Immunomodulation by specific human milk oligosaccharides and the relevance in allergy management

Towards advanced human *in vitro* mucosal immune models

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en de relevantie bij allergische ziektes

(*met een samenvatting in het Nederlands*)

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Contents

CHAPTER 1

General Introduction

Early life mucosal immune development

Early life is an essential period for the maturation of the immune system. The intestinal epithelium, innate and adaptive immune cells need to learn to interact efficiently in response to environmental triggers. Simultaneously, microbial colonization occurs after birth as well, impacting early life immune development. Exposure to pathogenic and commensal microorganisms promotes and influences adequate immune education [1]–[4]. Flawed early life immune maturation predisposes for immunological abnormalities later in life, including autoimmunity and allergic diseases [5].

Although lymphoid and myeloid cell development occurs prenatally, maturation of immune cell functioning and proper subset development takes place early in life [6]. After birth, the immune system is T helper (Th)2 skewed as a result from fetal tolerance [7], which prevents an immunological response against maternal antigens [8]. In addition, antigen presentation and antibody production functions are suboptimal, and the epithelial barriers are not fully formed yet [9]–[12]. During infancy, these barriers undergo structural and functional alterations to prevent transport of potentially harmful particles or pathogens over these barriers [9]. Underlying neonatal dendritic cells (DCs) are functionally inferior compared to adult DCs, due to a lower expression of major histocompatibility complex class II (MHCII) and costimulatory molecules such as CD80 and CD86 [12]. Furthermore, a limited production of type 1 driving cytokines by DCs in the first weeks of life may contribute to a lasting Th2 bias [13]. Interestingly, neonatal T cells are distinct from adult T cells in their enhanced capacity to perform regulatory and innate functions rather than canonical adaptive T cell responses [7]. These apparent differences in T cell responses translate into weakened early life B cell responses [14]. Neonatal B cell responses are further compromised by less efficient interactions with neonatal T cells due to their lower expression of costimulatory receptors [15]. Even though antibody production starts within 9 weeks after conception, lower rates of isotype switching and affinity maturation remain observable during the first years of life [11], [14], [16]. Without adequate immunogenic stimulation aimed to strengthen the development of regulatory and type 1 responses via exposure to e.g. bacterial components, these neonatal characteristics, in combination with the ongoing maturation of the microbiome and gut, places the infant at risk for the development of allergic sensitization and potentially other immune related disorders [1].

Immunological mechanisms underlying food allergy and allergic asthma

A clear link between early life microbial exposure and later allergy development has been suggested in multiple hypotheses, such as the 'hygiene hypothesis' and 'old friends hypothesis' [17], [18]. Parallel to increases in hygiene in Western societies, indeed significant increases in allergic patients have been reported, which is less or only later observed in less developed regions [19]. Next to decreased microbial exposure due to improved hygiene standards, many other environmental factors, such as air pollution and dietary changes, are involved in the development of allergic sensitization [20], [21]. These modern lifestyle changes are becoming globally present nowadays, therefore the differences in increased allergy prevalence between developed and less developed regions are becoming smaller. Currently, up to 10% of children in Western societies suffer from

food allergic diseases and/or allergic asthma [22]. This disturbingly high and increasing number of allergic individuals urges the need to improve the current understanding of the mechanisms involved in allergic sensitization and drive the development of preventive interventions. Allergic diseases display symptoms at different mucosal sites, but share a rather similar underlying immunological mechanism; a combined overview of the food allergic and allergic asthma immunological process will be described here.

Epithelial cells in the skin, gut and lungs are the first to encounter allergens derived from foods, medication or the environment. Allergens have the intrinsic capacity to stimulate or injure epithelial cells, promoting the secretion of the alarmins thymic stromal lymphopoietin (TSLP), interleukin (IL)25 and IL33 by these epithelial cells [23], [24]. Paracellular or transcellular transport of the allergen across the epithelial barrier or direct sampling by mucosal DCs allows allergen uptake by these DCs [25]. In response to specific cues from the local milieu, such as alarmins, DCs upregulate the presence of costimulatory molecules on their surface and enhance the production of certain cytokines [24], [26]. These, now matured, DCs are able to migrate to nearby lymph nodes in order to present their consumed allergen via their MHCII to naïve T helper cells (CD4+). During allergic sensitization, so in the presence of type 2 driving signals, the naïve T helper cells will develop into Th2 cells. These Th2 cells produce cytokines such as IL4 and IL13, which further enhance Th2 polarization, while instructing allergen specific B cells to undergo isotype switching and produce IgE antibodies [27]. Production and secretion of IgE is an important hallmark to allergic sensitization. Binding of allergen specific IgE to high-affinity Fcε receptors on mast cells can result in crosslinking during a subsequent allergen encounter. This crosslinking promotes the release of pre-stored granules containing proinflammatory and allergic symptom inducing mediators like histamine. Simultaneously, newly synthesized proinflammatory cytokines are released further strengthening the allergic immunological effector response and the pathophysiological characteristics even at other mucosal sites [28].

Research tools: *in vitro* models for allergy research

The increasing number of individuals suffering from allergic diseases places a major burden on the quality of patients' lives and national health systems. Therefore, there is an urging need for further molecular and cellular understanding of the mechanisms involved in allergic sensitization as well as strategies to prevent allergic sensitization. Advanced human *in vitro* models can be helpful tools to gain insight into these mechanisms, while reducing the need of animal models (figure 1). In addition, such models can be used to investigate potential preventive or treatment strategies to combat allergic diseases. Currently multiple *in vitro* models are available to aid in the diagnosis of an allergic condition [29], [30]. To study the process of allergic sensitization, often preclinical animal models are used [31], [32] or *in vitro* models using only one or two different cell types [33], [34]. Yet, allergic sensitization is the consequence of a complex interplay between multiple immune cells as described above. Furthermore, the use of animal models provides an incredible amount of valuable information but may lack some translational value due to e.g. interspecies and housing differences [35]–[37].

Previously, *in vitro* models of intestinal and airway epithelium have been studied in response to allergen exposure [38]–[40]. Although these models lack crosstalk with underlying mucosal

immune cells, they do provide useful information on epithelial responses to allergens potentially initiating allergic sensitization. More complex models have been developed, making use of both immune cells as well as epithelial cells to study the crosstalk between epithelium and immune cells [41]–[43]. Furthermore, studies have been performed using allergic patient samples [43], [44], focusing more on the effector response in allergic diseases rather than the intrinsic capacities of proteins to induce allergic sensitization. In this thesis the development of advanced human *in vitro* models is realized, which are relevant to study the development of allergic sensitization, creating a platform to investigate the sensitizing capacity of novel proteins but also potential allergy preventive strategies.

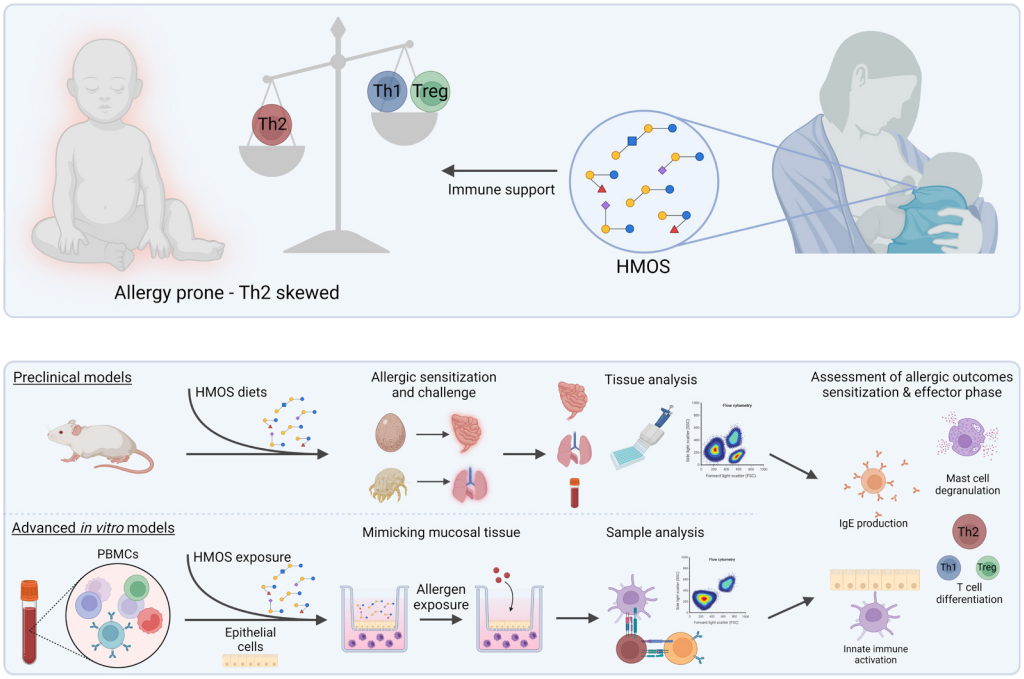

Figure 1. *Due to early life Th2 skewing of the immune system, infants are more prone to develop allergic diseases. HMOS present in human milk are known to possess immunomodulatory properties, suggested to support proper immune maturation in infants and therefore potentially contribute to preventing the onset of allergic sensitization. Using preclinical models and via developing advanced human in vitro models, it was aimed to study the potential allergy preventive effects of specific HMOS. Created with BioRender.com*

Potential dietary approaches for allergy prevention: the human milk oligosaccharides

Early life development of epithelial and mucosal homeostasis has gained interest as potential target in the prevention of allergic sensitization. The development of this homeostasis in infants is supported by breastfeeding [45], [46]. Already almost a century ago, the first association between breastfeeding and allergic outcomes was published [47]. Human milk, as golden standard of early life nutrition, contains many biological active components, which may contribute to these allergy preventive effects [48]. This thesis focusses on the impact of a unique group of human milk components: the non-digestible human milk oligosaccharides. Human milk oligosaccharides (HMOS) are the third most abundantly present solid component in human milk. HMOS concentrations are ranging from 20 to 25 g/L in colostrum and 5 to 15 g/L in mature milk. Partially because of their chemical structure and the high concentrations in which they are detected in human milk, biological functions are to be expected. Specific HMOS are involved in intestinal microbiota development, promoting intestinal barrier integrity, preventing bacterial infections, supporting immune, brain and cognitive functions [49]–[51]. Therefore, potential allergy preventive effects can be expected as well, either via promoting microbiome development or via their direct effect on structural cells and immune cells. However, linking specific biological functions to individual HMOS from whole human milk has been challenging. This is not only due to the more than 200 HMOS structures identified [52]–[54], but also because of the great diversity in structures found in human milk from different mothers caused by variations in e.g. genetic background, stage of lactation and environmental factors including the mother's diet [55], [56].

Although the basis of the HMOS structure is rather straightforward (further explained in Chapter 2), this uncomplicated blueprint results in the capacity to produce a vast amount of different HMOS structures. More than 200 individual structures have been characterized, with great variety in size and presence of functional fucose and sialic acid groups [52]–[54]. HMOS are indigestible for the infant, as they are resistant to digestive enzymes and gastric pH. Reaching the intestines completely intact, HMOS have minimal nutritional value [50], [51], [54]. However, they can be fermented by the intestinal microbiota into short chain fatty acids (SCFA) and a small fraction becomes systemically available [57]–[60]. The quantity and distribution of HMOS varies per individual and over the course of lactation [61]. Variations in fucosylated HMOS between women due to genetic differences depend on secretor and Lewis status [62]. The secretor and Lewis genes encode for the fucosyltransferases FUT2 and FUT3 respectively. Polymorphisms in these genes result in four distinguishable HMOS profiles. Secretor mothers (active FUT2 expression) produce milk containing relatively high levels of HMOS in general but especially 2'-fucosyllactose (2'FL) and lacto-N-fucopentaose I (LNFP I). Non-secretor's milk (inactive FUT2 expression) contains higher concentrations of 3-fucosyllactose (3FL) and lacto-N-fucopentaose I (LNFP II). More complexity to variations in HMOS is added by the Lewis blood group system. Lewis (a+b-) lactating mothers produce milk high in LNFP II and lacto-N-difucohexaose II (LNFD II), while Lewis (a-b+) milk contains LNFP I and lacto-N-difucohexaose I (LNDFH I), which are absent in milk from Lewis-negative individuals [52], [62]. The differences observed in HMOS composition between individuals are mainly explained by the variation in production of fucosylated HMOS

and may be of clinical importance [63]. However, geographical and seasonal factors have demonstrated to play a role in profiles of sialylated HMOS such as 3'-sialyllactose (3'SL) and 6'-sialyllactose (6'SL) [64]. Furthermore, mode of delivery, maternal BMI, ethnicity, maternal diet and gestational age may as well influence HMOS composition [63]. Differences over the course of lactation within individuals have been noticed. In general, HMOS levels in human milk decrease over time [61]. Focusing on specific HMOS, levels of 2'FL, LNFP I and difucosyllactose (DFL) decrease over the lactation period, while 3FL concentrations were found to increase over time [61], [65]. The large variety in HMOS concentrations and contents between mothers and within a mother, complicates observations of specific structure-function relationships in the total mixture of HMOS. In recent years, enzymatically and bacterial produced simple HMOS structures with high purity have become available [66]–[71]. These engineered HMOS, together with HMOS purified from human milk, enables research that focusses on structure-function relationship of HMOS and their immunomodulatory effects.

General aim

Considering the increasing prevalence and incidence of allergic diseases, this thesis aims to investigate the immunomodulatory effects of specific HMOS using several *in vitro* and *in vivo* approaches (summarized in Figure 1). Novel *in vitro* mucosal immune models were developed to study allergic sensitization, for both food derived as well as air-borne allergens. These advanced models provide a method to explore potential allergy preventive strategies. This thesis specifically focusses on the HMOS abundantly present in human milk and study their potential role in allergy development and possible preventive strategies. Studies were performed to elucidate the effects of single HMOS in the context of preventing allergic sensitization, contributing to the development and understanding of dietary strategies that promote the establishment of early life mucosal immune homeostasis. Furthermore, the findings presented in this thesis support the development and validation of complex *in vitro* models to study mucosal immunity during or in relation to allergic sensitization via either the gut or the lungs.

Thesis outline

Several pathways via which HMOS can exert their immunomodulatory effects and potential role in the prevention of allergic diseases are presented in **Chapter 2**. Besides the prebiotic functions of HMOS, also direct interactions between HMOS and epithelial as well as immune cells are described.

This direct interaction of HMOS with intestinal epithelial cells (IEC) and/or immune cells is studied in **Chapter 3**. The immunomodulatory effects of five HMOS were investigated in three different *in vitro* models, aimed to study the effects of HMOS in immune mediated epithelial barrier disruption and their immunomodulatory effect when added directly to activated peripheral blood mononuclear cells (PBMC). Furthermore, a coculture model was used enabling to study the crosstalk between IEC and immune cells, a model mimicking interactions that take place the intestinal mucosa. In this *in vitro* transwell coculture model of IEC with PBMC the effects of 2'FL and 3FL were studied in the presence of CpG oligodeoxynucleotides, a TLR9 ligand

mimicking bacterial derived single stranded DNA as common component of the microbiome. Finally, the contribution of IEC-derived regulatory mediators in response to 2'FL and 3FL exposure was examined.

A lack in validated complex *in vitro* models mimicking the human mucosal immune system to study sensitizing allergenicity risk of food proteins is identified. Therefore, intrinsic properties of tropomyosins with low (chicken) and high (shrimp) sensitizing capacity to disrupt and activate the intestinal epithelial barrier were studied in **Chapter 4**. Additionally, sequential *in vitro* models combining epithelial cells and innate and adaptive human blood derived immune cells were used to study the mucosal type 2 activation after exposure to these tropomyosins.

Continuing the development of advanced human *in vitro* models of the mucosal immune system, **Chapter 5** introduced a series of cocultures using epithelial and immune cells, representing key immunological events after allergen exposure, using hen's egg derived ovalbumin as model allergen. This series of cocultures included all relevant stages from epithelial cells up to mast cell degranulation.

To gain further insight in the immunomodulatory effects of the two main fucosylated HMOS 2'FL and 3FL in the context of allergy prevention, in this model for ovalbumin induced mucosal type 2 activation, epithelial cells were exposed to 2'FL and 3FL prior to ovalbumin stimulation, washed and exposed to monocyte derived dendritic cells (moDC) in **Chapter 6**. Subsequent immunological outcomes were studied using coculture of the IEC primed moDC and naïve T cells. Based on these findings and the fact that fermentation of HMOS would render SCFA production upon *in vivo* fermentation, the *in vitro* interaction between butyrate and 2'FL or 3FL was explored in **Chapter 7** in the same model. Furthermore, the *in vitro* effects observed after 2'FL or 3FL exposure were studied in a preclinical model for hen's egg allergy.

Next, an *in vitro* bronchial mucosal immune model, enabling to study the type 2 activating effects of aeroallergen house dust mite (HDM) exposure, was introduced in **Chapter 8**. As HMOS become systemically available in small quantities [58]–[60], the direct immunomodulatory effects of 2'FL and 3FL were studied in this *in vitro* model and tested via dietary intervention of 2'FL and 3FL in a preclinical *in vivo* model of HDM-induced acute allergic asthma.

Chapter 9 describes the effects of dietary intervention with two common and structurally similar sialylated HMOS, 3'SL and 6'SL, in a preclinical model of HDM-induced acute allergic asthma.

This thesis provides building blocks, novel insights and intervention options in order to understand and build preventive strategies for allergy development early in life, which is discussed in **Chapter 10**.

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CHAPTER 2

Immunomodulation by human milk oligosaccharides: the potential role in prevention of allergic diseases

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Abstract

The prevalence and incidence of allergic diseases is rising and these diseases have become the most common chronic diseases during childhood in Westernized countries. Early life forms a critical window predisposing for health or disease. Therefore, this can also be a window of opportunity for allergy prevention. Postnatally the gut needs to mature, and the microbiome is built which further drives the training of infant's immune system. Immunomodulatory components in breastmilk protect the infant in this crucial period by; providing nutrients that contain substrates for the microbiome, supporting intestinal barrier function, protecting against pathogenic infections, enhancing immune development and facilitating immune tolerance. The presence of a diverse human milk oligosaccharide (HMOS) mixture, containing several types of functional groups, points to engagement in several mechanisms related to immune and microbiome maturation in the infant's gastrointestinal tract. In recent years, several pathways impacted by HMOS have been elucidated, including their capacity to; fortify the microbiome composition, enhance production of short chain fatty acids, bind directly to pathogens and interact directly with the intestinal epithelium and immune cells. The exact mechanisms underlying the immune protective effects have not been fully elucidated yet. We hypothesize that HMOS may be involved in and can be utilized to provide protection from developing allergic diseases at a young age. In this review, we highlight several pathways involved in the immunomodulatory effects of HMOS and the potential role in prevention of allergic diseases. Recent studies have proposed possible mechanisms through which HMOS may contribute, either directly or indirectly, via microbiome modification, to induce oral tolerance. Future research should focus on the identification of specific pathways by which individual HMOS structures exert protective actions and thereby contribute to the capacity of the authentic HMOS mixture in early life allergy prevention.

Keywords: allergic diseases, early life nutrition, human milk oligosaccharides, mucosal immunity

Introduction

Human milk is unique in its composition as it covers all nutritional and physiological infant requirements during the first months of life [1]. Therefore, investigating the biological activity of components derived from human breast milk is an area of great interest, in order to identify specific components that support proper immune development in the infant when breastfeeding is not possible. The first indications of a link between breastfeeding and allergy outcome later in life has been published almost a century ago [2]. Since then, numerous studies have been conducted to substantiate this suspected link [3]–[8]. Breastmilk is the gold standard in early life nutrition, because of its large range of bio-active protective nutrients essential for healthy development of the microbiome and gastro-intestinal and immune maturation. However, it can also transfer allergens which may cause allergic reactions in atopic or allergic infants. Therefore the conflicting data presented by these studies demonstrate the importance of studies further evaluating the biological activities of specific constituents found in human milk [9], such as human milk oligosaccharides (HMOS).

HMOS are the third most abundant component of human breast milk after lactose and lipids. The concentration of total HMOS in human breast milk ranges from 5-15g/L, depending on the stage of lactation and genetic background of the mother [10], [11]. More than two hundred structurally different forms of HMOS have been identified [12]–[14]. Different structural and functional groups of HMOS have been related to various effects on several aspects of the immune system [15]–[19], highlighting the need for a diverse mixture of oligosaccharides in neonatal nutrition for optimal immune development.

Maturation of the immune system in the gastrointestinal tract is linked to proper systemic immunity and the establishment of effective oral tolerance for harmless food proteins and commensal bacteria of the host microbiome [20]. As microbial colonization coincides with a rapidly maturing immune system in infants, microbial dysbiosis may therefore disturb development of the gastro-intestinal tract and immune system [21]. Microbial dysbiosis and immature immune responses are thought to play a crucial role in e.g. necrotic enterocolitis (NEC), a disease characterized by inflammation and necrosis of the intestines affecting especially premature infants [22], whose immune system is not yet fully developed. Pathologies such as NEC and allergic diseases share common ground, as both have been linked to impaired microbial colonization and improper immune maturation.

One of the specific contributions of HMOS in human milk is its prebiotic capacity. Modulation of the infant's microbiome composition into a bifidogenic profile has been shown to have beneficial effects on infant health. Therefore, prebiotics, such as galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS), have shown several beneficial immune and microbiome developments in infants [23]–[25]. The specific combination of 90% short-chain (sc)GOS with 10% long-chain (lc)FOS resemble the molecular size distribution of the neutral HMOS fraction found in human milk [26]. Prebiotic supplementation with scGOS and lcFOS reduces the incidence of allergy development [26]–[31]. Murine models for both food allergy and house dust mite induced allergic asthma demonstrated the preventive effects of non-digestible oligosaccharides [29], [30]. Moreover, scGOS/lcFOS supplemented infant formula in neonates decreased the prevalence of atopic dermatitis and other allergic manifestations [26]–[28].

Currently, only a small number of *in vivo* studies have investigated immunomodulatory properties and immune development capacities of HMOS. Thus, there are a limited amount of studies that attribute immune development properties to HMOS and individual HMOS structures. Several studies describing immunomodulatory effects of scGOS and lcFOS have been included in this review as they may serve as a framework in which future research could focus on elucidating how immune related mechanisms may be affected by HMOS. In addition, almost no clinical trials have investigated the effects of HMOS supplementation, although the association between the presence of specific HMOS biologically available in human milk and the prevalence of infectious diseases [32]–[34] or allergic diseases [35]–[37] has been indicated. The possible biological functions of HMOS gain support from studies that show a potential protective effect of prebiotic administration in *in vitro* models, animal models and human studies against development of asthma or allergy [28], [35], [38], [39]. Most of the HMOS are not digested in the upper part of the gastrointestinal tract, but are fermented by local microbiota [40]. A large proportion of HMOS will reach the colon intact [40], where they can serve as prebiotics for the colonic microbiota of the infant. Although a large portion of HMOS is metabolized by gut microbiota, some cross the intestinal (sub)mucosa and enter systemic circulation [13], [41], [42], thereby potentially modulating systemic immune functions. This means that HMOS may influence immunity and potentially not only the intestinal microbiome but also the microbiome composition in the lungs, providing a possible explanation for the observation that breastfed infants are less likely to develop asthma during childhood [43]. In addition, reduced occurrence (up to 50% reduction) of atopic dermatitis, asthma, recurrent wheeze and food allergy in infants supplemented with prebiotics in early life has been observed [27], [28], [44]–[46]. Despite these observations, little is known regarding the systemic distribution of HMOS in the infant, and how it may influence processes outside the gastrointestinal tract.

The complexity and abundance of oligosaccharides in human milk is unique amongst mammals [47]. HMOS play an essential role in the postnatal growth and development of the mucosal immune system. HMOS are made up of monosaccharide units such as glucose (Glc), galactose (Gal), fucose (Fuc), N-acetylglucosamine (GlcNAc), and sialic acid with N-acetylneuramic acid (Neu5Ac). HMOS synthesis follows a distinct pattern of formation. Each structure has a Gal-Glc unit at the reducing terminus, also known as a lactose unit, containing a β1-4 glycosidic linkage. Elongation of lactose can occur by addition of Gal-GlcNAc units via a β1-3 or β1-6 glycosidic bond to form the linear or branched core structures (see Figure 1). The HMOS core structure can be further modified through the addition of Fuc or Neu5Ac residues [48].

The unique diversity of HMOS also includes galactosyllactoses, with structures based on the elongation of lactose and further galactose residues [49], [50]. These types of linkages are indigestible, but fermentable by specific bacteria; leading to the large number of approximately 200 distinct structures identified to date. Decoration of the core structure with sialic acid, results in an acidic structure, whereas all other HMOS, including those containing fucose groups, are considered neutral. The composition of HMOS produced by a mother is determined by genetic polymorphisms in genes encoding fucosyltransferases FUT2 (Secretor (Se) gene) and FUT3 (Lewis (Le) gene). Both genes are polymorphic, the individual expression of these genes are accountable for variable enzyme activity and corresponding variation in HMOS profiles in breast milk [11]. Recent data has even indicated that these genetic polymorphisms in mothers, impact

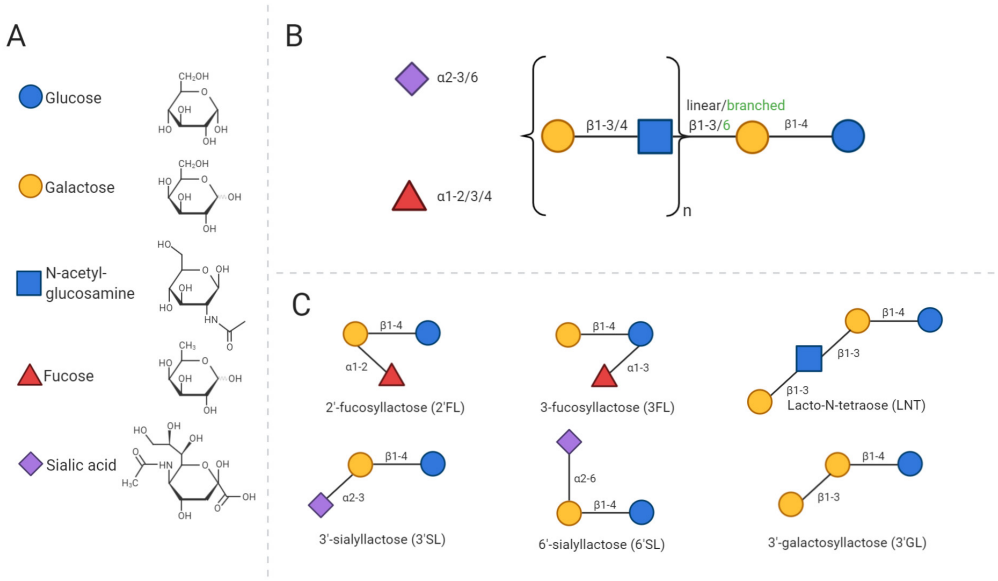

Figure 1. *General composition of human milk oligosaccharides and synthetic analogues. A) All HMOS consist of only 5 different monosaccharides. The chemical structures of these monosaccharides are presented in a D- configuration. B) The composition of HMOS follows a distinct structure. Elongation of the core structure and decoration with fucose and/or sialic acid residues leads to the large number of different structures discovered to date. C) As examples, six simple oligosaccharide structures are displayed. Created with BioRender.com*

immunologic outcome of their children later in life. This effect was demonstrated in children, with a hereditary high risk of developing allergic diseases, who were fed breast milk of FUT2 expressing mothers which decreased the incidence of allergic manifestation of these children at 2 years of age [36]. However, from this study it cannot be concluded that solely this genetic polymorphism is related to the allergic outcome of the infant, as many genetic, nutritional and environmental factors contribute to the immune development in neonates.

Synthetically manufactured HMOS or HMOS produced by genetically engineered bacteria, such as 2'-fucosyllactose (2'FL) [51], 3-fucosyllactose (3FL) [52], [53], lacto-N-neotetraose (LNnT) [54], 3'-sialyllactose (3'SL), 6'-sialyllactose (6'SL) [55] and 3'-galactosyllactose (3'GL) [56] have become commercial available just recently. This provides the opportunity to study specific pathways by which individual HMOS structures exert their protective immunologic effects in infants.

Allergic sensitization and the role of the epithelial barrier

The prevalence of allergic diseases is rising tremendously, particularly in Westernized regions [57]. An allergic disease is an immunological result of complex interactions between genetic, environmental and lifestyle factors mainly triggered by harmless substances [58]. Reduced microbial exposure and diversity is one of the many factors that may contribute to the rise in allergic disease prevalence. In allergic sensitization, a harmless, for example food-derived or airborne protein, crosses the mucosal lining and is presented by antigen presenting cells that

drive T helper 2 (Th2) biased immunity contributing to IgE isotype switching of B-cells. Mucosal surfaces with epithelial barriers provide the body with protection from external factors, ensuring that only specific components and nutrients can pass through the epithelium and enter systemic circulation. Allergic sensitization has been linked to dysfunction of the epithelial barrier, both in the intestine and skin [59], [60]. Epithelial barrier integrity depends, among other factors, on the mucus layer covering the single layer of epithelial cells. The mucus layer in the intestines prevents the majority of pathogens and intestinal contents from making direct contact with the epithelial cells [61]. In humans, the most abundant protein present in the intestinal mucus layer is mucin 2, which is secreted by goblet cells [62]. Several factors, including the microbiota, can influence the composition and therefore the protective effects of the mucus [63]. Gut maturation takes place the first couple of weeks after birth rendering a leaky barrier in the first weeks of life [64]. This can help to organize oral tolerance induction, but it also provides a risk for allergic sensitization.

Tight junctions strengthen apical connections between epithelial cells that cover the underlying connective tissue, thereby contributing to barrier function. Epithelial tight junction proteins tightly regulate paracellular compartments, preventing transport of large molecules, such as proteins and lipids or microbes and microbial products into the underlying tissue [65]. These tight junctions are apically present and are crucial for epithelial barrier integrity. Upon epithelial injury, antigens can cross the epithelium more easily. Cytokines, such as interleukin-8 (IL8), IL25, IL33 and thymic stromal lymphopoietin (TSLP), are produced by the epithelial cells as a response to stress and damage [66]. These epithelial cell secreted cytokines influence neighboring dendritic cells (DCs) [67]. Generally, DCs in the gastrointestinal tract are hyporesponsive and favor tolerogenic response to prevent unnecessary inflammatory responses to antigens and microbes [68]. IL25, IL33 and TSLP stimulate the uptake and processing of foreign antigens by DCs and drive these DCs to promote development of Th2 cells from naïve T cells [69], [70]. Consequently, IL4 and IL13 produced by the Th2 cells induces the activation and class-switching of B cells to produce allergen-specific IgE [67]. The secreted IgE will bind to the high-affinity Fc receptors on the surface of mast cells. Upon a consequent encounter, the allergen crosslinks the IgE bound to the mast cells, triggering the mast cell to degranulate and release inflammatory mediators, such as histamine, causing the symptoms of allergic disease [71].

Newborns may be particularly susceptible to developing allergic diseases since the immune system after birth is dominated by Th2 responsiveness [72]. Immune maturation involves shifting towards a more T helper 1 (Th1) prone and regulatory type, which favors the development of adequate immune protection and balanced immune responses [73]. The importance of the epithelial barrier and mucosal homeostasis in prevention of allergic sensitization has sparked interest. HMOS may help to support this function by stimulating proper epithelial maturation and microbial colonization [74]–[76].

HMOS shape the microbiota of neonates

The first 1000 days of life are critical for the development of a diverse, stable gut microbiome [77]–[79]. The initial microbial composition of the gut is determined by host genetics and environmental factors, such as health status, mode of delivery and diet [80]. The first bacteria to colonize neonate's intestines are *Enterobacteriaceae* and *Staphylococcus* [81], followed by bifidobacteria and

lactic acid bacteria [82]. Proper colonization is essential for optimal development and health, as the establishment of a rich and diverse microbiome is related to a decreased prevalence of allergic [83], metabolic and other immunologic diseases later in life [84], [85].

HMOS promote the growth of beneficial bacteria, such as *Bifidobacterium* and *Lactobacillus* species [86], [87]. Therefore, HMOS are known for their prebiotic effects and as players in shaping the microbiota of infants as depicted in Figure 2. The microbiota supporting effects of HMOS were observed when the gut colonization in breast-fed and formula-fed infants was compared, while addition of scGOS/lcFOS to formula milk was found to bring the microbiome composition closer to that of breastfed infants [88], [89]. The microbiota are capable of fermenting oligosaccharides, however the capacity to degrade HMOS is strain-specific and depends on the presence of several genes [90], [91]. Several strains of *Bifidobacterium* are well adapted to digest purified natural HMOS into metabolites such as short chain fatty acids (SCFA) [91]–[94]. Glycosyl hydrolases (GH), expressed by bifidobacteria, cleave monosaccharides from the HMOS and making them available for utilization by the microbe [95]. This enzymatic degradation can either occur by membrane-associated extracellular GHs [96] or, as is the case for *Bifidobacterium infantis*, intact HMOS are transported into the cell by Solute Binding Proteins [97] and broken down by GHs inside the cytoplasm [98]. The available monosaccharides are assimilated in central metabolic pathways and consequently release large volumes of e.g. SCFAs [99].

Both *B. longum* and *B. bifidum*, the major intestinal bacteria found in breastfed infants, are remarkably well equipped to metabolize HMOS. In contrast, *B. adolescentis* is often associated with the adult intestinal microbiota, and is a less effective HMOS metabolizer [82], [92], [94]. In contrast to *Bifidobacterium* spp., *Bacteroides* spp. are not specifically adapted to metabolize HMOS, but degradation of plant polysaccharides by *Bacteroides* spp. has been indicated [91]. As plant-derived oligosaccharides are structurally comparable to human oligosaccharides, the capacity of multiple *Bacteroides* strains to metabolize HMOS is not unexpected [90]. Providing a substrate for commensal gut bacteria results in a competitive growth advantage for these bacteria, enhancing proper colonization in the infants intestine and reducing growth conditions for and colonization by pathogenic bacteria [100], [101].

Unlike several species of commensal gut bacteria discussed previously, certain pathogenic species do not use HMOS as carbohydrate source for growth, including *Clostridium difficile, Enterococcus faecalis* and *Escherichia coli* [90]. In addition, HMOS can actively bind to several pathogenic microbes and thereby possibly prevent adhesion as first step of infection [102]. Infant formula can be supplemented with the prebiotics scGOS and lcFOS in order to promote the growth of various *Bifidobacterium* and *Lactobacillus* strains [103]. However, these oligosaccharides do not contain terminal fucose or sialic acid residues, hence missing out biological function of HMOS related to these specific functional groups [104].

Proper colonization of the gut promotes intestinal barrier function and immune maturation [105]. The establishment of a rich and diverse microbiome is related to a decreased prevalence of allergic diseases [83]. Prebiotics like HMOS can support the growth and function of commensal bacteria and therefore possibly enhance gut microbial diversity. The association between microbial diversity and development of allergic diseases [84], [106] and the role of HMOS in this context, has yet to be elucidated.

Metabolites of HMOS influence intestinal barrier integrity and immune function

As described in previous section, HMOS are digested by intestinal bacteria, resulting in various metabolites, among which SCFA are well known for immunomodulatory properties. The fermentation of major HMOS by bifidobacteria and lactobacilli into SCFA is very efficient [82], hence these bacteria are the dominant suppliers of SCFA in the infant's colon. Butyrate, propionate and acetate are SCFA metabolites that have gained interest in recent years due to their proposed health benefits. Butyrate is mainly utilized by the epithelial cells, whereas acetate and propionate can be transported across the epithelial barrier to become systemically available in low levels via the bloodstream as depicted in Figure 2 [107].

Upon absorption by the colonic epithelial cells, SCFA promote several functions of the epithelial barrier. The mucus layer covering the epithelial cells is essential to maintain epithelial barrier integrity. SCFA enhance the mucus secretion by upregulating the expression of mucin 2 [108]. Acetate, produced in high levels by *Bifidobacterium* and *Bacteroides* species, increases the expression of genes related to mucus and support goblet cell differentiation [109]–[111]. In addition, SCFA are known to protect against inflammatory insults and fortify the tight junction barrier [112]. Promoting and enhancing the epithelial integrity may be of relevance in preventing allergic diseases, as a disrupted intestinal epithelial layer could lead to a compromised local tolerance response in which food allergens are able to reach underlying immune cells intact [113].

In addition, SCFA interact with DC and T cells and therefore modulate inflammatory immune responses. Many of the protective effects of SCFA have been attributed to the interaction with G protein-coupled receptors (GPR) present on intestinal epithelial cells and immune cells [114]. Moreover, GPR-independent regulation of the immune response via T cell modulation has been shown in a murine model [115]. In this model, SCFA regulate cytokine production via mammalian target of rapamycin (mTOR) by inhibiting histone deacetylase (HDAC) in T cells. In a previous study, butyrate effectively inhibited several HDACs in various cells, among which those that promote the transcription of FoxP3 in T cells, leading to increased expression of this hallmark transcription factor of regulatory T (Treg) cells [116], [117]. In addition, inhibition of maturation and differentiation of macrophages and DCs has been demonstrated [118]. Suppression of inflammatory responses by butyrate was shown to involve inhibition of the NF-κB pathway in inflammatory cells such as macrophages in the lamina propria [119].

Interestingly, recently it was found that the microbiome of infants who develop allergic diseases during childhood have a reduced genetic potential for butyrate production from complex carbohydrates, supporting the importance of SCFA production in protecting the infant from developing allergic diseases [120]. Therefore supporting the microbial development may be of interest in infants more susceptible to developing allergic diseases [121], [122]. Altogether, as bacterial metabolites of HMOS, SCFA may contribute to the immunomodulatory and protective effects against allergic disease development.

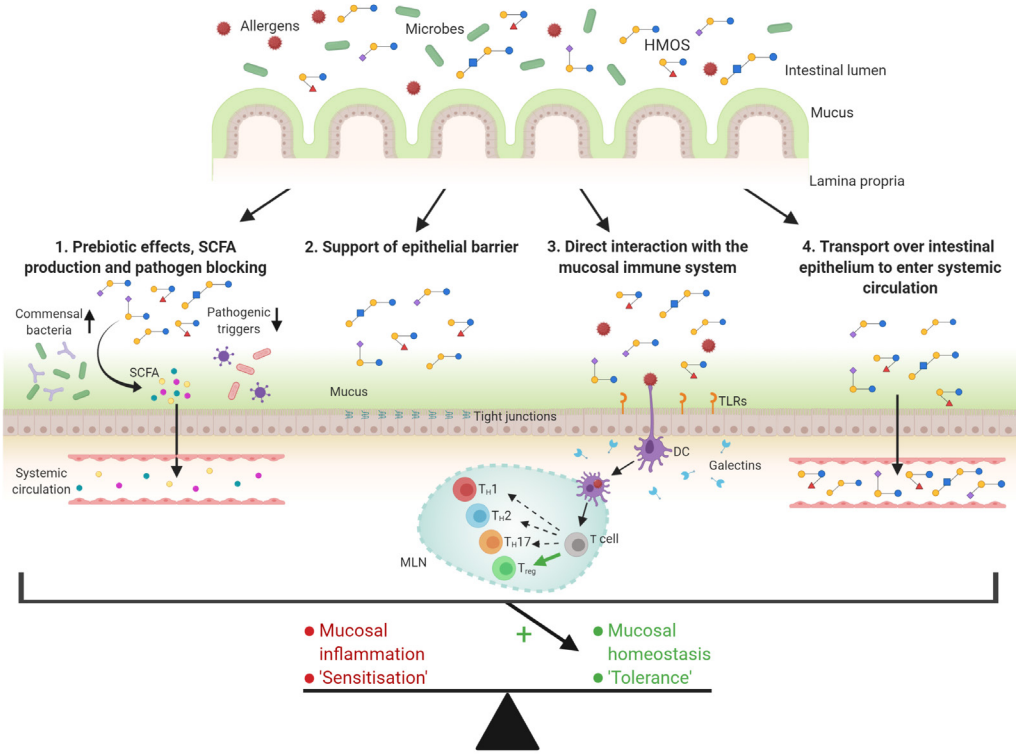

Figure 2. *Overview of the possible functions of HMOS related to the prevention of allergic diseases. The diversity in structures suggests engagement in several mechanisms related to maturation of the infant's gastrointestinal tract. 1) HMOS have shown to function as prebiotics and therefore stimulate growth of commensal bacteria. In addition, HMOS have shown to bind pathogens, thereby preventing binding of these pathogens to the intestinal epithelium itself and possible consequent infections. SCFAs produced during HMOS fermentation can enhance epithelial barrier integrity and locally and systemically modify immune responses. 2) HMOS can promote mucus production and epithelial tight junction integrity, thereby supporting the physical barrier between the intestinal epithelium and the gut content. 3) Several mechanisms by which HMOS directly affect the immune function have been described. Modulation of the response of DCs is one of those described mechanisms which may be relevant for the instruction of protective mucosal immune development. 4) Transportation of a small fraction of HMOS over the intestinal epithelium, results in systemic availability of these structures. This suggests an immunomodulatory role for HMOS, also beyond the gastrointestinal tract. All these HMOS related mechanisms can potentially enhance tolerance induction and therefore possibly prevent allergic diseases. Adjusted from Ayechu-Muruzabal et al., 2018 [48]. Created with BioRender.com*

HMOS strengthening the intestinal epithelial integrity

Beyond their fermentation products, HMOS themselves may directly provide protection from intestinal epithelial barrier dysfunction [123], by promoting epithelial barrier maturation and mucus production [75] (illustrated in Figure 2). A mixture of human milk derived HMOS was shown to increase mucus production after 24h of *in vitro* treatment in two different intestinal epithelial cell lines. The improved mucus production was linked to an upregulation of *Muc2*. In addition, apart from increased mucus production, HMOS could protect against pathogen induced barrier disruption as determined by means of transepithelial electrical resistance (TEER) [124]. Furthermore, pollution induced loss of epithelial barrier integrity could be prevented by scGOS and 3'GL as measured in both TEER values and luciferase yellow flux across the intestinal epithelial monolayer in Caco-2 cells [125], [126]. It was also demonstrated that supplementation with scGOS resulted in a significant increased rate of tight junction reassembly [125]. Interestingly, the galactosyllactose with a β1-3 glycosidic linkage was effective in protecting the intestinal barrier function, whereas the galactosyllactose with an α1-3 glycosidic linkage did not prevent the deoxynivalenol (DON)-induced disrupted intestinal barrier [126]. The protective effect of 3'GL on the intestinal epithelial barrier under challenge is structure-specific, which supports the notion that it is critical to understand the function and diversity of the structures within the total pool of HMOS, including the specific benefits of 3'GL within early life nutrition. These studies show that HMOS may directly promote proper development of the intestinal barrier, which strengthens the physical barrier between the intestinal epithelium and the gut content, contributing to lower antigenic load and mucosal homeostasis, which may help to decrease sensitization to food allergens.

In addition to this, the immunologic effects that are mediated through interaction between the intestinal epithelium and the underlying mucosal immune system should be addressed. Administration of synthetic HMOS 6'SL to antigen-antibody complex activated intestinal epithelial cells *in vitro* and resulted in a dose-dependent decrease of IL8 and CCL20 secretion. Whereas, administration of 2'FL selectively reduced the secretion of CCL20 from the two cell lines used in this study [38]. Similarly, a decrease of cytokine and chemokine production was observed upon TNFα stimulation of these cells after 6'SL exposure. Furthermore, comparable outcomes were observed for 3'GL, 4'GL and 6'GL in an *in vitro* model for the infant intestinal epithelium [50]. However, this decrease in cytokine production was not observed when two different intestinal cell lines were exposed to 2'FL [38]. Additionally, it was observed that 3'SL, which is an isomer of 6'SL, downregulated the production of pro-inflammatory cytokines in Caco-2 intestinal cells by inhibition of the NF-κB pathway in a PPARγ dependent manner [127]. These observations indicate that different functional groups and structures of HMOS exert the anti-inflammatory effects via different mechanisms. Silencing exaggerated or unwanted epithelial cell activation is essential for maintaining mucosal homeostasis.

Data indicated that mice, fed a diet supplemented with GOS for two weeks prior to exposure to DON, maintain their normal cellular distribution, as measured by villus height in the proximal small intestine [125]. A study in suckling rats investigated the effects of 2'FL on mucosal immunomodulation [19]. After treatment with 2'FL for 16 days an overall lower presence of inflammatory cytokines in the intestines compared to a reference group was observed,

whereas the ratio of Th1/Th2 cytokines remained unchanged. In addition, the height and area under the villi present in the intestines was significantly increased upon supplementation with 2'FL, pointing to a positive effects of this prebiotic on intestinal growth [19]. This is linked to the observation that 2'FL and scGOS/lcFOS in early life alter gut microbiome development while supporting vaccination responses [18], [128], [129].

In the light of NEC, especially sialylated oligosaccharides have shown promising outcomes *in vivo* in prevention and development of necrotic intestinal lesions [123]. Several studies in neonatal rats have reported reduced pathology scores upon intervention with HMOS mixture [130], or single HMOS alone [131], [132]. Although sialylated oligosaccharides have been identified as the protective agents [130], intervention with 2'FL has also resulted in a reduced pathology score in rats [131]. Dietary supplementation of 2'FL in preterm pigs had no significant effects on intestinal structure, digestive function and the development of NEC [133]. Nonetheless, pooled HMOS, rather than single HMOS, have consistently shown to be most effective in preventing development of NEC [123].

