food protein can occur upon exposure via the skin and even lungs or upon oral exposure. When the process of oral tolerance induction is interrupted, a Th2-driven immune response against the food protein is initiated instead. In the GI tract, a monolayer of intestinal epithelial cells (IECs) separates the luminal content from the underlying mucosal immune system. IEC control homeostasis and contribute to oral tolerance induction but upon activation, IEC drive the establishment of allergic sensitization [12]. The initial response of the intestinal epithelium to a potential allergen plays a central role in the subsequent activation of type 2-driven inflammation, mediated by alarmins such as IL33, TSLP, and IL25 [13]. These alarmins prime DCs to become type 2 instructing cells, characterized by increased expression of costimulatory factors such as CD80, CD86, and OX40L while producing mediators such as CCL22 [14]. In addition, alarmins inhibit type 1 and regulatory type polarizing signals from DCs such as IL12p70, IL15, and IL10 [15]. Upon arrival in mesenteric lymph nodes, type 2 instructing DCs encourage the proliferation of Th2 cells instead of instructing the development of regulatory T cells (Treg) or anergy. These Th2 cells are required for the development of allergic sensitization as they instruct IgE isotype switching of allergen-specific B cells [13]. Modulation of the initial epithelial response to potential allergens may contribute to altered crosstalk between IEC and DC, dominated by suppressed secretion of alarmins and an increase in regulatory factors such as TGFβ, galectin-9, and retinaldehyde dehydrogenase (RALDH) [10]. These factors can contribute to maintaining intestinal homeostasis by supporting regulatory T cell development and, therefore, possibly play a role in preventing allergic sensitization. In this respect, the composition and activity of the intestinal microbiota or the availability of specific dietary components such as fibers may promote the development of oral tolerance and thereby influence the risk of developing allergies [16].

Human milk oligosaccharides (HMOS) potentially possess such modulatory properties and may contribute to the development of homeostasis in the intestines. HMOS are important for the development of the infant's microbiome and consequent immune maturation. Moreover, HMOS come directly in contact with epithelial cells and immune cells and modulate immune properties both locally as well as systemically [16]. With >200 HMOS structures identified, the fucosylated HMOS form a distinguished group of HMOS and are among the most common HMOS found in human milk [17]. Depending on the expression of an active form of the gene FUT2, the production of 2′-fucosyllactose (2′FL) is mediated [18]. However, ~22% of European mothers do not express an active form of FUT2 [19, 20]; these mothers are enzymatically impaired to generate an α1-2 glycosidic linkage of the fucose group, resulting in the exclusive production of 3-fucosyllactose (3FL). Although 2′FL (Fuc-α1,2-Gal-β1,4-Glc) and 3FL (Gal-β1,4-Glc-α1,3-Fuc) are structurally very similar, they may differentially shape the neonate's immune system based on the notification that they can differentially bind to distinct cellular receptors [21, 22]. Recently, manufactured 2′FL and 3FL have become available, making it possible to study the structure-specific immunomodulatory properties of these HMOS.

In the current study, a human in vitro model for OVA-induced mucosal inflammation by using OVA-exposed HT-29 cells was developed. The crosstalk between these OVA-preexposed IECs (OVA-IECs) and human monocyte-derived DCs was studied. Next, the OVA-IEC-DCs were cocultured with allogenic human naïve CD4+ T cells to study the functional effect on T-cell development, mimicking events occurring in the lymph nodes to which the DCs migrate upon luminal allergen sampling. These steps are key events during allergic sensitization and are relatively poorly understood in food allergy [7]. Focusing on these sequential steps known to be required for driving type 2 allergic sensitization, the effects of epithelial OVA exposure were deciphered. In addition, the differential

immunomodulatory effects of the HMOS 2′FL and 3FL were explored in the model. Complex in vitro models, such as those presented here, contribute to elucidating the processes involved by enabling separate analysis of each key event in mucosal immune activation, which in this case is exemplified by using the food allergen OVA.

Materials and Methods

HT-29 Cell Culturing

As the IEC model, the human colon adenocarcinoma HT-29 cell line (passages 148–156) was used. The cells were cultured in culture flasks (Greiner, Germany) with McCoy's 5A medium (Gibco, USA) supplemented with 10% FCS (Gibco), penicillin (100 U/mL), and streptomycin (100 μg/mL) (Sigma-Aldrich, UK) as cell culture medium. The HT-29 cells were kept in an incubator with a 5% CO_2 influx at 37°C. The medium was refreshed every 2–3 days, and the cells were passaged at 80–90% confluency by trypsinization.

HT-29-Cell OVA Stimulation and HMOS Exposure

HT-29 cells were diluted 5 times based on surface area and transferred to a 48-well flat-bottom plate (500 μL/well). The cells were cultured for 6 days until 100% confluency. On the 6th day, the HT-29 cells were preincubated with 0.1% (wt/vol%) 2′FL, 3FL (both HMOS are synthetically derived from lactose, Carbosynth, UK) dissolved in McCoy's 5A medium for 24 h. The next day, the medium was refreshed containing new HMOS and the cells were exposed to 100 μg/mL OVA (Albumin from chicken egg white – lyophilized powder, ≥98% agarose electrophoresis; Sigma-Aldrich). After 24 h, 500 μL supernatant was collected, and the cells were lysed with 350 μL RLT buffer (Qiagen, Germany) containing β-mercaptoethanol (Sigma-Aldrich). Supernatants were stored at −20°C and cells at −70°C until further use. Endotoxin contamination was measured by a LAL assay (Charles River, USA) according to the manufacturers protocol (2′FL [3.0 pg/mg], 3FL [6.26 pg/mg], OVA [960 ng/mg]).

PBMC Isolation

Isolation of human PBMCs from buffy coats from healthy donors (Dutch Blood Bank, The Netherlands) was performed by density gradient centrifugation in Leucosep-tubes (Greiner). Next, the cells were washed two to three times with PBS (Lonza, Switzerland) containing 2% FCS. The remaining erythrocytes were lysed with red blood cell lysis buffer (4.14 g NH4Cl, 0.5 g KHCO3, 18.6 mg Na2EDTA in 500 mL demi water, sterile filtered, pH = 7.4) for 5 min on ice. Isolated PBMCs were counted and resuspended in a concentration of 2×10^6 cells/mL in RPMI 1640 with 2.5% FCS, penicillin (100 U/mL), and streptomycin (100 μg/mL).

Monocyte-Derived Dendritic-Cell Isolation and Culture

Monocytes were isolated from PBMCs through magnetic separation via negative selection (Miltenyi Biotec, Germany). For this purpose, FcR blocking reagent was added to the PBMCs prior to adding the Biotin-Antibody Cocktail per the manufacturer's instruction. PBMCs were gently mixed and incubated on ice for 10 min. After another 15 min incubation on ice with Biotin MicroBeads, the cells were added to LS columns that were placed in a QuadroMACS separator (Miltenyi Biotech). Collected effluent contained the enriched monocyte fraction. The isolation procedure yielded 87–92% pure monocyte suspensions as determined by flow cytometric analysis of the percentage of cells expressing CD14. The monocytes (1×10^6 cells/mL) were then cultured for 7 days in RPMI 1640 (Lonza) with 10% FCS, penicillin (100 U/mL) and streptomycin (100 μg/mL). Human recombinant IL4 (100 ng/mL) and GM-CSF (60 ng/mL) (Prospec, Israel) were used to differentiate monocytes into immature monocyte-derived dendritic cells (moDCs). The medium was changed on days 2, 4, and 6. On day 7, cytokines were washed away and the moDCs were collected for further use. The frequency of CD11c+HLA-DR+ expressing moDC was 41 ± 9.5% across donors.

Naïve T-Cell Isolation

Naïve CD4+ T cells were isolated from PBMCs using the Naïve CD4+ T-cell Isolation Kit II (Miltenyi Biotec), allowing for negative selection of the naïve Th cells, according to the manufacturer's protocol, and were resuspended (1×10^6 cells/mL) in T-cell medium (IMDM with 10% FCS, penicillin [100 U/mL], streptomycin [100 μg/mL], 20 μg/mL apo-transferrin [Sigma-Aldrich], and 50 μM β-mercaptoethanol) until further use. The isolation procedure yielded a 60–83% naïve Th-cell suspension.

HT-29 Cell, HT-29-moDC, moDC-T-Cell Model

This model is used to simulate crosstalk between epithelial cells and dendritic cells, and subsequently coculture of dendritic cells and naïve T cells during allergic sensitization. The model is developed to mimic the natural order of occurrence and will also be discussed in this sequence (see Fig. 1).

HT-29-moDC Coculture

Six days prior to the experiment, HT-29 cells were seeded in a 5 times dilution (based on surface area) in 12-well transwell inserts (polyester membrane, 0.4 μm pores) (Corning Incorporated, USA). The cells were incubated at 37°C, 5% CO_2, and the cell culture medium was refreshed every other day. On the 6th day medium was refreshed again and cells were apically exposed to OVA (100 μg/mL) (Sigma-Aldrich) for 24 h. In the case of the experiments with HMOS, HT-29 cells were preincubated with 2′FL, 3FL (0.1% wt/vol dissolved in McCoy's 5A medium) (Carbosynth, UK) on day 5 for 24 h. The next day, the medium was refreshed containing new HMOS and the cells were exposed to OVA (100 μg/mL) (Sigma-Aldrich). After 24 h of OVA exposure, the apical medium with stimuli was washed away and a new McCoy's 5A medium without stimuli was added. 5×10^5 moDC in 1,500 μL RPMI 1640 medium were transferred into a new 12-well plate. The transwell inserts with washed HT-29 cells were transferred to designated moDC wells. After 48 h of IEC/moDC coculture at 37°C, the inserts containing IEC were removed. Basolateral content was centrifuged and moDCs were collected for phenotyping by flow cytometry and subsequent coculture with naïve T cells. Cell-free supernatants were stored at −20°C for cytokine secretion analysis.