Moreover it has been shown that HMOS provision early in life can protect against the development of autoimmune diabetes in NOD-mice [134]. The number of *in vivo* studies looking into the immunomodulatory effects of single HMOS are rather limited, and currently restricted to only the simple short chain structures. In a murine model for hen's egg allergy, 2'FL or 6'SL were found to reduce allergy symptoms in association with the induction of IL-10+ Treg cells [39]. Prebiotic mixtures, such as scGOS and lcFOS, have been studied more extensively for immunomodulatory effects *in vivo*, showing promising results with regards to preventing allergic diseases, such allergic asthma and food allergy and these effects also link to the induction of Treg responses [135]–[138]. This implies a need for additional *in vivo* studies to gain insight in the properties of (single) HMOS to modulate gut maturation and the development of the mucosal immune system. Combining these studies, the direct effects of HMOS on the intestinal epithelial integrity and activation status and possibly the mucosal immune system are only started to be elucidated. The exact mechanisms and pathways involved are not yet fully understood. However, some of the receptors involved in HMOS signaling are identified and will be discussed in the following section.

HMOS bind to and act as receptors

One potential role of HMOS to modulate the infant's immune system is through receptor binding properties. In fact, multiple classes of human receptors have been described to interact with specific structures of HMOS, as summarized in Table 1. These receptors are mainly expressed by innate, adaptive immune cells and epithelial cells, they may therefore play a key role in mucosal immunomodulatory effects of HMOS [139].

Glycan receptors

Glycan-binding receptors, also known as lectins, are particularly effective in binding HMOS. Many of the receptors belonging to the lectin family are involved in modulation of immune pathways. Lectin receptors consist of several subcategories, such as: membrane bound C-type lectins, sialic acid binding immunoglobulin-like lectins (Siglecs) and soluble type galectins.

Table 1. *Overview of HMOS binding receptors, potentially involved in immunomodulation. Adapted from Triantis et al., 2018 [139].*

HMOS identified as ligands	Receptor	Expression of receptor on	Function of receptor	Reference
2'FL, 3FL, LNFP-III, LNFP-IV, LNDFH-I	DC-SIGN	Antigen presenting cells	Antigen presentation	[140]-[142]
3'SL & 6'SL	Siglec 5, 9	Neutrophils, monocytes, dendritic cells	Immune signaling	[140], [143]
LNnT, LNT, LNFP-II, LNFP-III, LNDFH	Galectin 1, 2, 3, 7, 8, 9	Intestinal cells, lymphocytes, antigen presenting cells	Immune signaling	[144]-[146]
2'FL & 3'SL	TLR4	Most cell types, mainly immune cells	Pathogen detection	[15], [16]

The C-type lectin receptor dendritic cell-specific ICAM-grabbing non-integrin (DC-SIGN) is present on the surface of DCs and macrophages. It is usually involved in phagocytosis of pathogens upon recognizing pathogen-related glycoproteins. DC-SIGN has an affinity for HMOS containing α-linked fucose residues [140]. A high affinity for 2'FL and 3FL (2 major structures of HMOS) may be of distinct physiological relevance in modulating immune responses in infants. DC-SIGN is expressed by cells in the gastrointestinal tract [141] and this receptor can promote allergen uptake by DCs. This may lead to subsequent Th2 cell polarization as seen in patients with atopic dermatitis [147]. Therefore, even though DC-SIGN can confer protective regulatory immunity in a pre-clinical model for auto-immune disease [148], DC-SIGN signaling may be involved in the sensitization phase of allergic diseases as allergens are capable of DC activation via DC-SIGN binding [149]. An HMOS mixture derived from human milk was found to lower the expression of DC-SIGN on DC [142]. This indicates that HMOS may be able to reduce DC-SIGN driven allergic sensitization through suppression of DC-SIGN expression on DC and via blocking the DC-SIGN receptor.

Siglecs are expressed by several immune cells that are involved in allergic effector responses, such as eosinophils and mast cells. Siglecs have been associated with binding of sialylated HMOS, although previous results show only affinity of siglec-1 -5, -7, -9 and -10 to 3'SL and 6'SL [143], and more recent data show a more limited binding affinity of Siglecs for HMOS [140]. Siglec-9 provides low binding affinity for 3'SL and 6'SL, while siglec-5 has very low affinity for only 3'SL. This study found no other Siglecs to bind sialylated HMOS [140]. Hence, the presence of sialic acid alone is not sufficient to ensure functional binding to a Siglec receptor [140]. Siglec-7 and siglec-8 have been associated with allergy related immune

mechanism [150], [151] making these potential targets for immune modulation by HMOS in relation to allergy prevention.

Galectins are another group of β-galactoside-binding receptors that bind carbohydrate moieties or glycan structures present on proteins. Moreover, galectins are expressed on and/or secreted by several immune cells and intestinal epithelial cells [152]. These receptors can directly forward signals into the cell upon binding to a ligand, but galectins can also be secreted from cells [153]. In the secreted form, galectins can act as ligands and bind to receptors, such as TIM-3 and CD44 on other mucosal immune cells [154]. Galectins such as galectin-9 have shown to induce Treg cells [155]–[157]. The binding of HMOS to galectins may directly modify galectin release and affect interactions of galectins with other cells, potentially resulting in immune modulation. Of the thirty-two different HMOS structures tested for binding to four galectins (galectin-1, -3, -7 and -9) [144], a total of twenty-five of these structures were recognized by all four galectins. Significant differences in affinity for each HMOS were observed, i.e. 2'FL, 3'SL and LNnT were shown to bind galectins, whereas 3FL and 6'SL did not. 2'FL, the most common HMOS in human milk, binds with moderate-to-high affinity to all four galectins, while 3FL a structure very similar to 2'FL, not or weakly binds to any of the four galectins included in the study [144]. Similar results were obtained in a different report, including galectin-1, -3 and -7 [145]. These findings are supported by a previous study [146], suggesting that all included galectins showed affinity for LNnT, but had no affinity for 6'SL. This study also highlighted the evolutionary conserved binding affinity of galectins for glycans. Galectin-9 is a particularly promising target in allergy prevention strategies, as exposure of intestinal epithelial cells to scGOS/lcFOS together with bacterial CpG DNA or synthetic CpG ODN promoted the secretion of galectin-9 *in vitro*, which resulted in enhanced secretion of IFNγ and IL10 production by underlying immune cells [155], [158]. These cytokines are related to a regulatory type of Th1 polarization and suppress Th2 cell activation. Experiments with dietary interventions including scGOS/lcFOS enhanced local and/or systemic galectin-9 levels in murine and human allergy in association with symptom reduction [138]. Furthermore, galectins can become systemically available and dampen allergic effector responses as shown in a murine model of food allergy [138].

Pattern recognition receptors

Toll-like receptors (TLR) are a family of receptors known to sense common molecules of pathogenic or commensal microorganisms, such as TLR4 ligand lipopolysaccharide (LPS) or TLR9 ligand bacterial CpG DNA. Decreased formation of the three-component complex TLR4, CD14 and LPS, inhibits subsequent pro-inflammatory immune signaling [159]. Xiao *et al.* showed an increase in LPS receptor TLR4 mRNA expression upon stimulation with pooled HMOS isolated from human milk in monocytic derived dendritic cells (moDC) *in vitro*, yet protein levels of this receptor were not increased [142]. In addition to affecting TLR4 transcription, HMOS suppress the expression of cluster of differentiation (CD)14, a coreceptor of TLR which is necessary to recognize LPS. 2'FL significantly suppresses CD14 in intestinal epithelial cells [16]. In contrast to suppression of inflammation via TLR4 by 2'FL, pro-inflammatory properties related to TLR4 modulation have been described for synthetic 3'SL. In a TLR4-dependent manner, 3'SL was shown to induce intestinal inflammation [15]. This pro-inflammatory effect of 3'SL can be explained by mimicking possible structural aspects of pathogenic bacteria, thereby

educating and preparing the immune system for possible pathogenic encounters later in life. However, the phenotypical changes of DCs by 3'SL may have been due to LPS contamination of the oligosaccharide during synthesis, since pre-exposure to LPS may contribute to TLR4 silencing [160]. However, LPS-containing bacteria are normal components of a healthy intestinal microbiome [161]. In this respect, the low level of endotoxins present in purified HMOS used in *in vivo* studies would be minimal compared to the vast amount of endotoxin triggers the infant receives directly after birth. The contradicting results regarding HMOS-induced modulation of TLR4 show that we are only beginning to elucidate the possible immunomodulatory effects of HMOS. In addition, as synthetic (s)HMOS are either derived from enzymatically-processed lactose or produced by *E.coli*. In the latter situation a second possible immune trigger from bacterial byproducts may add to the biological effects of sHMOS structure. The origin of HMOS may influence the immunomodulatory effect, therefore an overview of the source and main outcomes of the studies referred to in this review is provided in Table 2.

Pathogen binding

Besides binding to receptors on the cell membrane, HMOS can act as soluble receptors and bind to several pathogenic bacteria, thereby preventing binding to the intestinal epithelium and subsequent infection [102]. Both *in vitro* and *in vivo* studies show that 2'FL attenuated *Campylobacter jejuni* infection [17], [162]. However, Coppa *et al.* did not find inhibition of adhesion of *Escherichia coli, Vibrio cholerae* and *Salmonella fyris* in an *in vitro* intestinal epithelial setting with 2'FL [102]. Nonetheless, inhibition of adhesion was observed with 3'SL, 6'SL and 3FL and combinations of these sHMOS. There was a diminished growth of *Streptococcus agalactiae* (group B *Streptococcus*) upon incubation with human pooled natural HMOS, that was attributed to the neutral fraction of the HMOS [163].This effect was supported by other studies, as pooled HMOS inhibited growth of group B *Streptococcus* (GBS) and prevented biofilm formation, although the effects of single HMOS were GBS strain specific [164]–[166]. In this study, the effects of HMOS were compared to scGOS. scGOS did not diminish the growth of GBS [163], showing that the structures in scGOS in this respect do not exert similar effects as the mentioned HMOS subtypes. These studies indicate that HMOS can also function as decoy receptors, thereby inhibiting growth and adhesion of pathogens in the gastrointestinal tract.

As antibiotic resistance is a growing problem, alternative antibacterial treatments are being investigated [167], including the use of HMOS to potentiate antibiotic functioning [168]. It has been recently demonstrated that when exposed to HMOS, GBS becomes sensitive for trimethoprim, an antibiotic to which these bacteria are normally resistant. A significant decrease in metabolic pathways related to membrane construction was observed [169]. Furthermore, HMOS were able to sensitize GBS to several antibiotics, such as erythromycin, gentamycin and clindamycin. In addition, an increased sensitivity to gentamycin, when combined with HMOS, in *Staphylococcus aureus* and *Acinetobacter baumanii* was also observed. However, these potentiating effects were obtained for β-lactams and glycopeptides [170]. Next to the above reported antibacterial properties, similarly some viral inhibiting interactions have been described [171]. These interactions include binding of 2'FL to conserved epitopes, which are involved in binding to host cells, on norovirus [172], [173]. Next to 2'FL, also 3'SL and 6'SL showed to inhibit cell binding in a rotavirus *in vitro* model [174]. Some promising results of HMOS intervention have even been observed for influenza and HIV infections [171].

HMOS interact with immune cells

HMOS have been detected in the blood, feces and urine of breastfed term and preterm infants [175]–[178]. In breastfed infants, HMOS concentrations in urine appear to be around ten times higher than in serum [178], which can be explained by clearance of substances from a larger volume of blood and accumulation in a small volume of urine. Direct effects have been demonstrated *in vitro* in bone marrow-derived dendritic cells (BMDC) treated with 2'FL. There was an increase in the percentage of CD40+ and CD86+ BMDCs upon exposure to 2'FL [18]. Direct modulation of human moDCs was not found for 2'FL, 6'SL and scGOS [179], but the idea of possible moDC modulation via other HMOS cannot be excluded. BMDC exposed to 2'FL and stimulated by influenza vaccination had a greater capacity to induce CD4+ T cell proliferation in fresh whole splenocytes [18]. Low concentrations of a mixture of acidic HMOS, purified from human milk, can alter cytokine production in cord blood mononuclear cells (CBMC) [180]. The production of IFNγ and IL10 in CBMCs was increased upon exposure to acidic HMOS, while IL13 production remained unaltered, pointing to skewing of the balance towards a regulatory type Th1 response. Similar effects were observed in a prior study exposing CBMC to acidic HMOS, which resulted in decreased IL13 production in T cells [181]. Mast cell function and direct effects of HMOS on mast cell degranulation were investigated in a murine food allergy model [39]. *In vitro* exposure of bone marrow-derived mast cells to 6'SL resulted in significant inhibition of IgE-dependent mast cell degranulation, but only at a relatively high concentration of 1 mg/mL. However, in this same study, 2'FL did not significantly inhibit mast cell activation. Both 6'SL and 2'FL induce IL10+ Treg cells and thereby indirectly stabilize the degranulation of mast cells, in association with reduced food allergy symptoms [39]. Hence, HMOS may have the capacity to modulate the immune response via various mechanisms, as indicated by the direct effects of HMOS on several immune cell types.

In the above described murine model for food allergy, 2'FL and 6'SL reduced food allergy symptoms via inducing Treg cells and modulating mast cells [39]. After 2'FL and 6'SL treatment during challenge in ovalbumin sensitized mice enhanced the capacity of CD4+CD25+ Treg cells to inhibit mast cell degranulation *ex vivo* [39], indicating that specific sHMOS support Treg cell function. Similar results were found using scGOS and lcFOS in combination with acidic oligosaccharides or *B. breve* in prevention of food- [29], [182] or asthma-allergy in mice [31], [183]. In piglets, either sow-reared or formula fed, peripheral blood mononuclear cells (PBMCs) were isolated [184]. PBMCs from formula fed piglets showed more proliferation than sow-reared piglets upon LPS stimulation *ex vivo*, while *ex vivo* addition of sHMOS 2'FL normalized this increased proliferation. The percentage of T helper cells was higher in formula fed piglets compared to sow-reared piglets. *Ex vivo* added synthetic fucosylated and sialylated oligosaccharides downsized the expansion of the TH cell population in the formula fed piglets, while the cytotoxic T cell population remained unaffected by *ex vivo* sHMOS treatment [184]. These results indicate that fucosylated and sialylated oligosaccharides may possess immune regulatory properties, potentially modulating an allergic inflammatory response.

Although clinical trials in this area of research are scarce, data from an initial study indicate that addition of 2'FL to infant formula lowers concentrations of pro-inflammatory cytokines in plasma compared to infants fed a control formula [185]. In addition, the decrease of these cytokines in the 2'FL supplemented infants was comparable to the low level of inflammatory

cytokines that was measured in plasma of breastfed infants [185]. As such, it should be carefully considered whether the effects observed in any of the *in vivo* and clinical studies are caused by a direct effect of the HMOS or indirect immunomodulatory effects as a result of microbiome modulation.

A convincing body of evidence is missing to ascribe clear immune development properties to HMOS and individual HMOS structures, since only a small number of *in vivo* studies describe immunomodulatory properties and immune maturation. In addition, the exact properties of the different groups of HMOS to modulate the immune system are not clear. Therefore, several studies illustrating immunomodulatory effects of scGOS and lcFOS have been described here and summarized in Table 2, as they may propose a framework in which future research could focus to elucidate immune related mechanisms affected by HMOS. As synthetically produced HMOS have become available recently, studying these may contribute to acquiring knowledge of the exact properties of HMOS and their specific functional groups in more detail and promote research focusing on allergy prevention. Development of adequate *in vitro* models for allergic sensitization including intestinal epithelial cells and/or dendritic cells, may help understanding the direct immunomodulatory effects of HMOS and their possible role in allergy prevention.

Conclusion

The increasing prevalence of allergic diseases has sparked interest in the role of early life nutrition and allergy development. Dietary components drive early life microbiome development as well as gut and immune maturation. HMOS in breast milk exhibit various microbiome modulating as well as mucosal immune maturation properties, which are not yet fully understood. However, in recent years several pathways involved in the effects of HMOS have been elucidated, including their capacities to fortify the microbiome composition and the release of fermentation products including SCFAs, as well as direct binding to pathogens and interactions with the gastrointestinal epithelium and local and systemic immune cells (as illustrated in Figure 2). Specific structural groups of HMOS may target several aspects of the immune system and modify immune function, thereby highlighting the need for further research on this topic. In addition, a more diverse mixture of oligosaccharide structures in neonatal formula nutrition may more closely resemble the HMOS composition as available in human breast milk and provide extra benefit for the child. Future research should focus on uncovering the mechanisms and pathways by which HMOS and the specific functional groups present in these HMOS may exert immunomodulatory actions. Ultimately, it would be of utmost value to identify whether specific HMOS structures are capable of contributing to early life allergy prevention.

Table 2. *Overview of studies included in this review, which describe effects of non-digestible oligosaccharides (NDO) on immune function. As HMOS has different origin which may influence the immunological outcome, when possible the origin of the used HMOS was noted. Biological isolated HMOS = 1 ,chemically synthesized = 2 , bacterial fermentation/synthesis = 3 or source unknown. Studies are sorted based on model subgroup (in vivo e.g.), NDO and year of publication.*

Reference	Model	NDO	Main effect of intervention
	In vitro		
Gnoth *et al.* **2001 [42]**	Caco-2 cells	isolated HMOS	Neutral HMOS are transported across intestinal epithelia via receptor-mediated transcytosis as well as by paracellular flux, while acidic HMOS are translocated solely via paracellular pathways
Eiwegger *et al.* **2004 [180]**	cord blood T cells	isolated HMOS	Acidic HMOS increased the percentage of IFNɣ and IL13 producing T cells as well as CD25+ T cells. IgE and IgG1 production was unaffected.
Coppa *et al.* **2006 [102]**	Caco-2 cells	isolated HMOS	Acidic HMOS showed anti-adhesive effects on all 3 intestinal pathogens. Neutral HMOS showed anti-adhesive effects on 2 out of 3 tested pathogens.
He *et al.* **2014 [49]**	fetal small intestinal samples	isolated HMOS	HMOS from colostrum samples were able to attenuate mucosal response to surface inflammatory stimuli, and enhanced maturation of intestinal mucosa
Xiao *et al.* **2019 [142]**	human moDCs	isolated HMOS	HMOs limited LPS maturation of moDCs. HMOS-conditioned moDCs promoted T_{reg} generation.
Newburg *et al.* **2016 [50]**	T84 cells, H4 cells, NCM-460	isolated HMOS & GOS	HMOS attenuated surface inflammatory stimuli. HMOS and GOS attenuated NF-κB signaling.
Eiwegger *et al.* **2010 [181]**	Caco-2 cells	isolated HMOS & scGOS + lcFOS & AOS	Acidic HMOS increased IFNɣ and IL10 secretion and suppressed Th2 cytokine production in T cells from peanut allergic patients.
He *et al.* **2016 [16]**	T84 cells, H4 cells	isolated HMOS, 2'FL^3, LNFP-I^3, 3'SL^3 & 6'SL^3	HMOS and 2'FL inhibited LPS-TLR4 signaling via suppressed CD14 expression. No significant results for any of the other tested NDOs.
Akbari *et al.* **2015 [125]**	Caco-2 cells	GOS	GOS improved tight junction assembly and DON induced loss of transepithelial resistance was prevented.

Reference	Model	NDO	Main effect of intervention
	In vitro		
De Kivit *et al.* **2013 [155]**	T84 cells, HT-29 cells	scGOS + lcFOS	scGOS + lcFOS in combination with *B. breve* M-16V increased epithelial expression and secretion of galectin-9, and enhanced Th1 and Treg polarization.
Hayen *et al.* **2018 [158]**	HT-29 cells	scGOS + lcFOS & scFOS + lcFOS	Both mixtures induced enhanced IFNγ and IL10, but suppressed IL13 and TNFα secretion. scFOS + lcFOS enhanced Th1 and Treg response in a peanut-specific co-culture (HT-29/PBMC) model.
Zenhom *et al.* **2011 [127]**	Caco-2 cells	FOS & 3'SL[3]	Both decreased levels of inflammation, as IL12 secretion and mRNA expression of IL12p35, IL8, and TNFα was reduced in a dose- and time-dependent manner.
Perdijk *et al.* **2018 [179]**	human moDCs	GOS, 2'FL[1] & 6'SL[1]	None of the oligosaccharides influenced DC differentiation and LPS-induced maturation.
Yu *et al.* **2016 [17]**	Hep-2 cells, HT-29 cells	2'FL[2]	2'FL attenuated *C. jejuni* invasion in both cell lines.
Perdijk *et al.* **2018 [160]**	human moDCs	3'SL[1]	3'SL mediated NF-κB activation via TLR4 induction was explained by LPS contamination.
Zehra *et al.* **2018 [38]**	T84 cells, HT-29 cells	2'FL[2] & 6'SL[2]	2'FL inhibited CCL20 secretion from epithelium upon antigen-antibody complex stimulation. 6'SL inhibited IL8 and CCL20 secretion from epithelium upon antigen-antibody complex stimulation.
Holscher *et al.* **2014 [74]**	Caco-2Bbe cells, HT-29 cells	LNnT[3], 2'FL[3] & 6'SL[3]	All HMOS inhibited cell proliferation in undifferentiated cell cultures. 2'FL increased alkaline phosphatase and sucrase activity. LNnT increased transepithelial resistance.
Varasteh *et al.* **2019 [126]**	Caco-2 cells	3'GL[3], 4'GL[3] & 6'GL[3]	3'GL prevented loss of transepithelial resistance upon DON exposure, 4'GL and 6'GL had no effect.

Reference	Model	NDO	Main effect of intervention
	Preclinical		
Xiao *et al.* **2018 [134]**	Mice	isolated HMOS	HMOS intervention delayed and suppressed type 1 diabetes development and reduced development of severe pancreatic insulitis in NOD-mice.
Wu *et al.* **2019 [124]**	Mice	isolated HMOS	HMOS increased mucin expression, whereas intestinal permeability was decreased.
Jantscher-Krenn *et al.* **2012 [130]**	Mice	isolated HMOS & GOS	HMOS reduced NEC pathology scores, the effects were attributed to DSLNT in the HMOS mixture.
Yu *et al.* **2014 [132]**	Rats	isolated HMOS, GOS & synthetic disialylated-GOS	HMOS and sialylated-GOS reduced NEC pathology scores. GOS had no effect on NEC development.
Autran *et al.* **2016 [131]**	Rats	isolated HMOS, GOS & synthetic disialylated-GOS	HMOS and sialylated-GOS reduced NEC pathology scores. GOS had no effect on NEC development.
Comstock *et al.* **2014 [184]**	Pigs	isolated HMOS, 2'FL^3, 3FL^3, 3'SL^3, 6'SL^3, LNFP-III^3 & LNnT^3	HMOS stimulation IL10 production by PBMCs. Fucosylated HMOS decreased proliferation of HMOS. Sialylated HMOS increased PBMC proliferation, although less CD4+ cells were observed.
Akbari *et al.* **2015 [125]**	Mice	GOS	GOS treatment stabilized villus height upon DON exposure.
Verheijden *et al.* **2015 [30]**	Mice	GOS	GOS prevented induction of airway eosinophilia and Th2 related cytokine concentrations in lung, similar to budesonide treatment in house-dust mite allergy.
Verheijden *et al.* **2015 [136]**	Mice	GOS	GOS decreased IL33 secretion and expression in HDM-induced asthma.
Verheijden *et al.* **2015 [183]**	Mice	GOS	GOS decreased CCL5 and IL13 concentration in lung tissue from HDM-induced allergic asthma mice, similar to budesonide treatment.
Djouzi *et al.* **1997 [23]**	Rats	GOS & FOS	GOS and FOS decreased pH in caecum, increased total SCFA concentration.
Verheijden *et al.* **2016 [31]**	Mice	scFOS + lcFOS	scFOS + lcFOS in combination with *B. breve* M-16V prevented house-dust mite induced airway inflammation.

Reference	Model	NDO	Main effect of intervention
	Preclinical		
De Kivit *et al.* **2012 [138]**	Mice	scGOS + lcFOS	scGOS + lcFOS in combination with *B. breve* M-16V induced reduced acute allergic skin response, and higher concentrations of galectin-9, which was associated with allergy prevention.
De Kivit *et al.* **2017 [182]**	Mice	scGOS + lcFOS	scGOS + lcFOS in combination with *B. breve* M-16V in an ovalbumin allergic mouse model, reduced allergic symptoms and increased galectin-9 serum levels. DC activation and Th2 frequency were normalized in allergic mice.
Schouten *et al.* **2012 [135]**	Mice	scGOS + lcFOS + AOS	Prebiotic mixtures enhanced percentages of Th1 cells and decreased Th2 cell percentages were observed. Strong reduction in allergic skin reaction. CD25+ Treg cells were involved in the tolerance induction effect.
Kerperien *et al.* **2014 [29]**	Mice	scGOS + lcFOS & AOS	Only NDO mixtures reduced allergic skin response, whey-IgG1 levels, Th2 and Th17 mRNA expression, and increased Foxp3+ cells.
Kerperien *et al.* **2018 [137]**	Mice	scGOS + lcFOS + AOS	Prebiotic mixtures increased mRNA expression of IL10, TGFβ and Foxp3, and acute allergic skin response was 50% lower in whey allergic mice when fed the prebiotic mixture. These protective effect were depended on IL10 and TGFβ.
Xiao *et al.* **2019 [128]**	Mice	scGOS + lcFOS + 2'FL[2]	NDOs enhanced influenza vaccine response, higher levels of IgG1, IgG2a, and activated B cells were observed.
van den Elsen *et al.* **2019 [129**	Mice	scGOS + lcFOS + 2'FL[2]	NDOs improved vaccine-specific antibody response and modulated gut microbiota composition.
Yu *et al.* **2016 [17]**	Mice	2'FL[2]	2'FL attenuated *C. jejuni* colonization, weight loss and inflammatory cytokines.
Cilieborg *et al.* **2016 [132]**	Pigs	2'FL[3]	2'FL intervention did not result in observed differences in bacterial colonization, intestinal function and NEC pathology.
Xiao *et al.* **2018 [18]**	Mice	2'FL[2]	2'FL improved humoral and cellular immune response to influenza vaccination.
Azagra-Boronat *et al.* **2019 [19]**	Rats	2'FL[3]	2'FL increased plasma IgE and IgA levels. Increased intestinal villus height. Higher *Lactobacillus* proportion in cecum.
Weiss *et al.* **2012 [104]**	Mice	3'SL[3]	3'SL induced higher degree of resistance to dextran sulfate sodium-induced colitis.

Kurakevich *et al.* **2013 [15]**	Mice	3'SL[3]	3'SL increased colitis, via TLR4 signaling.
Castillo-Courtade *et al.* **2015 [39]**	Mice	2'FL[2] & 6'SL[2]	2'FL and 6'SL attenuated ovalbumin induced allergic symptoms like diarrhea, hypothermia, mast cell number in the intestine, and increased induction of IL10 producing Treg cells.
Reference	**Model**	**NDO**	**Main effect of intervention**
	Clinical		
Newburg *et al.* **2004 [32]**	Infants	HMOS in human milk	Higher 2'FL and LNF-I to 3FL and LNF-II ratios in human milk correlated with more protection against diarrhea in infants.
Sjögren *et al.* **2007 [35]**	Infants	HMOS in human milk	Neutral HMOS concentration in human milk is not related to maternal allergy status nor allergy development in children.
Bode *et al.* **2012 [33]**	Infants	HMOS in human milk	Higher concentrations of HMOS in human milk were correlated to decreased risk of HIV transmission from mother to child. However, higher concentrations of 3'SL were found in HIV transmitting woman.
Wang *et al.* **2015 [77]**	Infants	HMOS in human milk	Breastfed infants had relative higher abundances of Bacteroides, and lower proportions of *Clostridium*, *Streptococcus*, *Enterococcus* and *Veillonella* than infants fed formula milk.
Kuhn *et al.* **2015 [34]**	Infants	HMOS in human milk	Higher concentrations of 2'FL and LNF-I were found in human milk from HIV non-transmitting woman.
Sprenger *et al.* **2017 [36]**	Infants	HMOS in human milk	FUT-2 associated oligosaccharides in human milk in infants at high risk of allergy development, and born via C-section are associated with lower risk of IgE-associated eczema.
Seppo *et al.* **2017 [37]**	Infants	HMOS in human milk	Low LNFP-III concentrations in human milk was related to an increased likelihood to develop cow's milk allergy, compared high concentrations of LNFP-III in infants.

Reference	Model	NDO	Main effect of intervention
	Clinical		
Grüber *et al.* **2010 [44]**	Infants	neutral oligosaccharides + AOS	Prebiotic supplemented formula resulted in a significant lower rate of atopic dermatitis compared normal formula in infants. Incidence of atopic dermatitis in prebiotic supplemented infants was in a similar range compared to breast fed infants.
Moro *et al.* **2006 [27]**	Infants	GOS & FOS	GOS and FOS dose-dependently increased in *Bifidobacteria* and *Lactobacilli*, in infants receiving prebiotic supplemented formula compared to non-supplemented formula.
Arslanoglu *et al.* **2008 [28]**	Infants	scGOS + lcFOS	Infants receiving scGOS + lcFOS had a lower incidence of allergic manifestations, in addition, fewer physician-diagnosed respiratory tract infections, fever episodes, and antibiotic prescriptions were recorded.
De Kivit *et al.* **2012 [138]**	Infants	scGOS + lcFOS	scGOS + lcFOS in combination with *B. breve* M-16V induced higher serum galectin-9 levels, which is associated with allergy prevention.
Goehring *et al.* **2016 [185]**	Infants	GOS + 2'FL[3]	GOS + 2'FL supplemented formula fed infants had similar plasma inflammatory cytokine concentrations compared to breast fed infants. Infants fed with the GOS diet had significantly increased levels of inflammatory cytokines present in plasma.

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CHAPTER 3

Specific human milk oligosaccharides differentially promote Th1 and regulatory responses in a CpG-activated epithelial/ immune cell coculture

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Abstract

Proper early life immune development creates a basis for a healthy and resilient immune system, which balances immune tolerance and activation. Deviations in neonatal immune maturation can have life-long effects, such as development of allergic diseases. Evidence suggests that human milk oligosaccharides (HMOS) possess immunomodulatory properties essential for neonatal immune maturation. To understand the immunomodulatory properties of enzymatic or bacterial produced HMOS, the effects of five HMOS (2′FL, 3FL, 3′SL, 6′SL and LNnT), present in human milk have been studied. A PBMC immune model, the IEC barrier model and IEC/PBMC transwell coculture models were used, representing critical steps in mucosal immune development. HMOS were applied to IEC cocultured with activated PBMCs. In the presence of CpG, 2′FL and 3FL enhanced IFNγ ($p < 0.01$), IL10 ($p < 0.0001$) and galectin-9 ($p < 0.001$) secretion when added to IEC; 2′FL and 3FL decreased Th2 cell development while 3FL enhanced Treg polarization ($p < 0.05$). IEC were required for this 3FL mediated Treg polarization, which was not explained by epithelial-derived galectin-9, TGFβ nor retinoic acid secretion. The most pronounced immunomodulatory effects, linking to enhanced type 1 and regulatory mediator secretion, were observed for 2′FL and 3FL. Future studies are needed to further understand the complex interplay between HMO and early life mucosal immune development.

Keywords: early life nutrition, human milk oligosaccharides, intestinal epithelial cells, mucosal immunity, non-digestible oligosaccharides

Introduction

The third most abundant solid component in human milk are the human milk oligosaccharides (HMOS). HMOS are highly variable and complex fibers with over 150 different structures identified [1]. Each mother possesses a unique combination of HMOS depending on genetics, diet, environment and stage of lactation [2], [3]. Neonates are unable to digest HMOS, but these can be fermented by the intestinal microbiome [4]. However, HMOS can also cross the mucosal linings since they can be detected in the serum and urine of breastfed infants [5]–[7]. More evidence is gathering that HMOS possess immunomodulatory properties important for neonatal immune maturation via the intestinal microbiome and/or by directly affecting both mucosal and systemic immunity [8]. Deviations in neonatal immune maturation may have life-long effects, such as predisposing the infant to develop immune related disorders, such as allergic diseases [9].

The presence of HMOS is limited in commercial formula feeding; however, infant formulas are mostly supplemented with prebiotic non-digestible oligosaccharides such as long chain fructo-oligosaccharides (lcFOS) and short chain galacto-oligosaccharides (scGOS). These prebiotic fibers can suppress the production of pro-inflammatory cytokines and promote the diversity of the microbiome, similar but not identical to breastfed infants [10], [11]. Recent developments in large-scale chemical production of HMOS have presented the potential of supplementing formula with several types of manufactured HMOS [12], potentially further improving immune development in formula fed infants. Several HMOS can be manufactured by bacterial production, such as genetically modified bacteria [13]–[16] as well as enzymatic conversion of lactose [17], [18]. Although the final products should be similar, contaminants, such as endotoxins, may influence immunological responses to these products.

2′-Fucosyllactose (2′FL) is the most abundant HMOS present in human milk; however, not all mothers express this HMOS and produce 3-fucosyllactose (3FL) instead. This is due to a variation in the FUT2 (fucosyltransferase 2) gene that adds a fucose group via α1-2 linkage onto galactose [19]. In addition, the secretion of 2′FL decreases during lactation while 3FL secretion increases over time [20]. Therefore, it would be of interest to investigate whether 3FL has similar or complementary immunomodulatory properties compared to 2′FL. Despite the structural resemblance, the binding of the fucose group to glucose, as opposed to galactose, may result in differential immunological outcomes as binding affinity to immune receptors is affected by these structural differences [21], [22]. In addition, similar differences are observed in the structurally comparable sialylated HMOS 3′-sialyllactose (3′SL) and 6′-sialyllactose (6′SL). The secretion of 3′SL in human milk increases over time while 6′SL concentrations are found to decline [20]. Although a large proportion of the HMOS contain a fucose or sialic acid group, many of the HMOS structures are composed without these terminating groups; lacto-N-neotetraose (LNnT) is an example of such undecorated HMOS [1].

The early life induction of tolerance and strengthening of Th1 type immunity is of importance due to the infant's Th2-skewed immune system, which may pose the neonate at risk to develop allergies [23]. A dominant presence of Th2 type cytokines, such as IL13, disturbs intestinal epithelial integrity, increasing the leakage of allergens into the lamina propria [24]. Maintaining barrier integrity and stimulation of tolerogenic signals induces the regulation of immune responses through the tolerogenic polarization of the intestinal epithelial cells (IEC) and crosstalk

with underlying immune cells. Tolerogenic signals result in the release of TGFβ, galectin-9 and retinoic acid by IECs, which promotes the development of regulatory T (Treg) cells, homeostasis and protection of mucosal surfaces [25], [26].

A specific mixture of non-digestible oligosaccharides (9:1 mixture of scGOS and lcFOS) co-incubated with synthetic CpG, a TLR9 ligand used to mimic the presence of intestinal bacterial DNA, increased the secretion of galectin-9 from IEC and subsequently promoted the release of IFNγ and IL10 by peripheral blood mononuclear cells (PBMCs) in an *in vitro* IEC/PBMC coculture model [27]. Next to the scGOS/lcFOS mixture, 2′FL promotes Th1 type and regulatory immune development as well by increasing galectin-9 and TGFβ release from the intestinal epithelium upon exposure to the oligosaccharides and CpG [28]. In addition, a coculture of IEC exposed to non-digestible oligosaccharides and CpG with activated PBMCs resulted in enhanced IFNγ and IL10 and reduced IL13 secretion [28].

In this current manuscript, we studied the immunomodulatory effects of five enzymatically produced HMOS (see Table 1), which are present in human milk, using three different *in vitro* models for the intestinal barrier and (mucosal) immune functioning (Figure 1). In addition, the immunomodulatory effects of enzymatically produced 2′FL and 3FL were compared to bacterial produced 2′FL and 3FL to study the impact of production method on the immunomodulatory effects. Finally, the role of IEC in the immunomodulatory effects of the HMOS was investigated. The secretion of cytokines from both IEC and PBMCs and phenotype of PBMCs was determined to assess functional immune outcomes.

Materials and methods

Isolation of human peripheral blood mononuclear cells

Human PBMCs were purified from buffy coats from healthy donors (Sanquin, Amsterdam, The Netherlands). Cells were separated by density gradient centrifugation (1000× g, 13 min) and washed with PBS containing 2% FCS. An enriched cells fraction was harvested, and erythrocytes were lysed for 5 min using a red blood cell lysis buffer (4.14 g NH_4Cl, 0.5 g $KHCO_3$, 18.6 mg Na_2EDTA in 500 mL demi water, sterile filtered, pH = 7.14). PBMCs were carefully resuspended in RPMI1640 (Gibco, Waltham, MA, USA) supplemented with 2.5% FCS, penicillin (100 U/mL) and streptomycin (100 µg/mL).

Preparation of HMOS

Lyophilized enzymatically (lactose-derived) produced 2′FL, 3FL LNnT, 3′SL and 6′SL (Carbosynth, Berkshire, UK) and bacterially (*E. coli*) produced 2′FL and 3FL (Jennewein Biotechnologie GmbH, Rheinbreitbach, Germany) (Table 1) were dissolved in plain McCoy's 5A or DMEM/F12 medium (Gibco), consequently sterile filtered (0.2 µm filter) and stored at −20 °C until further use.

PBMC immune model

Freshly isolated PBMCs (2 × 10^6 cells/mL) were activated with anti-CD3 (150 ng/mL, clone CLB-T3/2) and anti-CD28 (100 ng/mL, clone CLB-C28) (Sanquin, Amsterdam, The Netherlands). A fraction (estimated to be up to 4%) of the ingested HMOS could be transported

over the intestinal epithelium and become systemically available [6,7,29]. Therefore PBMCs were directly exposed to 0.01%, 0.05% or 0.1% (w/v) concentrations of HMOS for 24 h to mimic the systemic availability of low HMOS concentrations. After 24 h, viability of the PBMCs was assessed, and supernatants were collected and stored at −20 °C to determine cytokine levels.

IEC barrier model

T84 cells were grown in 75 cm^2 flasks until 80% confluency in DMEM/F12 medium (Gibco, Waltham, MA, USA) supplemented with Glutamax (Invitrogen, Waltham, MA, USA), 10% fetal calf serum (FCS) and the antibiotics penicillin (100 U/mL) and streptomycin (100 g/mL) (Sigma-Aldrich, Saint Louis, MO, USA). After trypsinization, samples were 5 times diluted and seeded in 12 well transwell inserts (Costar Corning Incorporated, Saint Louis, MO, USA). Cells were grown for 4 weeks to reach confluency and establish differentiation. Differentiation and barrier integrity of cells was assessed by trans-epithelial electrical resistance (TEER) using a Millicell ERS-2 Volt-ohm meter (Merck Millipore, Burlington, NJ, USA). IEC were exposed to 10 ng/mL IL4, IL5, IL9 or IL13 (Prospec, Ness-Ziona, Israël) to explore induction of type 2 mediated barrier disruption. HMOS were added 24 h prior to barrier disruption by 10 ng/mL IL13. The degree of barrier disruption was measured TEER in the following 48 h.

Table 1. *Origin, purity and endotoxin content of the HMOS used in the experiments.*

Structure	Origin	Purity	Endotoxin (EU/mg)
2′FL	Enzymatic	>95%	0.03
3FL	Enzymatic	96.8%	0.0626
2′FL	Bacterial	92.7%	0.0175
3FL	Bacterial	94.1%	0.025
LNnT	Enzymatic	99.9%	0.0616
3′SL	Enzymatic	95.8%	0.0516
6′SL	Enzymatic	98.1%	1.023

IEC/PBMC coculture model

The human colon cancer cell line HT-29 ((HTB38) ATCC, Manassas, VA, USA) was used as a model for intestinal epithelium in the IEC/PBMC coculture model. The HT-29 cells were cultured in McCoy 5A medium (Gibco, Waltham, MA, USA) supplemented with 10% FCS and the antibiotics penicillin (100 U/mL) and streptomycin (100 µg/mL) (Sigma-Aldrich, Saint Louis, MO, USA) in 75 cm^2 cell culture flasks (Greiner Bio-One, Alphen aan den Rijn, The Netherlands). Cells were incubated at 37 °C and 5% CO_2. Medium was refreshed every 2–3 days, and cells were cultured until 80–90%, and upon trypsinization, 5× diluted cell suspensions were seeded in 12 wells transwell inserts (Costar Corning Incorporated, Saint Louis, MO, USA). Cells were grown for 6 days to reach confluency under normal culturing conditions. After 24 h of preincubation with the 5 selected HMOS (at 0.1% and 0.5% concentrations), the cell culture medium, including HMOS, was refreshed, and IEC were basolateral exposed to freshly isolated PBMCs (2×10^6 cells/mL) activated with anti-CD3 (150 ng/mL, clone CLB-T3/2)

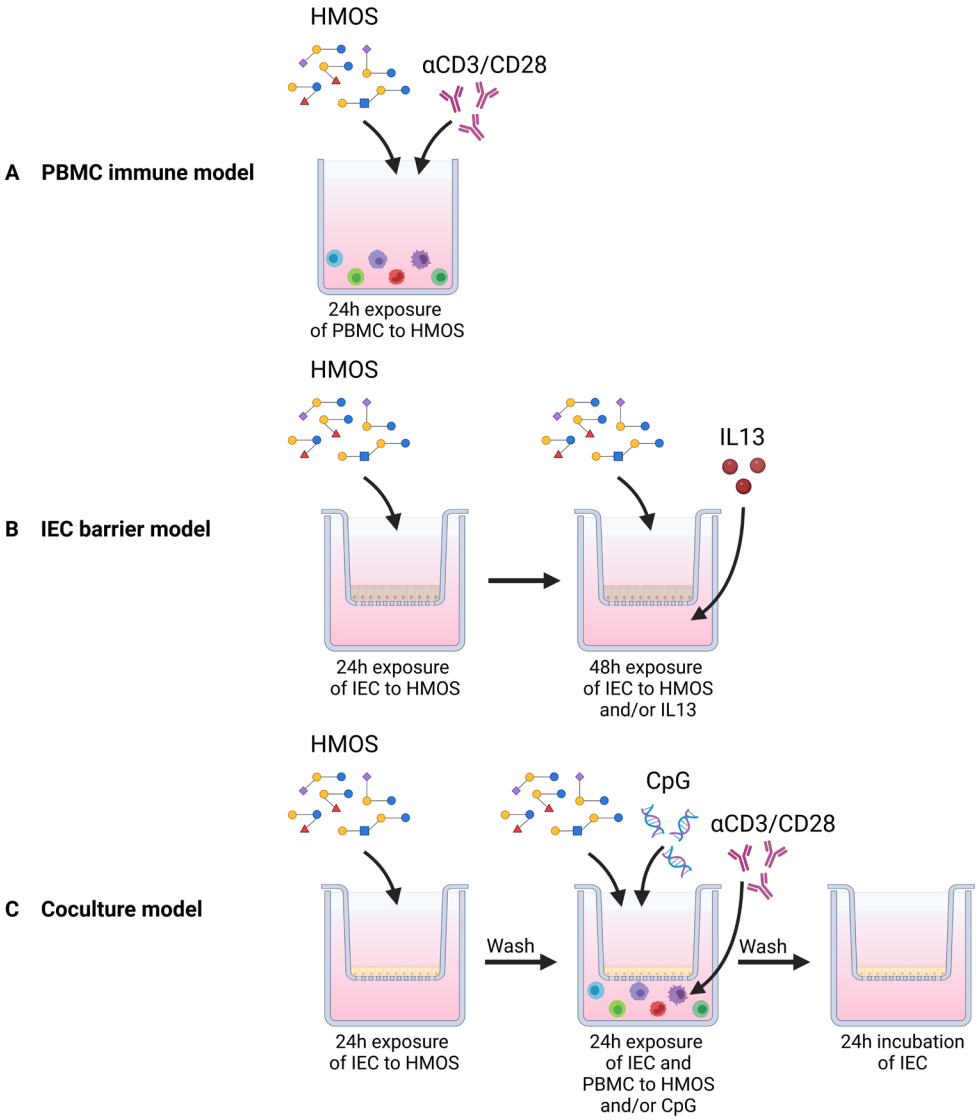

Figure 1. *Schematic overview of the different models described in this manuscript. (A) The PBMC immune model allowed for direct exposure of activated PBMCs to HMOS. Freshly isolated PBMC were activated using anti-CD3 and anti-CD28, while being exposed to HMOS for 24 h. (B) In the IEC barrier model, T84 cells were cultured in transwell inserts for 4 weeks. Cells were apically pre-exposed to HMOS for 24 h prior to basolateral addition of IL13 to induce barrier disruption. (C) For the IEC/PBMC coculture model, human intestinal IEC (HT29 cells) were cultured on transwell inserts until confluency was reached. IEC were apically exposed to HMOS for 24 h. After preincubation with HMOS, the apical an basolateral medium was refreshed. αCD3/CD28-activated PBMCs were added to basolateral compartment. IEC were apically exposed to HMOS again either in presence or absence of CpG. After 24 h of coculture, basolateral supernatant was collected to measure cytokine secretion and PBMCs were collected for phenotypical analysis. IEC were again washed and incubated for another 24 h in fresh medium to measure epithelial mediator release into the basolateral compartment. Created with Biorender.com*

and anti-CD28 (100 ng/mL, clone CLB-C28) (Sanquin, Amsterdam, The Netherlands). IEC was exposed to apically added CpG (0.5 μM CpG oligodeoxynucleotide (ODN) M362 type C, Invivogen, San Diego, CA, USA). Basolateral supernatant was collected and stored at –20 °C until further analysis after 24 h coculturing of IEC with PBMCs. Subsequently, IEC were separated from the PBMCs and washed with PBS before transferring to a new plate. IEC were cultured for an additional 24 h in fresh medium in the absence of HMOS, and basolateral supernatants were stored at –20 °C for TGFβ and galectin-9 secretion analysis. Phenotype of PBMCs was determined with flow cytometry immediately after coculture.