DC-T-Cell Coculture

Isolated naïve T cells were cocultured (in a 10:1 ratio [T:DC]) with IEC-primed moDCs, from the IEC-moDC coculture, in a 24 well flat-bottom plate for 5 days at 37°C in T-cell medium (1×10^6 cells/mL). Naïve T cells were stimulated with IL2 (5 ng/mL)

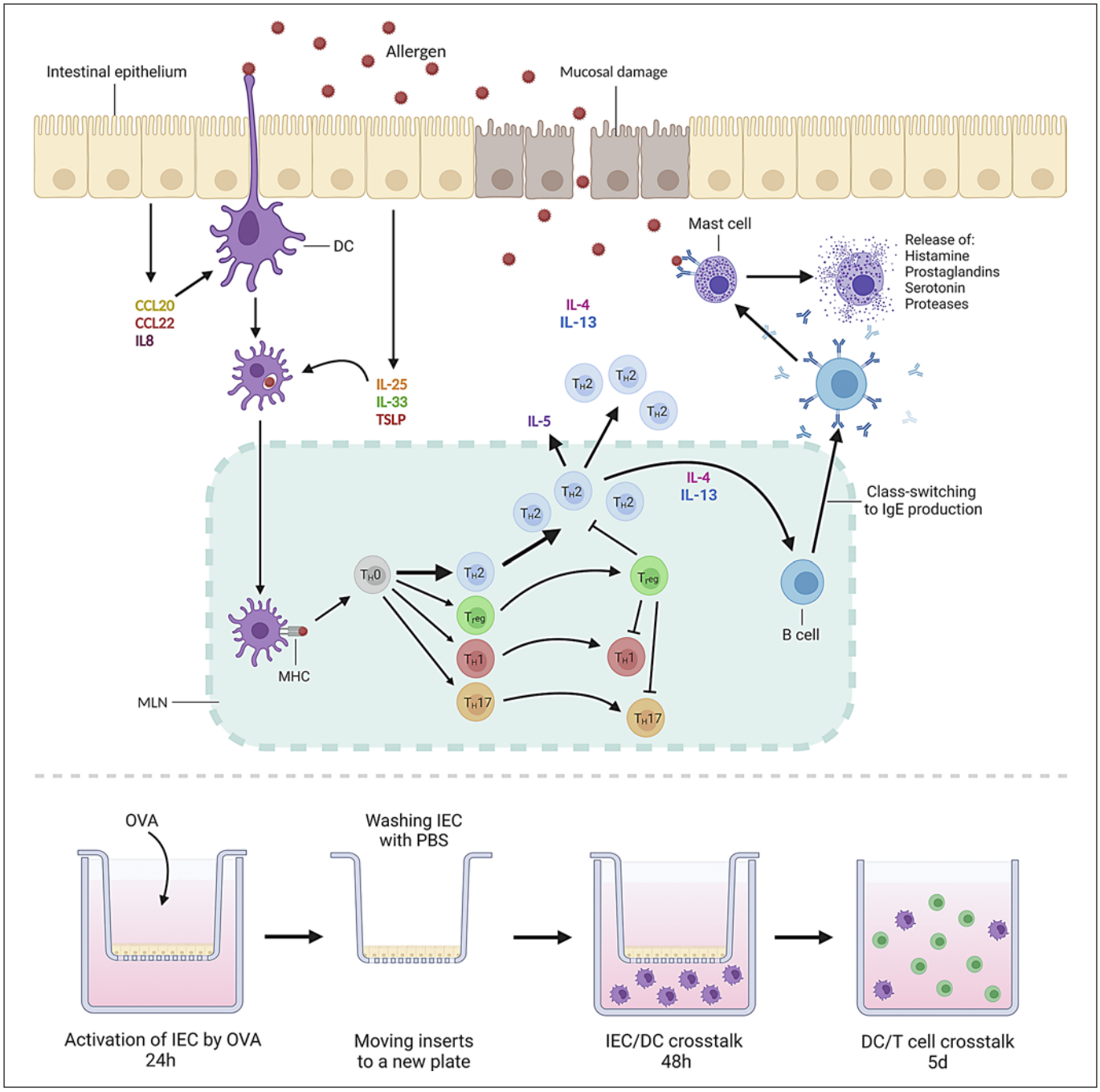

Fig. 1. Overview of the immunological mechanism of the sensitization cascade leading to food allergy, including major cell types and soluble mediators. Upon ingestion of a potential allergen, the epithelium will produce alarmins, such as IL33 and TSLP, which prime DCs to instruct a type 2 dominated response in T cells. These Th2 cells induce class switching in B cells to produce IgE, which will bind to mast cells. During a subsequent encounter with the allergen, the mast cells will degranulate, releasing symptom-inducing mediators such as histamine. The first steps of this mechanism are mimicked in this novel-developed sequential in vitro coculture model for gut sensitization of which a schematic overview is represented. The epithelial cells are exposed to OVA and washed. The OVA-preexposed epithelial cells are cocultured with moDC for 48 h to prime the DC. Next, these OVA-IEC-primed DCs are cocultured with naïve CD4+ T cells to instruct Th cell polarization. Created with Biorender.com.

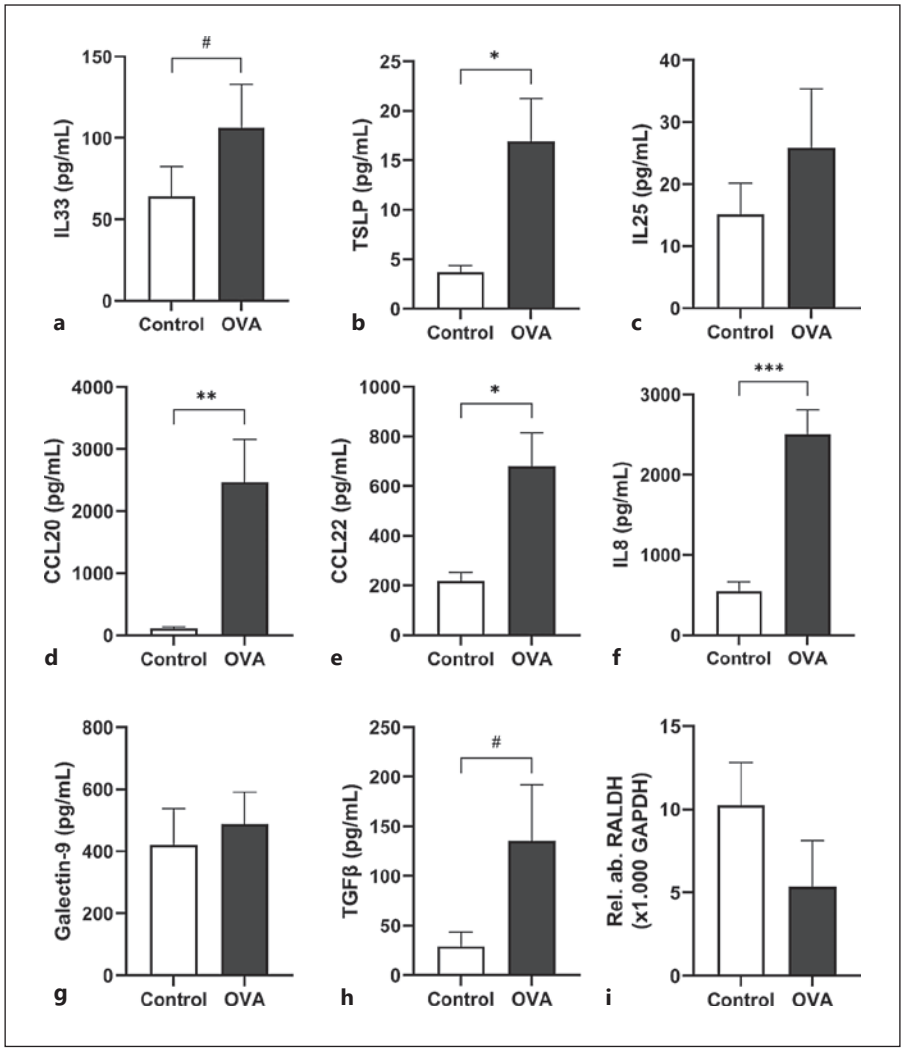

Fig. 2. Cytokine and chemokine release from IECs after apical stimulation with 100 μg/mL OVA for 24 h. IL33 ($p = 0.0929$) (**a**), TSLP (**b**), IL25 (**c**), CCL20 (**d**), CCL22 (**e**), IL8 (**f**), Galectin-9 (**g**), TGFβ ($p = 0.0844$) supernatant concentrations (**h**), and RALDH relative mRNA abundance (**i**) were measured 24 h after stimulation. Data is analyzed by paired *t* test, $n = 9$, mean ± SEM ($^{\#}p < 0.1$, $^{*}p < 0.5$, $^{**}p < 0.01$, $^{***}p < 0.001$).

(Prospec) and anti-CD3 (150 ng/mL, clone CLB-T3/2) (Sanquin) prior to coculture allowing generic activation to be further directed by the primed allogenic moDCs. During these 5 days of coculture, the medium was not refreshed. After incubation, the supernatant was collected and stored at −20°C for cytokine secretion analysis.

Enzyme-Linked Immunosorbent Assay

Supernatants collected from IEC, IEC/moDC, and moDC/T-cell cultures were analyzed for chemokine and cytokine secretion with Enzyme-Linked Immunosorbent Assays. ELISA kits were used to determine IL6, IL8, IL10, IL13, IL17, TGFβ, IFNγ (Thermo Fischer Scientific, USA), IL23, IL25, CCL22, IL12p70 (R&D Systems, USA), IL5, and IL15 (BioLegend, USA) secretion following the manufacturers' protocols. Galectin-9 was measured using an antibody pair (R&D Systems), using 0.75 μg/mL of both the affinity-purified polyclonal antibody and biotinylated affinity-purified polyclonal antibody. High-binding 96-well plates (Costar Corning, USA) were coated with cytokine-specific capture antibodies and stored at 4°C overnight. Nonspecific binding was prevented by blocking with 1% BSA in PBS for 1 h, after which the samples were diluted accordingly and added to the 96-well plates for 2 h at room temperature or overnight at 4°C. After washing, the appropriate detection antibodies were added. The plates were washed again, and wells were incubated with Streptavidin-HRP or Avidin-HRP for 30 min, and substrate solution (TMB, Thermo Fischer Scientific) was added after the final washings. The reaction was stopped by adding 1M H_2SO_4. Optical density was measured at 450 nm, with a correction at 655 nm, using a GloMax® Discover Microplate Reader (Promega, USA). The concentration was calculated from calibration curves in each plate using GloMax® software.

cDNA Synthesis and Real-Time qPCR

OVA-activated and/or 2′FL- or 3FL-preincubated HT-29 cells (IEC) were lysed in 350 μL RNA lysis buffer (provided with RNA isolation kit) and stored at −70°C until cDNA synthesis. mRNA was isolated using a RNeasy mini kit (Qiagen, Germany) following the manufacturer's protocol. Total mRNA content was measured using a NanoDrop (ND-1000 Spectrophotometer [Thermo Fisher Scientific]). The purity of mRNA samples was calculated using the

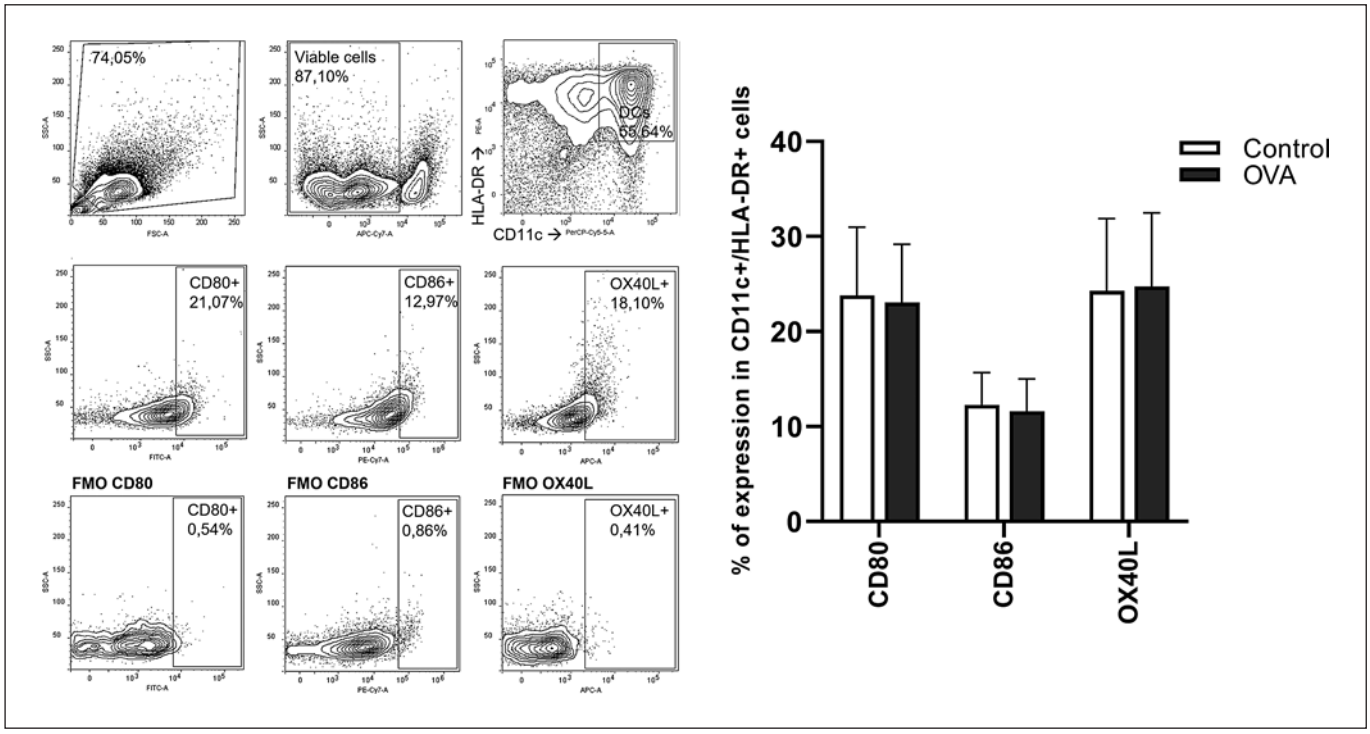

Fig. 3. Phenotype of moDCs after 48 h coculture with OVA-IECs. Percentage of surface expression of CD11c and HLA-DR was examined as a representation of a DC phenotype, within this population the percentage of expression of CD80, CD86, and OX40L was measured. Viability of the moDC was unaffected by coculture with OVA-stimulated IECs (Control = 70.7% ± 10.7, OVA = 66.9% ± 9.8) Data is analyzed by paired *t* test, $n = 6$, mean ± SEM.

260/280 nm and 260/230 nm ratios; ratios between 1.8 and 2.0 were considered high purity. cDNA was synthesized from mRNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories, USA) and a T100 Thermal Cycler (Bio-Rad Laboratories). Primers were in-house designed and manufactured by Bio-Rad Laboratories. The specificity and efficiency of primers were analyzed with dilution series of pooled cDNA samples and a temperature gradient to confirm melting curves and determine optimal annealing temperatures. Real-time PCR reactions were performed using a mastermix of Sybr green (Bio-Rad), nucleas-free water (Bio-Rad), a forward and reverse primer, and isolated cDNA in a 96-well hard-shell plate (Bio-Rad). The samples were heated at 95°C for 3 min, denatured for 10 s at 95°C, followed by 30 s of annealing at the appropriate temperature, after completion of 40 cycles, samples were heated again for 10 min at 95°C and cooled down in a CFX96 Real-Time Systems (Bio-Rad). GAPDH (Bio-Rad) was used as a reference gene. The relative mRNA expression was calculated using the formula: relative mRNA abundance = 100.000 × (2 Ct [GAPDH] – Ct [target mRNA]) [23].

Flow Cytometry

The moDCs that were collected after IEC/moDC culture were transferred to 96-well plates (Costar Corning). After washing the cells with PBS, the viability of the cells was measured with Fixable Viability Dye 780-APC Cyanine 7 (eBioscience). Blocking buffer (PBS with 2.5% FCS and human Fc block (BD Biosciences, USA) was added for 30 min at 4°C to prevent nonspecific binding of antibodies. Next, extracellular staining was performed using titrated volumes of the following antibodies: CD11c-PerCP eFluor 710 (clone 3.9), HLA-DR-PE (clone LN3), CD80-FITC (clone 2D10.4), CD86-PE Cyanine 7 (clone IT2.2), and OX40L-APC (clone RM134L). After 30 min of staining at 4°C, cells were washed, resuspended, and flow cytometric measurements were performed using BD FACS Canto II (Becton Dickinson, USA), and acquired data were analyzed using FlowLogic software (Inivai Technologies, Australia).

Statistical Analysis

Statistical analyses were performed using Graphpad Prism 9 software. Data were analyzed using paired *t* test or one-way ANOVA followed by Bonferroni's post hoc test on selected pairwise comparisons. If data did not fit a normal distribution, logarithmic transformation was applied prior to further analysis. $p < 0.05$ is considered statistically significant, and data is represented as mean ± SEM of $n = 3$, $n = 6$, or $n = 9$ independent repeats per dataset.

Results

The Allergen OVA Induces IEC Activation

The IEC activating properties of the high allergenic food protein OVA were investigated in an intestinal in

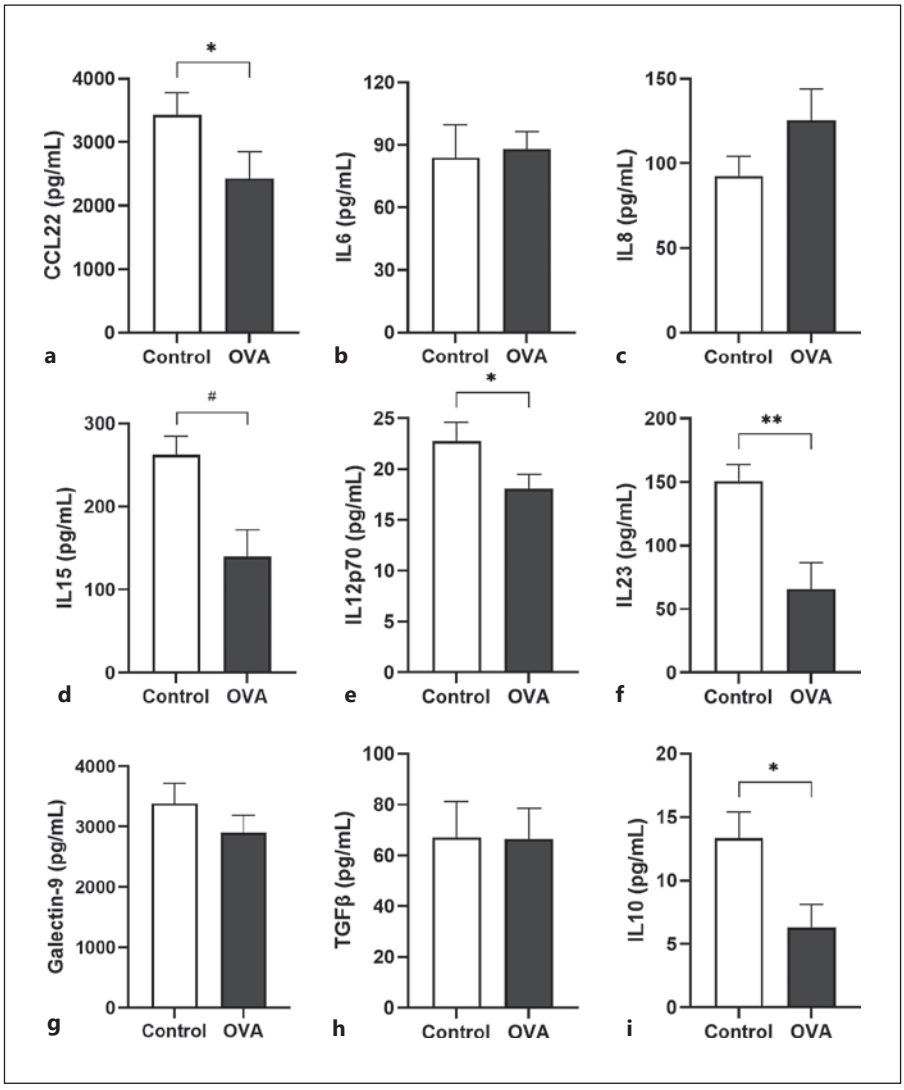

Fig. 4. Chemokine and cytokine secretion from 48 h IEC/moDC coculture after 24 h stimulation of IECs with OVA. Supernatant concentrations of CCL22 (**a**), IL6 (**b**), IL8 (**c**), IL15 ($p = 0.0707$) (**d**), IL12p70 (**e**), IL23 (**f**), Galectin-9 (**g**), TGFβ (**h**), and IL10 (**i**) were measured. Data is analyzed by paired *t* test, $n = 6$, mean ± SEM (*$p < 0.05$).

vitro setting, by subjecting IEC apically to the allergen OVA mimicking the in vivo situation (Fig. 1). After 24 h, OVA-induced IEC inflammatory response was measured in collected supernatants. OVA exposure promoted the release of several cytokines by the HT-29 cells (Fig. 2). Secretion of the alarmins IL33 and TSLP, but not IL25, were significantly increased (Fig. 2a–c). In addition, the release of chemoattractants CCL20, CCL22, and IL8 (Fig. 2d–f) were significantly elevated. Simultaneously, the secretion of immunoregulatory TGFβ (Fig. 2h) was also found to increase, which was not observed for galectin-9 (Fig. 2g) and RALDH mRNA expression (Fig. 2i). These results demonstrate that OVA is capable of inducing the release of inflammatory mediators in IEC, including alarmins known to play a role in the induction of allergic sensitization.

OVA-IECs Decrease Inflammatory Mediator Release during moDC Coculture

The HT-29/moDC transwell model represents the epithelial cell and DC crosstalk as takes place in vivo (Fig. 1). After 24 h exposure to OVA, the OVA exposed IEC (OVA-IEC) were washed, IEC containing transwell filters were placed in a new plate and moDCs were added to the basolateral compartment. In this model, the effects of the activated OVA-IEC on the crosstalk with basolateral moDCs after 48 h were studied. The frequency of CD11c+HLA-DR+ surface marker expression of OVA-IEC conditioned moDCs was not affected compared to control-IEC conditioned moDCs (Fig. 3). Percentage of cells expressing the costimulatory markers CD80, CD86, and OX40L was unaffected, as well as MFI levels of expression (data not shown). In addition, several secreted