Enzyme-linked immunosorbent assay

Stored supernatants were analyzed to quantify cytokine secretion according to the manufacturer's protocol. Commercially available kits were used to determine IFNγ, IL9, IL13, TGFβ, TNFα (Thermo Fisher Scientific, Saint Louis, MO, USA), IL10 (U-Cytech, Utrecht, The Netherlands) and retinoic acid (MyBioSource, San Diego, CA, USA).

Galectin-9 was measured using an antibody pair (R&D Systems, Minneapolis, MN, USA). High-binding Costar 9018 plates were coated with 0.75 μg/mL affinity-purified polyclonal antibody overnight at 4 °C. After washing, non-specific binding sites were blocked with 1% BSA in PBS for 1 h before samples were incubated for 2 h at room temperature. Plates were washed before the addition of 0.75 μg/mL biotinylated galectin-9 affinity-purified polyclonal antibody. After a 1 h incubation at room temperature, plates were washed again and streptavidin-HRP (R&D Systems, Minneapolis, MN, USA) was added for 30 min. Next, tetramethylbenzidine (TMB, Thermo Fisher Scientific, Saint Louis, MO, USA) was used as a substrate, and H2SO4 was used to stop the reaction. Optical density was measured using a Promega GloMax microplate reader at 450–655 nm.

Flow cytometry analysis

After 24 h of IEC/PBMC co-culture, PBMCs were collected and stained for analysis with flow cytometry. Viability of the cells was determined using Fixable Viability Dye 780-APC Cyanine 7 (eBioscience, Saint Louis, MO, USA). Immunophenotyping and intracellular cytokine staining was performed using antibodies with appropriate isotypes (eBioscience, Saint Louis, MO, USA; Invitrogen, Saint Louis, MO, USA) (for the list of antibodies, clones and dilutions, see Table S1). Nonspecific binding was prevented by blocking for 15 min with PBS containing 2.5% FCS and Human FC Block (BD Biosciences, Franklin Lakes, NJ, USA) before extracellular and intracellular staining. Cells were fixated and permeabilized with the FoxP3/Transcription Factor Staining Buffer Set or Intracellular Staining Buffer Set (eBioscience, Saint Louis, MO, USA). PBMC measurements was performed using BD FACS Canto II (BD Biosciences, Franklin Lakes, NJ, USA), and data were analyzed by Flowlogic software, version 8.4 (Inivai Technologies, Melbourne, Australia).

Statistical analysis

Data were analyzed using Graphpad Prism software (v8.4.0). Analysis was performed by One-Way ANOVA followed by Bonferroni multiple comparison or Dunnett post hoc test. When data did not fit a normal distribution, square root or logarithmic transformations were performed prior

to analysis. Data are represented as mean ± SEM using healthy, independent PBMC donors for n = 3, n = 6 or n = 9 independent experimental replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Results

Direct immunomodulatory effects of specific HMOS, leading to Th1 type and regulatory cytokine responses from activated PBMCs

First, the direct immunomodulatory effects of the HMOS on freshly isolated human PBMCs were studied, as HMOS are able to cross intestinal epithelial cells to interact with receptors on immune cells [30]. The effects of exposure to low concentrations (0.01%, 0.05% and 0.1%) of HMOS on non-activated PBMCs are shown in Table 2. After exposure to HMOS, PBMCs secreted in general higher levels of IFNγ, which was significantly enhanced with 0.1% 3FL exposure (Table 2). Furthermore, an increase in IL10 secretion was observed after 0.1% 6′SL exposure. The HMOS 2′FL, 3FL, 3′SL and LNnT reduced the levels of galectin-9 in PBMC supernatant compared to the medium control while 6′SL did not. Upon activation using anti-CD3 and -CD28, the PBMCs increased the secretion of IFNγ, TNFα, IL13, IL9 and IL10 (Figure 2). The activated PBMCs were exposed to HMOS for 24 h, mimicking systemic concentrations [7]. An analysis of secreted cytokines revealed that exposing activated PBMCs to 2′FL enhanced the secretion of IL9 and IL10 (Figure 2D,E) while the secretion of other cytokines was hardly affected compared to the activated control. In addition, 3FL exposure resulted in an increase in IL10 (Figure 2E) as well, and a dose dependent increased the secretion of IFNγ (Figure 2A) while TNFα levels were decreased (Figure 2B). Both sialylated HMOS, 3′SL and 6′SL, induced an increase in IL10 secretion from PBMCs, and even though 3′SL showed an inclining pattern for IFNγ and IL9 and 6′SL for TNFα, this did not reach significance (Figure 2K). in contrast, the undecorated LNnT only reduced the secretion of proinflammatory TNFα and basal levels of galectin-9 (Figure 2N,R).

IL13 induced barrier disruption in T84 cells is not prevented by HMOS pre-exposure

Knowing the important role of the epithelial barrier in tolerance induction and strengthening of Th1 immunity to compensate for Th2 skewing in early life, the differential effects of type 2 inflammatory mediators on barrier disruption using T84 cells was studied. Confluent and differentiated T84 cells were basolateral exposed to the type 2 related cytokines IL4, IL5, IL9 and IL13 for 48 h. Barrier integrity, determined by TEER, was decreased upon exposure to IL4 and IL13, yet IL5 and IL9 did not affect the barrier integrity (Figure 3A). As IL4 and IL13 are known to bind similar receptors (e.g., type II IL4Rα) [31], further experiments were conducted using only IL13. Subsequently, confluent and differentiated T84 cells were apically pre-exposed to 0.1% or 0.5% 2′FL, 3FL, 3′SL, 6′SL or LNnT for 24 h. HMOS were refreshed while IL13 was added to the basolateral compartment for 48 h. The development of TEER values was followed after IL13 exposure. As depicted in Figure 3B–D, none of the HMOS affected basic barrier properties of the T84 cells in the absence of IL13 nor prevented the IL13 induced barrier disruption.

Table 2. *Effects of HMOS on mediator secretion from nonactivated PBMCs. All conditions were compared to Control using a One-Way ANOVA wit Dunnett post-hoc test (n=6, [1] p = 0.0740, [2] p = 0.0813, * p < 0.05, ** p < 0.01, *** p < 0.001).*

(pg/mL)	Control	0.01% 2'FL	0.05% 2'FL	0.1% 2'FL	0.01% 3FL	0.05% 3FL	0.1% 3FL	0.01% 3'SL	0.05% 3'SL	0.1% 3'SL	0.01% 6'SL	0.05% 6'SL	0.1% 6'SL	0.01% LNnT	0.05% LNnT	0.1% LNnT
IFNγ	5.7 ± 3.5	18.5 ± 11.8	41.3 ± 23.6	145.7 ± 108.3	40.7 ± 35.4	182.5 ± 87.9[1]	193.5 ± 101.8 *	77.2 ± 36.2	125.4 ± 45.7	63.5 ± 23.5	5.7 ± 3.6	144.2 ± 65.6	78.2 ± 37.8	19.8 ± 12.1	38.3 ± 15.3	164.6 ± 94.6
TNFα	1424 ± 802.8	1195 ± 746.0	1185 ± 476.4	703.8 ± 258.2	633.6 ± 260.2	699.4 ± 142.6	905.1 ± 257.6	1083 ± 455.4	826.5 ± 332.6	673.8 ± 248.5	2526 ± 1922	2175 ± 1129	2757 ± 1501	700.5 ± 282.1	908.7 ± 499.5	980.5 ± 528.1
IL13	236.4 ± 91.4	128.2 ± 39.6	123.8 ± 50.5	167.7 ± 71.1	128.2 ± 56.4	181.6 ± 55.5	184.0 ± 57.0	101.0 ± 57.0	160.2 ± 93.4	82.9 ± 32.1	253.3 ± 100.4	129.4 ± 37.5	119.3 ± 24.4	158.1 ± 68.0	105.5 ± 33.6	139.3 ± 35.8
IL9	8.5 ± 1.9	17.8 ± 7.0	33.2 ± 16.9 [2]	22.8 ± 4.7	23.9 ± 8.7	16.2 ± 4.7	16.3 ± 6.4	20.8 ± 6.3	24.8 ± 7.3	17.1 ± 5.2	15.4 ± 3.5	20.3 ± 4.1	26.2 ± 9.9	27.2 ± 7.2	21.4 ± 7.2	18.9 ± 6.5
IL10	113.9 ± 63.7	134.4 ± 61.8	164.4 ± 92.0	398.7 ± 160.8	68.4 ± 46.2	164.1 ± 136.0	161.5 ± 90.4	296.4 ± 163.5	269.5 ± 142.6	250.2 ± 144.1	111.5 ± 41.0	442.7 ± 139.6	612.9 ± 127.4 *	156.3 ± 53.5	58.1 ± 36.6	153.6 ± 111.8
Gal-9	11755 ± 2229	5386 ± 1669 *	3646 ± 1693 **	4740 ± 1435 *	2221 ± 655.1 ***	3041 ± 1131 **	2777 ± 924.6 ***	1483 ± 332.8 ***	1815 ± 575.6 ***	4325 ± 2077 *	9040 ± 1097	9219 ± 3542	4470 ± 1395 *	2604 ± 1087 ***	3828 ± 1111 ***	3122± 1090 ***

Crosstalk between IEC and PBMC is differentially affected by 2′FL and 3FL

To further investigate the immunomodulatory effects of the HMOS 2′FL and 3FL, the well-studied HT29-activated PBMC coculture model was used [27,28,32]. Two sources (enzymatic modified lactose or bacterially produced) of 2′FL and 3FL were studied. IEC were apically exposed to the HMOS for 24 h before the apical addition of CpG and basolateral addition of anti-CD3/CD28 activated PBMC to allow cellular crosstalk. In this coculture model, it was previously shown that only under CpG-activated conditions epithelial cells were able to enhance galectin-9 expression and secretion. This correlated with increased IFNγ and IL10 secretion of underlying PBMC, which was further increased by non-digestible oligosaccharides [27,33].

In the absence of CpG, the enzymatic 2′FL exposure did not result in altered cytokine secretion. However, in the presence of CpG, 2′FL enhanced IFNγ and IL10 secretion in the IEC/PBMC coculture, as well as IEC-derived galectin-9 from after the coculture as compared to exposure to medium and/or CpG alone (Figure 4A,C,F). Exposure to 0.1% 2′FL further increased the secretion of IFNγ and IL10 compared to the CpG control while 0.5% 2′FL further enhanced galectin-9 secretion compared to the CpG control. CpG alone only slightly enhanced IL10 secretion compared to the medium controls (Figure 4C). On the other hand, 0.5% 3FL enhanced IFNγ secretion from the IEC/PBMC coculture and TGFβ release by IEC derived from the coculture in the absence of CpG (Figure 4A,E). When combined with CpG, both concentrations of the 3FL enhanced secretion of IL10 from IEC/PBMC coculture and galectin-9 from IEC compared to the medium controls (Figure 4C,F).

When the coculture was performed with bacterially derived 2′FL and 3FL, different cytokine secretion profiles were found. Both concentrations of 2′FL enhanced IFNγ and IL13, both in the presence or absence of CpG compared to the medium or CpG alone (Figure 4G,H). In addition, 0.5% 2′FL also significantly enhanced TNFα levels independent of CpG exposure and IL10 in the presence of CpG (Figure 4I,J). Bacterially derived 3FL did not show any immunomodulatory effects but increased TGFβ secretion from IEC at 0.5% only when combined with CpG (Figure 4K).

These data indicate that 2′FL and 3FL differentially enhance Th1 type, Th2 type and regulatory cytokine secretion from IEC and PBMC coculture depending on the 2′FL and 3FL production method. Therefore, the production method affects the immunomodulatory properties of HMOS and should be taken into account when designing experiments and interpreting their outcomes. As the most profound direct immunomodulatory effects were found for 2′FL and 3FL, this manuscript will further focus on these HMOS structures. The findings for 3′SL, 6′SL and LNnT are presented in Figure S1.

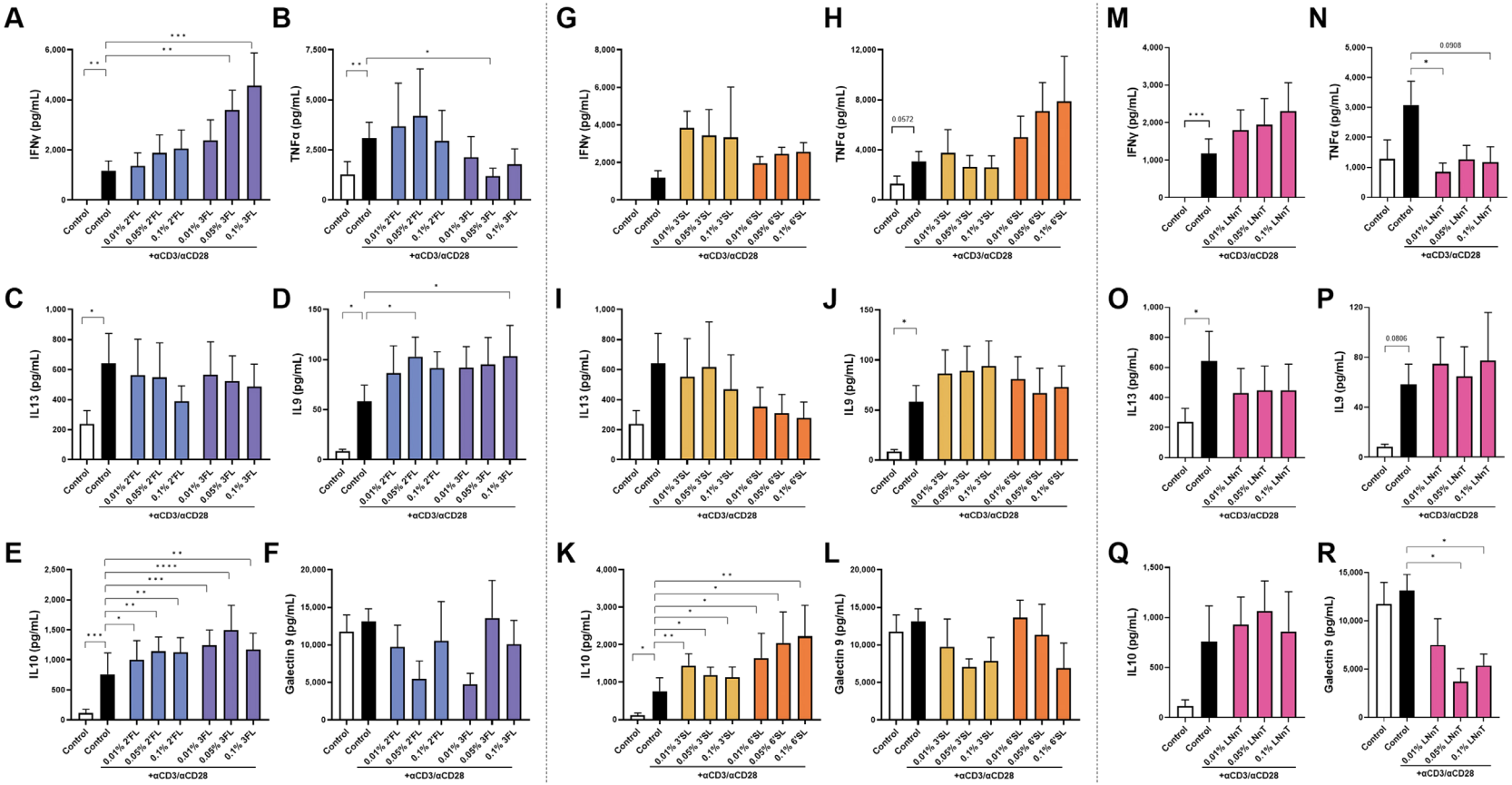

Figure 2. *PBMCs were exposed directly low concentrations of all HMOS for 24 h while activated using αCD3 and αCD28. Concentrations of (**A**,**G**,**M**) IFNγ, (**B**,**H**,**N**) TNFα, (**C**,**I**,**O**) IL13, (**D**,**J**,**P**) IL9, (**E**,**K**,**Q**) IL10 and (**F**,**L**,**R**) galectin-9 were assessed in the supernatant. Data is analyzed by One-Way ANOVA followed by a Dunnett post-hoc test, n = 6 independent experiments using different donors, mean ± SEM (* p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.).*

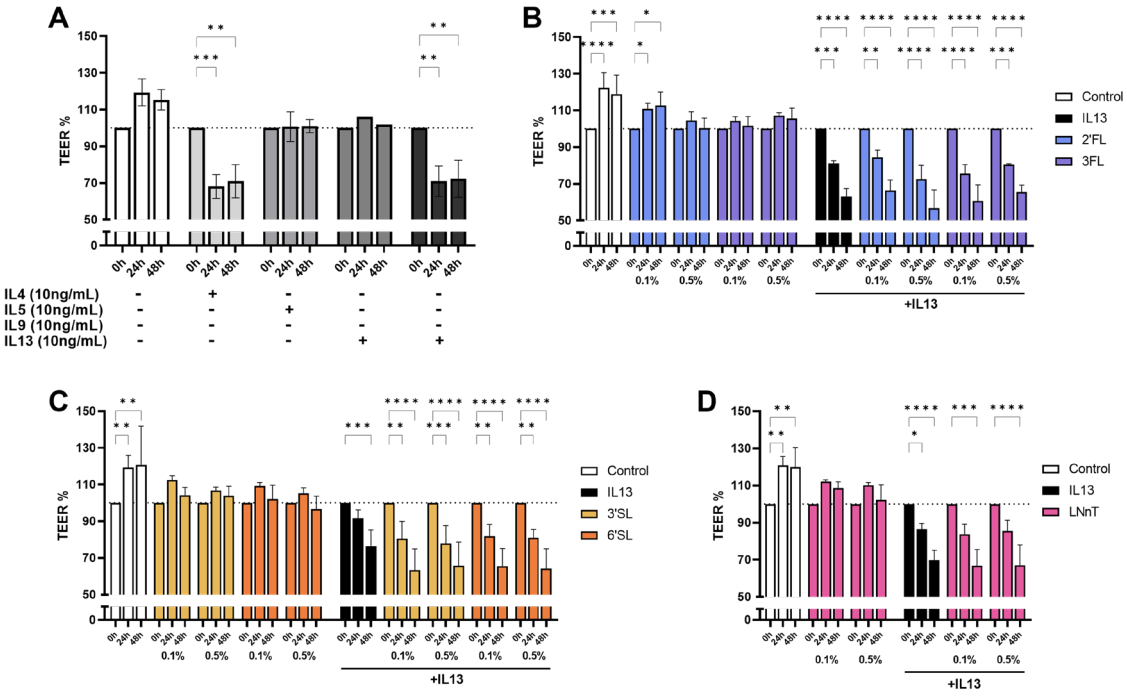

Figure 3. *Effects of HMOS on IL13-mediated IEC barrier disruption. T84 cells were grown on transwells and (A) exposed to the Th2-type cytokines IL4, IL5, IL9 and IL13 after which transepithelial electrical resistance (TEER) was measured at 24 h and 48 h. To investigate preventive effects of HMOS on barrier disruption by IL13, IEC were preincubated with (B) 2'FL and 3FL, (C) 3'SL and 6'SL, and (D) LNnT 24 h prior to exposure to IL13. Data is analyzed by Two-Way ANOVA followed by a Bonferroni post-hoc test, n = 6, mean ± SEM (* p < 0.05, ** p < 0.01, *** p < 0.001, ****p < 0.0001).*

Enzymatic 3FL decreases Th2 type development and enhances the polarization of Tregs

After 24 h of IEC/PBMC coculture, PBMCs were collected for flow cytometric analysis. Percentages of Th1 type (CXCR3 + in CD4 + cells), Th2 type (CRTH2 + in CD4 + cells) and Tregs (FoxP3 + in CD25 + CD4 + cells) were determined. A representative example of flow cytometry gating is shown in Figure 5G. The percentage of Th1 type cells was not affected by exposure to 2′FL or 3FL irrespective of the presence of CpG (Figure 5A,D). However, the percentage of Th2 type cells was decreased by enzymatically derived 0.1% 2′FL and both concentrations 3FL, but not by their bacterial analogues, compared to CpG exposed cells (Figure 5B,E). In addition, bacterially derived 0.5% 2′FL enhanced the frequency of the Treg population (Figure 5F), which was not observed for the enzymatically derived 2′FL (Figure 5C). Furthermore, only the enzymatically derived 0.5% 3FL increased the percentage of Treg cells (Figure 5C).

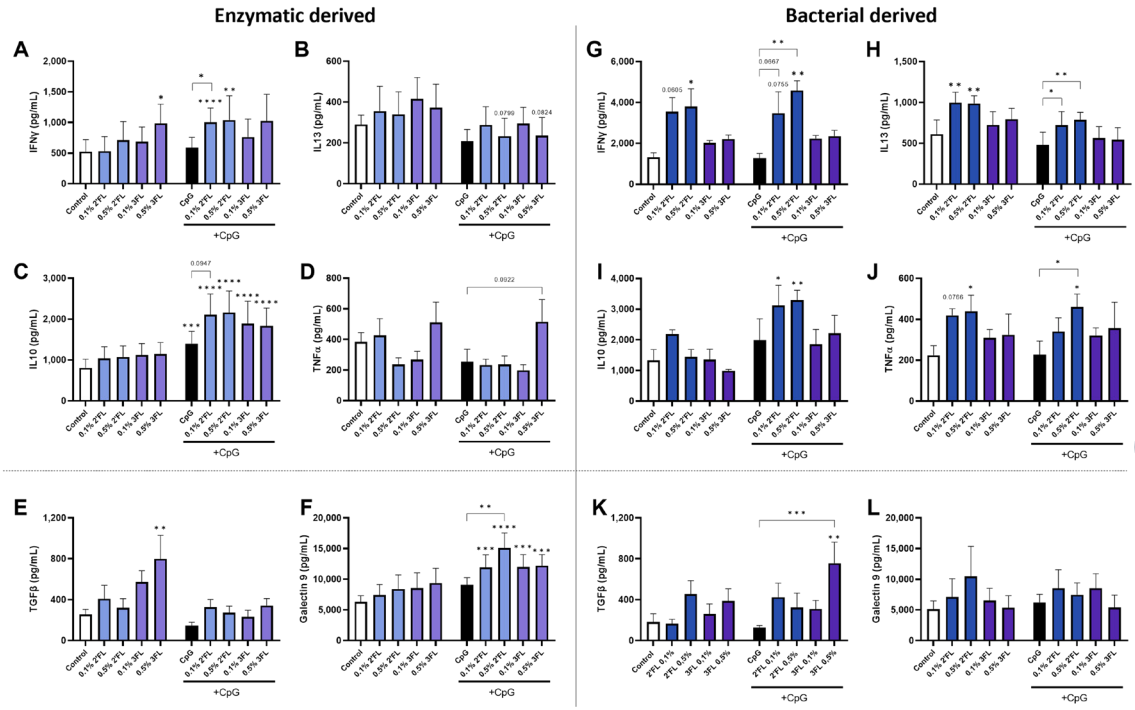

Figure 4. *24h after preincubation of IEC with HMOS, IEC were cocultured with activated PBMCs for another 24 h, while fresh HMOS and/or CpG were added to the apical compartment of the transwell. After this coculture, cytokine secretion was measured in the basolateral compartment. Inserts containing IEC were transferred to a new plate, HMOS and CpG were washed away and IEC were cultured for another 24 h to detect epithelial derived TGFβ and galectin-9 in the basolateral compartment. Release of (A) IFNγ, (B) IL13, (C) IL10 and (D) TNFα upon coculture of IEC and activated PBMC while exposing IEC to enzymatic derived 2'FL and 3FL, as well as IEC derived (E) TGFβ and (F) galectin-9 after coculture. In addition, release of (G) IFNγ, (H) IL13, (I) IL10 and (J) TNFα upon coculture of IEC and activated PBMC while exposing IEC to bacterial derived 2'FL and 3FL, as well as IEC derived (K) TGFβ and (L) galectin-9 IEC after coculture. Data is analyzed by One-Way ANOVA followed by a Bonferroni post-hoc test, n = 9 for synthetically produced HMOS, n = 3 for bacterial produced HMOS, mean ± SEM (* p < 0.05, ** p < 0.01, *** p < 0.001, ****p < 0.0001).*

IEC are required for 3FL mediated Treg polarization and IEC enhance 2′FL and 3FL mediated Th1 type and Th2 type cytokine responses

Epithelial-derived regulatory mediators such as galectin-9, TGFβ and retinoic acid are known to contribute to the differentiation of Tregs [34–36]. Therefore, the role of IEC in the immunomodulatory effect of enzymatic 2′FL and 3FL was investigated by comparing HMOS exposure in the absence and presence of HT29 cells in the IEC/PBMC coculture model.

The differentiation of Th1 (CXCR3 + CD4 cells) and Th2 (CRTH2 + CD4) cells was not significantly affected during exposure to either 2′FL or 3FL combined with CpG in the absence of IEC compared to the presence of IEC (Figure 6A,B). However, the improved Treg development upon the exposure of IEC to CpG and 3FL was abrogated when IEC were not present (Figure 6C). The combined exposure to CpG and 2′FL or 3FL enhanced the secretion of IFNγ and IL13

in the presence of IEC compared to exposure to CpG alone. This effect was lost when IEC were not present (Figure 6D,E). On the other hand, the secretion of IL10 in response to CpG plus 2′FL or 3FL was significantly further enhanced when IEC were not present (Figure 6F). After the coculture, IEC were washed and set apart for another 24 h to measure epithelial-derived regulatory mediators. Galectin-9 and TGFβ concentrations were significantly increased upon the exposure of IEC to 2′FL, and 3FL showed a similar pattern compared to control IEC exposed to CpG alone (Figure 6G,H). Retinoic acid produced by the IEC was also detected, but no significant differences were found in the used conditions (Figure 6I). The epithelial-derived mediators in Figure 6G–I are not displayed in the conditions without IEC; the medium background levels are indicated with a dotted line.

Discussion

HMOS are thought to play a pivotal role in the development of the gut microbiome, maturation of the gastrointestinal tract and shaping of a resilient and innate and adaptive immunity in infants [30]. The relative high abundance of fucosylated and sialylated oligosaccharides in human milk especially is unique among mammals [37]. HMOS promote the intestinal barrier integrity and support innate and adaptive immune responses [30], [38]–[40]. Aside from acting on the immune system via the microbiome, HMOS have also been found to directly interact with immune cells [41]. These direct effects are difficult to distinguish in *in vivo* models and clinical studies due to the presence of the microbiome. Furthermore, the large structural variety of HMOS makes identifying structure–function relationships from pooled HMOS challenging, although improved immune maturation has been attributed to the total mixture of HMOS present in human milk [41]–[44]. Using an established transwell mucosal immune model combining both IEC and activated PBMC coculture and the presence of a bacterial or allergic trigger, differential immunomodulatory properties of specific HMOS have been identified. The current study was designed to explore the immunomodulatory effects of 5 different HMOS: 2′FL, 3FL, 3′SL, 6′SL and LNnT, which are currently produced in larger quantities.

Antigen exposure to the mucosal immune system may lead to (mucosal) immune activation, as may be the case in early infancy due to immature gut barrier function. This further drives immune maturation and tolerance development, or alternatively may prime for developing immune disorders like food allergy in the case of a type 2 prone immune polarization [45]. To study whether the HMOS are able to modify the direction of immune activation, PBMCs were exposed to a low dosage of HMOS, as it is known that a small fraction of the ingested HMOS can become systemically available after ingestion [7], [46], [47]. Only limited effects on cytokine secretion from non-activated PBMCs were observed after HMOS exposure (Table 2). Therefore, PBMCs were activated using anti-CD3 and -CD28 antibodies, which initiate inflammatory and regulatory cytokine release via activating the T cells within the PBMCs. Both fucosylated and sialylated HMOS significantly enhanced the secretion of the regulatory cytokine IL10 in activated PBMCs, which was not observed in non-activated PBMCs. Previously, it was shown that monocyte-derived dendritic cells exposed to pooled HMOS enhance IL10 secretion [41]. However, enhanced IL10 secretion for the undecorated LNnT was not observed, which did reduce the release of proinflammatory TNFα. Furthermore, 3FL, but not the structurally similar

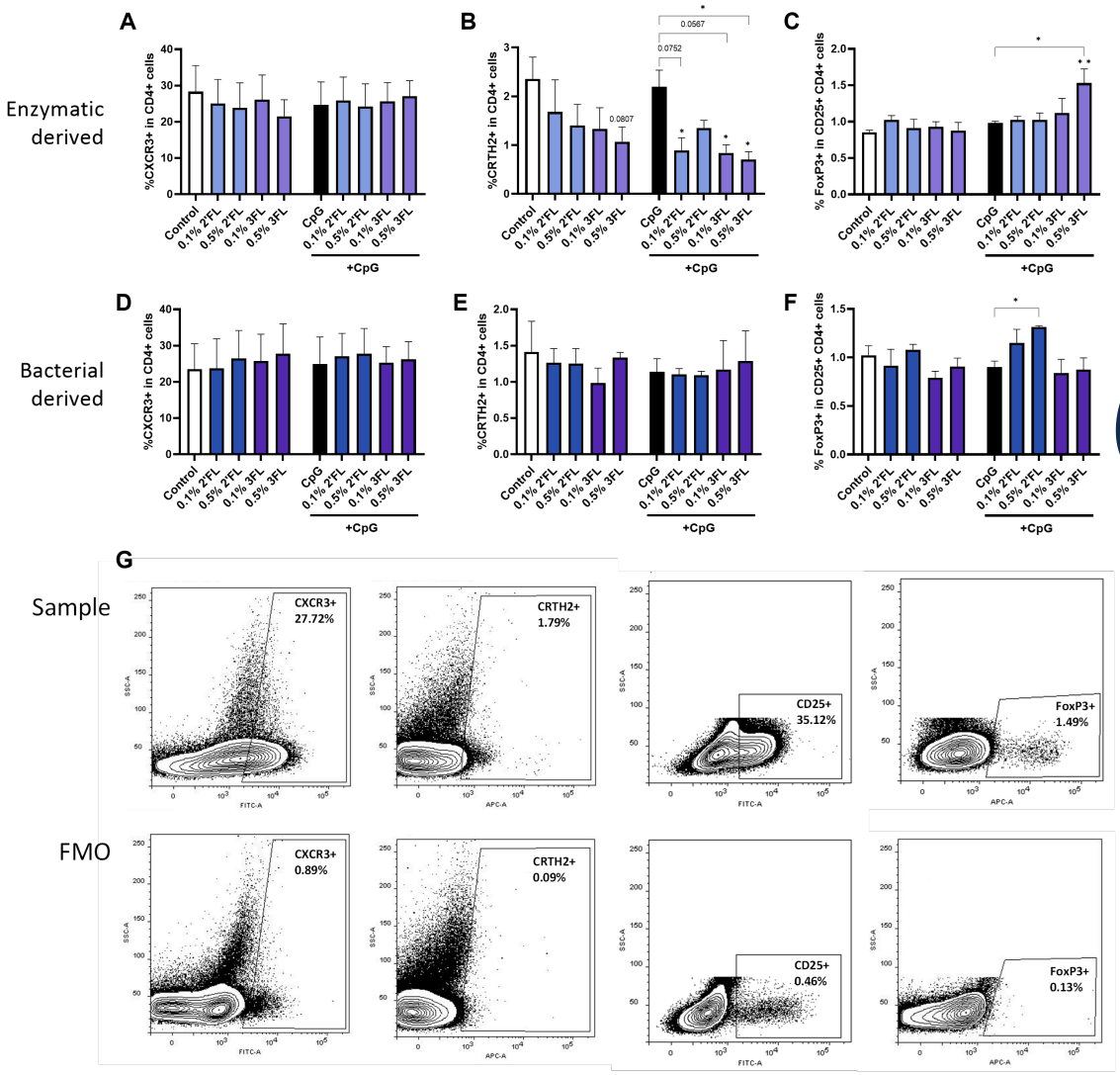

Figure 5. *After 24 h coculture of HMOS and CpG exposed IEC cocultured with activated PBMCs, basolateral PBMCs were collected for phenotypical analysis by flow cytometry. Expression (A, D) of CXCR3, as well as (B, E) CRTH2 in CD4+ cells was assessed to represent Th1-type and Th2-type cells. Expression of (C, F) FoxP3 + in CD25 + CD4 + cells was assessed, representing Treg cells. The upper row displays results of enzymatic derived 2'FL and 3FL, while the lower row displays bacterial derived 2'FL and 3FL. (G) A representative sample and corresponding FMO controls displaying the gating strategy of Th cell subset markers. Data is analyzed by One-Way ANOVA followed by a Bonferroni post-hoc test, n = 3, mean ± SEM (* p < 0.05, ** p < 0.01).*

2′FL, enhanced secretion of type 1 IFNγ while also reducing TNFα release, potentially promoting a regulatory Th1 response important for immune maturation. These fucosylated HMOS are structurally similar and comparable immunomodulatory effects could be hypothesized. However, these present data demonstrate that the structurally similar 2′FL and 3FL interact differently with PBMCs, which is in line with differences in receptor affinity profiles described for these fucosylated HMOS as previously reviewed [30].

Although the high abundance of fucosylated oligosaccharides distinguishes human milk from milk from other mammalian species, sialylated oligosaccharides are present in relatively high levels in the milk of most mammals [48]. Sialylated HMOS have been linked to cognitive development and health via the direct supply of sialic acid to the brain or indirectly via the gut–brain axis [49]. Therefore, investigating their immunomodulatory properties is relevant to gain insights into potential mechanistic routes. Although a few studies have looked into the effects of 3′SL and 6′SL on epithelial cells [50], [51], the direct immunomodulatory effects have been questioned [52], [53]. This current study demonstrates an increase in IL10 release while proinflammatory cytokines were unaffected by 3′SL and 6′SL in activated PBMCs. These effects were only observed after the activation of PBMCs; in non-activated PBMCs galectin-9 levels dropped with 3′SL exposure and only 0.1% 6′SL enhanced IL10 secretion. Combined with the relatively limited effects in the IEC/PBMC mucosal coculture model (Figure S1), these data indeed indicate minimal immunomodulatory effects, in the currently used models, mediated by these specific sialylated HMOS. Nevertheless, a role within the complex mixture of HMOS or other cell models cannot be excluded.

The final HMOS structure investigated in this study is LNnT, a neutral, less abundant and undecorated HMOS. LNnT has been studied mainly in preclinical and clinical settings; however, *in vitro* findings show that LNnT has affinity for galectins, including galectin-9, which has a regulatory function [21], [25], [54]. Here, LNnT lowered TNFα and galectin-9 when added directly to activated PBMC; however, these effects were lost when immunomodulatory effects of LNnT were studied in the mucosal immune coculture of IEC/PBMC (Figure S1). Preclinical and clinical studies demonstrate positive effects from LNnT supplementation on microbiota composition [55], which is supported by only a few *in vitro* studies reporting improved epithelial maturation and proliferation during exposure to LNnT [50,56]. However, to the best of our knowledge, this is one of the first studies investigating direct effects of LNnT on immune cells.

HMOS support the intestinal epithelial barrier of Caco-2:HT29-MTX cultures under healthy and inflammatory conditions [57] and may, therefore, support gut maturation in early infancy as well. For example, 2′FL reduces the secretion of proinflammatory cytokines while enhancing epithelial barrier integrity during LPS or chemotherapy exposure *in vitro* [57]–[59], yet 3FL increased the expression of MUC2, encoding the most abundant protein in intestinal mucus, in LS174T cells during TNFα and IL13 exposure [60]. In the present study, we could not detect effects on the epithelial tight junction barrier integrity with allergic phenotype (i.e., challenged with IL13), nor in the absence of an inflammatory trigger. However, studies investigating HMOS and barrier integrity mainly focus on fermentation products of HMOS rather than the direct effects of HMOS themselves, nor include a type 2 inflammatory milieu, which is relevant during early life [61], [62]. The current study does not indicate a direct effect of the diverse HMOS structures on epithelial integrity, and no protection against type 2 related barrier disruption was observed.

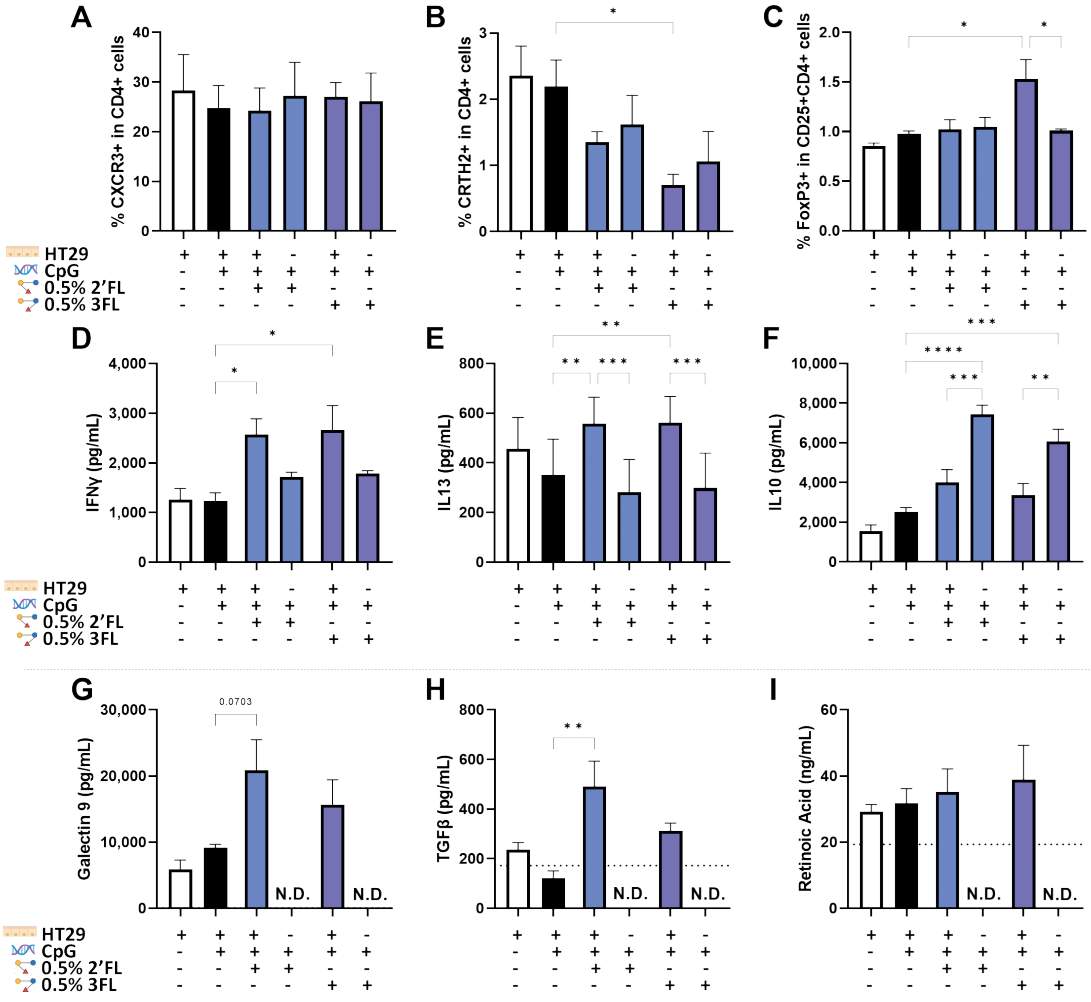

Figure 6. *The role of IEC in HMOS plus CpG mediated immunomodulation. This was assessed by comparing PBMC that were exposed to 2'FL and 3FL combined with CpG in absence or presence of IEC in the transwell. Th1-type, Th2-type and Treg cells were assessed by expressing (A) CXCR3 in CD4 + cells, (B) CRTH2 in CD4+ cells and (C) FoxP3 in CD25 + CD4+ cells. In addition, secretion of (D) IFNγ, (E) IL13, and (F) IL10 in the basolateral compartment was determined after exposure to 2'FL and 3FL combined with CpG in absence or presence of IEC. In addition, epithelial-derived (G) galectin-9, (H) TGFβ and (I) retinoic acid were measured when IEC were set apart after coculture. Basal concentrations in culture medium are indicated with the dotted line, N.D. is mentioned when concentrations were not determined. Data is analyzed by One-Way ANOVA followed by a Bonferroni post-hoc test, n = 3, mean ± SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).*

Beyond providing a barrier, intestinal epithelial cells however actively contribute to shaping of mucosal immune function as well. Therefore, we investigated the effects of HMOS on the immunological crosstalk between human IEC and immune cells using a validated HT-29/PBMC coculture model, which was developed for this purpose [27], [28], [63], [64]. In this model the two individual sialylated HMOS or LNnT did not give a clear immunomodulatory signature (Figure S1); therefore, next steps were focused on 2′FL and 3FL.

Recently, techniques have been developed to produce HMOS on larger scale, which makes these structures available for commercial purposes. Enzymatically and bacterially produced 2′FL and 3FL have been compared and it was demonstrated that the most pronounced immunomodulatory effects with these HMOS were shown on activated PBMCs. CpG is added in this model as an analogue for bacterial DNA (binding to TLR9) driving a regulatory type 1 response, which is amplified by several oligosaccharides as was previously investigated [27], [28], [32], [33]. In this manuscript, immunological responses were found to differ between enzymatic and bacterial produced HMOS. These differences could not be ascribed to differences in endotoxin contaminations in enzymatic or bacterial produced HMOS (presented in Table 1). The endotoxin contamination level of all used HMOS is low and not in a range to expect to significantly contribute to the immunological responses [33], [65]. In the presence of CpG, enzymatic 2′FL further enhanced regulatory and Th1 type cytokine release from PBMCs and galectin-9 secretion from IEC, while also diminishing Th2 cell development. Bacterial 2′FL enhanced the cytokine release in general while enhancing Treg development, in the presence of CpG. In the absence of CpG bacterial 2′FL enhanced the secretion of both type 1 and type 2 related cytokines including pro-inflammatory TNFα, while regulatory cytokine secretion and Th cell differentiation was unaffected. Bacterial derived 3FL did not have these immune stimulatory effects, but was found to enhance epithelial derived TGFβ secretion in the presence of CpG.

Similar to enzymatic produced 2′FL also enzymatic produced 3FL, enhanced IL10 in the IEC/PBMC coculture in the presence of CpG while increasing galectin-9 release by the epithelial cells. Even though 3FL did not further enhance IFNγ in the CpG conditions, 0.5% 3FL significantly enhanced Treg cell development combined with a reduction in Th2 cells. These data indicate that bacterial derived 2′FL generally drives immune activation while 3FL is less effective. By contrast, enzymatic 2′FL and 3FL selectively drive away from the allergic phenotype while enhancing either a regulatory type Th1 (2′FL) or regulatory T cell response (3FL) in association with increase epithelial derived galectin-9 secretion. Previous studies have indicated epithelial derived galectin-9 to correlate positively with IFNγ and/or IL10 release by the activated PBMC [27], [28], which may be one of the mechanisms by which 2′FL and 3FL exposed IEC are able to modify mucosal immune function and influence immune maturation. Thus, these results demonstrate different immunomodulatory outcomes from the structurally similar 2′FL and 3FL. Furthermore, the bacterial versus enzymatic origin of HMOS plays a role in immunological outcomes and should therefore be taken into account in future studies when investigating HMOS. To our knowledge this is the first study comparing manufactured HMOS from different origins. Future experiments should also include biological HMOS isolated from human milk as an internal standard for immunomodulation.

To further investigate the role of IEC in the immunomodulatory effects of 2′FL and 3FL combined with CpG, the IEC/PBMC coculture experiments were performed in the presence or absence of IEC in the transwell using only the enzymatic produced 2′FL and 3FL. Previously the

type 1 supporting effects of nondigestible oligosaccharides was shown to depend on the presence of IEC in the CpG exposed IEC/PBMC coculture model [27]. Indeed also in the current study, in the presence of CpG, 2′FL was found to enhance type 1 IFNγ release only in the presence of IEC. CpG can contribute to reduced type 2 IL13 secretion in the HT-29/PBMC coculture model [27], [63] which may be further enhanced by nondigestible oligosaccharides. This effect was not shown for 2′FL and 3FL, however it appeared that direct exposure of activated PBMC to CpG in absence of IEC resulted in a reduced type 2 response. CpG is a bacterial DNA surrogate known for its capacities to drive away from the allergic phenotype and immunoregulatory properties [33]. This may also explain the further increase in regulatory type IL10 secretion when 2′FL and 3FL plus CpG were exposed to activated PBMC in absence of IEC.

In particular, 3FL was found to decrease the Th2 type population while increasing the Treg population. Interestingly, this effect was depended on the presence of IEC, which was most prominent for Treg development. Mediators produced by epithelial cells in this coculture model may be required for the impact on polarization patterns in Th cells as was previously shown for epithelial derived galectin-9. Galectin-9 was found to contribute to increased IFNγ, IL10 secretion and Treg development in activated PBMC [27], [52]. Therefore, we measured the epithelial-derived regulatory mediator galectin-9. Furthermore, TGFβ and retinoic acid are known to be able to induce Treg formation [34]–[36]. Galectin-9 was indeed secreted by the epithelial cells, yet not significantly increased by 2′FL and 3FL on top of the CpG. However, as indicated galectin-9 is known to contribute to type 1 and regulatory type immune responses and may also play a role in the immunomodulatory effects of 2′FL and 3FL. In addition, in the 2′FL exposed conditions epithelial derived TGFβ was increased, which indicates that 2′FL indeed is able to enhance the regulatory function of epithelial cells. However, the increase in Treg population supported by 3FL in the presence of IEC and CpG could not be explained by enhanced release of TGFβ and/or retinoic acid by the epithelial cells. Future experiments should therefore focus on additional factors derived from IEC contributing to Treg development in underlying immune cells.

To validate the findings in the current manuscript, more complex mucosal immune models that mimic the sequential steps in mucosal immune activation should be used to further investigate the immunomodulatory effects of different HMOS. Furthermore, this study only investigated five relatively simple HMOS structures while the total pool of HMOS in human milk contains many more different structures. This leaves opportunities to investigate other single structures as well as mixtures of HMOS which could potentially lead to an optimized HMOS mixture suitable to be added to infant formula in order to promote the optimal immune development of infants that cannot receive breast milk.

Conclusions

HMOS are thought to possess immunomodulatory effects; however, it has only recently become possible to study specific HMOS structures that can be produced for commercial purposes. In this current the effects of five HMOS; 2′FL, 3FL, 3′SL, 6′SL and LNnT in several *in vitro* models for intestinal barrier and systemic or mucosal immune modulation have been investigated. Although none of the HMOS protected against type 2 mediated barrier disruption, these data demonstrate the most pronounced immunomodulatory effects for 2′FL and 3FL. This was affected by the HMOS origin and the presence or absence of IEC combined with CpG. 3′SL, 6′SL and LNnT had minimal immunomodulatory effects in the models tested. Enzymatically produced 3FL showed a regulatory type Th1 immune supportive effect while lowering general inflammation when added directly to activated PBMC. Both enzymatically produced 2′FL and 3FL showed regulatory effects when combined with CpG in the mucosal IEC/PBMC immune model, but in this model 2′FL also increase type Th1 immunity while 3FL enhanced Treg conversion. Future studies using more complex mucosal immune models and/or using more specific immune cell types are warranted to further study the direct immunomodulatory capacities of complete collections of HMOS in order to further understand their role in early life immune development and their potential application in infant formula.

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Supplement

Table S1. *Overview of antibodies used for flow cytometry. Appropriate isotype controls were used for FMO stainings. All antibodies were purchased from eBioscience or Biolegend and titrated prior to use.*

Marker	Fluorochrome	Clone	Isotype	Dilution
CD4	PerCP Cyanine 5.5	OKT4	m IgG2b, k	1:320
CD25	Alexa Fluor 488	BC96	m IgG1, k	1:150
CXCR3	Alexa Fluor 488	1C6/CXCR3	m IgG1, k	1:80
CRTH2	APC	BM16	r IgG2a, k	1:150
FoxP3	APC	PCH101	r IgG2a, k	1:320

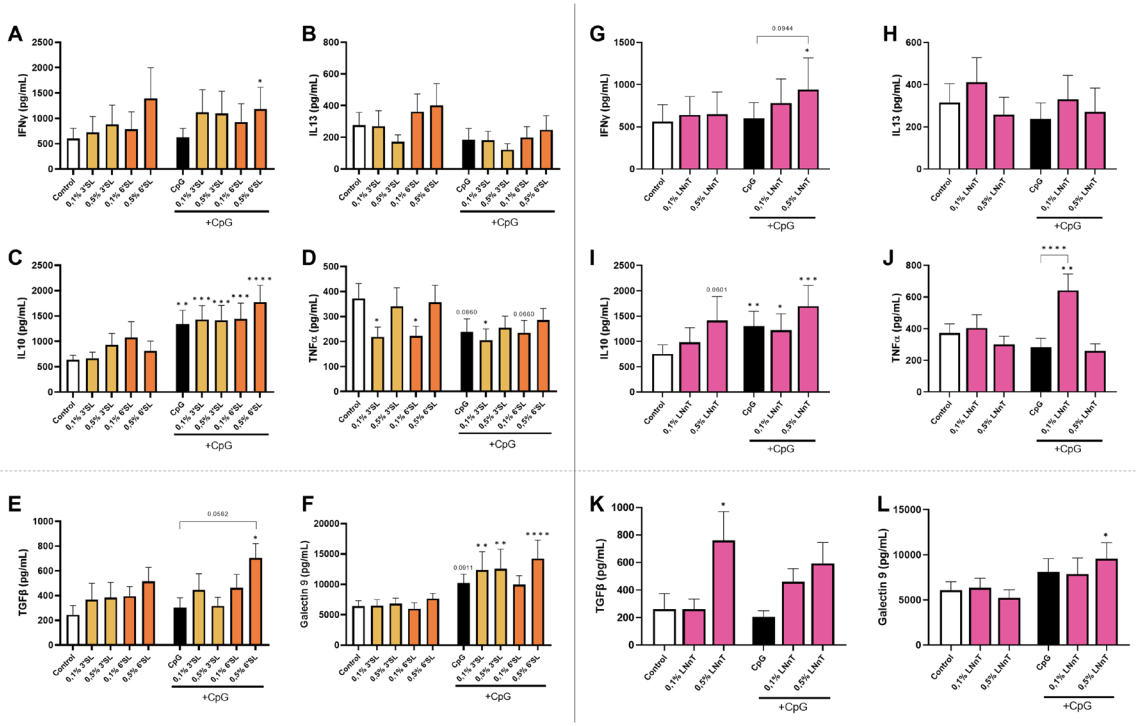

Figure S1. *24h After preincubation of IEC with HMOS, IEC were cocultured with activated PBMCs for another 24h while fresh HMOS and/ or CpG were added to the apical compartment of the transwell. After this coculture, cytokine secretion was measured in the basolateral compartment. Inserts containing IEC were transferred to a new plate, HMOS and CpG were washed away and IEC were cultured for another 24h to detect secreted TGFβ and galectin-9 in the basolateral compartment. Release of A) IFNγ, B) IL13, C) IL10 and D) TNFα upon coculture of IEC and activated PBMC with synthetic produced 3'SL and 6'SL, as well as IEC derived E) TGFβ and F) galectin-9 after coculture. In addition, release of G) IFNγ, H) IL13, I) IL10 and J) TNFα upon coculture of IEC and activated PBMC while exposing IEC to synthetic produced LNnT was determined, as well as IEC derived K) TGFβ and L) galectin-9 after coculture. Data is analyzed by One-Way ANOVA followed by a Bonferroni post-hoc test, n = 9 independent experiments using different PBMC donors, mean ± SEM (* p < 0.05, ** p < 0.01, *** p < 0.001, ****p < 0.0001).*

CHAPTER 4

Allergenic shrimp tropomyosin distinguishes from a non-allergenic chicken homolog by pronounced intestinal barrier disruption and downstream Th2 responses in epithelial and dendritic cell (co)culture

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Submitted

Abstract

Tropomyosins (TM) from vertebrates are generally non-allergenic, while invertebrate homologs are potent pan-allergens. This study aims to compare risk on sensitization between chicken TM and shrimp TM via affecting intestinal epithelial barrier integrity and type 2 mucosal immune activation. Epithelial activation and/or barrier effects upon exposure to 2-50 μg/mL chicken TM, shrimp TM or ovalbumin (OVA) as control allergen, were studied using Caco-2, HT-29MTX, or HT-29 intestinal epithelial cells. Monocyte-derived dendritic cells (moDC), cocultured with HT-29 cells or moDC alone, were exposed to 50 μg/mL chicken TM or shrimp TM. Primed moDC were cocultured with naïve Th cells. Intestinal barrier integrity (TEER), gene expression, cytokine secretion and immune cell phenotypes were determined in these human *in vitro* models. Shrimp TM, but not chicken TM or OVA exposure, profoundly disrupted intestinal barrier integrity and increased alarmin genes expression in Caco-2 cells. Proinflammatory cytokine secretion in HT-29 cells was only enhanced upon shrimp TM or OVA, but not chicken TM, exposure. Shrimp TM enhanced the maturation of moDC and chemokine secretion in the presence or absence of HT-29 cells, while only in the absence of epithelial cells chicken TM activated moDC. Direct exposure of moDC to shrimp TM increased IL13 and TNFα secretion by Th cells cocultured with these primed moDC, while shrimp TM exposure via HT-29 cells cocultured with moDC sequentially increased IL13 expression in Th cells. Shrimp TM, but not chicken TM, disrupted the epithelial barrier while triggering type 2 mucosal immune activation, both key events in allergic sensitization.

Keywords: epithelial barrier, food allergy, mucosal immunology, sensitizing allergenicity, tropomyosins

Introduction

Food allergic diseases are a growing health problem in Western societies. A recent systematic review and meta-analysis described that up to almost 10% of children are burdened with physician diagnosed food allergy [1]. Most food allergic reactions are induced by peanut-, cow's milk-, egg-, soy-, wheat, tree nut-, fish-, and shellfish-containing products [2], but numerous other food allergens have been identified [3]. Allergenic food proteins often possess intrinsic properties to promote allergic sensitization, including intestinal epithelial activation and permeabilization [4]–[9]. This mucosal immune activation can lead to the activation of dendritic cells (DC) and subsequent polarization of a type 2 T cell response which drives the development of allergen-specific IgE production and IgE-mediated clinical reactions upon subsequent encounter with the allergen [10]. Currently, there is a lack of *in vitro* models enabling to discriminate between proteins in food products with low or high risk for allergic sensitization [11], [12].

Tropomyosins are functional proteins present in all eukaryotic cells and key regulators of muscle contraction [13]. Typically in most vertebrate species, including chicken, these tropomyosins have a low sensitization and allergenicity risk [14], [15]. However, tropomyosins that can be found in arthropods, such as crustaceans and insects, or in mollusks are important allergens. These proteins consist of an evolutionary highly conserved dimeric α-helical structure, shown by the 54-60% shared sequence between vertebrate and invertebrate tropomyosins [16]. Recently it was described that a vertebrate (chicken) and invertebrate (shrimp) tropomyosin differ in gastric digestion, but both bound to IgE from shrimp-allergic patients. However, a positive skin prick test was only observed with shrimp tropomyosin in these patients [17]. In several types of existing assays, the allergic effector response is characterized and associated with allergenic potential that would indicate towards the risk of an allergic reaction. However, sensitizing allergenicity identifies the possible intrinsic property of a protein to activate type 2 driving immune cascades and, thus, increase the risk of allergic sensitization and food allergy development. The first step in this cascade is the breaching and/or activation of the epithelial barrier followed by activation of DC and a type 2 T cell response [18]. A previous study has shown this response for ovalbumin (OVA) as model allergen, which was able to activate intestinal epithelial cells (IEC) and/or monocyte derived dendritic cells (moDC) [19].

This study aims to investigate the differential capacity of chicken TM (low sensitizing capacity) versus shrimp TM (high sensitizing capacity) in epithelial barrier disruption and activation, and in mucosal type 2 activation. Human *in vitro* models were used, including intestinal epithelial cells and blood derived immune cells, which may provide tools to study the intrinsic sensitizing properties of current and future dietary proteins.

Materials & methods

Allergenic proteins

High-allergenic recombinant black tiger shrimp tropomyosin (Pen m 1; shrimp TM) and low-allergenic recombinant chicken tropomyosin (chicken TM) were expressed in *E. coli* and purified as previously described [17], [20]. A detailed description of cloning, expression and isolation can be found in the Supplemental Methods. A 'Control' condition was used in every cell

culture experiment, this control condition was not exposed to any additional protein component. Ovalbumin (OVA) was used as an allergenic protein model known to induce activation of IEC and/or moDC to drive sequential mucosal type 2 responses [19], [21].

Caco-2, HT-29MTX and HT-29 cell culture

Several human IEC model cell lines (colon carcinogenic origin) were used. Caco-2 cells were used for epithelial barrier studies, HT-29 cells were used to study epithelial cytokine responses. Caco-2 cells (passages 28-33 and 45-47) from the American Type Culture Collection (ATCC, USA) were seeded at a density of $1x10^5$ cells/well onto 12 well or $1.67x10^4$ cells/well onto 24 well transwell inserts (0.4 μm pore size; Costar, Corning, USA) and grown 2-3 weeks post confluency before use for barrier studies. HT29-MTX cells (methotrexate selected HT29 clone, gifted by Dr. Giblin from Teagasc Food Research Centre, Moorepark, Fermoy, Ireland) were seeded in a 3:1 ratio (Caco-2:HT-29MTX) at a density of $3x10^4$ cells/well onto 12 well transwell inserts (0.4 μm pore size; Costar, Corning). Additionally, Caco-2 cells were seeded and cultured for 21 days or HT-29 cells were seeded and cultured into confluent layers for 6 days in 96 wells plates. More information can be found in the Supplemental Methods.

Transepithelial electrical resistance (TEER) and cytotoxicity assay

Barrier permeability of transwell cultured Caco-2 or mixed Caco-2/HT-29MTX monolayer, featuring characteristics of polarized enterocytes and mucous producing cells, was measured using a Millicell ERS-2 electrical-resistance system (Millipore, USA) prior to and during allergen exposure and TEER was expressed as ohm•cm2.

The LDH (lactate dehydrogenase) activity cytotoxicity assay was carried out in the supernatants of exposed Caco-2 cells by using the CyQUANT™ LDH Cytotoxicity Assay Kit (Invitrogen) according to the manufacturer's instructions and expressed as absorbance value (OD490-655nm).

Caco-2 and PBMC coculture

Healthy volunteer donor peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient separation (Ficoll-Histopaque, GE Healthcare, Barcelona Spain) from heparinized venous blood samples from 5 non-allergic subjects obtained from the Institute of Food Science Research (CIAL, Spain) blood library. Written informed consent was obtained from all participants, according to the procedures approved by the Bioethics Committee of the CIAL.

PBMCs ($2x10^6$ cells/well) were added to the basolateral compartment of 12-well transwell plates where Caco-2 cells (at passage 28-33) where previously fully differentiated. Chicken TM, shrimp TM or OVA were apically added to the Caco-2 and PBMCs coculture and incubated for 8 h at 37°C and 5% CO_2. Caco-2 cells were preserved at -80°C in RA-1 buffer (Macherey-Nagel, Düren, Germany) for gene expression analyses by qPCR (See Supplemental Methods).

HT-29-moDC or moDC and sequential DC/T cell coculture

This coculture model to study mucosal immunity was previously described in more detail [19]. In brief, healthy donor PBMC from volunteers who had given written informed consent for research purposes, were obtained from the Dutch Blood bank (Amsterdam, The Netherlands). Monocytes and naïve Th cells were isolated using negative selection by magnetic beads. Monocytes were

differentiated into immature moDC for 6 days using GM-CSF and IL4, and Th cells were stored in liquid nitrogen. After HT-29 cells reached confluence in the transwells, $5x10^5$ moDC were added to the basolateral compartment for 48h. The epithelial cells in the HT-29/moDC coculture, were apically exposed to chicken TM or shrimp TM. Simultaneously, wells containing moDC without IEC, were also exposed to chicken TM or shrimp TM for 48h, allowing direct interaction with the moDC. Subsequently, moDC were collected for analysis by flow cytometry (FACS CantoII, BD Biosciences, USA) and coculture with allogenic naïve T cells (stimulated with αCD3 and IL2). moDC and T cells (in a 1:10 ratio) were cocultured for 96h. Cells were collected for flow cytometric analysis and supernatants were collected to measure cytokines using ELISA (R&D systems, USA) or multiplex array (Meso Scale Discovery, USA) (See Supplemental Methods).

Statistics

Statistical analysis was performed using Graphpad Prism (version 9.4.1). Data was analyzed using One-Way ANOVA or Friedmann test when data did not fit a normal distribution. All conditions were compared to the control condition, therefore a Dunnett's post hoc test (if normally distributed) or Dunn's post hoc test was performed. A Two-Way ANOVA was applied to analyze TEER data measured at several time points during protein exposure. $p<0.05$ is considered statistically significant, and data is represented as mean ± standard error of mean (SEM) of n=3 or n=5 independent repeats of a full dataset per *in vitro* model.

Results

Shrimp TM decreases epithelial barrier and induces expression of alarmins

First the effects of chicken TM, shrimp TM or OVA exposure on intestinal epithelial barrier integrity were investigated in transwell Caco-2 models. 48h Exposure of Caco-2 cells (Figure 1A) to chicken TM or OVA resulted in an overall increased TEER compared to medium Controls for most concentrations (Figure 1B, D, E). By contrast, all shrimp TM doses, except for 2 µg/mL, resulted in an overall decrease of TEER (Figure 1C). However, at the 6h timepoint only a decrease in TEER by 10, 25 and 50 µg/mL shrimp TM was observed, which was not detected for similar concentrations of chicken TM and OVA. Secretion of interleukin (IL)8, as marker of epithelial activation, was increased in all conditions (Figure 1F), however in this model also some increase in cytotoxicity was measured when exposing Caco-2 to all allergens except the lowest concentrations used (Figure 1G). Subsequent experiments were performed with 50 µg/mL of tropomyosins to provoke an epithelial response, which allows comparison to the concentrations used for OVA-induced IEC activation. In addition, subsequent experiments did not reveal a decrease in viability upon exposure to 50 µg/mL tropomyosins (Supplemental Figure S1D, E).

Next, Caco-2/HT-29MTX cells were cultured in transwells and exposed to 50 µg/mL of chicken TM, shrimp TM or OVA for 8h to validate the decrease in barrier integrity found in Caco-2 cells (Figure 1H). Similar to the Caco-2 cells, TEER was decreased after exposure to shrimp TM in Caco-2/HT-29MTX cultures compared to Control (medium control), this was not observed for chicken TM or OVA (Figure 1I). In addition, a coculture was performed combining

Caco-2 cells grown in 12 wells transwell plates with PBMCs in the basolateral compartment (Figure 1J). After 8h of incubation with chicken TM, shrimp TM or OVA, expression of type 2 associated alarmins *Il33, Il25*, and thymic stromal lymphopoietin (*Tslp*) was increased in Caco-2 cells after exposure to 25 µg/mL and 50 µg/mL shrimp TM (Figure S1A-C; Figure 1K-M). By contrast, 50 µg/mL chicken TM exposure only enhanced *Il33* expression and OVA did not enhance mRNA expression of these alarmins (Figure 1K). In addition, PBMC derived cytokines were measured but these remained below detection limit.

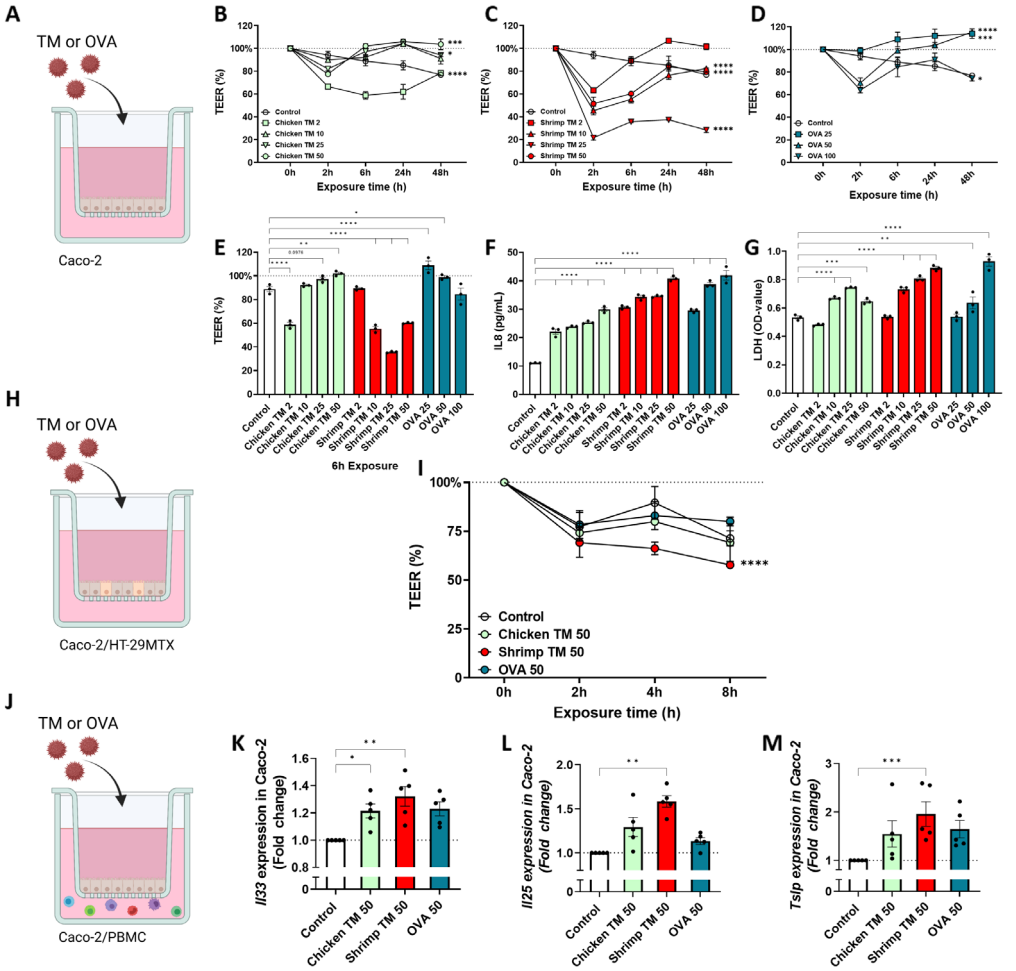

Figure 1*. Caco-2 cells were used as a model for studying allergen-specific effects on the intestinal epithelial barrier and A) they were exposed for 48h to increasing doses of B) chicken TM, C) shrimp TM or D) OVA while the TEER was measured at 0h, 2h, 6h, 24h, and 48h. E) TEER-values after 6h of exposure are presented as well as F) secreted IL8 and G) LDH-release after 48h of protein exposure. H) Cultured Caco-2/HT-29MTX cells were exposed to 50 µg/mL chicken TM, shrimp TM or OVA for 8h and I) TEER was measured at 0h, 2h, 4h, and 8h. J) A coculture of Caco-2 cells with PBMCs was performed to assess the fold change in gene expression of K) Il33, L) Il25, and M) Tslp 8h after 50 µg/mL chicken TM, shrimp TM or OVA exposure. Data was analyzed by Friedman test, One-Way or Two-Way ANOVA, n=3 or n=5, mean ± SEM (* p<0.05, ** p< 0.01, *** p<0.001, **** p< 0.0001).*

Cytokine and chemokine secretion is enhanced in HT-29 cells after allergen exposure

In order to study chicken TM, shrimp TM or OVA induced IEC activation and cytokine secretion, effect on Caco-2 and HT-29 cell lines were compared using multiplex analyses. Cells were grown in 96 well plates and exposed for 48h to 50 μg/mL of each protein (Figure 2A). No cytotoxicity was observed in these cultures (Supplemental Figure S1D-E). Despite increased mRNA expression of *Il33*, *Il25* and *Tslp* in transwell grown Caco-2 cells after shrimp TM exposure (Figure 1K-M), the secretion levels of these cytokines were not elevated after 48h exposure in the 96 wells plates (Figure 2B-D). Yet, CCL20 secretion was significantly increased in this condition (Figure 2G). On the other hand, enhanced secretion of IL25, IL1α, IL1β, CCL22, IL8, and TNFα was shown by HT-29 cells when exposed to shrimp TM, but not with chicken TM exposure which even lowered IL8 secretion (Figure 2C, E, F, H, I, K). Increased cytokine secretion by HT-29 cells was observed to a lesser extent after exposure to 25 μg/mL shrimp TM (Supplemental Figure S1F). OVA exposure also significantly enhanced secretion of ILβ, CCL22, and IL8 (Figure 2F, H, I). A dose-response experiment was performed in HT-29 cells and epithelial derived mediators were measured by ELISA. Secretion of IL8 and CCL20 from HT-29 cells in response to both shrimp TM or OVA exposure dose-dependently increased, while chicken TM did not affect these mediators (Supplemental Figure S2A-E).

Shrimp TM induces moDC activation in presence and absence of IEC

To study the sensitizing capacity of chicken TM and shrimp TM, these proteins were added to HT-29 cell/moDC (IEC-DC) coculture or moDC (DC) in absence of HT-29 cells for 48h (Figure 3A). After exposure, chicken TM increased the proportion of moDC expressing the costimulatory receptor CD80 (Figure 3B) as well as CCL22 secretion (Figure 3E). A small increase in CCL20 secretion was observed (Figure 3F), but only during direct exposure of moDC. Exposure to shrimp TM also increased the percentage of moDC expressing CD80 (Figure 3B) and secretion of CCL20, CCL22, and IL8 (Figure 3E-G) when exposed to IEC-DC or directly to moDC. During direct shrimp TM exposure to moDC, the percentage of cells expressing OX40L was also increased (Figure 3D).

Shrimp TM promotes a type 2 T cell response via moDC

After exposing IEC-DC and moDC to chicken TM or shrimp TM, the primed moDC were collected for a subsequent coculture with allogenic naïve Th cells for 4 days. Viability was assessed after each culture step (Supplemental Figure 3). The viability of IEC was minorly reduced (by 25%) due to exposure to 50 μg/mL shrimp TM, yet the viability of DC was reduced by 53%. However, shrimp TM exposure to IEC-DC did not affect the viability of the underlying moDC (Supplemental Figure S3). After the moDC/T cell coculture, the T cell response was assessed based on Th subset development and cytokine secretion. The percentage of Th2 cells, based on CRTH2 expression, was not affected by coculture with chicken TM or shrimp TM primed moDC (Figure 4B). The percentage of cells expressing intracellular type 2 IL13 was enhanced in T cells cocultured with shrimp TM primed IEC-DC (Figure 4C), while secretion of IL13 was enhanced in T cells cocultures with shrimp TM primed DC (Figure 4D). Furthermore, the proportion of CXCR3+ Th1 cells, was decreased upon coculture with shrimp TM primed moDC (Figure 4E).

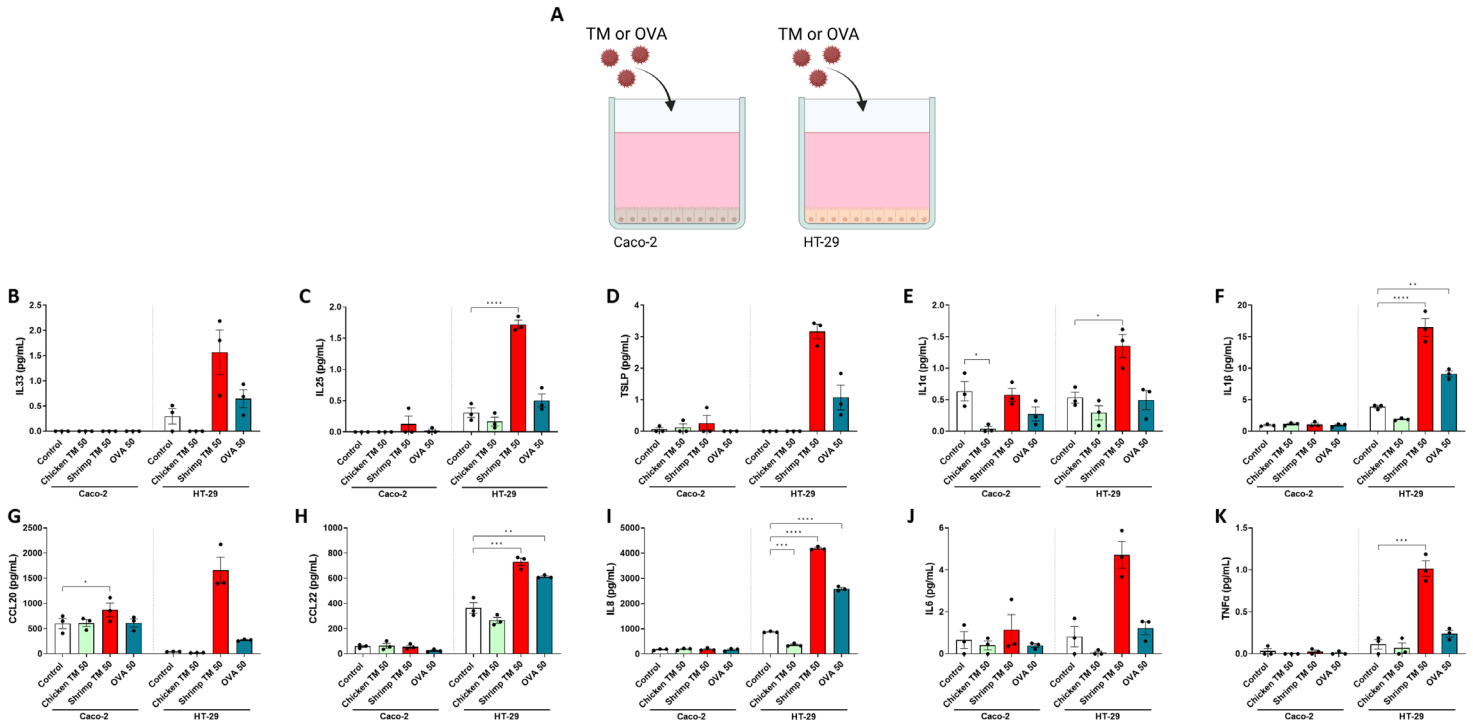

Figure 2. *A) Caco-2 and HT29 cells were culture in 96 well flatbottom culture plates prior to 48h exposure to 50 µg/mL chicken TM, shrimp TM or OVA. Supernatants were collected to measure B) IL33, C) IL25, D) TSLP, E) IL1α , F) IL1β, G) CCL20, H) CCL22, I) IL8, J) IL6 and K) TNFα secretion by multiplex array. Data was analyzed per cell line by One-Way ANOVA or Friedman test if data did not fit a normal distribution, , n=3, mean ± SEM (* p<0.05, ** p< 0.01, *** p<0.001, **** p< 0.0001).*

Although the secretion of type 1 IFNγ was not significantly changed upon coculture with allergen primed moDC (Figure 4F). Secretion of IL17 was decreased upon coculture with chicken TM or shrimp TM primed IEC-DC or moDC (Figure 4G). Furthermore, TNFα and IL21 secretion was significantly enhanced upon coculture of T cells with shrimp TM primed moDC (Figure 4H, J), while the secretion of regulatory IL10 (Figure 4I) was not affected. Supplemental Figure S4 shows a heatmap of overall data.

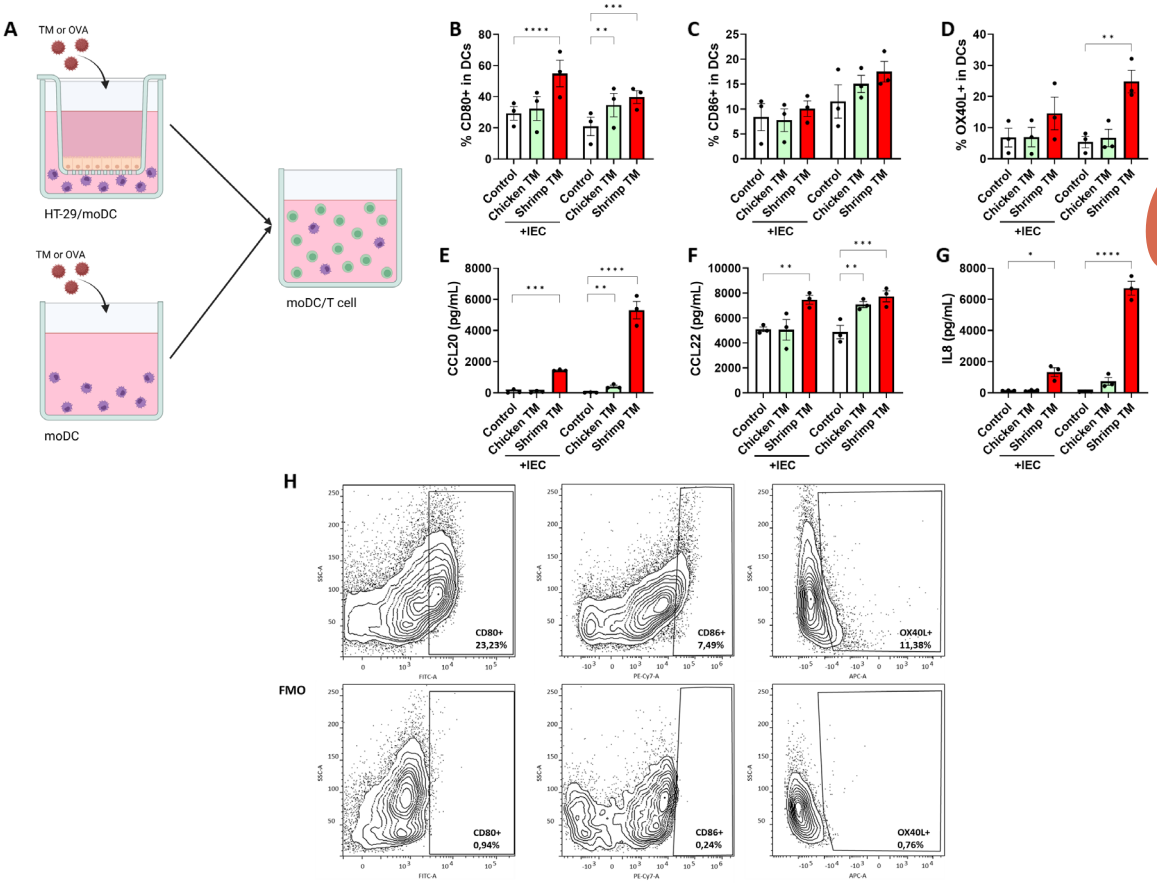

Figure 3. *A) HT-29 cells cocultured with moDC or moDC alone were exposed to 50 µg/mL chicken TM or shrimp TM for 48h, and the primed DC were subsequently cocultured with allogenic naïve T cells for 4 days. After HT-29 cell and/or moDC coculture, expression of the costimulatory molecules B) CD80, C) CD86 and D) OX40L was measured by flow cytometry. In addition, supernatant concentrations of E) CCL20, F) CCL22, and G) IL8 were measured. H) The flow cytometry gating strategy is given with a representative sample and corresponding FMOs. Data is analyzed by One-Way ANOVA, n=3, mean ± SEM (* p<0.05, ** p< 0.01, *** p<0.001, **** p< 0.0001).*

Discussion

Shellfish allergies belong to the top 8 of food allergies [22] and tropomyosins are common allergenic proteins in shellfish. Although highly conserved in the animal kingdom, a clear difference between low- and high-allergenic potential is found between respectively vertebrate and invertebrate tropomyosins [15]. In this study we aimed to investigate the effects of a recombinant chicken tropomyosin and recombinant shrimp tropomyosin on barrier integrity and mucosal immune activation to reveal their difference in sensitizing allergenicity. Hen's egg derived OVA was used as control allergen, as it is among the top three most common food allergy inducers [2]. Previously, it was shown that OVA promotes a type 2 immune response *in vitro*, demonstrating its sensitizing capacity [19], [23].

Proper intestinal epithelial integrity contributes to homeostasis and may prevent allergic sensitization [24]–[26]. A decrease in epithelial barrier integrity allows penetrations of allergens and potentially promotes the development of type 2 immune responses [27], [28]. Next to environmental triggers disrupting the intestinal epithelial barrier and/or activating the epithelial lining, various allergenic proteins are known for their intrinsic capacity to activate epithelial cells and/or their protease activity [29]–[31]. However, recently it was established that both shrimp TM as well as OVA interact with the intestinal epithelium via upregulation of the Hippo signaling pathway, which promotes epithelial instability and production of type 2 cytokines [32]. In the current study shrimp TM was found to reduce barrier integrity after 6h exposure in Caco-2 cell cultures and 8h exposure in Caco-2/HT29MTX cell cultures, which was not observed for the chicken TM or OVA. Previous studies exposing Caco-2 cells in transwell found that intestinal permeability remained intact as TEER values stayed constant, while OVA was taken up by the cells and transported over the epithelium [33], [34]. Beyond barrier disruption, allergens may also activate epithelial cells to produce alarmins and inflammatory mediators that can instruct underlying immune cells. For example Der p 10, a tropomyosin from house dust mites, is known to interact with epithelial expressed Dectin-1 [35]. Similar to Der p 10, OVA may be able to bind to Dectin-1, which normally protects against allergy development [36]. In allergic individuals, abnormal epithelial Dectin-1 expression via altered epigenetic regulation is observed [36], [37], connecting epithelial functioning to allergic sensitization. Similar interactions can be hypothesized for other tropomyosins due to structural homology, however there is no data available to verify this.

Epithelial injury often coincides with the release of alarmins such as IL33, TSLP and IL25 and pro-inflammatory IL8 secretion. In this study an increased fold change in alarmin gene expression was observed in Caco-2 cells after Shrimp TM exposure (Figure 1). This is in line with the increased mRNA expression of *Tslp* and *Il33* in the intestine of mice that were sensitized against a shrimp TM (Litopenaeus vannamei) as described by Fu *et al.*[38]. Furthermore, it is known that epithelial exposure to allergenic proteins promotes the secretion of multiple cytokines, such as of IL33, IL25, TSLP and IL1α or IL1β [39], [40]. Compared to Caco-2, the HT-29 cells were more responsive to the applied proteins allowing discrimination between low and high sensitizing TM (further visualized in Supplemental Figure S4A). Albeit from carcinogenic origin, Caco-2 and HT-29 are both commonly used model epithelial cell lines. Caco-2 can differentiate in culture and form ciliated polarized monolayers representing fully differentiated villus tip enterocytes. HT-29 cells do not differentiate in culture and are resembling crypt epithelial cells. Hence, these cells

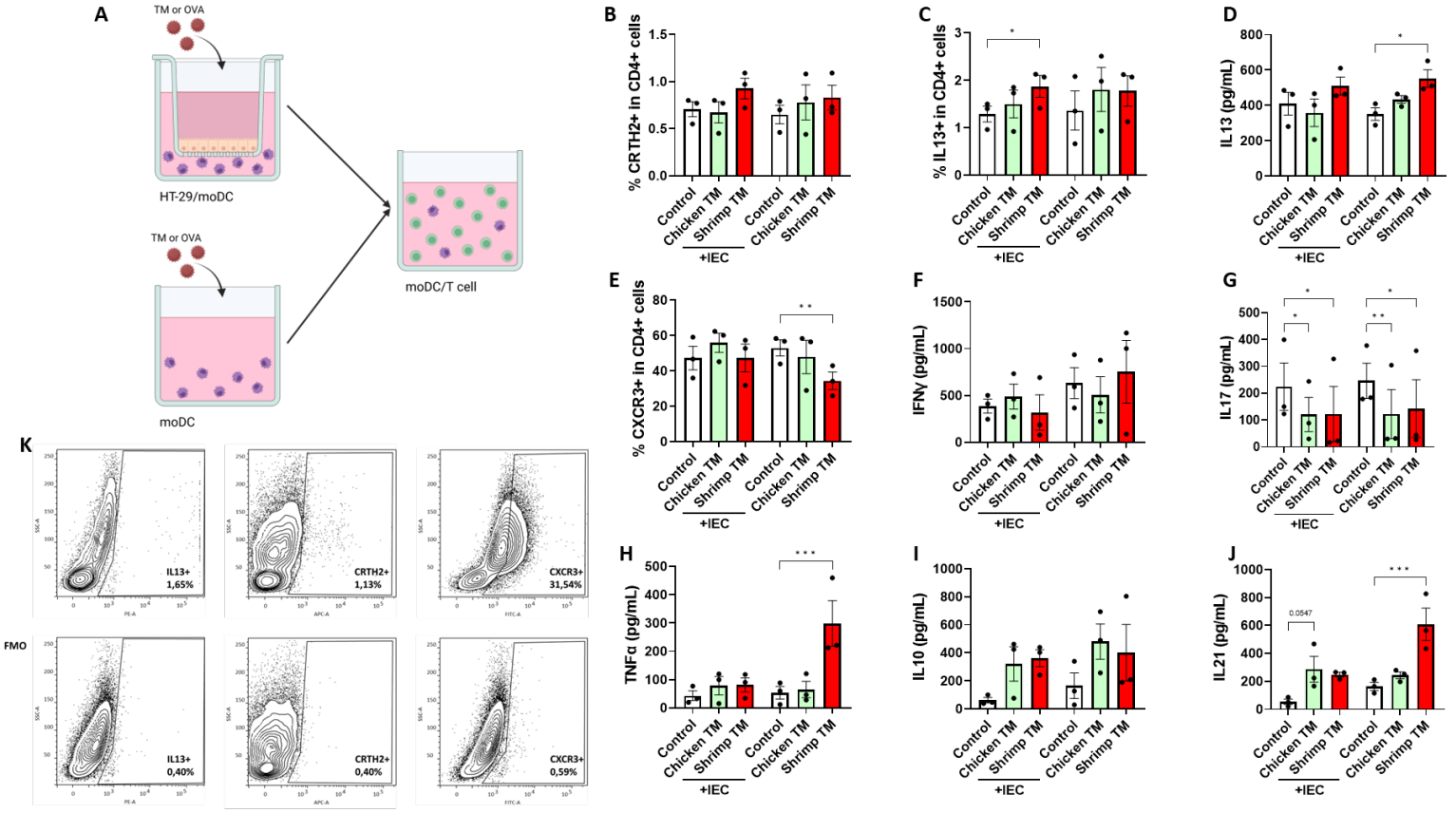

Figure 4. *A) HT-29 cells cocultured with moDC or moDC alone were exposed to 50 µg/mL chicken TM or shrimp TM for 48h, and the primed DC were subsequently cocultured with allogenic naïve T cells for 4 days. Cocultured DC and T cells were collected to analyze T cell subsets and supernatants were collected to measure secreted cytokines. The percentage of B) CRTH2 and C) IL13 expressing T cells as well as level of secreted IL 13 were measured as part of the type 2 response. The percentage of E) CXCR3 expressing T cells and IFNγ was measured as part of the type 1 response. Furthermore, secreted G) IL17, H) TNFα, I) IL10, and J) IL21 were measured. K) The flow cytometry gating strategy is given with a representative sample and corresponding FMOs. Data is analyzed by One-Way ANOVA, n=3, mean ± SEM (* p<0.05, ** p< 0.01, *** p<0.001).*

have different phenotypes as well as receptor expression and signaling cascades may differ between these cells [41], [42]. This may underly the difference in sensitivity of these cell lines for activation and concomitant cytokine release upon protein exposure. Here, increased secretion of several of these cytokines from HT-29 cells was measured after exposure to Shrimp TM, but not chicken TM, further emphasizing the differential epithelial interaction between the tropomyosins. The HT-29 cells were able to discriminate between proteins with high and low sensitizing allergenicity since the chicken TM, which has a low sensitizing risk, did not provoke increase in any of the mediators. Even though it is known that allergenic proteins can induce cytokine secretion from intestinal epithelial cells, the exact interactions with the epithelium are often poorly understood [43]. Future studies could further elaborate on these effects by using primary intestinal epithelial cells or human organoids.

After IEC-DC or moDC were exposed for 48h to chicken TM or shrimp TM, primed DC were subsequently cocultured with naïve T cells to assess the functional immune response after TM exposure. The effects of OVA exposure in this sequential mucosal immune model were already recently published, revealing type 2 immune polarization when OVA was directly exposed to DC and to a lesser extent this was also the case for IEC-DC [19]. The current study shows the differential activating capacity of shrimp TM versus chicken TM in IEC and moDC (visually summarized in Supplemental Figure S4B). Shrimp TM enhanced maturation of DC, based on an enhanced frequency of DC expressing of CD80 and OX40L, and secretion of CCL20, CCL22 and IL8 by the DC. Except for the increase in OX40L expression, also IEC-DC shrimp TM exposure enhanced DC activation in a similar manner. Especially, expression of OX40L by DC and secretion of CCL22 are related to promote subsequent Th2 immune development [44], [45]. Although chicken TM exposure also promoted expression of CD80 and CCL20 as well as CCL22 chemokine secretion, this only occurred when moDC were directly exposed to chicken TM. In contrast to shrimp TM, chicken TM did not activate IEC-DC. This shows that also in interaction with moDC, the presence of IEC aids to differentiate between high and low sensitizing allergenicity of these tropomyosin sources.

Exposure to shrimp TM via IEC slightly altered the response of the DC compared to direct exposure of DC, however the primed DC strongly differ in their capacity to induce T cell polarization. Shrimp TM-DC provoked increased IL13 secretion, while lowering Th1 development, whereas shrimp TM-IEC-DC induced intracellular IL13 expression in T cells. Interestingly, shrimp TM-DC enhanced proinflammatory TNFα and IL21 release on top of the type 2 immune shift after T cell coculture. A similar immune restraining effect by IEC was previously observed for OVA in this model, even though OVA was capable of activating IEC as well [19]. Xu *et al.* exposed moDC to Bla g 7, an allergenic cockroach TM, and subsequently cocultured the moDC with T cells [46]. Bla g 7 exposure enhanced expression of Th2 driving TIM-4, CD80 and CD86 on moDC. These moDC were then capable of promoting a type 2 response during T cell coculture, which is comparable to our results for shrimp TM. Even though chicken TM was capable of activating DC (albeit less pronounced than shrimp TM), these DC were unable to instruct a type 2 response in T cells. Furthermore, beyond being capable of producing pro-inflammatory mediators, IEC are known for their capability to promote tolerance development and control intestinal homeostasis by dampening mucosal immune activation [47]. Indeed. as indicated in the heatmap (Supplemental Figure S4B), in general when compared to medium control, chicken TM did not provoke type

2 activation when exposed via epithelial cells, while shrimp TM did which my lead to allergic sensitization. However, in absence of epithelial cells, thus upon direct exposure to moDC shrimp TM provoked a strong inflammatory type 2 response. In the current study, the dose of 50 mg/mL shrimp TM, but not chicken TM, was found to cause loss of viability in DC and IEC to some extent (Supplemental Figure S3). This may have further contributed to the provocation of inflammatory responses leading to type 2 development of sequential T cell responses. In future studies, the mechanisms by which shrimp TM versus chicken TM differ in their intrinsic capacity to induce type 2 activation should be further addressed. These data demonstrate the differential immune activating capacity of structurally similar tropomyosins and emphasize the importance of understanding the underlying mechanism of allergic sensitization to improve allergenicity risk assessment of current and future dietary protein sources.