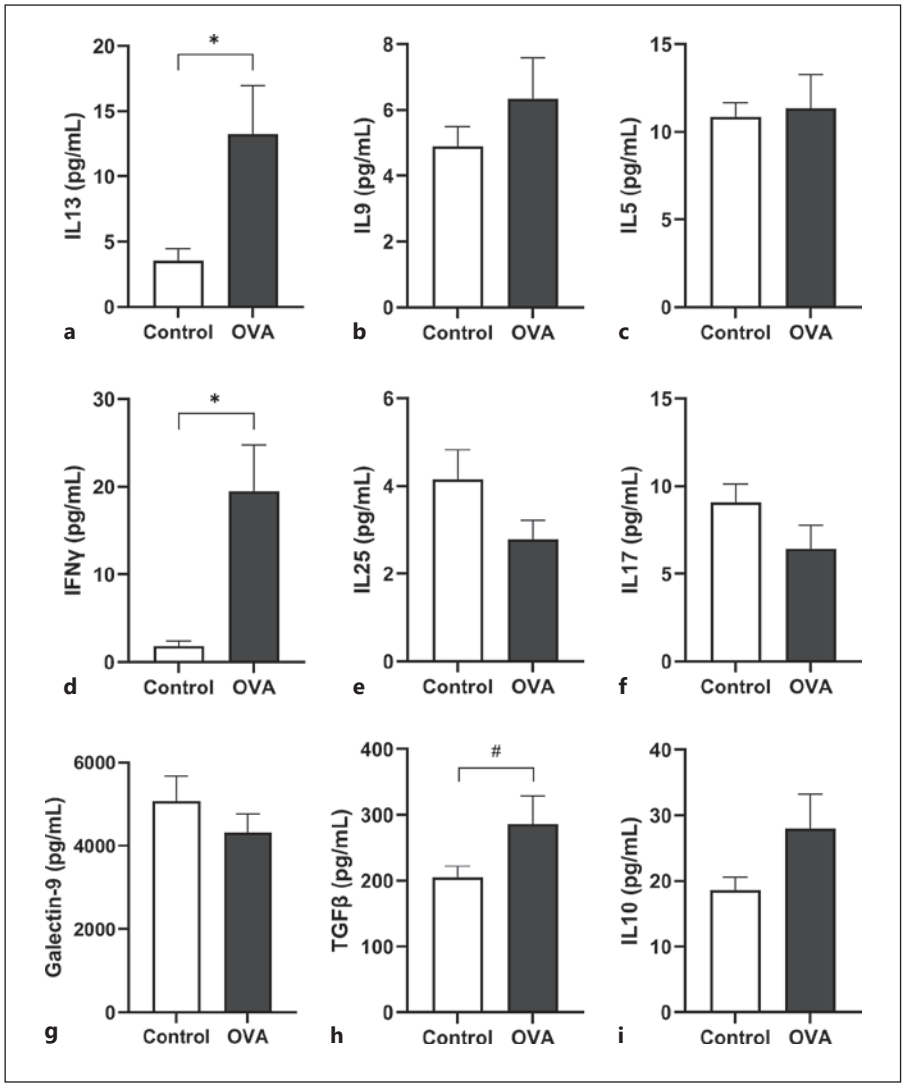

Fig. 5. Cytokine secretion from moDC/naïve T-cell coculture after priming of moDCs with OVA-stimulated IECs. Supernatant concentrations of IL13 (**a**), IL9 (**b**), IL5 (**c**), IFNγ (**d**), IL25 (**e**), IL17 (**f**), Galectin-9 (**g**), TGFβ ($p = 0.0970$) (**h**), and IL10 (**i**) were measured. Data is analyzed by paired *t* test, $n = 6$, mean ± SEM (*$p < 0.05$).

mediators were measured in the supernatant of the IEC-DC cocultures (Fig. 4). In the OVA-IEC exposed moDC cocultures a significant decrease in CCL22 (Fig. 4a), IL12p70 (Fig. 4e), IL23 (Fig. 4f), and IL10 (Fig. 4i) was observed compared to control-IEC exposed moDCs, while the levels of IL6, IL8, IL15, galectin-9, and TGF-β remained unaltered. Collectively, OVA-IEC-DCs did not show a matured phenotype since the expression of co-stimulatory molecules remained unaffected, while they secreted a smaller amount of several mediators during 48 h of incubation.

OVA-IEC-DCs Instruct IL13 and IFNγ Release in Allogenic Naïve Th Cells

To study the functionality of OVA-IEC-DCs, the primed moDCs were cocultured for 5 days with allogeneic naïve Th cells. Supernatants of the IEC-DC/T-cell coculture were collected for cytokine quantification. IL13, IL9, and IL5 were measured to reflect the development of type 2 allergic inflammation, whereas IFNγ represents type 1 inflammation, IL25 and IL17 reflect type 17 inflammation, and for a regulatory response galectin-9, TGFβ, and IL10 were measured (Fig. 5). Naïve T cells incubated with OVA-IEC-DCs secreted increased levels of IL13, and IFNγ, while TGFβ showed a similar tendency ($p = 0.0970$) compared to T cells cocultured with control-IEC-DCs (Fig. 5a, d, h). IL9, IL5, IL25, and IL17 (Fig. 5b, c, e, f) and also galectin-9 and IL10 were not affected, even though IL10 showed an inclining pattern (Fig. 5g, i). Since both type 2 and type 1 cytokines were induced and regulatory IL10 showed an increasing pattern, also the ratios were calculated to determine the immune balance. The

IL13/IL10, IFNγ/IL10, IL13/TGFβ, and IFNγ/TGFβ ratios (data not shown) were significantly higher when T cells were cocultured with OVA-IEC-DCs compared to control-IEC-DCs. These data reveal that OVA-exposed IECs affect moDC function resulting in the development of type 2 and type 1 immunity and a shift in immune balance at the expense of a regulatory response in naïve Th cells.

Exposing IECs to 2′FL or 3FL Induces Production of Regulatory Mediators from IECs, IEC-DCs, and DC/T Cells

In the developed model, the effects of HMOS 2′FL and 3FL were studied when exposed to IEC in the absence (2′FL-IEC or 3FL-IEC) or presence of OVA (2′FL-OVA-IEC or 3FL-OVA-IEC). After 48 h exposure to HMOS, with added OVA exposure in the last 24 h, the IECs were washed, IEC-containing transwell filters were placed in a new plate and moDCs were added to the basolateral compartment. Table 1 shows the effects of the primed 2′FL-IEC or 3FL-IEC in absence of OVA in the different steps of these models. After IEC-moDC coculture, moDCs were again combined with allogenic naïve Th cells for the final coculture step. Both 2′FL and 3FL did not affect the secretion of alarmins and chemoattractants but the secretion of IEC-derived TGFβ tended to increase. Upon subsequent IEC-moDC coculture, most mediators were produced similarly using 2′FL-IEC or 3FL-IEC compared to the control-IEC. However, 2′FL-IEC conditioned DCs (2′FL-IEC-DC) tended to increase TGFβ, while 3FL-IEC conditioned DCs (3FL-IEC-DC) cocultures showed decreased IL12p70 concentrations compared to control-IEC-DCs. During the subsequent IEC-moDC/T-cell coculture, again most cytokines levels were unaffected except for decreased concentrations of galectin-9 in both 2′FL-IEC-DC/T cell as well as 3FL-IEC-DC/T-cell cultures, while TGFβ was significantly increased. In addition, 3FL-IEC-DCs also increased IL10 and tended to increase IL25 secretion in the IEC-DC/T-cell coculture.

OVA-Induced IEC Activation Is Differentially Modulated by 2′FL and 3FL

Subsequently, the immunomodulatory effects of 2′FL and 3FL on OVA-IEC activation were studied. HT-29 cells were incubated for 24 h with HMOS prior to and during OVA exposure, and after 24 h inflammatory mediator release was assessed. Combined exposure of OVA and 2′FL or 3FL increased IL33 release compared to unstimulated HT-29 cells (Fig. 6a), IL33 concentrations

Table 1. Overview of cytokine and chemokine secretion (pg/mL unless indicated otherwise) or RALDH mRNA expression in the IEC-moDC-T-cell model when IECs were exposed to 2′FL and 3FL in absence of OVA

IEC	Control	2′FL	3FL	IEC/DC	Control	2′FL	3FL	DC/T cel	Control	2′FL	3FL
IL33	1.4±1.2	7.0±3.7	2.2±1.3	**CCL22 (OD-value)**	0.3±0.03	0.2±0.03	0.2±0.09	**IL13**	3.6±1.9	39.6±20.2	22.8±8.6
TSLP	8.4±2.9	11.5±2.5	9.5±4.0	**IL6**	9.2±5.2	10.7±5.2	16.0±3.9	**IL9**	4.1±2.1	10.0±7.9	8.7±4.7
IL25	20.8±6.5	20.4±6.3	19.3±6.5	**IL8**	86.3±45.4	45.0±22.4	31.1±12.0	**IL5**	10.8±1.8	14.0±2.2	21.4±8.6
CCL20	37.2±4.6	57.4±13.7	42.3±11.0	**IL15**	261.9±22.5	207.2±42.24	132.9±38.81	**IFNγ**	3.3±1.0	30.01±24.2	98.4±88.3
CCL22	159.8±28.7	191.3±34.9	180.6±29.9	**IL12p70**	47.5±3.8	47.0±5.2	7.5±1.9**	**IL25 (OD-value)**	0.33±0.05	0.37±0.1	0.69±0.1[4]
IL8	258.1±72.4	366.1±90.1	310.4±118.3	**IL23**	112.4±18.8	73.8±9.6	59.9±41.5	**IL17 (OD-value)**	0.07±0.02	0.2±0.01	0.7±0.3
Galectin-9	543.2±155.7	513.1±84.0	437.3±79.5	**Galectin-9**	3,643±203.4	3,409±278.8	3,759±592.5	**Galectin-9**	6,583±1,619	4,176±1,069*	4,601±948.2*
TGFβ	40.7±19.7	161.9±38.7[1]	165.8±44.8[2]	**TGFβ**	27.5±6.3	120.3±38.2[3]	32.3±13.0	**TGFβ**	263.7±27.0	614.1±28.2*	673.8±79.2*
RALDH (Rel. Ab.)	29.5±11.09	19.3±5.8	46.11±17.65	**IL10**	11.3±1.8	17.0±1.7	12.4±0.6	**IL10**	1.5±0.006	59.7±42.0	308.1±75.3*

Data is analyzed by one-way ANOVA and Dunnet's post hoc tests were used to analyze statistical differences, $n = 3$, mean±SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). [1] $p = 0.0943$. [2] $p = 0.0821$. [3] $p = 0.0756$. [4] $p = 0.0838$.

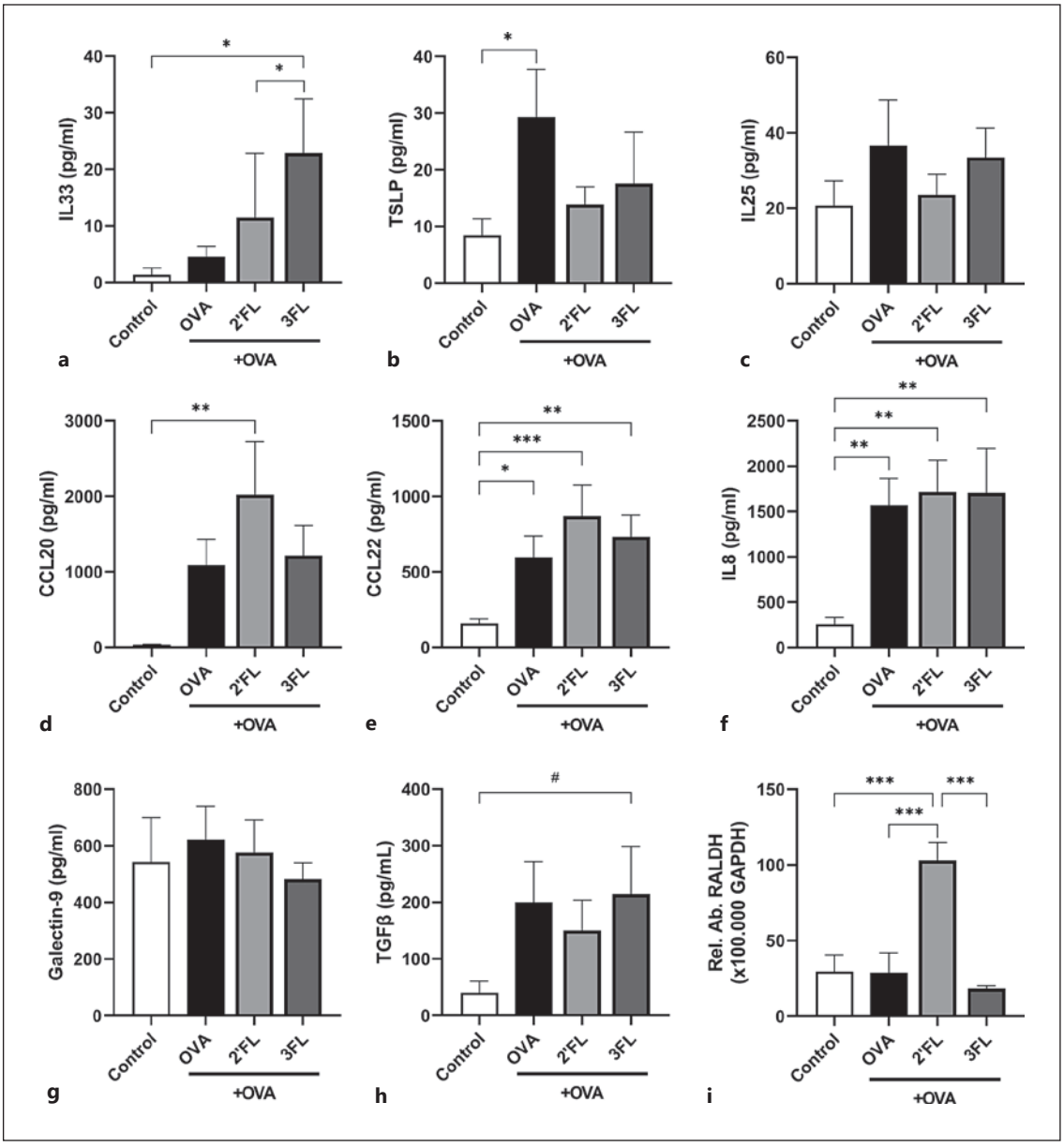

Fig. 6. Cytokine and chemokine release from IECs after 24 h apical preincubation with 2′FL or 3FL followed by apical stimulation with 100 μg/mL OVA for another 24 h. IL33 (**a**), TSLP (**b**), IL25 (**c**), CCL20 (**d**), CCL22 (**e**), IL8 (**f**), Galectin-9 (**g**), TGFβ ($p = 0.0796$) supernatant concentrations (**h**), and RALDH relative mRNA abundance (**i**) were measured 24 h after stimulation. Data is analyzed by one-way ANOVA and Bonferroni's post hoc tests, $n = 5$ or $n = 6$, mean ± SEM, log transformation was performed when data did not fit normal distribution ($^{\#}p < 0.1$, $^{*}p < 0.5$, $^{**}p < 0.01$, $^{***}p < 0.001$).

were higher in 3FL, and OVA exposed HT-29 cells supernatants compared to 2′FL-OVA-IEC supernatants. Secretion of TSLP (Fig. 6b) was significantly increased upon OVA stimulation compared to control, which is abolished by 2′FL and 3FL preincubation (Fig. 6b). Exposure to OVA and 2′FL increased secretion of CCL20 compared to control-IEC, while OVA enhanced CCL22 and IL8 secretion in the presence or absence of 2′FL or 3FL (Fig. 6d, e). 2′FL or 3FL preincubation did not change IL25 and galectin-9 levels, but exposure to OVA in the presence of 3FL tended to increase TGFβ (Fig. 6c, f, g). Interestingly, the relative mRNA expression of RALDH (Fig. 6i) was

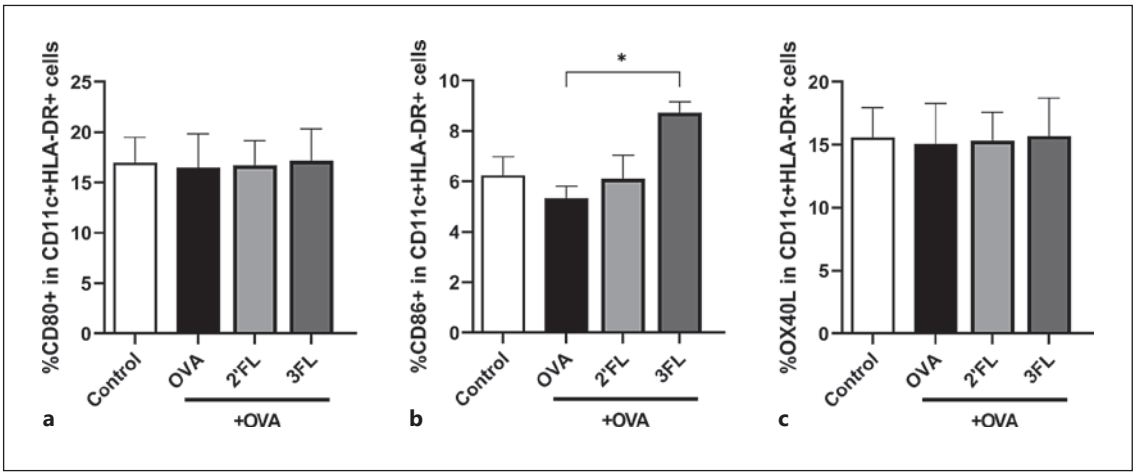

Fig. 7. a–c Percentage of surface expression of CD80, CD86, and OX40L in moDC (CD11c+/HLA-DR+ cells), cocultured with HMOS preincubated and OVA-stimulated IECs, was measured. Data is analyzed by one-way ANOVA and Bonferroni's post hoc tests, $n = 3$, mean ± SEM ($^*p < 0.5$).

only increased when HT-29 cells were exposed to 2′FL-OVA but not to OVA alone or 3FL-OVA. Also, 2′FL alone did not enhance RALDH expression (Table 1). The levels of secreted mediators indicate that 2′FL and 3FL differentially modulate the IEC response to OVA-induced inflammation.

3FL Increases CD86 Expression while Blocking Cytokine Release in OVA-IEC-DCs

Next, the effects of the HMOS on the crosstalk between OVA-primed HT-29 cells and moDCs were investigated by measuring effects on DC maturation by surface marker expression (Fig. 7) and cytokine secretion (Fig. 8). Surface expression of CD80 and OX40L (Fig. 7a, c) remained unaltered by the 2′FL and 3FL preincubation of OVA-IEC-DCs. However, an increased percentage of CD86 expressing cells was observed in the 3FL-OVA-IEC-DCs compared to the OVA-IEC-DCs, indicating enhanced DC maturation when primed with HT-29 exposed to OVA in the presence of 3FL (Fig. 7b).

In addition, CCL22 secretion (Fig. 8a) in the OVA-IEC-DC coculture was not affected by 2′FL or 3FL. However, 2′FL but not 3FL, significantly enhanced IL6 release, and 2FL-OVA-IEC-DCs showed an inclining pattern for IL15 and IL10 when compared to OVA-IEC-DCs (Fig. 8b, e, i). By contrast, 3FL-OVA-IEC-DCs had lower concentrations of IL12p70 and IL23 compared to control-OVA-IEC-DCs and/or control-IEC-DCs (Fig. 9d–f). Regulatory mediators galectin-9, TGFβ, and IL10 (Fig. 8h, i) were not significantly affected.

Hence, these results indicate that the differential modulation by 2′FL and 3FL of the OVA-induced IEC inflammatory response is mainly shown in the differences in cytokine release during subsequent IEC-moDC cocultures. 2′FL was capable of promoting IL6 release. By contrast, 3FL enhanced the expression of costimulatory marker CD86 on OVA-IEC-DC, while lowering the release of IL12p70 and IL23 in the coculture.

3FL Exposed OVA-IEC-Primed DCs Inhibit IL13 Release by T Cells, while 2′FL Boosts both Inflammatory and Regulatory Mediator Secretion

Next, the function of the 2′FL and 3FL-OVA-IEC-primed moDCs was studied using DC/T-cell cocultures to determine the effects on downstream naïve T-cell development. OVA-IEC-DCs and 2′FL-OVA-IEC-DCs increased the secretion of IL13 by naïve T cells, while this was hindered by 3FL-OVA-IEC-DC. Furthermore, 2′FL-OVA-IEC-DCs increased or tended to increase inflammatory IL9, IFNγ, and IL25 but also regulatory mediators TGFβ and IL10 secretion by T cells compared to DCs primed with control, OVA or 3FL-OVA-exposed IECs (Fig. 9a–i). Both 2′FL-OVA-IEC-DCs and 3FL-OVA-IEC-DCs increased IL17 compared to control-OVA-IEC-DCs exposed T cells. However, next to 2FL, also 3′FL enhanced regulatory IL10 secretion in parallel (Fig. 9f, i). On the other hand, galectin-9 concentrations were decreased by both 2′FL and 3FL (Fig. 9g).

These data together show that the initial differential response observed in HMOS preincubated and OVA

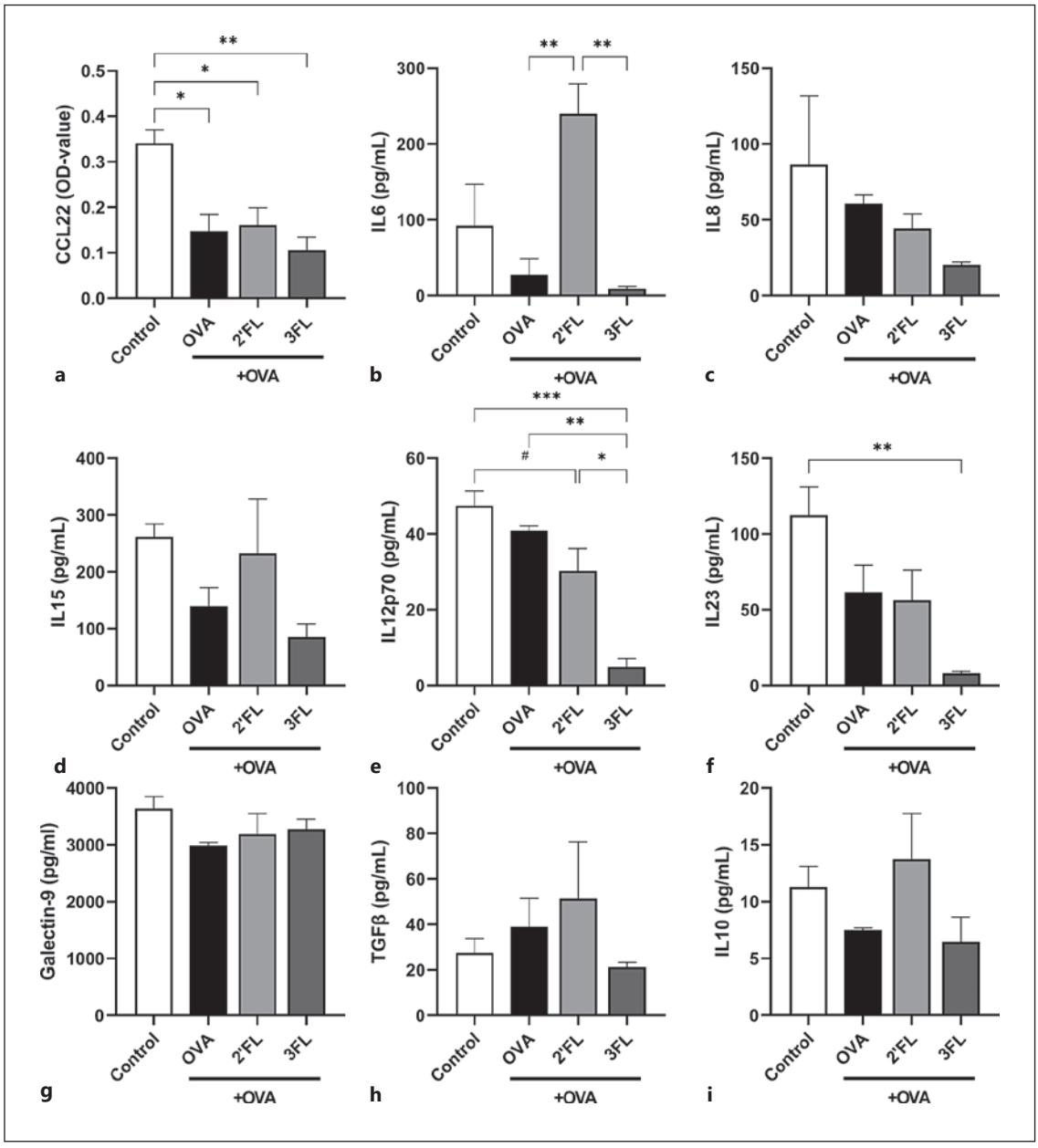

Fig. 8. Chemokine and cytokine release from IEC/moDC coculture after HMOS preincubation and OVA stimulation of IEC. Supernatant concentrations of CCL22 (**a**), IL6 (**b**), IL8 (**c**), IL15 (**d**), IL12p70 ($p = 0.0779$) (**e**), IL23 (**f**), Galectin-9 (**g**), TGFβ (**h**), and IL10 (**i**) were measured. Data is analyzed by one-way ANOVA and Bonferroni's post hoc tests, $n = 3$, mean ± SEM, log transformation was performed when data did not fit normal distribution ($^{\#}p < 0.1$, $^{*}p < 0.5$, $^{**}p < 0.01$, $^{***}p < 0.001$).