Conclusion

This study focused on the differential epithelial barrier disruption and mucosal immune activation by homologous tropomyosins with known differences in sensitizing allergenicity. The allergenic invertebrate shrimp tropomyosin provoked a proinflammatory response in IEC and/or DC, while disrupting epithelial barrier properties. The non-allergenic vertebrate chicken tropomyosin did not induce type 2 activation of IEC nor induced type 2 immunity. Future studies should elaborate on elucidating the mechanism underlying epithelial barrier disruption and mucosal immune activation by allergenic proteins, such as tropomyosins. This would allow for the further development of predictive tools to assess potential sensitizing capacity of food proteins.

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Supplemental methods & figures

Cloning and expression of recombinant chicken tropomyosin α-1 chain isoform X1

Recombinant chicken tropomyosin α-1 chain isoform X1 (UniProt acc. no. P04268) was expressed in *E coli* BL21 (DE3) with a C-terminal His_6-tag, similarly, as described previously [1], purified by immobilized metal affinity chromatography (IMAC) [2], and anion exchange chromatogtaphy (AEC) according to manufacturer's instructions.

Briefly, purchased tropomyosin DNA string (GeneArt, Thermo Fisher Scientific, Germany) was cloned with a C-terminal His_6-tag using vector pET23b (Novagen/Merck, Germany) and in-Fusion ecodry cloning Kit (Clontech, Takara Bio, USA). Competent *E. coli* BL21 (DE3) pLysS (Stratagene/Agilent Technologies, Germany) cells were transformed, and expression was chemically induced by adding isopropyl-beta-D-thiogalactopyranoside (IPTG). After lysis in a cell disruptor (Constant Systems Limited, Low March, UK) and centrifugation, recombinant chicken tropomyosin in supernatant was filtered through 0.45 µm asymmetrical polyether sulfone membrane (Thermo Fisher Scientific) and added to Ni-NTA Superflow bead (Qiagen, Germany) in equilibration buffer (20 mM Tris, pH 8, 10 mM NaCl), and incubated overnight at 4°C. After packing into an ECO10/120VOV column (YMC Europe GmbH, Germany) and washing in equilibration buffer (80 min at 0.3 mL/min flow rate), tropomyosin was eluted within 80 min, using 500 mM imidazole in equilibration buffer (0.3 mL/min flow rate). This was followed by subsequent purification by AEC on HiTrap Capto Q ImpRes column (Cytiva Europe, Germany) according to manufacturer's instructions. Both chromatography steps were run on an ÄKTA pure 25 M (Cytiva Europe, Germany). Purified chicken tropomyosin was dialyzed against low salt buffer (20 mM Tris, pH 8, 10 mM NaCl, 1 mM EDTA) in D-Tube Dialyzer Maxi 3.5 kDa molecular weight cutoff (Merck, Darmstadt, Germany) for further analysis.

Cloning and expression of recombinant shrimp tropomyosin

The shrimp tropomyosin gene, the gene encoding Pen m 1, was published by Motoyama *et al.* [3]. The corresponding cDNA (A1KYZ2) was ordered at Eurofins (Ebersberg, Germany) in the cloning vector pEX-A2. Specific Bam HI- and BgIII-sites were introduced for later subcloning into vector pQE-16. Transformation of TOP10 cells was done with pEX-A2-shrimp TM plasmid. TOP 10 cell clones were screened for the presence of pEX-A2-shrimp TM plasmid. The pEX-A2-shrimp plasmid was purified, subjected to a double digestion done using BamHI and BgIII and ligated into pQE-16. This new plasmid was transformed/secured into E.coli XL1-Blue cells. After DNA sequencing control, the pQE-16-shrimp TM plasmid was transformed into expression host E.coli M15. In production culture, the protein expression was induced using IPTG.

Bacterial pellets were lysed by resuspension with 12 mL lysis buffer (20 mM KH2PO4, 0.5 M NaCl, 10 mM Imidazole, pH 8) and sonicated twice by Biologics Model 150 VT Ultrasonic Homogenizer for 2 min, 40% pulse mode with 50% power on ice. Cells were incubated with lysozyme 1 mg/mL (Sigma, 62970) and benzonase (Merck, 101654) 1250 U per 0.6 L for 30 min on a tube rotator. After centrifugation, supernatants were filtered by Millex 0.22 µm and loaded on Profinia His-Trap (Biorad).

Recombinant protein was purified using a PROFINIA His-Trap device with a Protino® Ni-NTA Column 1 mL (MACHEREY-NAGEL, 745410.1), a purification system used to perform an immobilized metal affinity chromatography (IMAC). Recombinant Pen m 1 was eluted with 500 mM imidazole-containing buffer. Buffer exchange to PBS buffer was performed with fractions containing recombinant protein.

Physicochemical confirmation and characterization of recombinant tropomyosins

Chicken TM

The identity of recombinant chicken tropomyosin α-1 chain was analyzed by mass spectrometry (MS) as described earlier for natural pea 2S albumin nPA1 on a Synapt G2-Si [2]. Differing from this, MS^E data were searched against an in-house, UniProt (as of 2016) derived database consisting of reviewed entries of all species and the amino acid sequence of the recombinant tropomyosin.

Secondary structure elements were analyzed using UV-circular dichroism (CD) spectroscopy (Jasco J-810S, Jasco Germany GmbH, Pfungstadt, Germany) in low salt buffer. Hydrodynamic radii (R_H) were determined by dynamic light scattering (DLS, Zetasizer Nano-127 ZS, software v6.12, Malvern Intruments GmbH, Herrenberg, Germany) in low salt buffer.

Shrimp TM

The protein identity was verified by MS analyses. Trypsin-digested recombinant tropomyosin (Pen m 1) was spotted on a MALDI plate (Polished steel 384 MALDI target plate, Bruker) and 0.3 µL of matrix solution added (5 mg/mL alpha-cyano-4-hydroxycinnamic acid, HCCA, Bruker and 1 mg/mL 2,5-dihydroxybenoiquem DHB, Bruker in 50% Acetonitile containing 0.1% TFA) according to dried-droplet method. An external calibration was done before each analysis with trypsin digested bovine serum albumin (Brucker manufacturer's instructions). Protein mass finger print (PMF) was generated and compared to in silico digestions of TM in NCBInr database (Mascot server, Matrix Science). Confirmatory Edman sequencing was performed on a Procise 49X HT protein sequencer (Applied Biosystems). CD was used for studying secondary protein structures. Samples were measure in a cuvette of 0.1 cm path length using the Chirascan CD spectrometer (Applied Photophysics). Far-Ultraviolet CD spectra were recorded at 20°C starting with a wavelength of 180 nm to 260 nm (1 nm bandwidth, 0.5 seconds interval, 5 repeats). The read-out was converted with respective protein details into degrees*cm2*dmol–1 according to the manufacturer's instructions Dichroweb was used to analyze circular dichroism data.

Endotoxin removal and concentration determination

Chicken TM

After four cycles of endotoxin removal using EndoTrap red-kit (Lionex GmbH, Braunschweig, Germany) according to manufacturer's instructions, the final preparation of recombinant chicken tropomyosin and recombinant shrimp tropomyosin was gained in equilibration buffer (phosphate buffer, pH 7.4, 80 mM NaCl), and endotoxin was quantified using the LAL kinetic turbidimetric

assay according to Remillard *et al.* [4]. Protein was quantified against bovine serum albumin dilution series by densitometry (ImageJ) of Coomassie-stained SDS-PAGE gel.

Shrimp TM

The removal of endotoxins was achieved by endotoxin removal columns EndoTrap red 5/1 (Endotrap, Hyglos, Germany). The protein was eluted with 10 mM Na_2HPO_4, 80 mM NaCl, pH 7.4 and tested for endotoxin content according to the protocol of the Pierce LAL chromogenic endotoxin quantification kit (Thermo Scientific, Rockford, US), resulting to 165 EU/mg endotoxin. Protein was quantified by the Bradford method (Biorad, Nazareth, Belgium) using bovine serum albumin (Sigma, US) as standard protein (0.001-0.008 mg/ml) by measuring the absorption at 595 nm using a spectrophotometer (Ultraspec III, Pharmacia LKB).

Allergen preparations

Chicken TM

In Coomassie-stained SDS-PAGE, the purified recombinant His_6-tagged chicken α-1 tropomyosin appeared as a major protein band (estimated 90 % of total protein) around 36 kDa and according to a calculated molecular mass of 34.044 kDa, and comparable to the work previously published [1].

Purified recombinant chicken tropomyosin showed a typical α-helical signature (maximum at 195 nm, minima at 208 nm and 222 nm) in UV CD-spectroscopy, which was comparable to the data previously published [1]. In DLS analysis, a mean R_H of 7.76 (+/- 0.76) nm indicated some level of aggregation of the major peak (> 60 % mass) that was interpreted as monodisperse.

Sequence identity of His_6-tagged recombinant chicken tropomyosin (based on UniProt Acc. No. P04268) was confirmed with a protein score of 18798 by detecting 130 peptides with a mean mass error of 2.0 ppm, covering 58.0 % of the amino acid sequence. Chicken Tropomyosin was finally gained at 346 µg/mL in phosphate buffered saline (pH 7.4, 80 mM NaCl). The amount of residual LPS in recombinant chicken tropomyosin was quantified at 0.28 EU/mL after repetitive LPS removal.

Shrimp TM

To verify the purity of Pen m 1, a SDS-PAGE followed by silver stain was performed. A tropomyosin-like double band was visualized at 35 kDa. To summarize, a total amount of 2.5 mg pure, recombinant tiger prawn tropomyosin (Pen m 1) was made available.

MS-based analyses using PMF gave a sequence coverage of 91% sequence coverage to the previously in the laboratory cloned and translated cDNA sequence of tropomyosin of giant tiger prawn (database no. A1KYZ2).

Using CD analyses, recombinant shrimp TM showed an alpha-helical folded, reflected by typical curves with two characteristic minima at 208 nm and 222 nm. The ratio of the negative peak intensity '222 nm/208 nm' was 1.081 for recombinant Pen m 1, indicating that the TM had characteristic coil structures. Using the Dichroweb tool, the shrimp TM had an alpha-helical content of 68%.

Cell culture

Caco-2 cells (passage 28-33 & 45-47) were cultured for 21 days in DMEM medium supplemented with 10% fetal bovine serum (FBS), 1% l-glutamine, 1% sodium pyruvate, 1% penicillin/streptomycin/amphotericin and 1% non-essential amino acids (all from Biowest, France).

HT-29 cells (passage 158-161), obtained from ATCC, were grown until ~80% confluency in 25cm^2 cell culture flasks. McCoy's 5A medium (Gibco, USA), containing 10% heat-inactivated FBS, 1% penicillin and streptomycin (Sigma-Aldrich, UK) was used. Upon trypsinization, HT-29 cells were 5 times diluted based on surface area and seeded in transwell inserts (Corning Incorporated, USA) and cultured for 6 days into confluent layers be used for coculture experiments

Cytokine measurements

Cytokine levels were determined in collected cell-free supernatants. Concentrations of IL33, TSLP, IL25, CCL20, CCL22, IL8 and IL13, IFNγ, IL10, IL17, IL21 and TNFα, were measured by commercially available ELISA kits (R&D systems, USA or Invitrogen, USA) according to manufacturer's instructions. To compare cytokine secretion after OVA, TmL or TmH exposure of Caco-2 cells and HT-29 cells (cultured in 96 wells) a multiplex array was performed (Meso Scale Discovery, USA) to measure secreted levels of epithelial derived IL33, TSLP, IL25, IL1α, IL1β, IL6, IL8 CCL20, CCL22 and TNFα in undiluted supernatant, according to the manufacturer's instructions.

Flow cytometry

Flow cytometric analysis was performed on cocultured moDC and T cells. After collection of the cells, the cells were washed with PBS, stained with Fixable Viability Dye eFluor 780 (eBioscience, USA) and nonspecific binding was blocked. Staining of moDCs was performed using titrated amounts of CD11c-PerCP eFluor 710, HLA-DR-PE, CD80-FITC, CD86-PE-Cy7 and OX40L-APC. Staining of T cells was performed with titrated volumes of CD4-PerCP-Cy5.5, CXCR3-AF488, CRTH2-APC and IL13-PE. All antibodies were purchased from eBioscience or BD Biosciences. Flow cytometric data was collected using a BD FACS CantoII (BD Biosciences, USA) and analyzed using FlowLogic software (Inivai Technologies, Australia).

Gene transcription analysis

Total RNA was isolated using RNA Kit (Macherey-Nagel, Germany) and reverse transcribed into cDNA using PrimeScript RT Reagent Kit (Takara Bio Inc., Shiga, Japan). Primer pairs and thermal cycling conditions for q-PCR assays are described in Supplementary Table 1. Relative gene transcription was calculated by normalizing data to the transcription of the *Gadph* gene, using the $2^{-\Delta\Delta CT}$ method.

Supplementary Table 1 *Primer pair sequences for gene transcription analysis in Caco-2 cells. Fw, forward; rv, reverse.*

Gene	Primer pairs	Reference	Cycling conditions
Gapdh	fw 5' GAAGGTGAAGGTCGGAGTCAA 3' rv 5' ACGTACTCAGCGCCAGCATC 3'	[5]	Pre-Incubation 2 min 50°C Incubation 10 min 95°C **40 cycles**
Il33	*fw* 5' GAGCTAAGGCCACTGAGGAA 3' *rv* 5' TGGGCCTTTGAAGTTCCATA 3'	[6]	
Il25	*fw* 5' CCAGGTGGTTGCATTCTTGG 3' *rv* 5' TGGCTGTAGGTGTGGGTTCC 3'	[7]	
Tslp	*fw* 5' CTCTGGAGCATCAGGGAGAC 3' *rv* 5' CAATTCCACCCCAGTTTCAC 3'	[5]	

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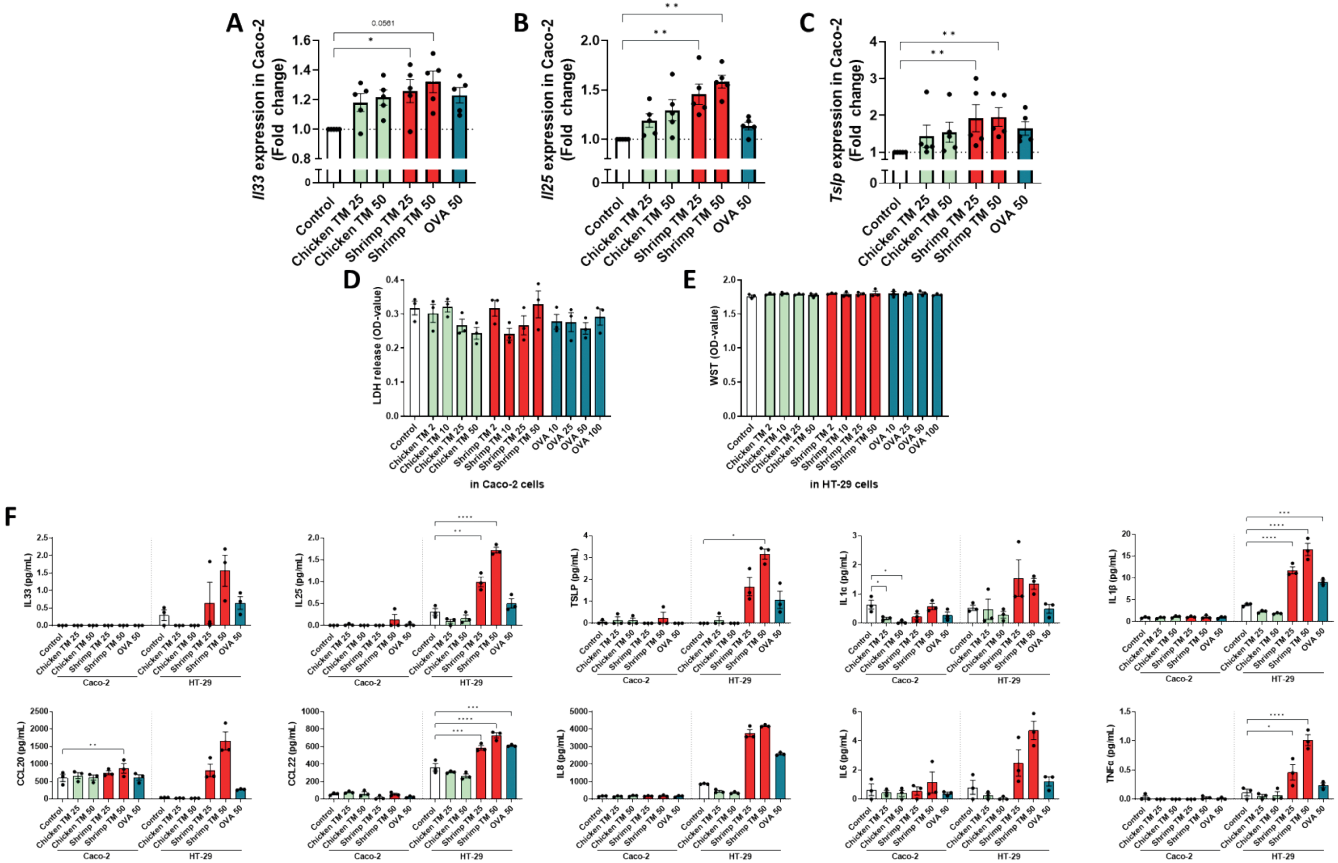
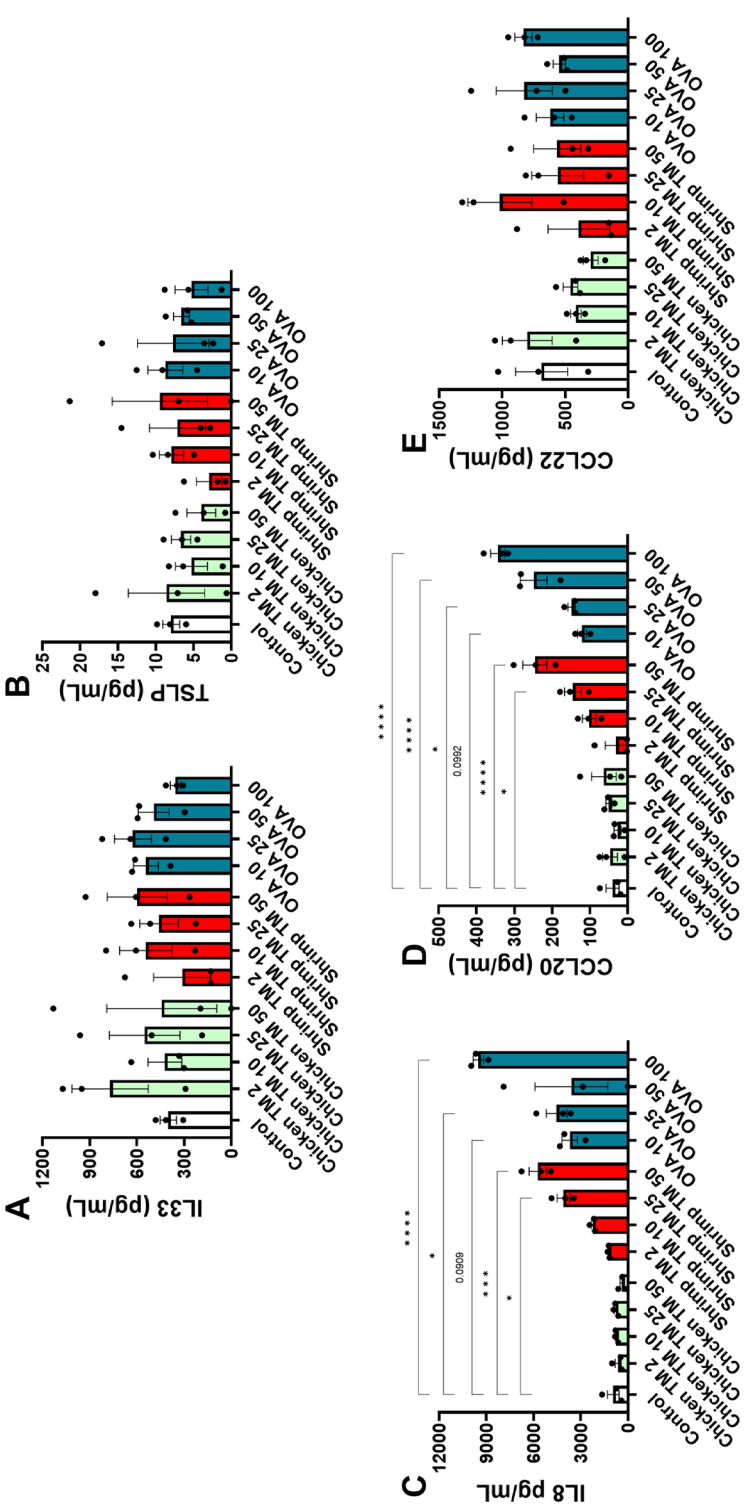
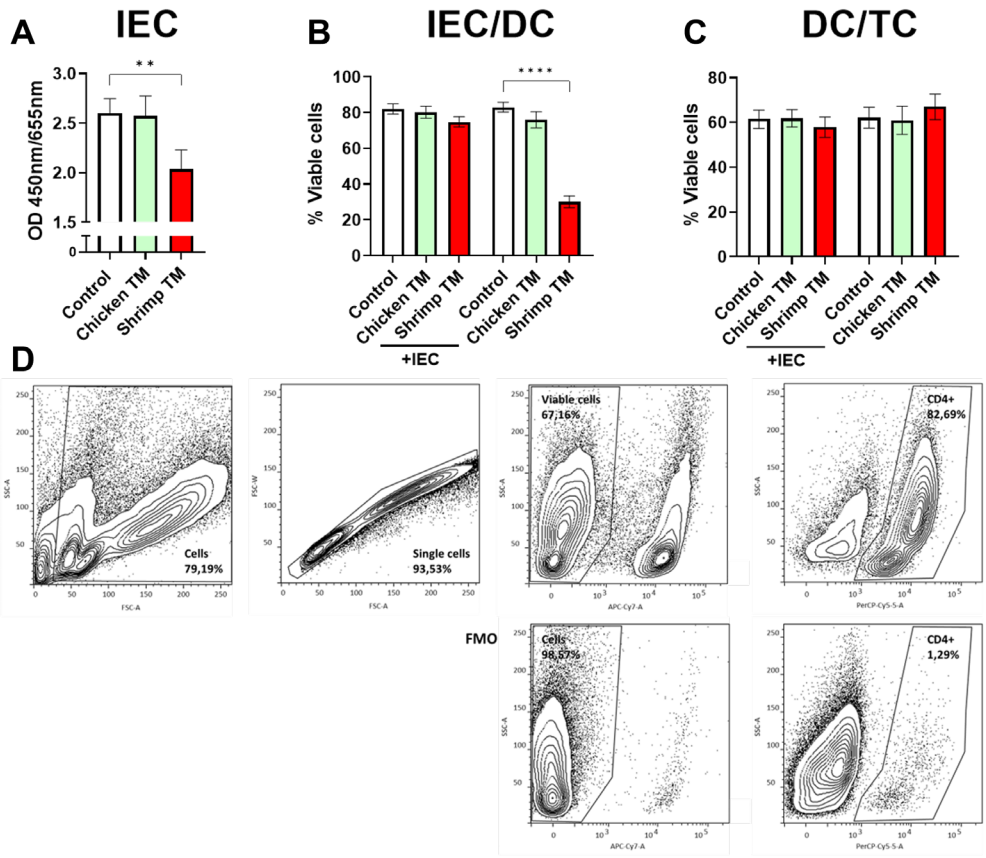


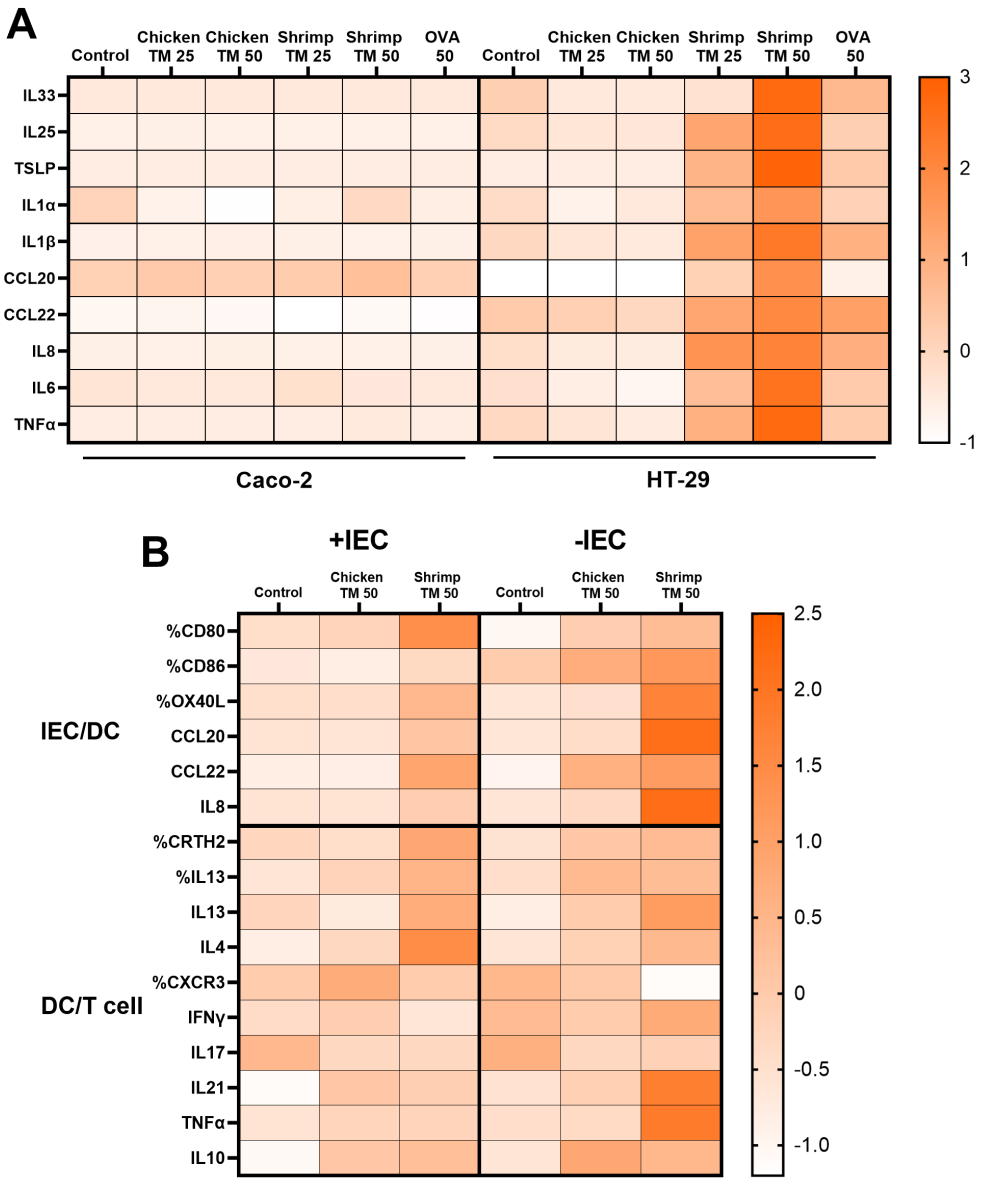
Supplemental Figure S1. *Fold change in gene expression was measured 8h after 25 or 50 µg/mL chicken TM, shrimp TM or OVA exposure in Caco-2 cells cocultured with PBMCs. mRNA expression of the alarmins A) Il33, B) Il25, and C) Tslp was increased upon exposure to either concentration of shrimp TM. D) LDH release and E) WST conversion by Caco-2 cells and HT-29 cells respectively was not affected 48h after exposure in 96 wells flat bottom plates to increasing doses of chicken TM, shrimp TM or OVA. F) A multiplex array was performed on supernatants from Caco-2 cells and HT-29 cells that were exposed to 25 and 50 µg/mL chicken TM or shrimp TM, or 50 µg/mL OVA. Next to the increased secretion of IL25, TSLP, IL1β, CCL22, and TNFα after exposing HT-29 cells to 50 µg/mL shrimp TM, a similar effect on cytokine secretion was seen after exposure to 25 µg/mL shrimp TM. Both chicken TM exposures decreased secretion of IL1α in Caco-2 cells, but exposure to this low-allergenic protein did not induce any other changes in cytokines secretion from Caco-2 cells or HT-29 cells. Data is analyzed by One-Way ANOVA or Friedman test when data did not fit a normal distribution, n=3 or n=5, mean ± SEM (* p<0.05, ** p< 0.01, *** p<0.001, **** p< 0.0001).*



Supplemental Figure S2. *HT-29 cells were exposed to increasing doses of chicken TM, shrimp TM or OVA for 48h in 96 wells flat bottom plates. Secreted A) IL33, B) TSLP, C) IL8, D) CCL20, and E) CCL22 were measured by ELISA. Exposure to chicken TM did not enhance cytokine secretion, exposure to shrimp TM and OVA induced a dose-dependent enhanced secretion of IL8 and CCL20. Data is analyzed by One-Way ANOVA, n=3, mean ± SEM (* p<0.05, ** p< 0.01, *** p<0.001, **** p< 0.0001).*



Supplemental Figure S3. *After culture of moDC with or without HT-29 cells and coculture of primed DC with T cells, viability was assessed. Viability of A) HT-29 cells after 50 µg/mL shrimp TM exposure. B) Viability of moDC was not affected by chicken TM or shrimp TM exposure when cocultured with HT-29 cells. However when moDC were directly exposed to shrimp TM, cell viability was significantly decreased. C) Coculture of the primed DC with T cells did not affect viability of the cells. D) Furthermore, the gating strategy used to determine the viable T helper cells population and corresponding FMOs are presented. Data is analyzed by One-Way ANOVA, n=3, mean ± SEM (* p<0.05, ** p< 0.01, *** p<0.001, **** p<0.0001).*



Supplemental Figure S4. *A visual representation, using z-scores, of A) the cytokine secretion from Caco-2 and HT29 cells cultured in 96 well flatbottom culture plates and, B) cytokine secretion and marker expression upon exposure to chicken TM or shrimp TM in the IEC/DC/T cell coculture model.*

CHAPTER 5

An advanced *in vitro* human mucosal immune model to predict food sensitizing allergenicity risk: a proof of concept using ovalbumin as model allergen

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Abstract

The global demand of sustainable food sources leads to introduction of novel foods on the market, which may pose a risk of inducing allergic sensitization. Currently there are no validated *in vitro* assays mimicking the human mucosal immune system to study allergenicity risk of novel food proteins. The aim of this study was to introduce a series of sequential human epithelial and immune cell cocultures mimicking key immune events after exposure to the common food allergen ovalbumin from intestinal epithelial cell (IEC) activation up to mast cell degranulation. This *in vitro* human mucosal food sensitizing allergenicity model combines crosstalk between IEC and monocyte-derived dendritic cells (moDC), followed by coculture of the primed moDCs with allogenic naïve CD4+ T cells. During subsequent coculture of primed CD4+ T cells with naïve B cells, IgE isotype-switching was monitored and supernatants were added to primary human mast cells to investigate degranulation upon IgE crosslinking. Mediator secretion and surface marker expression of immune cells were determined. Ovalbumin activates IEC and underlying moDCs, both resulting in downstream IgE isotype-switching. However, only direct exposure of moDCs to ovalbumin drives Th2 polarization and a humoral B cell response allowing for IgE mediated mast cell degranulation, IL13 and IL4 release in this sequential DC-T cell-B cell-mast cell model, indicating also an immunomodulatory role for IEC. This *in vitro* coculture model combines multiple key events involved in allergic sensitization from epithelial cell to mast cell, which can be applied to study the allergic mechanism and sensitizing capacity of proteins.

Keywords: advanced *in vitro* model, allergic sensitization, food allergy, mucosal immunity, allergenicity risk assessment, ovalbumin

Introduction

The European Food Safety Authority (EFSA) updated their international guidelines in 2019 with new insights to assess adverse immune responses of novel food derived proteins [1]. Food allergens may contain intrinsic properties to drive allergic sensitization, which can be designated as sensitizing allergenicity [2]. Biological tools to predict adverse immune outcomes such as allergic sensitization are becoming increasingly relevant for safety assessment of new products, e.g. based on novel proteins before launching on the market due to the increasing number of individuals suffering from food allergies [3].

Key immunological events during food allergic sensitization have been identified [2]. Allergic sensitization mainly starts at epithelial surfaces, upon contact with the allergenic food protein intestinal epithelial cells (IEC) become activated and start producing the type 2-driving alarmins IL25, IL33 and TSLP [4], [5]. These epithelial-released proinflammatory mediators condition mucosal dendritic cells (DC) while sampling proteins in the Peyer's patches (PP), gut lumen and/or lamina propria. Activated DCs can instruct naïve T and B cells in the PP or migrate to the mesenteric lymph nodes (MLN) for this purpose. Contact with an allergen and detection of local signals, including epithelial derived type 2 driving alarmins, promote the expression of MHC-II, costimulatory molecules and secretion of proinflammatory cytokines by DC [6]. Allergen activated DCs are acting in the PP or MLN to drive T-helper 2 (Th2) cell polarization. By producing cytokines such as IL4 and IL13, Th2 cells promote immunoglobulin E (IgE) isotype-switching in allergen-specific B cells in the PP and/or MLN [7]. Via the MLN the effector Th2 and B cells enter the blood stream and home back to the lamina propria [8]. Within the mucosa, the Th2 cells are further activated and B cells differentiate into plasma cells, respectively secreting type 2 cytokines and allergen-specific IgE. Secreted IgE will bind to the FcεRI receptor on mast cells and basophils in mucosal tissue, which sensitizes these cells for degranulation upon crosslinking of the FcεRI-bound IgE by the allergen during a second exposure. Degranulation results in release of symptom-inducing mediators such as histamine, prostaglandins, mast cell proteases and proinflammatory type 2 cytokines [9].

In addition to IEC, many innate and adaptive immune cells, cellular molecules and humoral mediators involved in the allergic response have been studied extensively. However, due to the complexity of the gut-associated lymphoid tissue and the sequential organization of the pathological mechanism in food allergy development, it is difficult to determine the exact role and kinetics of individual cell types in a complete organism for sensitizing allergenicity studies. In addition, for ethical reasons it is essential to limit the use of animals for research purposes. Therefore, a growing body of research is focusing on the development of advanced *in vitro* mucosal immune models to allow effective safety and allergenicity risk assessment of novel food proteins and to provide tools for further mechanistic studies without the use of animal models [10]. Recent attempts have aimed to develop sensitizing allergenicity models up till the T cell response [11], [12], however the use of murine cells may lack translational value to study food allergy from a human perspective. In a similar approach we recently showed the contribution of epithelial cells in driving the first steps of allergic sensitization [13]. In the current manuscript, we also used the common food allergen ovalbumin, the most abundant protein in hen's eggs [14], to develop a novel predictive and advanced *in vitro* human mucosal immune model. This model

includes all major cell types involved in allergic sensitization and the allergic effector response, while allowing individual analysis of each single cell type. Therefore, this sequential mucosal food sensitizing allergenicity model facilitates further mechanistic studies and may be used as a first screening method to test intrinsic sensitizing capacities of novel food proteins.

Methods

Isolation and culture of cells

The human intestinal HT-29 cell line (passages 158-161) was cultured in McCoy's 5A medium (Gibco, USA) containing 10% FCS (Gibco), 1% penicillin and streptomycin (pen/strep) (Sigma-Aldrich, UK). PBMCs were isolated from buffy coats from healthy donors, who gave consent that their donations could be used for research purposes (Dutch Blood Bank, The Netherlands), by density-gradient centrifugation in Leucosep tubes (Greiner). Subsequent isolation of monocytes, naïve T cells and naïve B cells was performed by negative selection and isolation of CD34+ stem cells by positive selection using appropriate magnetic separation kits according to the manufacturer's protocol (Miltenyi Biotec, Germany). Monocytes from 3 independent donors were cultured for 6 days in RPMI 1640 (Lonza, Switzerland) containing 10% FCS, 1% penicillin/streptomycin, 100 ng/ml IL4 and 60ng /ml GM-CSF (Prospec, Israël) to allow differentiation into immature monocyte-derived dendritic cells (moDCs). Naïve T and naïve B cells were isolated from 3 independent donors, therefore naïve B cells were stored at –80°C in FCS containing 10% DMSO until further use. Both naïve T and naïve B cells were cultured in IMDM (Sigma-Aldrich) containing 5% FCS, 1% penicillin/streptomycin, 20µg/ml apo-transferrin (Sigma-Aldrich) and 50µM β-mercaptoethanol (Sigma-Aldrich). Primary human mast cells were differentiated from CD34+ stem cells [15]. Purity of isolated monocytes, naïve T and B cells was assessed immediately after isolation, see Supplemental Figure 1.

Sequential mucosal food allergenicity model

This advanced sequential *in vitro* coculture model was used to mimic cross talk in natural order of occurrence between relevant cell types in food allergic responses. A schematic overview of coculture steps is displayed in Figure 1.

HT-29 cells were 5 times diluted based on surface area and seeded in transwell inserts (polyester membrane, 0.4 µm pores) (Corning Incorporated, USA). After 6 days HT29 cells reached confluency and $5x10^5$ immature moDCs (in 1.5 mL) were added to the basolateral compartment, alternatively moDCs were added to wells without IEC. OVA (100 µg/mL (Sigma-Aldrich)) was added apically (in 0.5 mL) to the IEC or empty transwell filter membranes for 48 hours.

Afterwards, viability of IEC was not affected as measured by a WST-assay to determine mitochondrial activity (Supplemental Figure 2) moDCs were collected for phenotyping by flow cytometry and coculture with allogenic naïve T helper cells (10:1 ratio (T cell : moDC)) in a 24 well flat-bottom plate for 4 days in the presence of 5 ng/mL IL2 (Prospec) and 150 ng/mL anti-CD3 (clone CLB-T3/2, Sanquin, The Netherlands), to allow generic T cell activation guided by the primed moDCs .

Following the moDC/T cell coculture, cells were collected again for phenotyping and cocultured with autologous (to T cells) $2x10^5$ naïve B cells (1:1 ratio) in a 24 well flat-bottom

plate in the presence of 5 µg/mL anti-IgM (Sigma-Aldrich), to allow generic B cell activation guided by the primed T cells.

The activation status of the B cells was assessed after 4 days by flow cytometry. Cell free supernatant was collected after 18 days and added to primary human mast cells in a 1:1 dilution with fresh mast cell culture medium. After 24h incubation the supernatant was washed away and IgE-mediated degranulation (at 1 hour) and type 2 cytokine production (after 18h) by mast cells were determined. Appropriate control conditions for each step of the model are shown in Supplemental Figure 3.

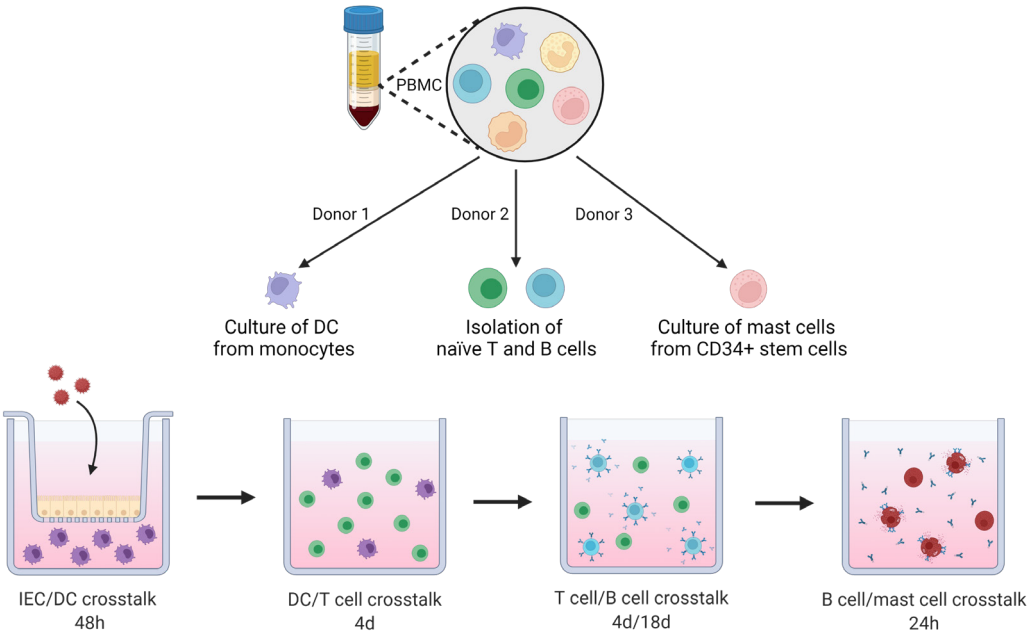

Figure 1. *Schematic overview of coculture steps in this novel sequential mucosal food sensitizing allergenicity model. PBMCs are isolated from 3 donors, from the first donor, moDC are cultured from isolated monocytes. The second donor provides naïve T and B cells. The third donor is used to isolate CD34+ stem cells which are differentiated into primary human mast cells. moDC are exposed to OVA in presence or absence of IEC for 48h, primed moDCs are collected and cocultured with naïve Th cells in a 1:10 ratio for 4 days. Next, the primed Th cells are cocultured with naïve B cells for 4 and 18 days. The B cell activation status was assessed after 4 days. After 18 days of coculture supernatant was collected to determine antibody secretion and for incubation with primary human mast cells overnight. IgE specific mast cell degranulation was measured as well as cytokine secretion during a final 18h incubation of the primed mast cells. This figure was created with BioRender.com.*

Enzyme-linked immunosorbent assay (ELISA)

Supernatants collected from IEC, IEC/moDC, moDC, moDC/T cell, T cell/B cell and mast cell cultures were analyzed for cytokine, chemokine and immunoglobulin secretion. Concentrations of IFNγ, IL4, IL8, IL10, IL12p70, IL13, IL17, IgE, IgG, TGFβ, TSLP (Invitrogen, USA), IL15 (Biolegend, USA), CCL20, CCL22, IL25, IL33 (R&D systems, USA) were measured according to manufacturer's instruction.

FACS

Phenotype of moDC, T cells and B cells after coculture was analyzed by flow cytometry. Collected cells were stained with Fixable Viability Dye 780-APC Cyanine 7 (eBioscience, USA), followed by blocking of nonspecific binding sites with human Fc block (BD Biosciences, USA) in PBS containing 1% bovine serum albumin (Roche, Switzerland). 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-Cy7 (clone IT2.2), OX40L-APC (clone RM134L), CD4-PerCP-Cy5.5 (clone OKT4), CXCR3-AF488 (clone 1C6/CXCR3), CRTH2-APC (clone BM16) , CD19-PE-Cy7 (HIB19) , CD4-PE (clone RPA-T4), CD25-AF488 (clone BC96) (purchased from eBioscience or BD Biosciences). Cells were permeabilized with the Intracellular Fixation & Permeabilization Buffer Set (eBioscience, USA) to allow staining with IL13-PE (clone JES10-5A2). Flow cytometric measurements were performed using BD FACS CantoII (Becton Dickinson, USA) and data was analyzed using FlowLogic software, (Inivai Technologies, Australia). Representative gating strategies are given in Supplemental Figure 4.

β-Hexosaminidase assays

After overnight incubation with B cell supernatant, mast cells were washed and incubated with mouse anti-human IgE (eBioscience) for 1 hour. Next, 158μM 4-methylumbelliferyl-β-d-glucopyranoside (4-MUG) was added to the cell-free supernatant for 1 hour. Enzymatic reaction was stopped with 0,1M glycine buffer (pH 7.8). 4-Methylumberriferone was quantified by measuring fluorescence at ex350nm/em460nm with a GloMax® Discover Microplate Reader (Promega, USA). The percentage of β-hexosaminidase release was calculated as percentage relative to a positive control (100% degranulation) (Triton X-100) and negative control (0% degranulation). Mast cells were washed and fresh medium was added, mast cells were incubated for an additional 18h to measure secretion of cytokines.

Statistical analysis

Statistical analyses were performed using GraphPad Prism Version 9.4.1. Data was analyzed by paired t-test. $p < 0.05$ is considered statistically significant, and data is represented as mean ± SEM of n=3 independent repeats per dataset.

Results

Ovalbumin induces increased maturation in moDCs in absence of IEC

At first a food allergen encounters the intestinal epithelial barrier, therefore *in vitro* activation of IEC by the second most common food allergen ovalbumin (OVA) was explored by exposing IEC in a flat-bottom plate for 48h to different doses of OVA to determine optimal concentration for epithelial activation (Supplemental Figure 5). Exposure to 100 μg/mL OVA resulted in the significant secretion of IL8 and CCL20, therefore this concentration was used in following experiments.