stimulated IEC, results in downstream altered T-cell responses. 2′FL-OVA-IEC priming of DCs shows a general increase in inflammatory and regulatory cytokine secretion, dominated by a regulatory IL10 component. Instead, 3FL-OVA-IEC priming of DCs prevents the secretion of type 2-related cytokines and enhances the regulatory response by increasing IL10 levels. Hence, improving the balance of both IL10 and TGFβ over effector type 2 and type 1 Th cell responses and enhancing the type 1 over type 2 response skewing away from the allergic phenotype.

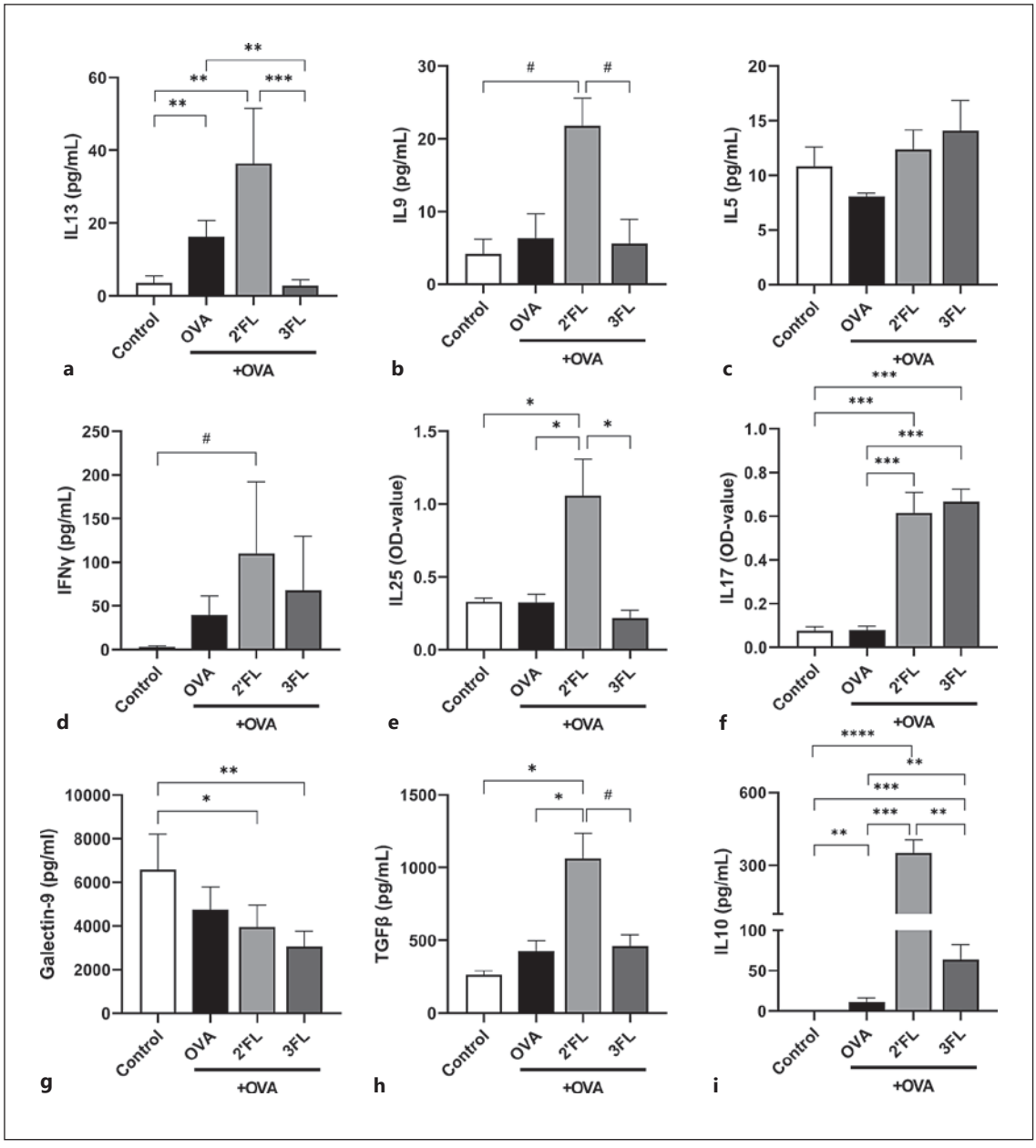

Fig. 9. Cytokine secretion from moDC/naïve T-cell coculture after priming of moDCs with HMOS preincubated and OVA-stimulated IECs. Supernatant concentrations of IL13 (**a**), IL9 ($p = 0.0664$; $p = 0.0963$) (**b**), IL5 (**c**), IFNγ ($p = 0.0630$) (**d**), IL25 (**e**), IL17 (**f**), Galectin-9 (**g**), TGFβ ($p = 0.0596$) (**h**), and IL10 (**i**) were measured. Data is analyzed by one-way ANOVA and Bonferroni's post hoc tests, $n = 3$, mean ± SEM, log transformation was performed when data did not fit normal distribution ($^{\#}p < 0.1$, $^{*}p < 0.5$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

Discussion

A rise in the incidence and prevalence of allergic disorders has been recognized, and food allergies are one of the first allergies to develop already early in life. Therefore, there is an increasing need for preventive strategies. Complex immunological human in vitro models are used to study the crosstalk between the intestinal epithelium and cells involved in mucosal immune development and regulation. Here, a novel approach is chosen in which epithelial exposure to the hallmark allergic protein OVA was shown to trigger the sequential steps known to drive

allergic sensitization (Fig. 1). The model does not aim to instruct allergen-specific T-cell responses but immune polarization based on epithelial activation induced by the food allergen. Hence, it indicates the intrinsic capacity of the allergenic protein to drive immune activation including type 2 immunity via epithelial cell activation. Such sequential human IEC-DC and DC/T-cell models can provide insight into key immunological events and potential targets for preventing allergic diseases. A recent study aimed to develop a similar model combining a murine epithelial cell line with murine DCs and T cells isolated from murine spleens [24]. Fu et al. [24] exposed the murine epithelial and the dendritic cells to OVA in a transwell system and transferred basolateral cell-free supernatant to murine T cells. Based on mRNA expression, they observed similar effects in response to OVA as we have demonstrated on mediator secretion. However, in the current manuscript we only exposed the IEC to OVA, which resulted in activation of the IEC. IECs were washed and cocultured with moDCs. Ensuring that in our model, direct effects of OVA on moDC are excluded, showing the contribution of OVA-activated IEC to sequential immune activation. Beyond the development of this OVA-induced novel human coculture model for mucosal immune activation, we also studied the effects of 2′FL or 3FL on the inflammatory effects induced by OVA on IEC and the subsequent modulation of the moDCs and allogeneic naïve T-cell response.

OVA exposure of HT-29 cells was found to activate these cells to release type 2 inflammatory mediators (Fig. 2), which are known to attract and instruct DC. An allergen from peach induces the expression of type 2 driving IL33, IL25, and TSLP by Caco-2 epithelial cells [25]; it is known that increased levels of IL33 and TSLP drive DCs to a proallergic phenotype [26, 27]. This indicates the intrinsic capacities of OVA to provoke epithelial activation, showing both a type 2 as well as a type 1 profile in mediator release. The observed increased secretion of the chemoattractants CCL20 and IL8 is known to recruit innate immune cells, including DC, to the site of inflammation [9]. Elevated levels of galectin-9, TGFβ, and RALDH are part of maintaining this mucosal homeostasis by inducing Treg formation [23, 28–30]. Upon OVA exposure, TGFβ levels were increased, while galectin-9 and RALDH levels were not affected. The increase of TGFβ alone, to compensate for the increase in inflammatory mediators, may not be sufficient to restore local homeostasis during OVA exposure. This was observed since moDCs cocultured with these OVA-primed IECs were found to drive immunity instead of tolerance at the T-cell level.

DC2 type maturation is characterized by induced expression of the surface markers OX40L, CD80, CD83, and CD86 [31, 32]. Even though OVA-IECs produced many inflammatory mediators, including type 2 driving cytokines (Fig. 2), coculture of the OVA preexposed IECs with immature moDCs did not induce phenotypic DC2 type maturation. However, the secretion of type 1 driving mediators IL15, IL12p70, type 17 driving IL23, and regulatory IL10 secretion were reduced in the OVA-IEC-DC coculture, as was also the case for type 2 driving CCL22, thus the function of these DCs was changed. Indeed, during subsequent coculture of these OVA-IEC-DCs with naïve Th cells, OVA-IEC-instructed DCs were capable of differentially inducing a distinct T-cell cytokine response.

Strikingly, during the following coculture of the OVA-IEC-primed DCs with allogenic naïve T cells showed the functional response of these DCs, as they enhanced type 2 IL13 as well as type 1 IFNγ secretion by the Th cells (Fig. 5). OVA lowered type 1, 2, 17 and regulatory type mediator production in the OVA-IEC-DC coculture setting. However, the overall balance may have contributed to the instruction of both a type 2 as well as a type 1 effector T-cell response [15, 33, 34]. The developed model omits the presence of the allergen during DC and T-cell interaction and instead makes use of generic TCR-cell activation [32] since to show an allergen-specific response it would require allergen-specific Th memory cells from hen's egg-allergic patients allowing allergen-specific autologous DC/T-cell activation [35]. Interestingly, the cellular crosstalk between OVA exposed HT-29 cells functionally affected moDCs, which instructed T cells to elicit these type 2 and type 1 immune activation of the T cells. Hence, activation of the HT-29 cells by OVA is sufficient to instruct DCs to drive immune activation at the T-cell level, which indicates a generic immunopolarizing effect. Epithelial-derived mediators are known to instruct DC development [31], indicating the relevance of the observed effects in this novel developed human in vitro model of allergic sensitization in the intestine. This underpins the relevance of using epithelial cells as the main component in driving allergy development in models that study the sensitizing capacity of proteins.