Activation of IEC and/or moDCs in transwells by apically administered OVA was assessed by cytokine secretion and expression of costimulatory markers. Apical exposure of IEC in the

transwell to OVA resulted in an enhanced basolateral secretion of IL33 and TSLP (Figure 2A, C). When IEC were exposed to OVA in presence of moDCs (OVA-IEC-DC), secretion of IL25 and TSLP was increased (Figure 2B). These epithelium derived cytokines were not measured in cultures with only moDCs. Coculture of IEC with moDCs (IEC-DC) resulted in an increased percentage of moDCs expressing CD80 and OX40L in response to OVA (Figure 2D, F), while exposing moDCs to OVA in absence of IEC (OVA-DC) tended to increase the frequency of moDCs expressing all maturation related costimulatory molecules (Figure 2D-F). Representative FACS plots including FMOs are shown in Figure 2G. Appropriate control conditions are shown in Supplemental Figure 3.

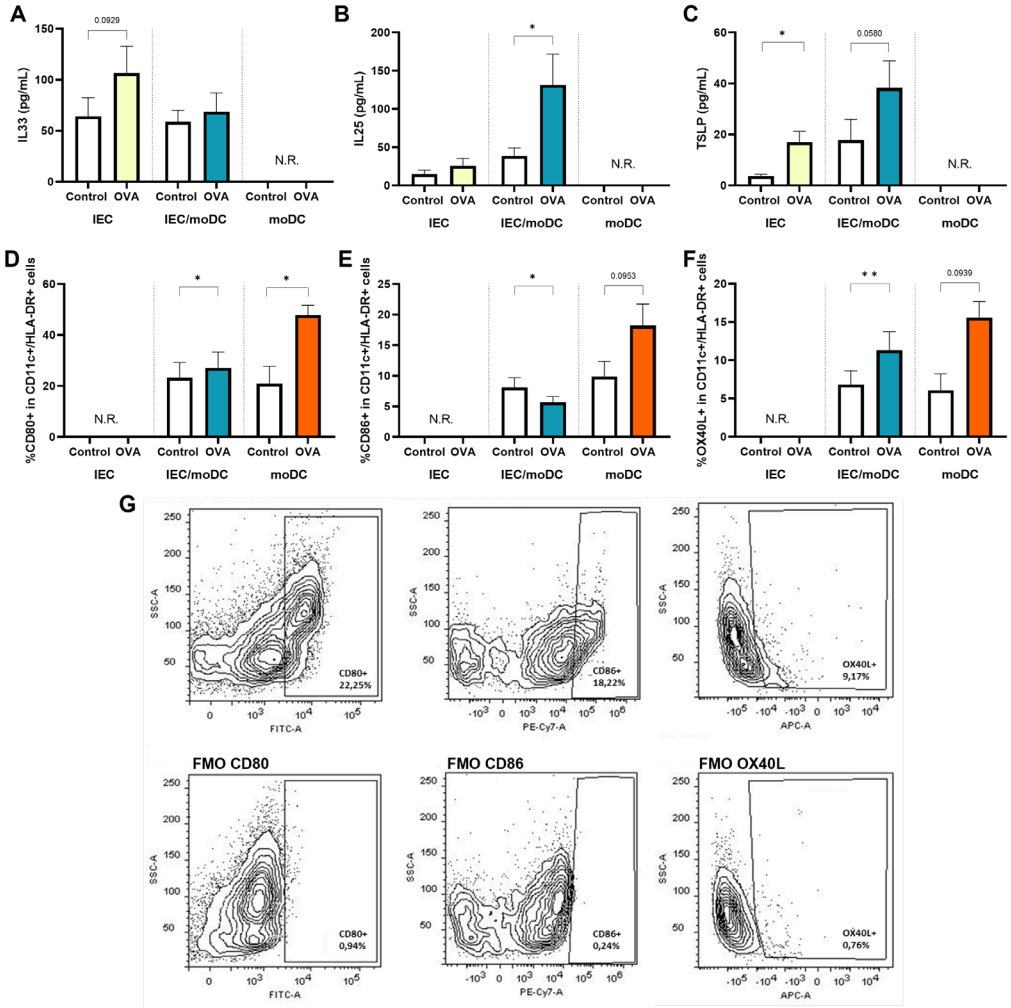

Figure 2. *After 48h OVA exposure of IEC, IEC-moDC or moDC, secretion of epithelial derived alarmins A) IL33, B) IL25, and C) TSLP was determined. In addition, the percentage of D) CD80+, E) CD86+ and F) OX40L+ expressing moDCs (defined as CD11c+ and HLA-DR+) was measured. A representative sample and corresponding FMO controls are shown in G). Data is analyzed by paired t-test, n=3, mean ± SEM (* p<0.05, ** p<0.01).*

OVA enhances secretion of CCL20, CCL22 and IL8 irrespective of IEC presence

Cytokines and chemokines produced by IEC and/or moDCs during OVA exposure were measured. Both in presence or absence of IEC, secretion of CCL20, CCL22 and IL8 was increased (Figure 3A-C). IL15 (Figure 3D) secretion was not significantly affected during OVA exposure, while OVA-IEC-DC showed an inclining trend for IL12p70 (Figure 3E) and OVA-DC tended to decrease TGFβ levels (Figure 3F).

Type 2 mediators are increased in T cells after coculture with OVA-DC

To investigate the immunological function of the OVA or OVA-IEC exposed moDCs, cells were cocultured for 4 days with allogenic naïve Th cells. The T cells were exposed to anti-CD3 and IL2 to allow a generic TCR activation, to be guided by the primed moDC. Secretion of IL13 and IL4, and the percentage of Th2 cells containing IL13 (Figure 4A, B, D) was increased in Th cells that were cocultured with OVA-DC compared to unexposed DC. This increase was not observed upon coculture with OVA-IEC-DC. The percentage of Th2 cells (CRTH2+) was not affected (Figure 4E), but the percentage of Th1 cells (CXCR3+) tended to increase when T cells were coculture with OVA-IEC-DC, while a decreasing trend was observed upon coculture with OVA-DC (Figure 4F). Th1 type IFNγ (Figure 4C) and Th17 type IL17 (data not shown) secretion remained unaffected. While Th cells primed with OVA-DC showed increased type 2 cytokine release, levels of regulatory IL10 were increased after coculture OVA-IEC-DC with T cells (data not shown).

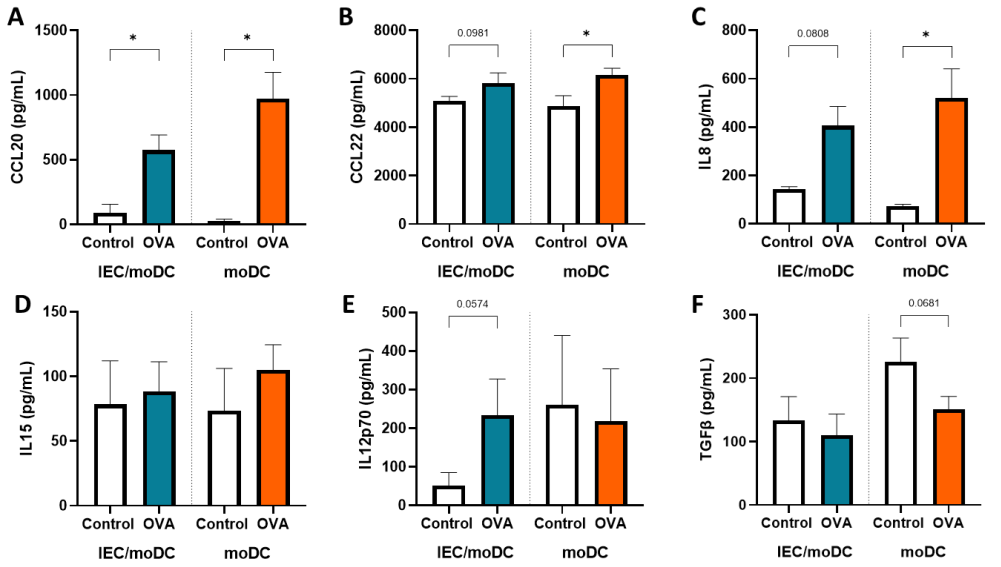

Figure 3. *After 48h OVA exposure of IEC-moDC or moDC alone, secretion of A) CCL20, B) CCL22, C) IL8, D) IL15, E) IL12p70, and F) TGFβ was determined in the basolateral compartment. Data is analyzed by paired t-test, n=3, mean ± SEM (* p<0.05).*

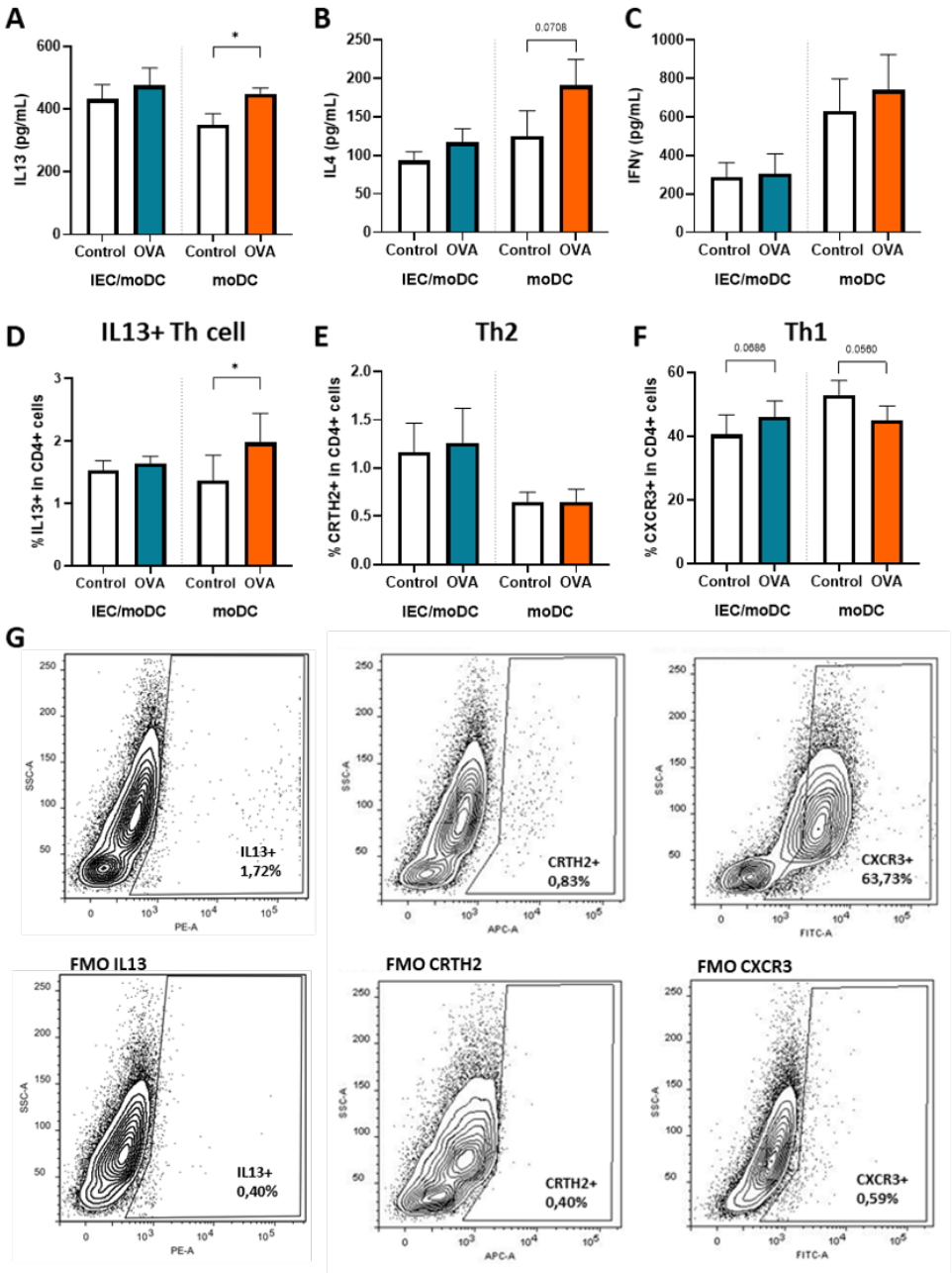

Figure 4. *After the T cells were cocultured with OVA primed IEC-moDC or OVA primed moDC, subsequently the primed T-cells were cocultured with autologous naïve B cells. Activation of B cells was assessed at day 4 by flow cytometric analysis of expression of CD25, A) a representative sample and appropriate FMO control are shown. B) Percentage of CD25 expressing B cells are displayed for the used conditions. Secretion of C) IgG and D) IgE was determined in the supernatant after 18 days of coculture. These cell free 18 day supernatants were also overnight incubated with primary human mast cells. E) Degranulation upon crosslinking with anti-IgE was determined by means of β-hexosaminidase release as well as overnight F) IL13, and G) IL4 secretion by the primed and degranulated mast cells. Data is analyzed by paired t-test, n=3, mean ± SEM (* p<0.05).*

Both OVA-DC-T cells and OVA-IEC-DC-T cells induce IgE secretion in B cells

To study whether DC-instructed Th cells were capable of inducing immunoglobulin production by B cells, OVA-DC and OVA-IEC-DC cocultured Th cells (OVA-DC-T and OVA-IEC-DC-T respectively) were incubated with autologous naïve B cells for 18 days. The B cells were stimulated with anti-IgM to allow a generic BCR activation, to be guided by the primed T cells. The activation status of the B cells was determined after 4 days, immunoglobulin secretion was measured after 18 days. OVA-IEC-DC-T cell coculture with naïve B cells (OVA-IEC-DC-T-B) increased the percentage of CD25+ activated B cells and IgE secretion (Figure 5A, C). OVA-DC-T cells did not induce CD25 expression in B cells (OVA-DC-T-B), but tended to enhance IgG and IgE secretion (Figure 5B, C). Subsequently, mast cells were primed with supernatant from OVA-IEC-DC-T-B and OVA-DC-T-B collected after 18 days of T/B cell coculture. Degranulation upon crosslinking with anti-IgE as indicated by % β-hexosaminidase release was measured and, after mast cells were kept in culture for another 18h, IL13 and IL4 secretion were quantified. The IgE-mediated mast cell degranulation, as well as IL13 and IL4 secretion were significantly increased when mast cells were primed with the OVA-DC-T-B supernatant, but not with the OVA-IEC-DC-T-B supernatant compared to their respective controls (Figure 5D, E).

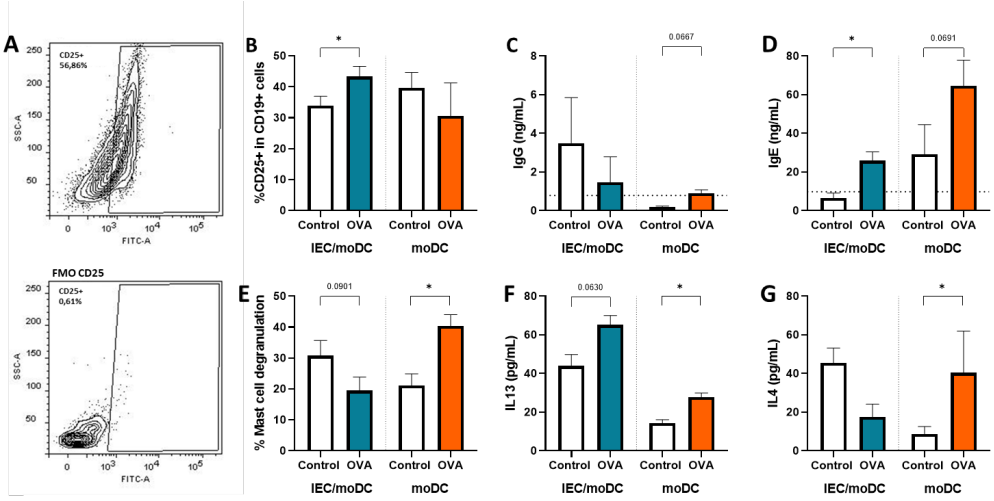

Figure 5. *OVA-IEC-moDC or OVA-moDC were coculture with allogenic naïve T cells for 4 days. After the coculture period, secreted A) IL13, B) IL4, C) IFNγ were measured in the basolateral supernatant. Furthermore, cells were analyzed by flow cytometry to determine the percentage of CD4+ cells expressing D) IL13, E) CRTH2, and F) CXCR3. A representative sample and corresponding FMO controls are displayed in G). Data is analyzed by paired t-test, n=3, mean ± SEM (* p<0.05).*

Discussion

An increasing number of individuals is suffering from food allergies. With the appearance of novel food proteins in our diets, safety testing to identify proteins with high potential to cause allergic sensitization is becoming more relevant before these products enter the market [1]. We aimed to develop the first fully human *in vitro* model to mimic the sequential steps that can lead to allergic sensitization via the mucosal immune system including the effector phase. For this approach, the hallmark food allergen ovalbumin was chosen for exposure to moDCs in absence and presence of IECs. Subsequent steps consisted of crosstalk between OVA-primed moDCs and naïve Th cells, followed by a coculture of primed naïve Th cells with naïve B cells to investigate immunoglobulin production and the capability of the T cells to instruct IgE isotype switching. Finally, the effector phase was reproduced by studying mast cell degranulation upon culture with B cell supernatant, completing the series of key events involved in the food allergic sensitization and effector response. Allergen-specific responses are difficult to achieve since they require cells with already developed memory responses to a determined protein as was shown for peanut [16]. However, the current model is based on moDCs, naïve T and B cells from healthy donors, which could also be applied for novel introduced proteins. This model shows the generic type 2 driving capacity by food allergenic proteins (sensitizing allergenicity), in this case OVA, to provoke allergic sensitization and effector responses without the requirement of using cells from patients allergic to the protein of interest. An overview of our findings is presented in Figure 6.

Exposing the HT-29 cell line, as IEC, to ovalbumin enhanced secretion of type 2 driving alarmins. Contributions of these alarmins in modulating DC function enabling them to drive Th2 cell development have been previously studied [4], [17]–[19]. Murine studies revealed that increased epithelial IL33 secretion is sufficient for *in vivo* DC activation leading to peanut allergy, independent of IL25 and TSLP [17]. However, although the authors suggest that IL33 is required to induce upregulation of OX40L in DC, we observed an increased OX40L expression in the absence of increased IL33 secretion in OVA-IEC-DC. CD86 upregulation is involved in allergic sensitization as well [20], [21] and we showed a tendency to increased CD86 expression in response to OVA in absence of IEC and a significant release of Th2-polarizing CCL22 [22] and inflammatory IL8 [10], [23] by the moDCs together with a decrease in regulatory TGFβ. Although increased secretion of alarmins and enhanced expression of OX40L on DC is related to inhibited type 1 instructing IL12p70 release [17], both IL12p70 and also type 1 driving IL15 secretion remained unaltered. The current data show that ovalbumin can provoke type 2 activation of both IEC as well as DC. Previously we confirmed that OVA-IEC could drive functional type 2 differentiation in DC, but in the latter study IEC were exposed to OVA for 24h before the IEC/moDC coculture [13]. The current study shows both OVA-IEC-DC as well as OVA-DC to obtain a type 2 moDC phenotype, but their phenotype differs depending on the presence of IEC which are known to shape the innate immune response. The present study uses a relatively high, yet physiological relevant OVA concentration, future studies could focus on exposure to different OVA concentrations and the following mucosal immune responses.

Subsequent coculture of OVA-IEC-DC and OVA-DC with allogenic naïve Th cells demonstrated differential functional polarizing outcomes of these DC. More conventional *in vitro* allergy models mostly do not include IEC [16], [24], [25], while the presence of IEC is

important in the initiation of the allergic response [13], [26], [27]. Interestingly, OVA-IEC-DC did not alter type 1 or type 2 related cytokine secretion by T-cells, while OVA-DC were capable of driving Th2 polarization as indicated by enhanced IL13 secretion and an increased percentage of IL13-expressing Th cells. Based on the differences in functional immunological outcomes, we hypothesized that even though OVA exposure via IEC enhanced CD80 and OX40L expression in OVA-IEC-DC, in these DC CD86 expression was reduced. This may have resulted in a lesser Th2 driving capacity of these DC, while IL10 secretion was increased (data not shown). Interestingly, the expression of CD86 can be suppressed by epithelial-derived factors resulting in more tolerogenic T cell effects [28]. Thus the initial presence of IEC during allergen exposure may have facilitated not only the release of type 2 activating mediators but also regulatory factors that suppressed full DC activation in this model. Yet, previous *in vitro* studies demonstrated a type 2 instructing effect from epithelial cells, which can be due to differences in model conditions [11]–[13]. However, the current study shows a stronger type 2 response to OVA in absence of IEC, while OVA-IEC-DC contribute to enhanced regulatory IL10 secretion during coculture with T cells as indicator for a more tolerogenic response to the allergen. Furthermore, in a previous study we investigated the contribution of OVA pre-exposed IEC in type 2 development via instructing moDC [13]. In the current study we use the same OVA source which contains some contamination with endotoxins. In spite of this, the OVA exposed moDC were capable of driving type 2 responses both at the level of T cells and mast cells comparable to DC2 (as shown in Supplemental Figure 3). Future studies should therefore further look into the possible contribution of endotoxins in the process of OVA induced allergic sensitization, which may also be applicable under physiologic conditions in mucosal tissues of the intestine.

A Th2-dominant environment is essential to trigger IgE production in B cells. B cells were stimulated with anti-IgM to induce aggregation of the BCR [29] as surrogate signal otherwise provoked by an allergen. The OVA-DC-T cells, which showed phenotypical Th2 polarization, tended to enhance IgE secretion by B cells and the B cell supernatant was leading to anti-IgE provoked primary human mast cell degranulation, as well as both IL13 and IL4 release by these mast cells. The OVA-IEC-DC-T cells did not induce Th2 polarization, but coculture of these T-cells with B cells enhanced CD25 expression in these B cells, indicating activation. In addition, IgE secretion was significantly increased in OVA-IEC-DC-T-B cells. Therefore, even though these OVA-IEC-DC -T cells did not show typical Th2 polarization, which is generally considered to be necessary for IgE isotype switching [30], these cells were capable of inducing IgE secretion in B cells. Some studies involving human B cells reported that IL21 produced by follicular Th cells also promotes IgE isotype-switching [31], [32], more recently this IgE boosting effect was attributed to a balance between IL21 and IL4, and strong stimulation via CD40 [33]. However, in contrast with the OVA-DC-T-B cell supernatant, the IgE present in the IEC-OVA-DC-T-B cell supernatant did not lead to mast cell degranulation upon anti-IgE crosslinking, not IL13 and IL4 secretion. This sequential model to identify the food sensitizing allergenicity risk has the advantage that also this last effector step is implemented. The model indicates if a food protein is capable of instructing DC to induce T-cell activation, contributing to a humoral response in B-cells (IgE isotype switch)m which is functionally capable of eliciting an IgE mediated effector response. Indeed, beyond IgE, B cells may produce other humoral or regulatory mediators such as IL10 and TGFβ which can prevent mast cell degranulation [34], [35]. Furthermore,

both binding of IgE and IgG to the FcεRI and several types of FcγR respectively or altered immunoglobulin glycosylation, may provide inhibitory signals affecting the anti-IgE induced mast cell degranulation [36], [37]. Therefore, future studies should focus on elucidating the role of regulatory mediators such as IL10 and TGFβ from T and B cells on IgE-mediated mast cell degranulation. This may contribute to the predictive value of the model when studying food sensitizing allergenicity risk.

The OVA-DC-T-B cell supernatant did increase anti-IgE induced mast cell degranulation as well as IL13 and IL4 secretion. Indeed, degranulation and type 2 mediator release by mast cells are the consequences of IgE induced mast cell activation and known to contribute not only to allergic symptom development, but also to further drive allergy development [37]. Here, ovalbumin exposure to moDCs in absence of IEC was capable of fully driving sequential type 2 sensitization even facilitating IgE mediated mast cell activation, while the latter was prevented when DC function was modulated by IEC (Figure 6).

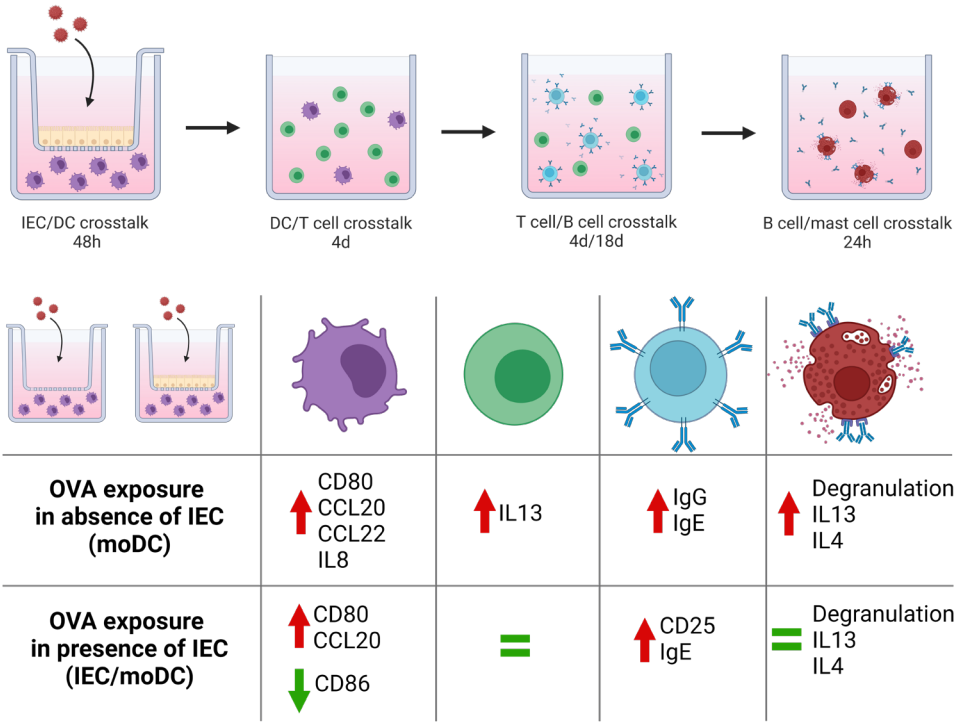

Figure 6. *Schematic overview of the coculture steps from our novel sequential mucosal food sensitizing allergenicity model and findings both in absence and presence of IEC during OVA exposure. OVA exposure in the absence of IEC resulted in mast cell activation downstream in the model, while the presence of IEC during OVA exposure did not result in mast cell activation despite the presence of IgE. This figure was created with BioRender.com.*

Although several *in vitro* models to study the development of food allergy have been published [11], [12], [38], to our knowledge this is the first method to fully describe the sequential steps from allergic sensitization towards effector cell activation in a novel developed human *in vitro* model.

Here, healthy donors were used and an allogenic DC-T cell interaction was performed, effects from sex and age differences between donors were not taken into account. Future studies should focus on investigating immunological outcomes from different concentration of OVA, different allergens as well as further refinement using immune cells from allergic donors, which may allow analysis of allergen specific immune activation in an autologous moDC-T cell coculture setting [16]. Furthermore, further characterization of the immune polarization should be investigated, e.g. the development of Th17 and Tfh cells. Basophils and innate lymphoid cells are also involved in early steps of allergic sensitization and implementing these cells or their mediators may have added value. As curative treatments for food allergic disorders are not available, novel preventive strategies could be studied in this model as well. In addition, this method could be used as a starting point to develop similar models for different mucosal and/or barrier sites, such as the lungs and skin, which are continuously controlling the balance between establishing immunity or tolerance when exposed to immune activating components.

Conclusion

The introduction of novel food products provides a demand for validated human *in vitro* assays which mimic the mucosal immune system allowing to study the sensitizing capacities of novel food proteins. We introduced a sequential mucosal food sensitizing allergenicity model using ovalbumin to provoke epithelial cell and DC activation, mimicking key events of the food allergic sensitization and effector response. This method demonstrated that ovalbumin-induced mucosal immune activation via the epithelium results in downstream IgE production, but not in mast cell degranulation. Direct exposure of moDCs to ovalbumin drives a Th2 and B cell activation, facilitating IgE mediated mast cell degranulation and cytokine release. These opposing effects indicate both an activating as well as a tolerogenic role for the intestinal epithelium in response to food allergen ovalbumin. This *in vitro* model combines multiple key events involved in allergic sensitization which can be applied to study mechanisms in allergy development and the sensitizing allergenicity of proteins.

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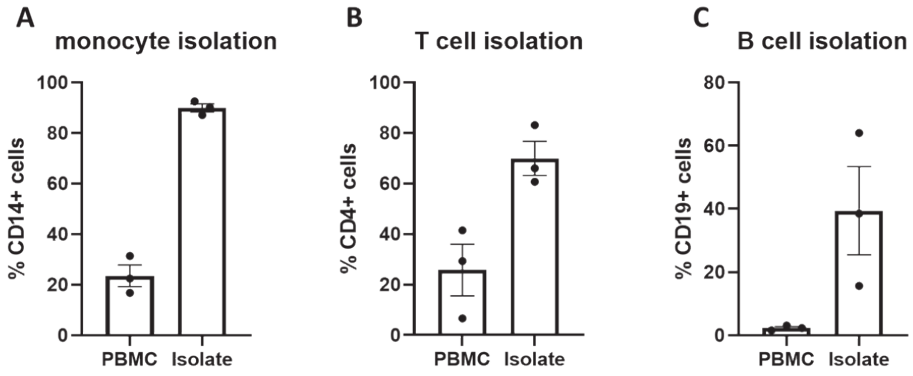
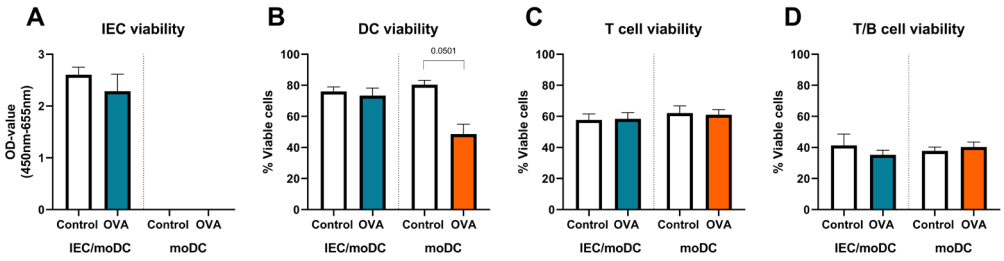
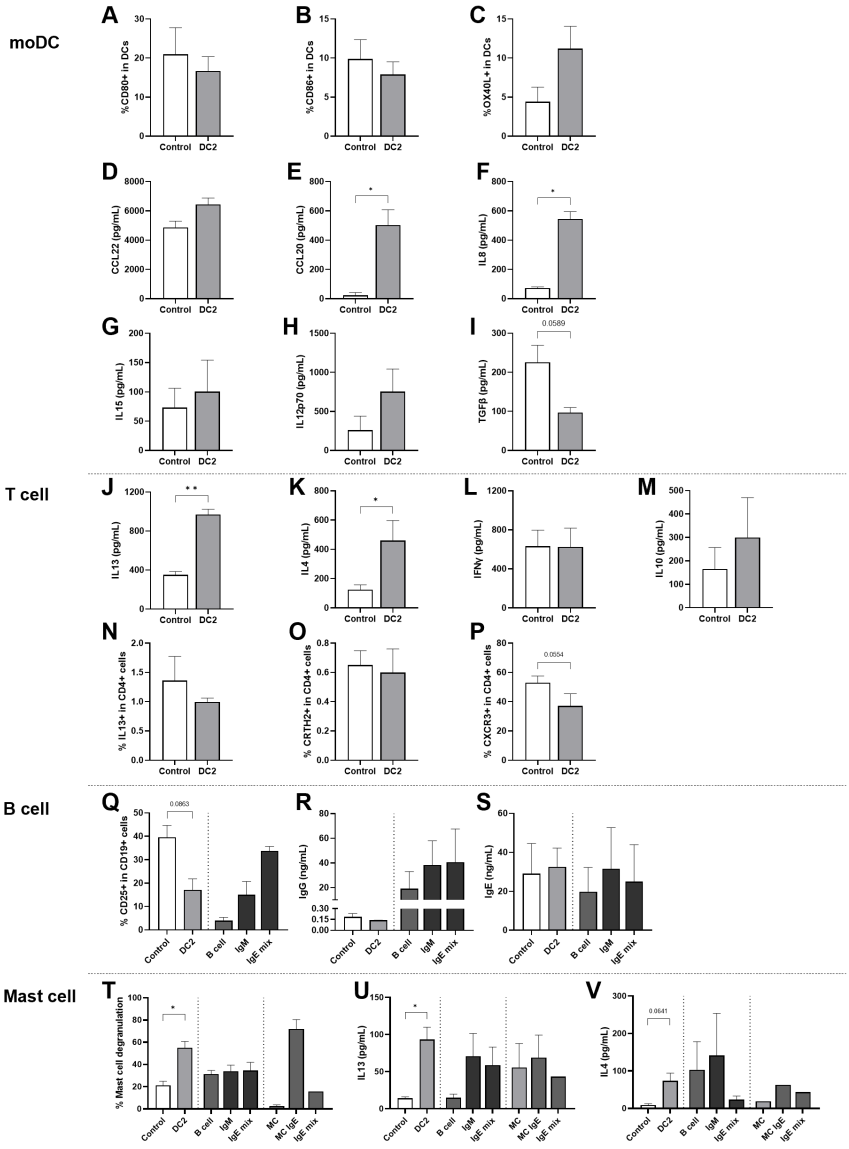
Supplemental figures



Supplemental Figure 1. *Immediately after isolation, purity of A) monocytes, B) naïve T cells and C) naïve B cells was assessed by flow cytometry using the markers CD14, CD4 and CD19 respectively.*

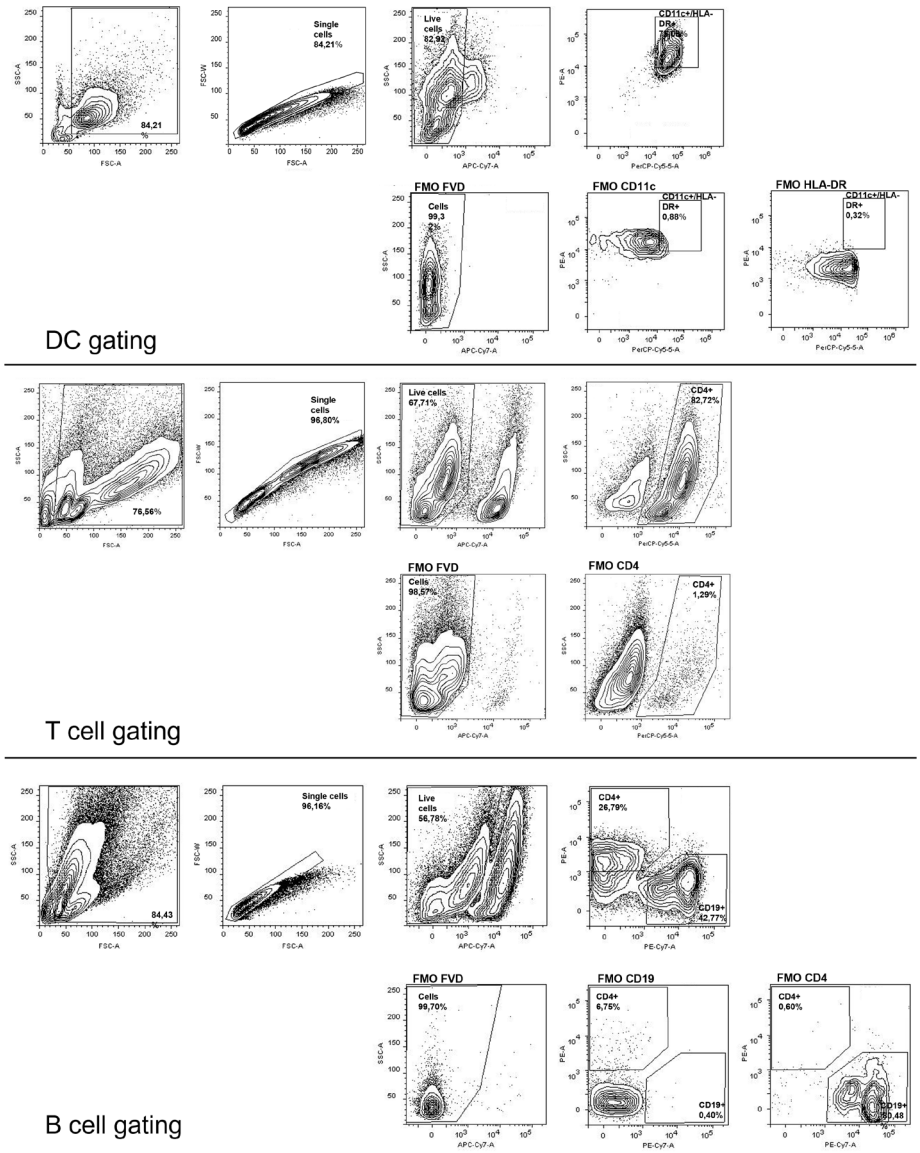


Supplemental Figure 2. *Viability of the cells was assessed after each coculture step. A) Mitochondrial activity of the IEC was determined by WST-assay. Viability of B) DCs, C) DC-T cell and D) T cell–B cell coculture was assessed by flow cytometry.*

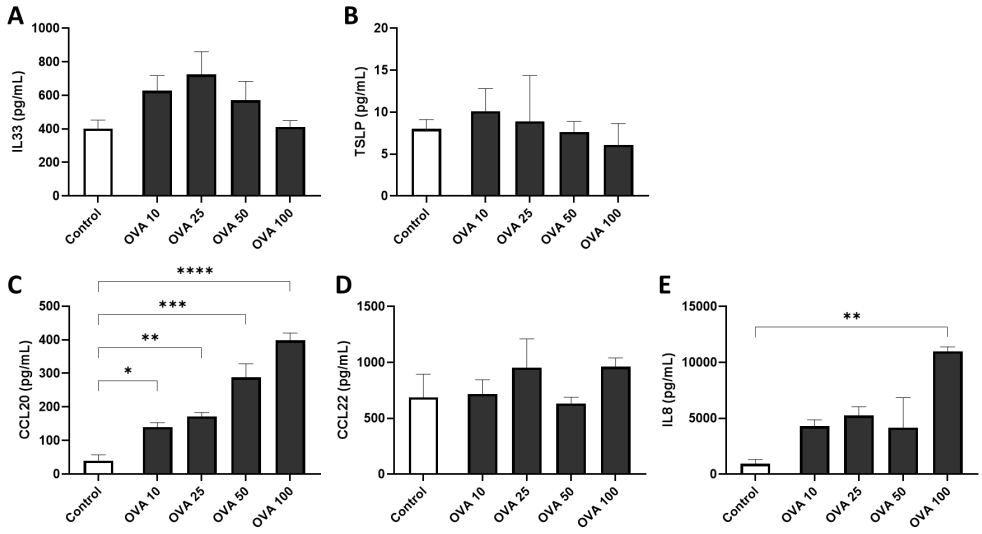


Supplemental Figure 3. *Overview of the control conditions used in the sequential mucosal food allergy model. A DC2 driving cytokine mix (consisting of 50ng/mL TNFα, 25ng/mL IL1β, 10ng/mL IL6 and 1μg/mL prostaglandin E2) was applied for 48h to the moDCs to induce maturation into type 2 DCs. Expression of the costimulatory markers A) CD80, B) CD86, and C) OX40L was determined by flow cytometry as well as secretion of D) CCL22, E) CCL20, F) IL8, G) IL15, H) IL12p70, and I) TGFβ in the collected supernatant. After sequential coculture of moDCs with naïve T cells, secretion of K) IL13, L) IL4, L) IFNγ, and M) IL10 was measured in the collected supernatant. Furthermore, T cell expression of N) IL13, O) CRTH2 and P) CXCR3 was assessed by flow cytometry. Following the succeeding coculture of primed T cells with naïve B cells, the activation status of B cells within this T cell/B cell coculture was measured after 4 days as indicated by expression of Q) CD25. In figure Q, R and S additional controls are included (black bars), these are controls for B-cell responsiveness (not cocultured with T-cells) and composed of non-activated B cells (B cell), anti-IgM activated B cells (IgM) and B-cells exposed to an IgE isotype switching mix (IgE-mix, consisting of 5μg/mL anti-IgM, 5ng/mL anti-CD40 and 20 ng/mL IL4). After 18 days of (T cell/)B cell (co)culture secretion of R) IgG and S) IgE*

was measured in the collected supernatant. Next, the collected supernatant from the B cell experiments was incubated for 24h with primary human mast cells, after which T) the percentage of mast cell degranulation was calculated upon anti-IgE incubation, and following another 18h overnight incubation, secretion of mast cell derived U) IL13 and V) IL4 was quantified. In figure T,U and V, the last three bars (dark grey) represent controls for the mast cell responsiveness with non-stimulated mast cells as negative control (MC), IgE-exposed mast cells were used as positive control (MC IgE) and IgE mix (5µg/mL anti-IgM, 5ng/mL anti-CD40 and 20 ng/mL IL4) stimulated mast cells were used to control for non-IgE mediated mast cell responses (IgE mix) since the IgE mix B-cell supernatant that was added to the mast cells also contained these stimuli. These last three MC control conditions were not overnight incubated with supernatants derived from (T/)B cells. Control and DC2 conditions are analyzed by paired t-test, n=3, mean ± SEM (p<0.5, ** p<0.01).*



Supplemental Figure 4. *On top the gating strategy to determine DC populations, in the middle the gating strategy to determine T cell populations and on the bottom the gating strategy to determine B cell populations are shown using representative samples and corresponding FMO controls.*



Supplemental Figure 5. *Optimal dose of OVA was determined by exposing 3 different passages of IEC to a dose-response in 96 wells plate. Confluent HT29 cells were exposed for 48h to 10, 25, 50 or 100µg/ml OVA in 200µL medium. After 48h, the supernatant was collected and stored for cytokine analysis by ELISA. The levels of A) IL33 and B) TSLP were unaffected by any of the OVA concentrations. C) CCL20 levels followed a dose-dependent significant increase, which was not observed for D) CCL22. Secretion of E) IL8 was only significantly enhanced after exposure to 100µg/ml OVA. Based on these findings, following experiments were conducted with 100µg/ml OVA. Data is analyzed by One-Way ANOVA and Dunnett posthoc test, n=3, mean ± SEM (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001).*

CHAPTER 6

Ovalbumin induced epithelial activation directs moDC 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 (DC) to instruct Th2-cell polarization. Food allergens trigger intestinal epithelial cell (IEC) activation. Human milk oligosaccharides (HMOS) may temper the allergic phenotype by shaping mucosal immune responses. We investigated *in vitro* mucosal immune development after allergen exposure by combining OVA pre-exposed IEC with monocyte-derived DC (OVA-IEC-DC), and subsequent coculture of OVA-IEC-DC with Th-cells. IEC were additionally preincubated with 2'FL or 3FL. OVA activation increased IEC cytokine secretion. OVA-IEC-DC 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-DC boosted inflammatory and regulatory T-cell development. 3FL-OVA-IEC lowered IL12p70 and IL23 in DC 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 IEC 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-DC silenced IL13, shifting the balance towards IL17 and IL10. This model demonstrates the contribution of IEC in OVA Th2-type immunity. 2'FL and 3FL modulate the OVA-induced activation in this novel model to study allergic sensitization.

Keywords: allergic sensitization, food allergy, human milk oligosaccharides, *in vitro* models, mucosal immunity

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 egg, cow's milk and peanut [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 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 potential 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, dendritic cells (DC) 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 GI tract defends the host against pathogenic intruders, while it is also essential for the establishment of oral tolerance for harmless food proteins. In case of food allergy, oral tolerance is hampered for specific food allergens such as the major hen's egg allergen ovalbumin. Ovalbumin is an important food allergen as it can escape gastro-intestinal (GI) digestion and has an intrinsic capacity to induce immune activation [11]. Allergic sensitization for 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 (IEC) 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 subsequent activation of type 2 driven inflammation, mediated by alarmins, such as IL33, TSLP and IL25 [13]. These alarmins prime DC 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 DC such as IL12p70, IL15 and IL10 [15]. Upon arrival in mesenteric lymph nodes, type 2 instructing DC encourage 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 an altered crosstalk between IEC and DC, dominated by suppressed secretion of alarmins and increase of regulatory factors such as TGFβ, galectin-9, and retinaldehyde dehydrogenase (RALDH) [10]. These factors can contribute to maintain intestinal homeostasis by supporting regulatory T cell (Treg) 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 or 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 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, 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, 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-a1,2-Gal-b1,4-Glc) and 3FL (Gal-β1,4-Glc-α1,3-Fuc) are structurally very similar, they may differentially shape the neonates 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 ovalbumin induced mucosal inflammation by using ovalbumin (OVA) exposed HT-29 cells, is developed. The crosstalk between these OVA pre-exposed IEC (OVA-IEC) and human monocyte-derived DC was studied. Next the OVA-IEC-DC 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 DC 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 ovalbumin 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 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 ovalbumin.

Materials and methods

HT-29 cell culturing

As IEC model, 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 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 flatbottom plate (500 µL/well). The cells were cultured for 6 days until 100% confluency. On the 6th day

the HT-29 cells were pre-incubated with 0.1% w/v 2'FL, 3FL (both HMOS are synthetically derived from lactose, Carbosynth, UK) dissolved in McCoy's 5A medium for 24 hours. The next day, 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 hours, 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 NH_4Cl, 0.5 g $KHCO_3$, 18.6 mg Na_2EDTA 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 $2x10^6$ 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 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 an LS columns that was 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 ($1x10^6$ 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 (moDC). The medium was changed on day 2, 4 and 6. On day 7, cytokines were washed away and the moDC 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 ($1x10^6$ 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 cells 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 Figure 1).