A vast amount of allergy development originates early in life, which may be redirected by specific HMOS. Therefore, the effects of HMOS 2′FL and 3FL on the OVA-induced type 2 inflammation were explored in the IEC-moDC-T-cell model. Preincubating IECs with 2′FL or 3FL for 24 h prior to OVA exposure differentially modified the OVA-induced mediator secretion (Fig. 6). 2′FL incubation resulted in an increased secretion of CCL20

and expression of RALDH while hindering the increase in TSLP, possibly enhancing downstream regulatory mechanisms. Alike 2′FL, 3FL prevented the rise in TSLP but further enhanced IL33 levels. Although IL33 is mainly known for its proinflammatory, type 2 driving role, more evidence is gathering that IL33 may also be involved in enhancing Treg formation [36] and therefore possibly in the prevention of inflammatory responses. Both 2′FL and 3FL differentially modified the OVA-induced HT-29 cell activation, which subsequently influenced the outcome of the moDC development and T-cell response.

Silencing of the costimulatory markers, like CD86, has been used as a novel therapeutic approach to dampen allergic responses [37] and a previous study demonstrated that HMOS could reduce the expression of these markers [38]. Yet, in our current study surface marker expression remained unaffected in the 2′FL-IEC-DC. However, a strong increase in IL6 release was observed by these DCs. IL6 release by DCs is known to enhance differentiation into T helper subsets while inhibiting the development and activity of Treg cells [39]. On the other hand, incubation with 3FL increased the expression of CD86 in OVA-IEC-DCs and almost completely silenced IL6, IL8, IL12p70, and IL23 secretion by DC. Perdijk et al. [40] demonstrated that incubating moDCs in the presence of TGFβ induced a tolerogenic phenotype, characterized by an increased expression of CD86 and a reduced secretion of IL6, IL8, and IL12p70. These findings correlate with our observed effects since high levels of TGFβ were released also from 3FL-OVA-IEC.

The functional consequences of IEC exposure to 2′FL and 3FL prior to OVA activation were further studied via the downstream effects of the OVA-IEC-primed DCs on naïve T-cell development. Here we showed 2′FL-OVA-IEC-DCs to induce a general immunostimulatory effect which is counterbalanced by an increase in regulatory IL10 and TGFβ release from the differentiated T cells (Fig. 9), which was also observed in the absence of OVA (Table 1). Previously, Ayechu-Muruzabal et al. [10] showed, in a dissimilar transwell coculture model, that 2′FL induces increased secretion of IFNγ and IL10 under type 1 inflammatory conditions, while no changes in IL13 were observed. Yet we observe an increase in type 2 IL13 alongside a general increase in type 9 as well as type 1, type 17, and regulatory cytokine release in the DC/T-cell coculture. Nonetheless, the 2′FL modulation in our experiments is dominated by a regulatory response over inflammatory, based on the decreased IL13/IL10, IL13/TGFβ, IFNγ/IL10, and IFNγ/TGFβ ratios (data not shown). In previous studies 2′FL, similar to nondigestible oligosaccharides, was capable of enhancing galectin-9 levels in IEC/PBMC cocultures; however, this was always in the presence of CpG-ODN [10, 41, 42]. Similar to those studies, 2′FL and 3FL do not induce galectin-9 release by IEC nor IEC/DC cultures themselves. However, in the current study, they lowered galectin-9 release in the supernatants of the DC/T cells, which may relate to the increased IL17 release since galectin-9 is known to suppress IL17 levels [23]. Thus, in contrast to the immunostimulatory effect of 2′FL, 3FL is capable of silencing IL13 secretion, while enhancing IL17 and IL10 release. Though IL17 is generally related to Th17 cells, a subset of IL17 secreting Tregs has been identified in vitro. The development of functional IL17 + Tregs is induced by IL6 but inhibited by TGFβ [43]. Unlike 2′FL, 3FL-IEC-DCs exposed T cells did not enhance TGFβ levels but strongly increased IL-10 secretion. This may indicate that 3FL suppresses the development of a type 2 response, in association with enhanced regulatory response in this sequential coculture model mimicking allergic sensitization taking place in the intestinal mucosa.

The expression of 2′FL or 3FL in breast milk depends on maternal genetic polymorphisms in fucosyltransferases 2 (*FUT2*) which has been linked to different immunological outcomes in children [44]. Although 2′FL and 3FL are structurally related, their downstream effects were clearly distinguishable in this complex in vitro model. The differential effects of 2′FL and 3FL could be explained by observed differences in binding affinity for epithelial receptors or other types of interaction with IEC. For example, 2′FL and 3FL were found to differentially modulate mucus production (*MUC2* expression) by goblet cells both during homeostasis as well as under inflammatory conditions [45]. Furthermore, 2′FL binds to galectin-1, -3, -7, and -9, while 3FL shows no affinity for these receptors [21, 46]. Future studies are needed to further decipher the mechanisms by which these two HMOS differentially affect immune polarization in this sequential intestinal sensitization model.

There is a need for complex in vitro models to study mucosal immune activation induced by allergenic proteins. Here, we aimed to emphasize the importance of crosstalk between intestinal epithelial cells and underlying DC, which drives further T-cell development at the inductive sites such as the mesenteric lymph nodes. However, the use of HT-29 cells in this model can be considered suboptimal as this is a colon adenocarcinoma cell line, which does not completely represent all functional properties of the intestinal epithelium and future studies should consider the confirmation of these findings in other epithelial cell lines and the use of primary epithelial

cultures [47–49]. Nonetheless, HT-29 transwell coculture models have previously shown their predictive significance, for example by identifying galectin-9 as an immunomodulatory biomarker in an in vitro HT-29/PBMC coculture model [10, 23], which was confirmed in murine models for food allergy and a clinical study [41, 50].

Several studies have been exploring similar possibilities to develop complex in vitro models to study mucosal immune activation. As mentioned earlier, a comparable model as presented here, using murine cells has recently been described [24]. However, most models only study the effects of allergens on barrier properties of intestinal epithelial cells [8, 51], and immune interactions in the absence of IEC using innate and/or adaptive cells from animals, human volunteers, or allergic donors [32, 35, 52–54]. Some models combining Caco-2 cells with immune cells were used to study immune activation upon allergens or oligosaccharides crossing the intestinal barrier [25, 55–57]. Although these studies are all relevant to investigating parts of the allergic mechanism, our model combines several key components in the cascade driving allergic sensitization following the kinetics of events occurring at different compartments in the intestines [7]. We demonstrated the importance of allergen-induced epithelial activation and the crucial role of epithelial cells in the crosstalk with underlying immune cells during the initial process of immune activation and allergic sensitization. This model allows future studies to focus on unraveling mucosal immune mechanisms of high and low-allergenic food-derived proteins. In this way, it can be further evaluated for its value to discriminate between low and high allergenic novel food proteins. The model can be expanded by combining the IEC-DC-instructed Th cells with autologous B cells to study IgE isotype switching and subsequent mast-cell degranulation. In addition, further refinements may include the introduction of primary epithelial cells and immune cells derived from allergic donors. Furthermore, the model may be translated into an organ-on-a-chip aiming to identify the sensitizing capacities of novel proteins and possible immunomodulatory effects of dietary components. Further development and validation of this model should demonstrate its value in reducing the use of existing in vivo models.

Conclusion

This study investigated the immunological crosstalk in a novel human in vitro coculture model for OVA-induced type 2 inflammation (Fig. 1), in which IEC activation plays a key role in type 2 and type 1 T-cell development. OVA preexposed HT-29 cells direct underlying moDC to instruct a type 2 and type 1 characterized response in T cells. In addition, 2′FL and 3FL were able to differentially modulate the epithelial response to OVA-mediated epithelial activation. 2′FL-OVA-IECs imprinted moDC instructed a general increased inflammatory and regulatory cytokine secretion upon DC/T coculture. In contrast, when exposed to 3FL-OVA-IEC, the DC suppressed the type 2 response, while enhancing the regulatory response in the DC/T-cell coculture. Suggesting that, although structurally similar, 2′FL and 3FL have different immunomodulatory properties by modifying the crosstalk between intestinal epithelial cells and dendritic cells which drive adaptive immune responses.

Statement of Ethics

No ethical approval for the use of buffy coats from voluntary anonymous healthy blood donors was required.

Conflict of Interest Statement

Authors Johan Garssen and Belinda van't Land are partly employed by Danone Nutricia Research. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author Contributions

Study design: Marit Zuurveld and Linette E.M. Willemsen. Data collection: Marit Zuurveld, Pien C.J. Kilia-an, and Sophie E.L. van Grinsven. Data analysis and interpretation: Marit Zuurveld and Linette E.M. Willemsen. Drafting of the manuscript: Marit Zuurveld. Critical review of the manuscript: Gert Folkerts, Johan Garssen, Belinda van't Land, and Linette E.M. Willemsen. All the authors listed have approved for publication.

Data Availability Statement

All data generated or analyzed during this study can be inspected upon request, and further inquiries can be directed to the corresponding authors.

References

1 EAACI.Advocacy manifesto: tackling the allergy crisis in Europe – concerted policy action needed. The European Academy of Allergy and Clinical Immunology; 2015.
2 Loh W, Tang MLK. The epidemiology of food allergy in the global context. Int J Environ Res Public Health. 2018;15(9):2043.
3 Chen J, Hu Y, Allen KJ, Ho MHK, Li H. The prevalence of food allergy in infants in Chongqing, China. Pediatr Allergy Immunol. 2011;22(4):356–60.
4 Gray CL, Levin ME, Zar HJ, Potter PC, Khumalo NP, Volkwyn L, et al. Food allergy in South African children with atopic dermatitis. Pediatr Allergy Immunol. 2014;25(6):572–9.
5 Mullins RJ, Dear KBG, Tang MLK. Time trends in Australian hospital anaphylaxis admissions in 1998–1999 to 2011–2012. J Allergy Clin Immunol. 2015;136(2):367–75.
6 De Martinis M, Sirufo MM, Viscido A, Ginaldi L. Food allergy insights: a changing landscape. Arch Immunol Ther Exp. 2020;68(2):8–15.
7 van Bilsen JHM, Sienkiewicz-Szłapka E, Lozano-Ojalvo D, Willemsen LEM, Antunes CM, Molina E, et al. Application of the adverse outcome pathway (AOP) concept to structure the available in vivo and in vitro mechanistic data for allergic sensitization to food proteins. Clin Transl Allergy. 2017;7(1):13–8.
8 Gavrovic-Jankulovic M, Willemsen LEM. Epithelial models to study food allergen-induced barrier disruption and immune activation. Drug Discov Today Dis Model. 2015;17–18:29–36.
9 Overbeek SA, Kostadinova AI, Boks MA, Hayen SM, de Jager W, van't Land B, et al. Combined exposure of activated intestinal epithelial cells to nondigestible oligosaccharides and CpG-ODN suppresses Th2-associated CCL22 release while enhancing galectin-9, TGF β, and Th1 polarization. Mediators Inflamm. 2019;2019:8456829.
10 Ayechu-Muruzabal V, Overbeek SA, Kostadinova AI, Stahl B, Garssen J, van't Land B, et al. Exposure of intestinal epithelial cells to 2′-fucosyllactose and cpg enhances galectin release and instructs dendritic cells to drive th1 and regulatory-type immune development. Biomolecules. 2020;10(5):784.
11 Dhanapala P, De Silva C, Doran T, Suphioglu C. Cracking the egg: an insight into egg hypersensitivity. Mol Immunol. 2015;66(2):375–83.
12 Mowat AMI. To respond or not to respond: a personal perspective of intestinal tolerance. Nat Rev Immunol. 2018;18(6):405–15.
13 Divekar R, Kita H. Recent advances in epithelium-derived cytokines (IL-33, IL-25, and thymic stromal lymphopoietin) and allergic inflammation. Curr Opin Allergy Clin Immunol. 2015;15(1):98–103.
14 Yang D, Han Z, Oppenheim JJ. Alarmins and immunity. Immunol Rev. 2017;280(1):41–56.
15 De Jong EC, Smits HH, Kapsenberg ML. Dendritic cell-mediated T cell polarization. Springer Semin Immunopathol. 2005;26(3):289–307.
16 Zuurveld M, van Witzenburg NP, Garssen J, Folkerts G, Stahl B, van't Land B, et al. Immunomodulation by human milk oligosaccharides: the potential role in prevention of allergic diseases. Front Immunol. 2020 May;11:801.
17 Orczyk-Pawiłowicz M, Lis-Kuberka J. The impact of dietary fucosylated oligosaccharides and glycoproteins of human milk on infant well-being. Nutrients. 2020;12(4):1105.
18 Kunz C, Meyer C, Collado MC, Geiger L, García-Mantrana I, Bertua-Ríos B, et al. Influence of gestational age, secretor, and lewis blood group status on the oligosaccharide content of human milk. J Pediatr Gastroenterol Nutr. 2017;64(5):789–98.
19 Oriol R, Le Pendu J, Mollicone R. Genetics of ABO, H, Lewis, X and related antigens. Vox Sang. 1986;51(3):161–71.
20 Moreno A, Campi C, Escovich L, Borrás SG, Racca L, Racca A, et al. Analysis of the FUT2 gene and Secretor status in patients with oral lesions. Inmunologia. 2009;28(3):131–4.
21 Shams-Ud-Doha K, Kitova EN, Kitov PI, St-Pierre Y, Klassen JS. Human milk oligosaccharide specificities of human galectins. Comparison of electrospray ionization mass spectrometry and glycan microarray screening results. Anal Chem. 2017;89(9):4914–21.
22 El-Hawiet A, Chen Y, Shams-Ud-Doha K, Kitova EN, St-Pierre Y, Klassen JS. High-throughput label- and immobilization-free screening of human milk oligosaccharides against lectins. Anal Chem. 2017;89(17):8713–22.
23 De Kivit S, Kraneveld AD, Knippels LMJ, Van Kooyk Y, Garssen J, Willemsen LEM. Intestinal epithelium-derived galectin-9 is involved in the immunomodulating effects of nondigestible oligosaccharides. J Innate Immun. 2013;5(6):625–38.
24 Fu L, Lin W, Wang C, Wang Y. Establishment of a 3-dimensional intestinal cell model to simulate the intestinal mucosal immune system for food allergy investigations. Front Immunol. 2022 Mar;13:853443.
25 Tordesillas L, Gómez-Casado C, Garrido-Arandia M, Murua-García A, Palacín A, Varela J, et al. Transport of Pru p 3 across gastrointestinal epithelium: an essential step towards the induction of food allergy? Clin Exp Allergy. 2013;43(12):1374–83.
26 Ito T, Wang YH, Duramad O, Hori T, Delespesse GJ, Watanabe N, et al. TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. J Exp Med. 2005;202(9):1213–23.
27 Rank MA, Kobayashi T, Kozaki H, Bartemes KR, Squillace DL, Kita H. IL33-activated dendritic cells induce an atypical Th2-type response. J Allergy Clin Immunol. 2009;123(5):1047–54.
28 Bono MR, Tejon G, Flores-santibañez F, Fernandez D, Rosemblatt M, Sauma D. Retinoic acid as a modulator of T cell immunity. Nutrients. 2016;8(6):E349–15.
29 Rivas MN, Chatila TA. Regulatory T cells in allergic diseases. J Allergy Clin Immunol. 2017;138(3):639–52.
30 Ko HJ, Hong SW, Verma R, Jung J, Lee M, Kim N, et al. Dietary glucose consumption promotes RALDH activity in small intestinal CD103+CD11b+ dendritic cells. Front Immunol. 2020 Aug;11:1897.
31 Gilliet M, Soumelis V, Watanabe N, Hanabuchi S, Antonenko S, De Waal-Malefyt R, et al. Human dendritic cells activated by TSLP and CD40L induce proallergic cytotoxic T cells. J Exp Med. 2003;197(8):1059–63.
32 Hoppenbrouwers T, Fogliano V, Garssen J, Pellegrini N, Willemsen LEM, Wichers HJ. Specific polyunsaturated fatty acids can modulate in vitro human moDC2s and subsequent Th2 cytokine release. Front Immunol. 2020 May;11:748.
33 Koch S, Sopel N, Finotto S. Th9 and other IL-9-producing cells in allergic asthma. Semin Immunopathol. 2017;39(1):55–68.
34 Jankovic D, Kullberg MC, Noben-Trauth N, Caspar P, Paul WE, Sher A. Single cell analysis reveals that IL-4 receptor/Stat6 signaling is not required for the in vivo or in vitro development of CD4+ lymphocytes with a Th2 cytokine profile. J Immunol. 2000;164(6):3047–55.
35 Hayen SM, Knulst AC, Garssen J, Otten HG, Willemsen LEM. Fructo-oligosaccharides modify human DC maturation and peanut-induced autologous T-cell response of allergic patients in vitro. Front Immunol. 2021;11:600125.
36 Matta BM, Lott JM, Mathews LR, Liu Q, Rosborough BR, Blazar BR, et al. IL-33 is an unconventional Alarmin that stimulates IL-2 secretion by dendritic cells to selectively expand IL-33R/ST2+ regulatory T cells. J Immunol. 2014;193(8):4010–20.
37 Suzuki M, Yokota M, Matsumoto T, Ozaki S. Synergic effects of CD40 and CD86 silencing in dendritic cells on the control of allergic diseases. Int Arch Allergy Immunol. 2018;177(2):87–96.
38 Xiao L, van De Worp WRPH, Stassen R, van Maastrigt C, Kettelarij N, Stahl B, et al. Human milk oligosaccharides promote immune tolerance via direct interactions with human dendritic cells. Eur J Immunol. 2019;49(7):1001–14.
39 Xu YD, Cheng M, Shang PP, Yang YQ. Role of IL-6 in dendritic cell functions. J Leukoc Biol. 2022 Mar;111(3):695–709.

40 Perdijk O, van Neerven RJJ, Van den Brink E, Savelkoul HFJ, Brugman S. The oligosaccharides 6'-sialyllactose, 2'-fucosyllactose or galactooligosaccharides do not directly modulate human dendritic cell differentiation or maturation. PLoS One. 2018;13(7):e0200356.
41 De Kivit S, Kostadinova AI, Kerperien J, Morgan ME, Muruzabal VA, Hofman GA, et al. Dietary, nondigestible oligosaccharides and Bifidobacterium breve M-16V suppress allergic inflammation in intestine via targeting dendritic cell maturation. J Leukoc Biol. 2017;102(1):105–15.
42 Hayen SM, Otten HG, Overbeek SA, Knulst AC, Garssen J, Willemsen LEM. Exposure of intestinal epithelial cells to short- and long-chain fructo-oligosaccharides and CpG oligodeoxynucleotides enhances peanut-specific T Helper 1 polarization. Front Immunol. 2018;9:923.
43 Beriou G, Costantino CM, Ashley CW, Yang L, Kuchroo VK, Baecher-Allan C, et al. IL-17-producing human peripheral regulatory T cells retain suppressive function. Blood. 2009;113(18):4240–9.
44 Boix-Amorós A, Collado MC, van't Land B, Calvert A, Le Doare K, Garssen J, et al. Reviewing the evidence on breast milk composition and immunological outcomes. Nutr Rev. 2019;77(8):541–56.
45 Cheng L, Kong C, Walvoort MTC, Faas MM, de Vos P. Human milk oligosaccharides differently modulate goblet cells under homeostatic, proinflammatory conditions and ER stress. Mol Nutr Food Res. 2020;64(5):e1900976.
46 Hirabayashi J, Hashidate T, Arata Y, Nishi N, Nakamura T, Hirashima M, et al. Oligosaccharide specificity of galectins: a search by frontal affinity chromatography. Biochim Biophys Acta. 2002;1572(2–3):232–54.
47 Chantret I, Barbat A, Dussaulx E, Brattain MG, Zweibaum A. Epithelial polarity, villin expression, and enterocytic differentiation of cultured human colon carcinoma cells: a survey of twenty cell lines. Cancer Res. 1988;48(7):1936–42.
48 Lesuffleur T, Violette S, Vasile-Pandrea I, Dussaulx E, Barbat A, Muleris M, et al. Resistance to high concentrations of methotrexate and 5-fluorouracil of differentiated HT-29 colon-cancer cells is restricted to cells of enterocytic phenotype. Int J Cancer. 1998;76(3):383–92.
49 de Kivit S, van Hoffen E, Korthagen N, Garssen J, Willemsen LEM. Apical TLR ligation of intestinal epithelial cells drives a Th1-polarized regulatory or inflammatory type effector response in vitro. Immunobiology. 2011;216(4):518–27.
50 de Kivit S, Saeland E, Kraneveld AD, van de Kant HJG, Schouten B, Van Esch BCAM, et al. Galectin-9 induced by dietary synbiotics is involved in suppression of allergic symptoms in mice and humans. Allergy. 2012;67(3):343–52.
51 Smits M, Nooijen I, Redegeld F, Jong A, Le TM, Knulst A, et al. Digestion and transport across the intestinal epithelium affects the allergenicity of Ara h 1 and 3 but not of Ara h 2 and 6. Mol Nutr Food Res. 2021;65(6):e2000712.
52 Gómez E, Díaz-Perales A, Tordesillas L, Doña I, Torres MJ, Blázquez AB, et al. Effect of Pru p 3 on dendritic cell maturation and T-lymphocyte proliferation in peach allergic patients. Ann Allergy Asthma Immunol. 2012;109(1):52–8.
53 Eiwegger T, Stahl B, Haidl P, Schmitt J, Boehm G, Dehlink E, et al. Prebiotic oligosaccharides: In vitro evidence for gastrointestinal epithelial transfer and immunomodulatory properties. Pediatr Allergy Immunol. 2010;21(8):1179–88.
54 Ziegler A, Olzhausen J, Hamza E, Stojiljkovic A, Stoffel MH, Garbani M, et al. An allergen-fused dendritic cell-binding peptide enhances in vitro proliferation of equine T-cells and cytokine production. Vet Immunol Immunopathol. 2022;243:110351.
55 Perusko M, van Roest M, Stanic-Vucinic D, Simons PJ, Pieters RHH, Cirkovic Velickovic T, et al. Glycation of the major milk allergen β-Lactoglobulin changes its allergenicity by alterations in cellular uptake and degradation. Mol Nutr Food Res. 2018;62(17):e1800341.
56 Lee JB. Regulation of IgE-mediated food allergy by IL-9 producing mucosal mast cells and type 2 innate lymphoid cells. Immune Netw. 2016;16(4):211–8.
57 Martos G, Lopex-Exposito I, Bencharitiwong R, Berin C, Nowak-Wegrzyn A. Mechanisms underlying differential food-allergic response to heated egg. J Allergy Clin Immunol. 2011;127(4):990–7.e1–2.