HT-29-moDC coculture

6 Days prior to the experiment, HT-29 cells were seeded in a 5 times dilution (based on surface area) in 12 wells transwell inserts (polyester membrane, 0.4µm pores) (Corning Incorporated, USA). The cells were incubated at 37°C, 5% CO_2 and 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 24h. In case of the experiments with HMOS, HT-29 cells were pre-incubated with 2'FL, 3FL (0.1% w/v dissolved in McCoy's 5A medium) (Carbosynth, UK) on day 5 for 24 hours. The next day, medium was refreshed containing new HMOS and the cells were exposed to OVA (100 µg/mL) (Sigma-Aldrich). After 24 hours of OVA exposure, apical medium with stimuli was washed away and new McCoy's 5A medium without stimuli was added. $5x10^5$ moDC in 1500 µL RPMI 1640 medium were transferred into a new 12 wells plate. The transwells inserts with washed HT-29 cells were transferred to designated moDC wells. After 48 hours 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 moDC, from the IEC-moDC coculture, in a 24 well flat-bottom plate for 5 days at 37°C in T cell medium ($1x10^6$ cells/mL). Naïve T cells were stimulated with IL2 (5ng/mL) (Prospec) & anti-CD3 (150ng/mL, clone CLB-T3/2) (Sanquin) prior to coculture allowing generic activation to be further directed by the primed allogenic moDC. 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.

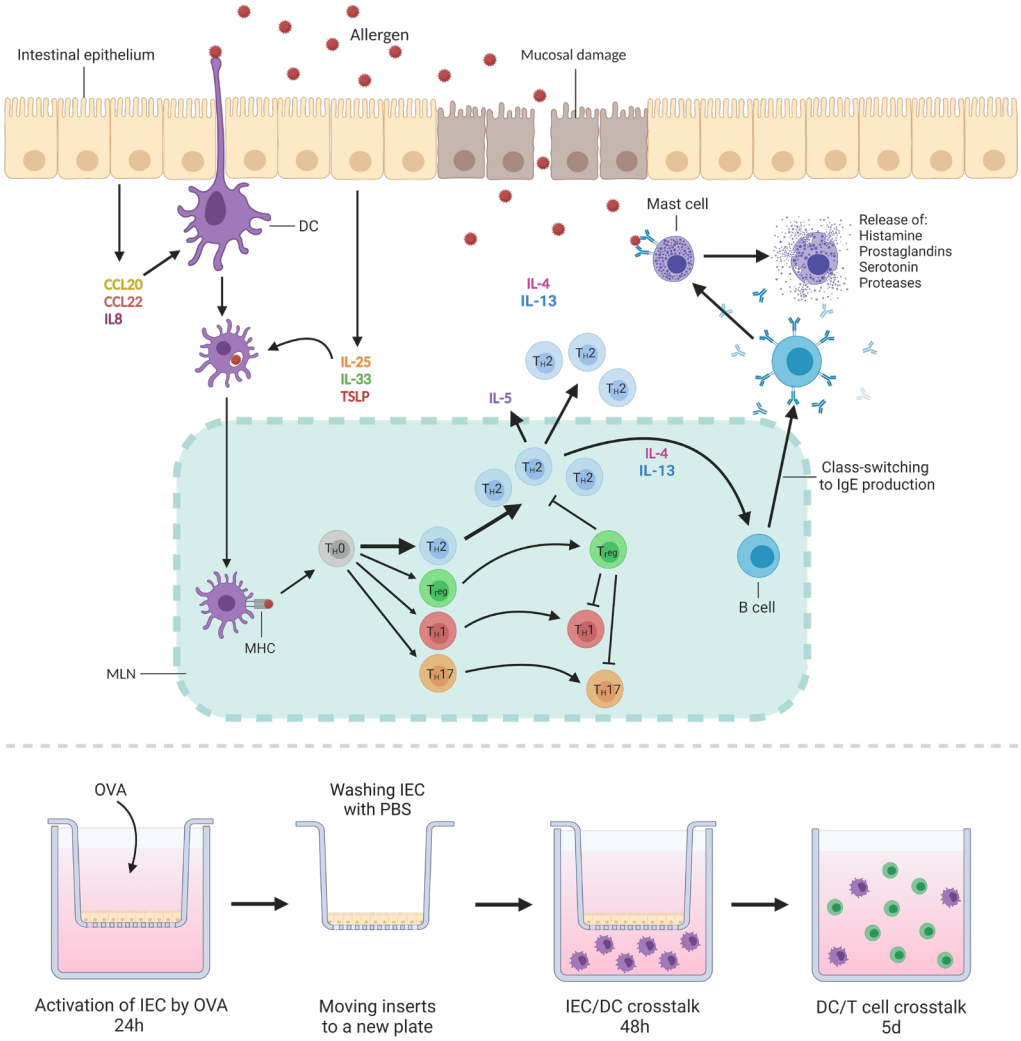

Figure 1. *Overview of the immunological mechanism of the sensitization cascade leading to food allergy, including major cell types and soluble mediators. Upon ingestion of a potential allergen the epithelium will produce alarmins, such as IL33 and TSLP, which prime DC to instruct a type 2 dominated 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 ovalbumin pre-exposed epithelial cells are cocultured with moDC during a 48h to prime the DC. Next, these OVA-IEC primed DC are cocultured with naïve CD4+ T cells to instruct Th cell polarization. Created with BioRender.com.*

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 (ThermoFischer 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. Non-specific binding was prevented by blocking with 1% BSA in PBS for 1h, after which the samples were diluted accordingly and added to the 96-well plates for 2 hours 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 minutes and substrate solution (TMB, ThermoFischer 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 655nm, 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 pre-incubated HT-29 cells (IEC) were lysed in 350 μL RNA lysis buffer (provided with RNA isolation kit) and stored in -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 260/280nm and 260/230 nm ratios, ratios between 1.8 and 2.0 were considered as 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 inhouse designed and manufactured by Bio-Rad Laboratories. Specificity and efficiency of primers was 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 (Biorad), nuclease free water (Biorad), a forward and reverse primer and isolated cDNA in a 96 well hard shell plate (Biorad). The samples were heated at 95°C for 3 minutes, denatured during 10 seconds at 95°C, followed by 30 seconds of annealing at the appropriate temperature, after completion of 40 cycles, samples were heated again during 10 minutes at 95°C and cooled down in a CFX96 Real-Time Systems (Biorad). GAPDH (Biorad) was used as 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 moDC that were collected after IEC/moDC culture were transferred to 96 wells plates (Costar Corning, USA). After washing the cells with PBS, 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 minutes at 4°C to prevent

non-specific binding of antibodies. Next, an 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 minutes 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 was analyzed using FlowLogic software (Inivai Technologies, Australia).

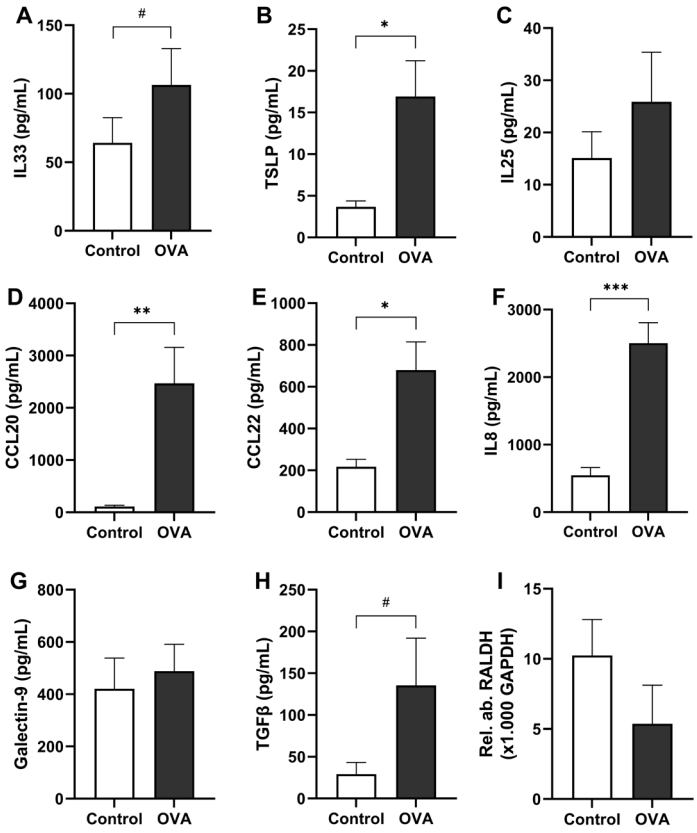

Figure 2. *Cytokine and chemokine release from IEC after apical stimulation with 100µg/mL ovalbumin for 24h. A) IL33 (p=0.0929), B) TSLP, C) IL25, D) CCL20, E) CCL22, F) IL8, G) Galectin-9, H) TGFβ (p=0.0844) supernatant concentrations and I) RALDH relative mRNA abundance were measured 24h after stimulation. Data is analyzed by paired t-test, n=9, mean ± SEM (# p<0.1, * p<0.5, ** p<0.01, *** p<0.001).*

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 ovalbumin induces IEC activation

The IEC activating properties of the high allergenic food protein ovalbumin were investigated in an intestinal *in vitro* setting, by subjecting IEC apically to the allergen ovalbumin mimicking the *in vivo* situation (Figure 1). After 24h OVA induced IEC inflammatory response was measured in collected supernatants. Ovalbumin exposure promoted the release of several cytokines by the HT-29 cells (Figure 2). Secretion of the alarmins IL33 and TSLP, but not IL25, were significantly increased (Figure 2A,B,C). In addition, release of chemoattractants CCL20, CCL22 and IL8 (Figure D-F) was significantly elevated. Similtaneously, the secretion of immunoregulatory TGFβ (Figure 2H) was also found to increase, which was not observed for galectin-9 (Figure 2G) and RALDH mRNA expression (Figure 2I). These results demonstrate that ovalbumin is capable of inducing the release of inflammatory mediators in IEC, including alarmins known to play a role in the induction of allergic sensitization.

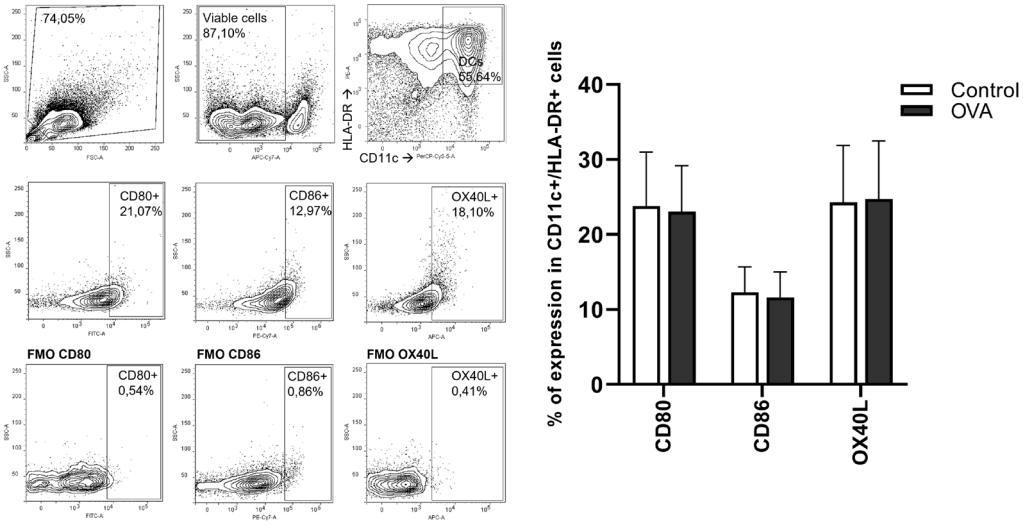

Figure 3. *Phenotype of moDC after 48h coculture with OVA-IEC. Percentage of surface expression of CD11c and HLA-DR was examined as representation of a DC phenotype, within this population the percentage of expression of CD80, CD86 and OX40L was measured. Viability of the moDC was unaffected by coculture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

OVA-IEC 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* (Figure 1). After 24h exposure to OVA, the OVA exposed IEC (OVA-IEC) were washed, IEC containing transwell filters were placed in a new plate and moDC were added to the basolateral compartment. In this model the effects of the activated OVA-IEC on the crosstalk with basolateral moDC after 48h was studied. The frequency of CD11c+HLA-DR+ surface marker expression of OVA-IEC conditioned moDC was not affected compared to control-IEC conditioned moDC (Figure 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 mediators were measured in the supernatant of the IEC-DC cocultures (Figure 4). In the OVA-IEC exposed moDC cocultures a significant decrease in CCL22 (Figure 4A), IL12p70 (Figure 4E), IL23 (Figure 4F) and IL10 (Figure 4I) was observed compared to control-IEC exposed moDC, while the levels of IL6, IL8, IL15, galectin-9 and TGFβ remained unaltered. Collectively, OVA-IEC-DC did not show a matured phenotype since the expression of costimulatory molecules remained unaffected, while they secreted smaller amount of several mediators during 48h of incubation.

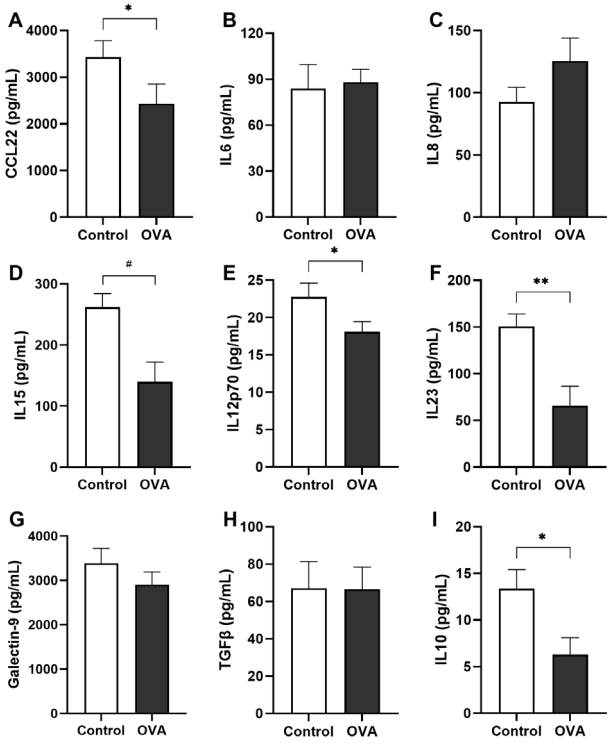

***Figure 4**. Chemokine and cytokine secretion from 48h IEC/moDC coculture after 24h stimulation of IEC with OVA. Supernatant concentrations of A) CCL22, B) IL6, C) IL8, D) IL15 (p=0.0707), E) IL12p70, F) IL23, G) Galectin-9, H) TGFβ and I) IL10 were measured. Data is analyzed by paired t-test, n=6, mean ± SEM (* p<0.05).*

OVA-IEC-DC instruct IL13 and IFNγ release in allogenic naïve Th cells

To study the functionality of OVA-IEC-DC, they were cocultured for 5 days with allogenic naïve Th cells. Supernatants of the IEC-DC/T cell coculture were collected for cytokine quantification. IL13, IL9 and IL5 were measured to reflect 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 (Figure 5). Naïve T cells incubated with OVA-IEC-DC secreted increased levels of IL13, and IFNγ, while TGFβ showed a similar tendency (p=0.0970) compared to T cells cocultured with control-IEC-DC (Figure 5A, D, H). IL9, IL5, IL25 and IL17 (Figure 5B, C, E, F) and also galectin-9 and IL10 were not affected, even though IL10 showed an inclining pattern (Figure 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-DC compared to control-IEC-DC. These data reveal that OVA exposed IEC 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.

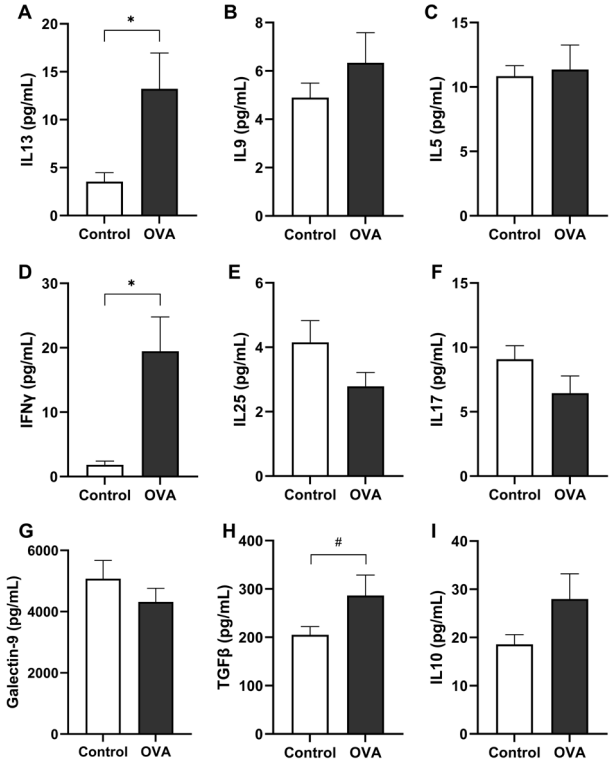

Figure 5. *Cytokine secretion from moDC/naïve T cell coculture after priming of moDC with OVA stimulated IEC. Supernatant concentrations of A) IL13, B) IL9, C) IL5, D) IFNγ, E) IL25, F) IL17, G) Galectin-9, H) TGFβ (p=0.0970) and I) IL10 were measured. Data is analyzed by paired t-test, n=6, mean ± SEM (* p<0.05).*

Exposing IEC to 2'FL or 3FL induces production of regulatory mediators from IEC, IEC-DC and DC/T cells

In the developed model the effects of HMOS 2'FL and 3FL were studied when exposed to IEC in absence (2'FL-IEC or 3FL-IEC) or presence of OVA (2'FL-OVA-IEC or 3FL-OVA-IEC). After 48h exposure to HMOS, with added OVA exposure in the last 24h, the IEC were washed, IEC containing transwell filters were placed in a new plate and moDC 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, moDC were again combined with allogenic naïve Th cells for the final coculture step. Both 2'FL and 3FL did not affect secretion of alarmins and chemoattractants, but 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 DC (2'FL-IEC-DC) tended to increase TGFβ, while 3FL-IEC conditioned DC (3FL-IEC-DC) cocultures showed decreased IL12p70 concentrations compared to control-IEC-DC. 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-DC also increased IL10 and tended to increase IL25 secretion in the IEC-DC/T cell coculture.

Ovalbumin 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 24h with HMOS prior to and during ovalbumin exposure, after 24h inflammatory mediator release was assessed. Combined exposure of OVA and 2'FL or 3FL increased IL33 release compared to unstimulated HT-29 cells (Figure 6A), IL33 concentrations were higher in 3FL and OVA exposed HT-29 cells supernatants compared to 2'FL-OVA-IEC supernatants. Secretion of TSLP (Figure 6B) was significantly increased upon ovalbumin stimulation compared to control, which is abolished by 2'FL and 3FL pre-incubation (Figure 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 (Figure 6D, E). 2'FL or 3FL pre-incubation did not change IL25 and galectin-9 levels, but exposure to OVA in the presence of 3FL tended to increase TGFβ (Figure 6C, F, G). Interestingly, the relative mRNA expression of RALDH (Figure 6I) was 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 ovalbumin induced inflammation.

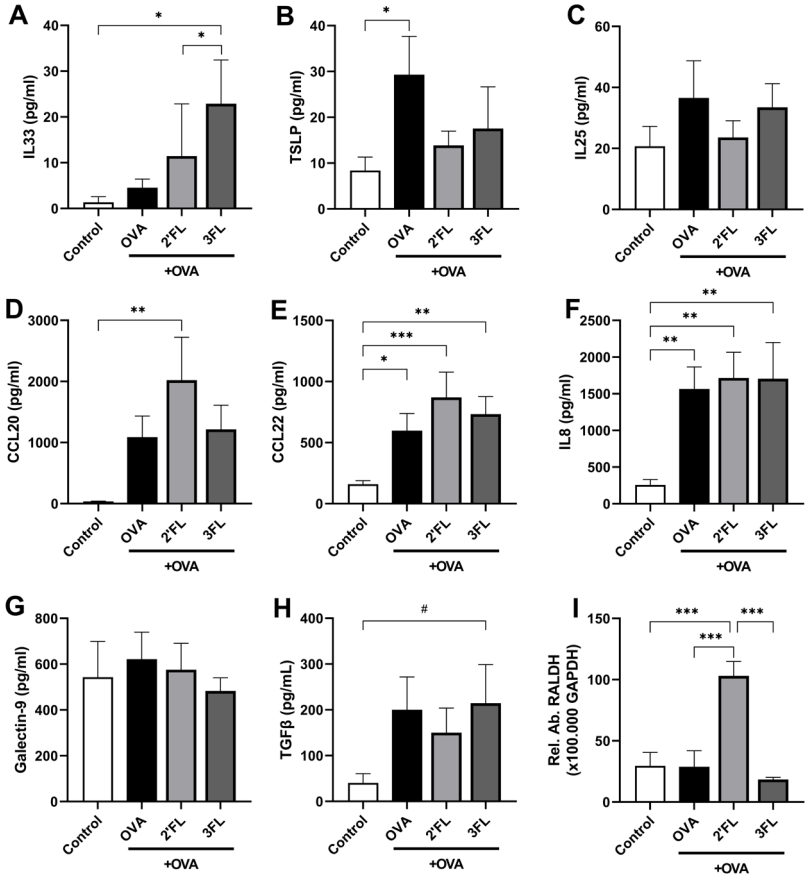

Figure 6. *Cytokine and chemokine release from IEC after 24h apical preincubation with 2'FL or 3FL followed by apical stimulation with 10μμg/mL OVA for another 24h. A) IL33, B) TSLP, C) IL25, D) CCL20, E) CCL22, F) IL8, G) Galectin-9, H) TGFβ (p=0.0796) supernatant concentrations and I) RALDH relative mRNA abundance were measured 24h 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).*

Table 2. *Overview of cytokine and chemokine secretion (pg/mL unless indicated otherwise) or RALDH mRNA expression in the IEC-moDC-T cell model when IEC were exposed to 2'FL and 3FL in absence of ovalbumin. Data is analyzed by One-Way-ANOVA and Dunnett'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*

IEC	Control	2'FL	3FL	IEC/DC	Control	2'FL	3FL	DC/T cell	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±42.2	132.9±38.8	**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**	3643±203.4	3409±278.8	3759±592.5	**Galectin-9**	6583±1619	4176±1069*	4601±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	46.1±17.7	**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*

3FL increases CD86 expression while blocking cytokine release in OVA-IEC-DC

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

In addition, CCL22 secretion (Figure 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-DC showed an inclining pattern for IL15 and IL10 when compared to OVA-IEC-DC (Figure 8B, E, I). By contrast, 3FL-OVA-IEC-DC had lower concentrations of IL12p70 and IL23 compared to control-OVA-IEC-DC and/or control-IEC-DC (Figure 9 D-F). Regulatory mediators galectin-9, TGFβ and IL10 (Figure 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.

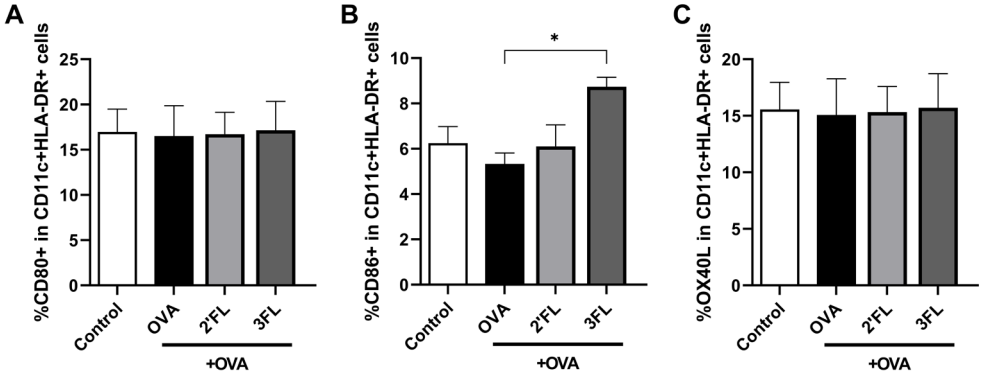

Figure 7. *Percentage of surface expression of CD80, CD86 and OX40L in moDC (CD11c+/HLA-DR+ cells), cocultured with HMOS preincubated and OVA stimulated IEC, was measured. Data is analyzed by One-way ANOVA and Bonferroni's post hoc tests, n=3, mean ± SEM (* p<0.5).*

3FL exposed OVA-IEC primed DC 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 moDC was studied using DC/T cell cocultures, to determine the effects on downstream naïve T cell development. OVA-IEC-DC and 2'FL-OVA-IEC-DC increased the secretion of IL13 by naïve T cells, while this was hindered by 3FL-OVA-IEC-DC. Furthermore, 2'FL-OVA-IEC-DC increased or tended to increase

inflammatory IL9, IFNγ, IL25, but also regulatory mediators TGFβ and IL10 secretion by T cells compared to DC primed with control, OVA or 3FL-OVA exposed IEC (Figure 9A-I). Both 2'FL-OVA-IEC-DC and 3FL-OVA-IEC-DC increased IL17 compared to control-OVA-IEC-DC exposed T cells. However, next to 2FL, also 3'FL enhanced regulatory IL10 secretion in parallel (Figure 9F, I). On the other hand, galectin-9 concentrations were decreased by both 2'FL and 3FL (Figure 9G).

These data together show that the initial differential response observed in HMOS preincubated and ovalbumin stimulated IEC, results in downstream altered T cell responses. 2'FL-OVA-IEC priming of DC shows a general increase in inflammatory and regulatory cytokine secretion, dominated by a regulatory IL10 component. Instead, 3FL-OVA-IEC priming of DC prevents secretion of type 2 related cytokines and enhances the regulatory response by increased IL10 levels. Hence, improving the balance of both IL10 and TGFβ over effector type 2 and type 1 Th cell responses and enhancing the type1 over type 2 response skewing away from the allergic phenotype.

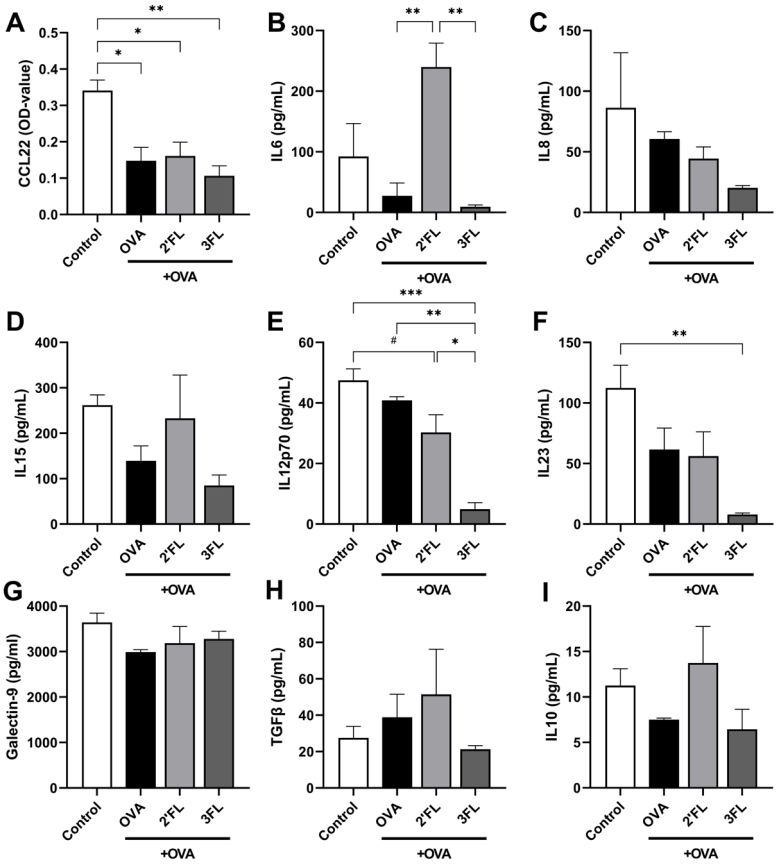

Figure 8. *Chemokine and cytokine release from IEC/moDC coculture after HMOS preincubation and OVA stimulation of IEC. Supernatant concentrations of A) CCL22, B) IL6, C) IL8, D) IL15, E) IL12p70 (p=0.0779), F) IL23, G) Galectin-9, H) TGFβ and I) IL10 were measured. Data is analyzed by One-way ANOVA and 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).*

Discussion

A rise in the incidence and prevalence in 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 ovalbumin was shown to trigger the sequential steps known to drive allergic sensitization (Figure 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, indicating 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 in 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 DC and T cells isolated from murine spleens [24]. Fu *et al.* exposed the murine epithelial and the dendritic cells to ovalbumin 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 ovalbumin as we have demonstrated on mediator secretion. However, in the current manuscript we only exposed the IEC to ovalbumin, which resulted in activation of the IEC, IEC were washed and cocultured with moDC. Ensuring that in our model direct effects of ovalbumin 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 ovalbumin on IEC and the subsequent modulation of the moDC and allogenic naïve T cell response.

Ovalbumin exposure of HT-29 cells was found to activate these cells to release type 2 inflammatory mediators (Figure 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 DC to a pro-allergic phenotype [26], [27]. This indicates the intrinsic capacities of ovalbumin 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 ovalbumin 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 ovalbumin exposure. This was observed since moDC cocultured with these OVA-primed IEC 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-IEC produced many inflammatory mediators, including type 2 driving cytokines (Figure 2), coculture of the OVA pre-exposed IEC with immature moDC 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 DC was changed. Indeed, during subsequent coculture of these OVA-IEC-DC with naïve Th cells, OVA-IEC instructed DC were are capable of differentially inducing a distinct T cell cytokine response.

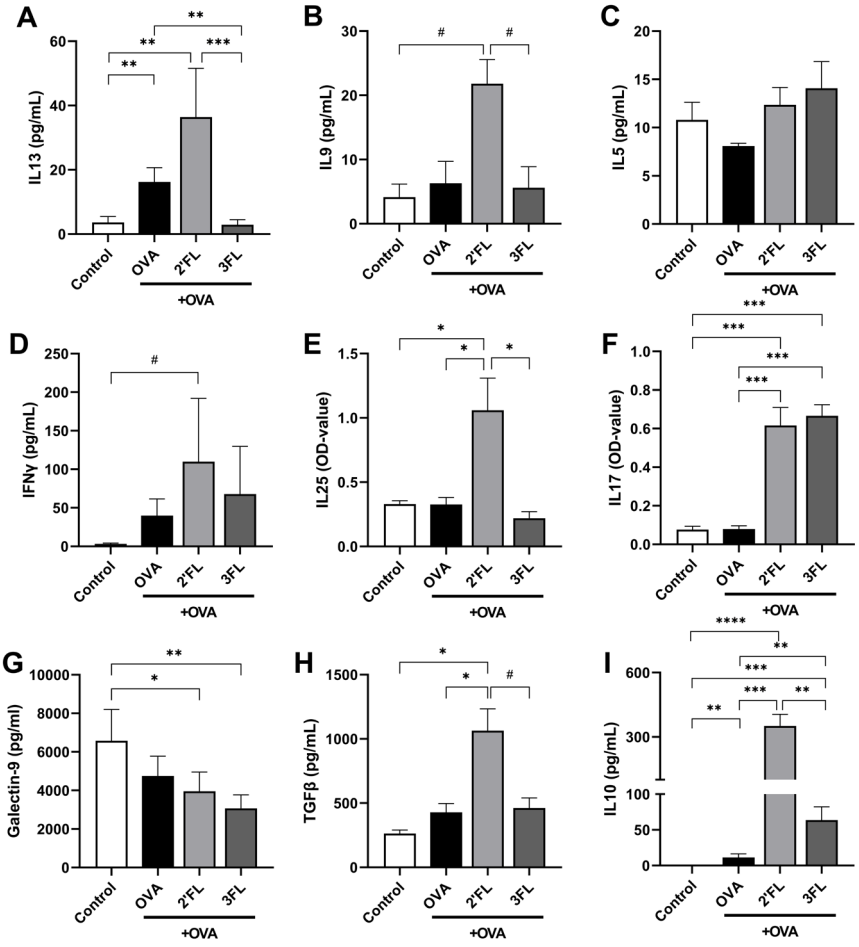

Figure 9. *Cytokine secretion from moDC/naïve T cell coculture after priming of moDC with HMOS preincubated and OVA stimulated IEC. Supernatant concentrations of A) IL13, B) IL9 (p=0.0664; p=0.0963), C) IL5, D) IFNγ (p=0.0630), E) IL25, F) IL17, G) Galectin-9, H) TGFβ (p=0.0596) and I) IL10 were measured. 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).*

Strikingly, during the following coculture of the OVA-IEC primed DC with allogenic naïve T cells showed the functional response of these DC, as they enhanced type 2 IL13 as well as type 1 IFNγ secretion by the Th cells (Figure 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 responses 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 ovalbumin exposed HT-29 cells functionally affected moDC 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 DC to drive immune activation at the T cell level, which indicates a generic immune polarizing 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 a 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 ovalbumin induced type 2 inflammation were explored in the IEC-moDC-T cell model. Preincubating IEC with 2'FL or 3FL for 24h prior to ovalbumin exposure, differentially modified the ovalbumin induced mediator secretion (Figure 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 prevention of inflammatory responses. Both 2'FL and 3FL differentially modified the OVA induced HT-29 cells 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 DC. IL6 release by DC is known to enhance differentiation into T helper subsets, while inhibiting development and activity of Treg cells [39]. On the other hand, incubation with 3FL increased the expression of CD86 in OVA-IEC-DC and almost completely silenced IL6, IL8, IL12p70 and IL23 secretion by DC. Perdijk *et al.* demonstrated that incubating moDC 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 [40]. 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 ovalbumin activation were further studied via the downstream effects of the OVA-IEC primed DC on naïve T cell development. Here we showed 2'FL-OVA-IEC-DC to induce a general immunostimulatory effect which is counter balanced by and increase in regulatory IL10 and TGFβ release from the differentiated T cells (Figure 9), which was also observed in the absence of ovalbumin (Table 1). Previously, Ayechu-Muruzabal *et al.* 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 [10]. 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 non-digestible 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 DC/T cells supernatants, 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-DC exposed T cells did not enhance TGFβ levels, but strongly increased IL10 secretion. This may indicate that 3FL suppresses the development of a type 2 response, in association with an 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 drive 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 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], 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 investigate 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 for 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 ovalbumin induced type 2 inflammation (Figure 1), in which IEC activation plays a key role in type 2 and type 1 T cell development. Ovalbumin pre-exposed 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 ovalbumin mediated epithelial activation. 2'FL-OVA-IEC 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.

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CHAPTER 7

Butyrate interacts with the effects of 2'FL and 3FL to modulate *in vitro* ovalbumin induced immune activation, and 2'FL lowers mucosal mast cell activation in a preclinical model for hen's egg allergy

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Abstract

Early life provides a window of opportunity to prevent the development of food allergy. With a prevalence of 0,5-2% in infants, hen's eggs allergy is one of the most common food allergies. Here, the immunomodulatory effects of human milk oligosaccharides (HMOS), 2'-fucosyllactose (2'FL) and 3-fucosyllactose (3FL), were studied in an *in vitro* mucosal immune model and an *in vivo* murine model for hen's egg (ovalbumin) allergy. Intestinal epithelial cells (IEC)/dendritic cells (DC) and DC/T cell cocultures were used to expose IEC to ovalbumin in an *in vitro* mucosal immune model. The effects of epithelial preincubation with 0,1% 2'FL or 3FL and/or 0,5mM butyrate were studied. 3-4 Weeks-old female C3H/HeOuJ mice were fed AIN93G diets containing 0,1%-0,5% 2'FL or 3FL two weeks prior to and during OVA sensitization and challenge. Allergic symptoms, systemic and local immune parameters were assessed. Exposing IEC to butyrate *in vitro* left IEC/DC/T cell crosstalk unaffected, while 2'FL and 3FL showed differential immunomodulatory effects. In 3FL exposed IEC-DC-T cells, the IFNγ and IL10 secretion was enhanced. This was observed upon preincubation of IEC to 2'FL and butyrate as well, but not 2'FL alone. In presence of OVA and 2'FL an increase of downstream type 1, type 2 and regulatory responses were detected. In contrast when adding OVA and 3FL a reduction in type 2 and increment of type 1 responses, independent of butyrate were detected. The presence of butyrate did not affect OVA activation, but when combined with 3FL an increase in IL6 release from DCs was seen ($p<0.001$). OVA allergic mice receiving 0,5% 3FL diet had a lower %Th2 cells in MLNs, but the humoral response was unaltered as compared to control mice. OVA-allergic mice receiving 0,1 or 0,5% 2'FL diets had lower serum levels of OVA-IgG2a ($p<0.05$) or the mast cell marker mMCP1, in association with increased cecal short chain fatty acids (SCFA) concentration ($p<0.05$). *In vitro* butyrate exposure promotes the development of a downstream type 1 and regulatory response observed after 2'FL exposure. 2'FL and 3FL differentially modulate ovalbumin induced mucosal inflammation predominantly independent of butyrate. Mice receiving dietary 3FL during ovalbumin sensitization and challenge had lowered Th2 activation, while the frequency of Treg cells was enhanced. By contrast, 2'FL improved the humoral immune response and suppressed mast cell activation in association with increased SCFA production in the murine model for hen's egg allergy.

Keywords: allergic sensitization, human milk oligosaccharides, mucosal immunology, short chain fatty acids

Introduction

Short chain fatty acids (SCFA) are known for their immunomodulatory effects and are formed during fermentation of non-digestible oligosaccharides (NDOs) by the intestinal microbiota. In early life human milk oligosaccharides (HMOS), present in breast milk, form the major source of NDOs. Various intestinal microbial species are equipped to metabolize HMOS into SCFA [1], [2]. Immunomodulatory properties have been attributed to HMOS and SCFA, including both direct effects on immune cells and indirect effects via the microbiome [3]. Some of these effects involve promoted mucus production, improved gut integrity, interaction with G protein-coupled receptors (GPRs), inhibition of histone deacetylases (HDACs) and the NF-κB pathway in IEC as well as improved Treg formation [4]–[9]. Many of these pathways are involved in allergic diseases also.

An increasing percentage of the human population is developing allergic diseases, especially early in life [10]. Belonging to the top three most common food allergens, 0,5% to 2% of infants become allergic to hen's egg, of which ovalbumin is one of the major allergens. Ovalbumin (OVA), also known as Gal d2, is a phospho-glycoprotein with a molecular weight of ~44,5 kDa [11]. As curative treatments for allergic diseases are not available yet, the focus has shifted to study potential preventive strategies. Early life has been identified as a window of opportunity for the prevention of food allergic diseases. During the first 1000 days of life the immune system matures and moves away from the pre- and neonatal associated Th2-dominated immune status, while the microbiome develops to an adult-like steady state [12]–[14].

Human milk contains uniquely and very specific high levels of oligosaccharides, indicating that these structures may have a biological function during the development of neonates [15]. Depending on e.g. the genetic background and stage of lactation the mother mainly excretes 2'FL or 3FL [16], [17]. Previously we showed that *in vitro* exposure to 2'FL and 3FL resulted in a differential immunomodulatory effect during *in vitro* OVA induced mucosal inflammation [18]. Specific bifidobacteria and lactobacilli are able to metabolize fucosylated HMOS into SCFA [19]. SCFAs, in particular butyrate, are considered to possess the most potent immunomodulatory properties [20], and restores or maintains homeostasis in mucosal immune responses [21]–[24]. Furthermore, neonates with high butyrate levels in fecal samples were less likely to develop allergic rhinitis, allergic asthma and food allergy during childhood [25], substantiating the potential protective role of butyrate in the development of allergic sensitization.

To the best of our knowledge interactions between HMOS and SCFA have not been studied so far *in vitro*, while the exposure occurs simultaneously *in vivo*. Therefore, this study investigated the potential interaction between butyrate and the most common fucosylated human milk oligosaccharides, 2'-fucosyllactose (2'FL) and 3-fucosyllactose (3FL), on intestinal epithelial function during homeostasis or upon OVA induced type 2 activation and subsequent dendritic cell maturation and T cell functioning in a mucosal coculture model. To validate these findings, 2'FL or 3FL supplemented diets were applied in a murine model for OVA induced food allergy. Systemic allergic symptoms after challenge as well as immunological parameters were measured, furthermore SCFA levels in the cecum were determined.

Materials and methods

HT-29 cell culturing

The human colon adenocarcinoma HT-29 cell line (passages 148-156) was used to model IEC. The cells were cultured in McCoy's 5A medium (Gibco, USA) supplemented with 10% fetal calf serum (Gibco), penicillin (100 U/mL) and streptomycin (100 µg/mL) (Sigma-Aldrich, UK). The IEC were cultured at 5% CO_2 influx and 37°C. Medium refreshments occurred every 2-3 days and the cells were trypsinized at 80-90% confluency to passage.

PBMC, monocyte and naïve T cell isolation and culture

Isolation of human PBMCs from buffy coats from healthy donors (Dutch Blood Bank, Netherlands) was performed by density gradient centrifugation (Greiner Bio-One, The Netherlands) as previously described [18]. Monocytes were isolated from PBMCs through magnetic separation via negative selection according to the manufacturer's instructions (Miltenyi Biotec, Germany). Subsequently, monocytes were cultured for 7 days in RPMI 1640 (Lonza, Switzerland) with 10% FCS, penicillin (100 U/mL) and streptomycin (100 µg/mL) at $2x10^6$ cells/mL. Human recombinant IL4 (100 ng/mL) and GM-CSF (60 ng/mL) (Prospec, Israel) were added to differentiate monocytes into monocyte derived dendritic cells (moDCs) for 5 days. Every other day half of the medium was refreshed and new cytokines were added until the moDCs were collected for coculture. Naïve CD4+ T cells were isolated from PBMCs using a negative selection MACS kit according to the manufacturer's instructions (Miltenyi Biotec). The collected T cells were resuspended in T cell medium (IMDM with 10% FCS, penicillin (100 U/mL), streptomycin (100 µg/mL), 20 µg/mL apo-transferrin (Sigma-Aldrich) and 50µM β-mercaptoethanol) and directly used.

IEC, IEC-moDC, moDC-T cell model description

This model is previously described [18] and can be used to study the cross talk between epithelial cells and dendritic cells, and subsequently dendritic cells and naïve T cells during allergic sensitization.

Sodium butyrate (Sigma-Aldrich) was dissolved in PBS (0.5M). 2'FL and 3FL, enzymatically produced from lactose, were purchased from Carbosynth (UK) and dissolved in PBS (100 mg/mL, 10% w/v%). HMOS and butyrate solutions were sterile filtered prior to use. In short, confluent HT29 cells in transwell plate were incubated with 2'FL or 3FL (0,1% w/v dissolved in McCoy's 5A medium) (Carbosynth, UK), and/or butyrate (0,5mM) (Sigma-Aldrich) for 24h (butyrate concentration is based on data shown in Supplemental Figure 1). Thereafter, medium was refreshed containing new HMOS and butyrate and designated conditions were exposed to ovalbumin (100 µg/mL) (Sigma-Aldrich) for another 24h. Basolateral medium was collected for cytokine analysis and after washing the HT29 cells with new medium, moDCs were added to the basolateral compartment for 48h. moDC were collected for FACS analysis or subsequent coculture with naïve T cells for 5 days. Control and OVA conditions in Figure 2 are shared with a previous dataset [18].

Animals

3-4 week old female C3H/HeOuJ mice were purchased from Charles River (Germany) and were randomly assigned to the control (n=6) or experimental groups (n=12). The mice were housed under sterile conditions (2 cages/group) with standard chip bedding, tissues and a plastic shelter on a 12h light/dark cycle with controlled temperature and humidity. The mice had ad libitum access to AING93 diet supplemented with or without 0,1% or 0,5% 2'FL or 3FL (2'FL and 3FL were purchased from Jennewein Biotechnologie GmbH, Germany & diets were produced by Sniff Spezialdiëten GMBH, Germany) and water. Supplementation of intervention diets with 2'FL or 3FL was isocaloric compensated with reduction in cellulose. Animal procedures were conducted in accordance with the Animal Welfare Body according to institutional guidelines for the care and use of laboratory animals as established by the Animal Ethics Committee of Utrecht University (AVD108002015262).

Animal procedures

A schematic overview of the experimental design is given in Figure 3A. Mice received the HMOS supplemented or control diets 2 weeks prior to the first sensitization. Oral sensitization occurred on experimental days 0, 7, 14, 21 and 28 with 20 mg ovalbumin (Grade V; Sigma Aldrich, USA) and 10µg cholera toxin (List Biological Laboratories, USA) in 500 µL sterile PBS. Sham mice (n=6) received cholera toxin alone. Five days after the final sensitization, mice were intradermally challenged in both ears with 12,5 µg OVA 25 µL PBS to determine acute allergic skin and systemic shock response. After the intradermal challenge, mice were orally challenged with 50 mg OVA in 500 µL PBS. Eighteen hours later, the mice were sacrificed and tissue samples were collected.

Assessment of clinical symptoms

The evaluate the severity of the allergic response to OVA, mice were intradermally challenged in both ears. Acute allergic skin response, anaphylactic shock symptoms and body temperature were determined by individuals blinded to the intervention groups. The acute allergic skin response was determined by measuring ear thickness of anesthetized mice prior to and 1h after intradermal challenge, subtracting the basal ear thickness from the ear thickness 1h after intradermal challenge results in the Δ ear swelling (µm).

cDNA synthesis and real-time qPCR

HT29 cells were cultured in 48 well plates for 6 days and preincubated for 24h with butyrate and/or 2'FL or 3FL prior to OVA exposure for 24h. HT29 cells were lysed in RNA lysis buffer (provided with RNA isolation kit) and stored at -70°C. Total mRNA content was isolated, cDNA was synthesized and real-time PCR was performed as previously described [18]. The relative expression of the gene of interest over housekeeping gene GAPDH was calculated: relative mRNA abundance = 100,000× 2 Ct[GAPDH mRNA] – Ct[target mRNA] [26].

Enzyme-linked immunosorbent assay

Supernatants collected from the different cell culture steps were analyzed for chemokine and cytokine secretion with Enzyme-Linked Immunosorbent Assays. ELISA kits were used to

determine IL6, IL10, IL13, IL17, IFNγ, TGFβ, TSLP (Thermo Fischer scientific, USA), CCL20, IL12p70 and IL33 (R&D systems, USA) secretion following the manufacturers protocols.

OVA-specific immunoglobulins were measured in murine serum. Serial dilutions of pooled serum were used to generate a standard curve. For OVA-specific IgE, 96 well high-binding plates (Costar Corning Incorporated, USA) were coated with 100 µl rat anti-mouse IgE (2 µg/ml in PBS) overnight. After blocking, diluted samples were incubated for 2h. 100µl Biotinylated OVA (1 µg/ml) was added for 90 minutes prior to incubation with streptavidin-HRP. For OVA-specific IgG1 and IgG2a, 96 well high-binding plates (Costar Corning Incorporated, USA) were coated with 100 µl OVA (10 µg/ml in PBS) overnight. After blocking, diluted samples were incubated for 2h. 100 µl Biotinylated anti-mouse IgG1 or anti-mouse IgG2a was added for 90 minutes prior to incubation with streptavidin-HRP. Colorimetric reaction was started by addition of 100 µl o-phenylenediamine dihydrochloride (0.4 mg/mL), the reaction was stopped by 75 µl of 4M H_2SO_4.

Flow cytometry

All cells collected for flow cytometric analysis were transferred to 96 wells plates (Costar Corning Incorporated). After washing the cells with PBS, viability of the cells was determined with Fixable Viability Dye 780-APC Cyanine 7 (eBioscience). Blocking buffer (PBS with 2.5% FCS and human Fc block (BD Biosciences, USA) or FcR blocking reagent mouse (Miltenyi Biotech, USA) was added for 15 minutes at 4°C to prevent non-specific binding of antibodies. Murine samples were stained using titrated volumes of the following antibodies: CD4-BV510 (clone RM4-5), CD69-PE-Cy7 (clone H1.2F3), CXCR3-PE (clone CXCR3 473), T1ST2-FITC (clone DJ8), CD25-PerCP-Cy5.5 (clone 3C7) and FoxP3-FITC (clone FJK-16s). After 30 minutes of staining at 4°C, stained cells were washed. Cells were resuspended and flow cytometric measurements were performed using BD FACS Canto II (Becton Dickinson, USA) and acquired data was analyzed using FlowLogic software (Inivai Technologies, Australia). Representative gating strategy is shown in Supplemental Figure 2.

Short chain fatty acid detection

Cecum contents were 5x diluted in ice cold PBS. 10 to 20 1.0 mm glass beats (BioSpec, USA) were added. Samples were homogenized on a vortex for 90 seconds. After centrifugation for 10 min (13,000 RPM) at 4°C, supernatants were collected and stored at -80°C until further analysis. SCFA levels were detected by gas chromatography (Shimadzu GC2010, Shimadzu Corporation, Japan).

Statistical analysis

Statistical analyses were performed using Graphpad Prism (Version 9.4.1) software.

In vitro data was analyzed by One-way ANOVA followed by Bonferroni's post hoc test on selected pairwise comparisons or Dunnett's multiple comparisons test when all conditions were compared to the OVA-exposed condition.

In vivo data was analyzed using an unpaired t-test to compare Sham and OVA groups. All intervention groups were compared to the OVA group using One-way ANOVA followed by Dunnett's multiple comparisons test. For SCFA analysis, all intervention groups were compared

to the OVA group and 0,1% 2'FL was compared to 0,1% 3FL as well as 0,5% 2'FL to 0,5% 3FL, therefore the One-way ANOVA was followed by a Bonferroni's post-hoc test with selected pairs.

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=6-10 animals per group or n= 3-6 independent paired *in vitro* repeats.

Results

Butyrate alone does not influence IEC/DC/T cell crosstalk, while 2'FL and 3FL responses are altered in the presence of butyrate during homeostasis

To investigate the potential interaction between butyrate exposure and two commonly expressed fucosylated HMOS, IEC were cultured in transwells and apically incubated for 48h with butyrate and/or 2'FL or 3FL. After incubation, IEC were washed and cocultured with moDCs for 2 days. The primed moDCs were cocultured with naïve T cells to assess functional immune outcomes.

Exposing IEC to butyrate (but-IEC) or HMOS did not affect IEC derived IL33, TSLP or IL25 secretion (Figure 1A-C) or RALDH expression (Figure 1D). However combined exposure of butyrate and 3FL (but-3FL-IEC) enhanced the relative expression of RALDH mRNA in IEC significantly as compared to the control cells (Figure 1D). After washing the exposed IEC and subsequent coculture with moDCs (IEC/DC), mediator secretion from IEC/DC (Figure 1E-H) was measured. The mediator release was not significantly affected after coculture of DC with either but-IEC or 2'FL-IEC. However, when butyrate incubation was combined with 2'FL (but-2'FL-IEC/DC), IL12p70 secretion tended to decrease (Figure 1E) while IL6 secretion increased (Figure 1F) as compared to moDC coculture with but-IEC. These data indicate that combined exposure of IEC to 2'FL and butyrate results in a different functional DC phenotype, compared to exposing the IEC to 2'FL or butyrate separately. Similar to the combination of 2'FL and butyrate, 3FL exposure alone already inhibited IL12p70 secretion (Figure 1E), which remained stable when combined with butyrate (but-3FL-IEC/DC). But-3FL-IEC/DC reduced secretion of regulatory IL10 compared to control (Figure 1H). Although TGFβ is known as important regulator in mucosal immunity, none of the incubations significantly affected secretion of TGFβ (Figure 1G).

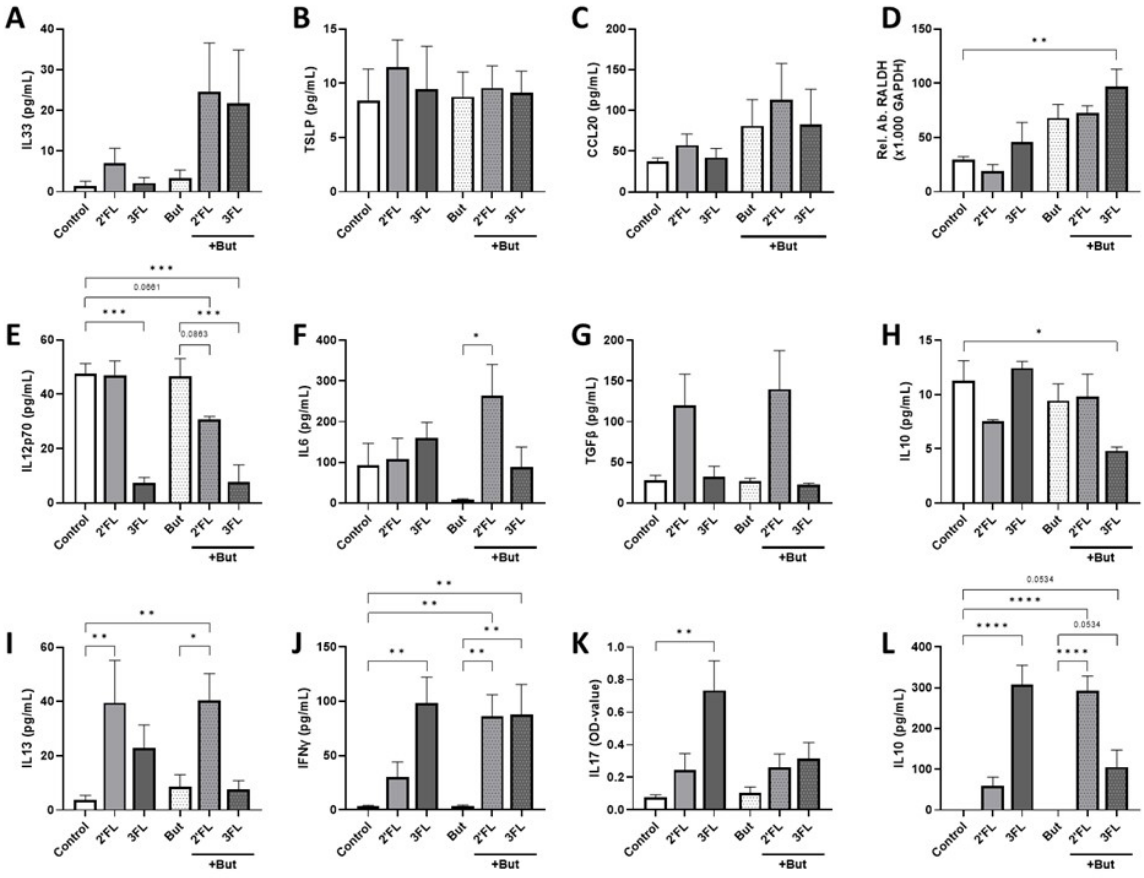


Figure 1. *Cytokine and chemokine release from IEC, IEC/moDC and moDC/T cell cocultures after 48h incubation of IEC with butyrate and/or 2'FL or 3FL. IEC supernatant concentrations of a) IL33, b) TSLP, c) CCL20 and d) relative abundance of RALDH mRNA were measured. Secretion of e) IL12p70, f) IL6, g) TGFβ and h) IL10 was assessed after IEC/moDC coculture. Secretion of the functional cytokines i) IL13 j) IFNγ k) IL17 and l) IL10 after moDC/T cell coculture were measured. Data is analyzed by One-Way ANOVA followed by Bonferroni's post hoc test, n=3-6, mean ± SEM (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).*

In order to study the functional outcomes of the differentially primed moDCs, these cells were cocultured with naïve T cells (IEC/DC/T cells). The T cell cytokine secretion was not affected by coculture with primed DC derived from but-IEC/DC compared to control (Figure 1I-L). In the absence of concurrent exposure to butyrate, coculture with 2'FL-IEC/DCs enhanced secretion of IL13 in T cells (Figure 1I), in contrast, 3FL-IEC/DC/T cells enhanced secretion of IFNγ, IL17 and IL10 (Figure 1J-L). But-2'FL-IEC/DC/T still showed increased IL13 secretion, yet in addition enhanced secretion of IFNγ and IL10 was observed as compared to control and butyrate alone. Furthermore, but-3FL-IEC/DC/T cells were found to maintain enhanced secretion of IFNγ and IL10 (p=0.0534), while the IL17 secretion remained low as compared to control and butyrate alone (Figure 1K,L).

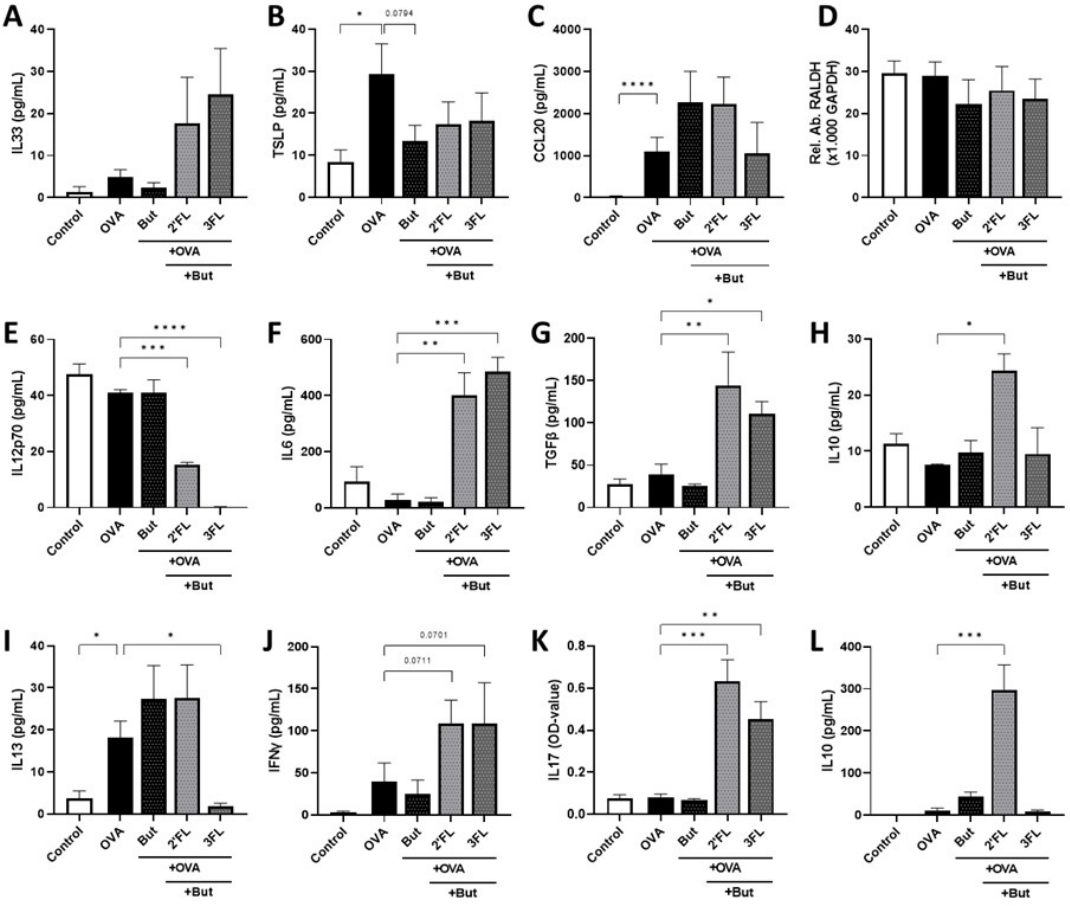


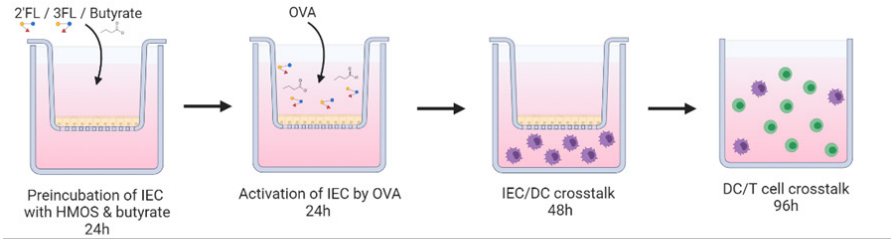
Figure 2. *Cytokine and chemokine release from IEC, IEC/moDC and moDC/T cell cocultures after 24h incubation of IEC with butyrate and/or 2'FL or 3FL and subsequent 24h exposure to OVA. IEC supernatant concentrations of a) IL33, b) TSLP, c) CCL20 and d) relative abundance of RALDH mRNA were measured. Phenotype of moDCs and mediator secretion was assessed after IEC/moDC coculture,. e) Secretion of e) IL12p70, f) IL6, g) TGFβ and h) IL10 was assessed after IEC/moDC coculture. Secretion of the functional cytokines i) IL13 j) IFNγ k) IL17 and l) IL10 after moDC/T cell coculture were measured. Data is analyzed by One-Way ANOVA followed by Dunnett's post hoc test as all groups were compared to , n=3-6, mean ± SEM (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).*

Butyrate supports 3FL mediated IL6 release from DCs and IFNγ secretion from T cells in an OVA induced type 2 inflammation model

In order to assess the impact of HMOS and interaction with butyrate in an inflammatory condition, we aimed to study the effects of butyrate and/or 2'FL or 3FL in a model of OVA induced epithelial inflammation. This model was recently used to demonstrate the differential immunomodulatory effects of 2'FL and 3FL [18], a summary of these results are displayed in Table 1 and a heatmap of all outcomes is given in Supplemental Figure 3. Incubating IEC with

butyrate prior to OVA (but-OVA-IEC) exposure tended to prevent OVA-mediated TSLP release (p=0.0794, Figure 2B), while the OVA induced CCL20 release remained unaffected by exposure to butyrate and HMOS (Figure 2C). The other IEC-related mediators (IL33 and RALDH; Figure 2A, D) were also not affected by preincubation with butyrate and/or 2'FL or 3FL prior to OVA exposure. However, 2'FL preincubation of OVA-IEC alone resulted in enhanced RALDH mRNA levels (Table 1), which was prevented by combined incubation with butyrate (Figure 2D) as compared to OVA exposed cells.

Table 1. *Overview of mediator secretion (pg/mL unless indicated otherwise) and relative RALDH mRNA expression in the IEC-moDC-T cell model when IEC were exposed to 2'FL and 3FL prior to ovalbumin stimulation. Data is analyzed by One-Way ANOVA followed by Dunnett's post hoc test compared to Control, n=3-6, mean ± SEM (1 p = 0.0877, [2] p = 0.0528, [3] p = 0.0879, [4] p = 0.0628 *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).*



	Control	OVA	OVA 2'FL	OVA 3FL
IEC				
IL33	1,4±1,2	4,9±1,7	11,5±11,4	22,9±9,6 *
TSLP	8,4±2,9	29,3±7,2 *	13,9±3,1	17,5±7,9
CCL20	37,2±4,5	1088±346,3 [1]	2021±701,4 **	1217±395,0 [2]
RALDH				
(Rel. Ab.)	29,5±3,0	28,9±3,4	103,1±11,7 ***	18,4±1,7
IEC/DC				
IL12p70	47,5±3,8	40,9±1,2	30,3±5,9 [3]	4,9±2,1 ***
IL6	92,3±52,0	27,2±20,9	240±39	9,2±3,1
TGFβ	27,5±6,3	38,9±22,0	51,5±24,9	21,3±2,0
IL10	11,3±1,8	7,5±0,2	13,7±4,0	6,4±2,2
DC/T cell				
IL13	3,6±1,9	18,2±4,0 **	36,4±10,2 **	2,9±1,6
IFNγ	3,3±1,0	39,9±21,7	109,8±82,1 [4]	67,9±51,9
IL17 (OD-value)	0,07±0,02	0,08±0,02	0,6±0,09 ***	0,7±0,06 ***
IL10	1,5±0,006	11,3±5,1 **	351,0±54,0 ****	63,7±18,6 ***

After removal of butyrate, 2'FL, 3FL and OVA from IEC by washing, the primed IEC were cocultured with moDCs. OVA exposure nor butyrate preincubation significantly affected the IEC/DC cytokine response (Figure 2E-H) compared to control. Preincubation with 3FL, but not 2'FL, resulted in a lower IL12p70 secretion in OVA-IEC/DC (Table 1) as compared to control, which was also observed in the absence of OVA (Figure 1E). Combined exposure to 2'FL and butyrate also resulted in lower levels of IL12p70 in OVA-IEC/DCs (but-2'FL-OVA-IEC/DCs), similar to but-3FL-OVA-IEC/DCs (Figure 2E), as compared to OVA-IEC/DC. IL6 secretion was enhanced after 2'FL preincubation of OVA-IEC/DCs (Table 1) and in but-2'FL-OVA-IEC/DCs (Figure 2F), this effect was observed also in absence of OVA but to a much lesser extent (Figure 1G). Furthermore, TGFβ secretion was not affected by butyrate or 2'FL preincubation alone, but was significantly increased in but-2'FL-OVA-IEC/DCs (Figure 2G). Interestingly, 3FL or butyrate preincubation separately did also not induce an increase in IL6 and TGFβ secretion by OVA-IEC/DCs (Table 1 and Figure 2F, G), however enhanced IL6 and TGFβ secretion was measured in but-3FL-OVA-IEC/DCs similar to but-2'FL-OVA-IEC/DCs as compared to OVA-IEC/DCs. IL10 secretion was only significantly enhanced in but-2'FL-OVA-IEC/DCs compared to OVA-IEC/DCs(Figure 2H), this increase was not observed in absence of OVA (Figure 1H) or butyrate (Table 1). This again shows the differential interaction between either 2'FL or 3FL with butyrate modifying the outcome of the IEC/DCs response.

After IEC/DC coculture, primed DCs were cocultured with allogenic naïve T helper cells to study functional immune outcomes. Although OVA-IEC/DC enhanced IL13 secretion during T cell coculture (Figure 2I), again preincubation of OVA-IEC with butyrate alone had no significant effects on downstream OVA induced T helper cell (but-OVA-IEC/DC/T cells) cytokine secretion (Figure 2I-L). 3FL preincubation prevented OVA-IEC/DC mediated IL13 secretion by T cells (Table 1), which was also observed in but-3FL-OVA-IEC/DC/T cells (Figure 2I). The previous observed tendency to enhanced IFNγ secretion in 2'FL-OVA-IEC/DC/T cells remained noticeable for but-2'FL-OVA-IEC/DC/T cells (p=0.0711) similar to but-3FL-OVA-IEC/DC/T cells (p=0.0701, Figure 2J) as compared to OVA-IEC/DC/T cells. These results were similar to the incubations in absence of OVA (Figure 1J). IL17 levels were enhanced by 2'FL-OVA-IEC/DC/T cells and 3FL-OVA-IEC/DC/T cells (Table 1), which remained significantly enhanced in the presence of butyrate (Figure 2K) as compared to OVA-IEC/DC/T cells. In absence of OVA, butyrate was able to dampen enhanced IL17 secretion after 3FL exposure (Figure 1K). Secretion of IL10 was significantly enhanced by but-2'FL-OVA-IEC/DC/T cells (Figure 2L) as compared to OVA-IEC/DC/T cells, similar to 2'FL-OVA-IEC/DC/T cells (Table 1). However, the significant increase in IL10 concentration in 3FL-OVA-IEC/DC/T cells (Table 1) was not present in but-3FL-OVA-IEC/DC/T cells, similar outcomes were found in absence of OVA (Figure 1L).

The observed differences in T cell functionality could affect subsequent B cell responses, which were not assessed in this *in vitro* model. Furthermore, the immunomodulatory effects of HMOS and SCFA may be different *in vivo* compared to this *in vitro* model. Therefore, a subsequent murine study was performed to assess the effects of HMOS on allergy development.

OVA-allergic mice receiving 2'FL or 3FL supplemented diets have altered immunoglobulin levels in serum

To further investigate the effects 2'FL and 3FL may have *in vivo* on allergy development, a murine model for OVA induced food allergy was used. Mice received HMOS supplemented diets starting 2 weeks prior sensitization to OVA and during the entire experiment (Figure 3A). Significant anaphylactic shock and ear swelling were observed in OVA-allergic mice 1h after intradermal challenge (Figure 3B,C). In addition, OVA-specific immunoglobulin levels in serum were observed in OVA-allergic mice 18h after oral challenge compared to non-allergic mice (Figure 3D-F). None of the HMOS supplemented diets impacted the allergic shock symptoms or OVA-specific IgE levels in serum (Figure 3D). However, mice receiving 0,5% 3FL diet tended to have a higher OVA-specific IgG1 level ($p=0.0837$) in serum as compared to OVA-allergic mice receiving control diets (Figure 3E), In contrast, mice receiving a 0,1% 2'FL supplemented diet showed significantly lower levels of OVA-specific IgG2a present in serum (Figure 3F) as compared to mice receiving control diets. This indicates an impact on B cell development during OVA sensitization, potentially modulating the allergic outcomes.

OVA-allergic mice receiving HMOS supplemented diets have altered T cell populations present in MLN

In order to study the T cell development, which guide B cell development and antibody production, mesenteric lymph nodes (MLNs) were collected 18h after oral challenge and local Th subsets were characterized by flow cytometry. Figure 4F shows a representative sample of T1ST2 in CD4+ cells from each experimental group and corresponding FMO control. The proportion of Th2 (T1ST2+ in CD4+ cells, Figure 4A) and activated Th2 cells (T1ST2+ in CD69+CD4+ cells, Figure 4B) were enhanced in OVA-allergic mice, percentages of Th1 cells (CXCR3+ in CD4+ cells, Figure 4C), Th17 cells (CCR6+RORγt+ in CD4+, Figure 4D) and Tregs (FoxP3+ in CD25+CD4+, Figure 4E) were not affected in OVA-allergic mice. MLNs from mice receiving 0,5% 3FL diets contained a lower percentage of Th2 and activated Th2 cells, together with a minor yet significant increase in Treg population compared to OVA-allergic mice.

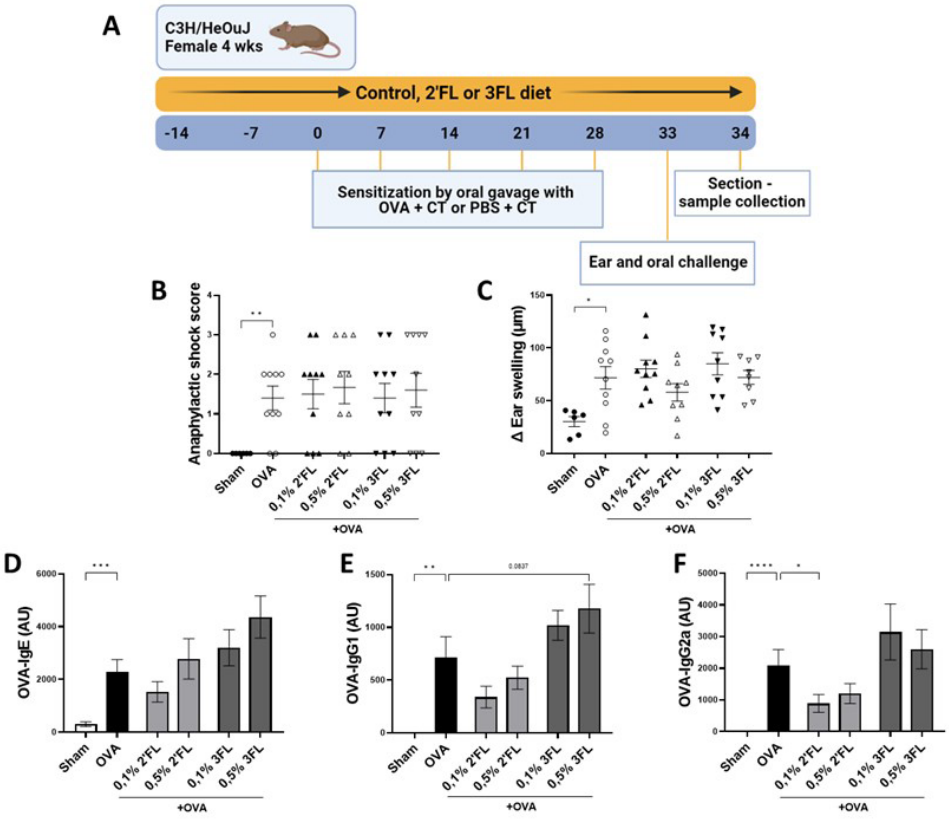


Figure 3. *The effects of dietary 2'FL and 3FL intervention on OVA food allergy in mice on anaphylactic shock and serum immunoglobulins. a) The design of the study is shown. b) The anaphylactic shock score and c) the increase in ear thickness 1h after intradermal challenge are shown. Relative levels of d) OVA-specific IgE, e) IgG1 and f) IgG2a were measured in serum 18h after oral challenge. Sham and OVA groups were analyzed by an unpaired t-test. All dietary intervention groups were compared to the OVA group using a One-Way ANOVA followed by Dunnett's post hoc test, n=6-12, mean ± SEM (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).*

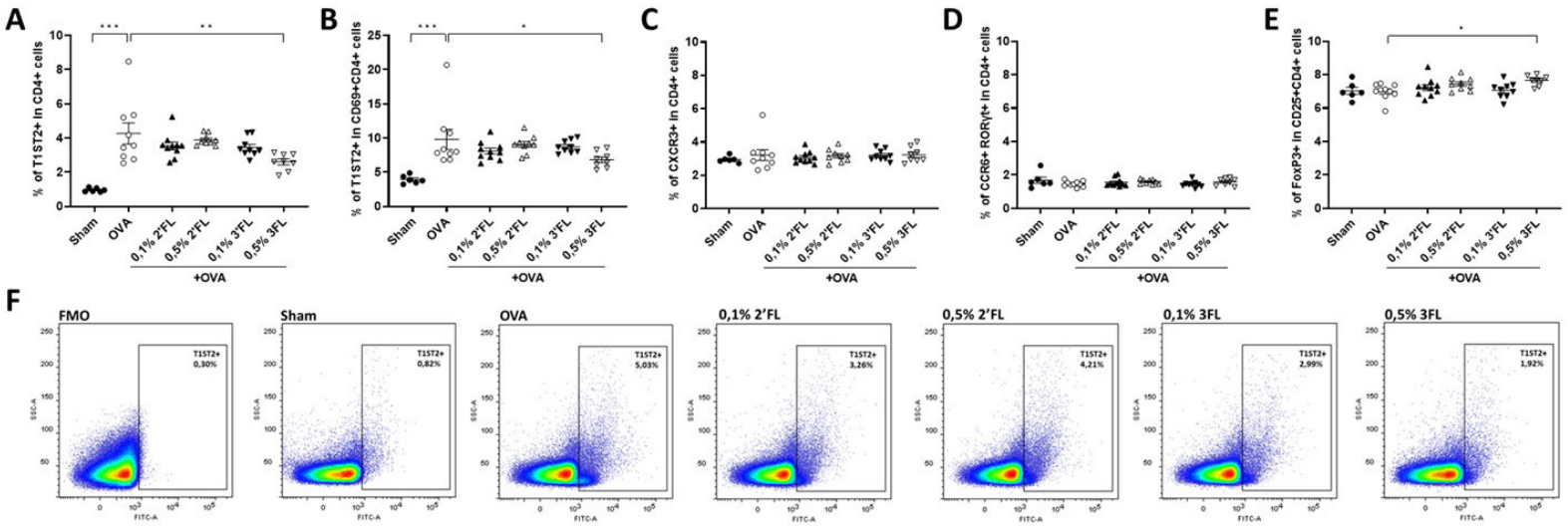


Figure 4. *Frequency of T helper subsets in MLNs. The percentage of a) CRTH2+ (Th2), b) CRTH2+ in CD69+ (activated Th2), c) CXCR3+ (Th1) and d) FoxP3+ in CD25+ (regulatory T) CD4+ cells was measured in MLNs 18h after oral challenge. e) Representative plots of CRTH2 gating strategy are shown. Sham and OVA groups were analyzed by an unpaired t-test. All dietary intervention groups were compared to the OVA group using a One-Way ANOVA followed by Dunnett's post hoc test, n=6-12, mean ± SEM (*p < 0.05, **p < 0.01, ***p < 0.001).*

HMOS supplemented diets impact mMCP1 concentration in serum and SCFA levels in cecum content of OVA-sensitized mice

As marker for mucosal mast cell degranulation after allergen exposure, mMCP1 levels were measured in serum. As expected, the mMCP1 concentration in serum was increased in OVA-allergic mice. Interestingly although no impact was on detected allergy symptoms like shock or ear swelling, in mice receiving the 0,5% 2'FL diet a significantly lower level of mMCP1 was detected, reaching levels observed in the non-allergic mice (Figure 5A). As mast cell degranulation can be affected by HDAC-inhibitors, including some SCFAs [22], SCFA levels were measured in cecum content of allergic and diet supplemented mice. The mice receiving the 0,5% 2'FL diets had significantly higher total SCFA levels (incl. acetate, propionate, butyrate, iso-butyrate, valeric acid and iso-valeric acid) in their cecum content (Figure 5B), while mice receiving 0,1% 3FL diets total SCFA levels in cecum content tended to be lower as compared to OVA-allergic mice on control diet (p=0.0590). Focusing on the most abundantly present SCFAs, mice receiving 0,1% 2'FL diet had higher levels of acetate and butyrate in the cecum content compared to mice receiving a 0,1% 3FL diet (Figure 5C, E). However, in mice receiving 0,5% supplemented diets higher propionate levels were measured (Figure 5D). These results are indicative for an immunomodulatory interaction between SCFA and HMOs, but also illustrate the complexity of location dependent immunity effects in an whole organism.

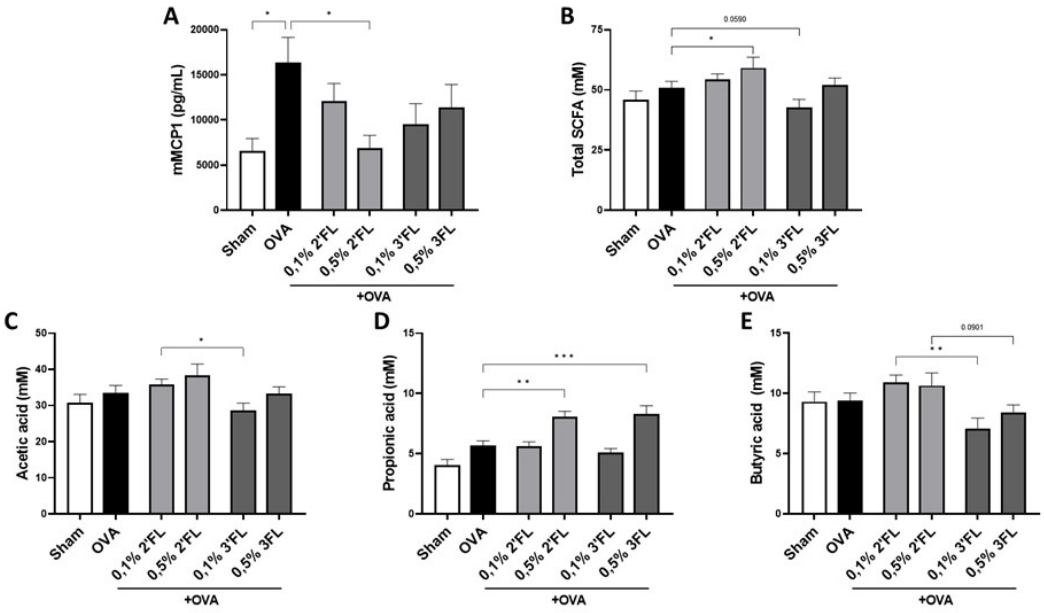


Figure 5. *Mucosal mast cell degranulation and presence of SCFA in cecum content. a) mMCP1 was measured in serum as marker for mucosal mast cell degranulation. b) Total SCFAs, c) acetic acid (acetate), d) propionic acid (propionate) and e) butyric acid (butyrate) levels were determined in cecum content. Sham and OVA groups were analyzed by an unpaired t-test (only for mMPC1). All dietary intervention groups were compared to the OVA group using a One-Way ANOVA followed by Dunnett's post hoc test, n=6-12, mean ± SEM (*p < 0.05, **p < 0.01, ***p < 0.001).*

Discussion

Infancy is an important stage of life during which immune maturation occurs, which may impact immune fitness and resilience throughout life. Exposure to HMOS via breastmilk contributes to maturation of the immune system e.g. via interactions with IEC, underlying immune cells and fermentation into other bioactive compounds such as SCFA [27]. As both HMOS and SCFA are abundantly present in early life, we hypothesized a potential interaction between 2'FL and 3FL with SCFA on the immunomodulatory effects. The interaction between HMOS and butyrate, as most potent immunomodulator amongst the SCFAs [20], was explored in an *in vitro* model for OVA induced epithelial inflammation. Furthermore, the impact of 2'FL and 3FL supplemented diets was validated *in vivo* using a murine model for OVA food allergy, in which both SCFA and HMO impact local mucosal immunity.

The *in vitro* mucosal immune model used in this manuscript was previously developed to study the effects of allergen induced epithelial inflammation. The subsequent type 2 skewing of the sequential immune activation, as well as immunomodulatory effects of the HMOS 2'FL and 3FL are demonstrated within this model [18]. Here, this coculture model was initially used to study the interaction between butyrate and 2'FL or 3FL in the absence of an inflammatory trigger (Figure 1). 2'FL and 3FL exposure modulated the subsequent epithelial cell crosstalk with moDCs in such a way that secretion of IL13 in 2'FL-IEC/DC/T cells was enhanced, while 3FL-IEC/DC/T cells showed a significant increase in IFNγ, IL17 and IL10, potentially steering away from the Th2 skewed phenotype. This phenomenon is of importance since a Th2 prone immune response is observed in early life [28], [29], and healthy immune maturation shifts the balance towards Th1 and Treg driven immunity.

Butyrate, produced upon fermentation of HMOS [30], is well known for its interactions with e.g. colonocytes and small intestine epithelial cells. This interaction occurs via binding to GPR41 and GPR43, or butyrate becomes intracellularly available via MCT-mediated transport and can act as HDAC-inhibitor or suppress NF-κB activation [20], [24], [31]. Here we did not observe any effects of butyrate alone in the homeostatic model upon IEC exposure to butyrate alone. Previous studies did not observe alterations in epithelial chemokine and cytokine secretion when the cells were exposed to butyrate in the absence of an inflammatory trigger as well [24], [32], [33].

Although human milk contains butyrate at physiological relevant levels (median concentration of 0.75 mM, depending on e.g. stage of lactation [34]), the HMOS present in human milk are fermented by specific intestinal bacteria into SCFAs [30] and will therefore naturally be present together in the infant's intestine. Here we also observed that combined exposure of butyrate with 2'FL enhanced secretion of hallmark indicator of type 1 immunity IFNγ, and regulatory IL10 in IEC/DC/T cells on top of the 2'FL associated IL13 increase observed in T cells instructed by primed DC from 2'FL-IEC/DC cultures. On the contrary, but-3FL-IEC/DC/T cell supernatants did not contain significant elevated levels of IL17 and IL10 as was observed for 3FL-IEC/DC/T cells, while the enhanced IFNγ concentration remained present. Indicating a differential effect of butyrate combined with either 2'FL or 3FL in this mucosal immune coculture model. This difference could be explained by e.g. different receptor-interactions observed between 2'FL and 3FL. For example, in TNFα activated FHs 74 Int cell cultures 3FL, but not 2'FL,

inhibits IL8 release via shedding of the TNF receptor 1 [35], thereby decreasing the number of available receptor on the cell. Furthermore, 2'FL inhibited IL8 release, IL8 and CD14 mRNA expression while the concentration of soluble CD14 increased in LPS-triggered T84 cells [36] Gram-negative pathogenic bacteria activate mucosal inflammation through lipopolysaccharide (LPS). Interestingly, these effects were not observed for 3FL. Although these studies were not performed in an allergy focused setting, they demonstrate that the isomers 2'FL and 3FL interact differently with epithelial cells and therefore potentially also differentially affect the response to allergic triggers.

In combination with OVA as an allergic inflammatory trigger, differential immunomodulatory effect by preincubation with 2'FL and 3FL were observed in this mucosal immune model previously [18]. A summary of these data is displayed in Table 1. Here, butyrate preincubation tended to prevent the OVA-mediated TSLP release from IEC, while the subsequent cocultures steps with moDCs and T cells remained unaffected (Figure 2). TSLP is an epithelial derived alarmin, known for its contributions to type 2 polarization of DCs and subsequent T cells [37], which is also shown in the T cell response downstream of the OVA-IEC/DC culture. Yet in this current study, even though butyrate prevented OVA induced TSLP increase, it was insufficient to significantly affect DC and T cell polarization during subsequent coculture steps. Furthermore, butyrate is a long known suppressor of NF-κB activation [24], [38], which downstream triggers IL8 release. In these *in vitro* models, butyrate was not able to suppress IL8 secretion after OVA exposure and IEC/DC coculture (data not shown).

When butyrate preincubation of IEC/DC was combined with either 2'FL or 3FL, similar to the homeostatic conditions without OVA exposure, distinct immunomodulatory effects were observed. A general boost in both inflammatory and regulatory cytokine secretion was observed previously in 2'FL-OVA-IEC/DC/T cells [18], which remained present in but-2'FL-OVA-IEC/DC/T cells although here type 1 immunity was further promoted. Therefore, although an interaction between butyrate and 2'FL was hypothesized and observed at the level of IEC/DC interaction in this OVA induced mucosal immune activation model, the effects observed after but-2'FL preincubation corresponded to a great extent with the effects observed after 2'FL preincubation alone. Nonetheless, combined preincubation with butyrate and 3FL enhanced secretion of IL6 and TGFβ significantly for but-3FL-OVA-IEC/DC, which was not observed for the separate butyrate or 3FL preincubations. This phenomenon was only observed in the presence of OVA. In the homeostatic model IL6 secretion remained unaltered by combined 3FL and butyrate exposure, while it IL6 increased only in small amounts by 2'FL and butyrate. Previously, it was described that in the presence of IL6 Treg function is suppressed [39] as well as the production of type 1 IFNγ [40], while the development of a type 2 [41] and type 17 response [42] is promoted. However, secretion of IFNγ tended to increase in but-3FL-or-2'FL-OVA-IEC/DC/T cells which was not observed for the separate butyrate or 3FL or 2'FL preincubation. Interestingly, IL6 and TGFβ together drive the development of an Th subset capable of secreting high levels of both IFNγ and IL17 in mice [43]. Indeed, but-3FL-or-2'FL-OVA-IEC/DC/T cell supernatants contained elevated levels of IL17, which was not the case in the homeostatic model. Thus, butyrate supported 3FL or 2'FL in driving type 1 and type 17 responses, in this inflammatory condition. In addition, OVA induced type 2 immunity was suppressed by 3FL and in combination with butyrate. Although IL6 was increased after 3FL preincubation combined

with butyrate, other factors may have prevented the development of type 2 development during preincubation with 3FL alone. This was not the case for butyrate and 2'FL, but in this condition IL10 secretion remained elevated which may also supports to counteract type 2 immunity. The initiation of this regulatory response was already shown at the level of the IEC/DC culture where solely in the condition of 2'FL and butyrate increased IL10 secretion was observed. IL10 secreted from DCs is required for the development of IL10 producing T cells and has been found to promote individual's outcomes during allergy immunotherapy [44], [45].

Overall, these *in vitro* studies indicated the 3FL may be capable of suppressing OVA induced type 2 responses, while enhancing type 1 and 17 immunity. In contrast, 2'FL could not suppress type 2 immune development, but beyond type 1 and type 17 also a regulatory response developed in the presence of butyrate. To validate our *in vitro* findings, a murine study was performed. Mice received 2'FL or 3FL supplemented diets 2 weeks prior to the start of sensitization to explore the allergy preventive effects of the supplemented diets. Although clinical symptoms were not alleviated by the dietary interventions, a shift in humoral response was observed (Figure 3). OVA-specific IgE levels were not affected in mice receiving 2'FL or 3FL supplemented diets, yet mice receiving the 0,1% 2'FL diet had lower levels of OVA-specific IgG2a in their serum. Murine IgG2a is functionally compared to human IgG1, which is associated with antiviral responses but can be produced upon allergen exposure also [46]. The production of murine IgG2a upon vaccination can be enhanced via dietary 2'FL [47], yet here we observe a decrease in IgG2a in OVA-allergic mice receiving dietary 2'FL. The modulation of B cell responses by dietary 2'FL therefore seems to be context or trigger dependent. Furthermore, mice receiving the 0,5% 3FL diet had higher levels of OVA-specific IgG1 in their serum. Murine IgG1 has been functionally paired to human IgG4 [48]human IgG4 fails to bind to C1q. Instead, it has been suggested that human IgG4 can block IgG1 and IgG3 hexamerization required for their binding to C1q and activating the complement. Here, we show that murine IgG1, which functionally resembles human IgG4 by not interacting with C1q, inhibits the binding of IgG2a, IgG2b, and IgG3 to C1q *in vitro*, and suppresses IgG2a-mediated complement activation in a hemolytic assay in an antigen-dependent and IgG subclass-specific manner. From this perspective, we discuss the potential of murine IgG1 and human IgG4 to block the complement activation as well as suppressive effects of sialylated IgG subclass Abs on FcγR-mediated immune cell activation. Accumulating evidence suggests that both mechanisms seem to be responsible for preventing uncontrolled IgG (auto, which is considered non-inflammatory and increased levels are found during successful allergen immunotherapy [46], [49].

As antibody secretion by B cells is regulated by interaction with T helper cells, local T helper subset development was assessed in MLNs (Figure 4). A significantly lower percentage of total Th2 and activated Th2 cells was found in mice receiving 0,5% 3FL diets, furthermore MLNs from these mice contained a higher proportion of regulatory T cells, while the Th1 and Th17 populations remained unaffected. This shift in local T helper response in mice receiving a 0,5% 3FL diet may be sufficient to explain the observed shift in the humoral response, as the production of regulatory cytokines combined with lower levels of type 2 cytokines has been linked to an enhanced IgG1 production in mice [50].

Binding of allergen specific IgE to mucosal mast cells is crucial to elicit mast cell degranulation upon subsequent exposure to the allergen. Many bioactive compounds, such as mMCP1, are

released during mast cell degranulation that are involved in symptom induction. mMCP1 in serum of mice receiving the 0,5% 2'FL diet was significantly decreased (Figure 5), which points to a lower degree of mucosal mast cell degranulation. Lower levels of mMCP1 in serum tended to correlate with less clinical symptoms as measured by the acute skin response (Spearman correlation, r^2=0.352, p=0.069), however no significant effects were observed in reducing clinical symptoms. IgE-crosslinking is essential to induce mast cell degranulation, yet we did not observe significant differences in serum OVA-specific IgE levels in mice receiving a 2'FL or 3FL supplemented diet. But many other factors are known to regulate mast cell stability, such as the presence of regulatory cytokines [51] or HDAC inhibitors such as butyrate and propionate [22]. The latter was further addressed by measuring SCFA in the cecum content of the mice. Mice receiving the 0,5% 2'FL diet had higher SCFA levels in their cecum content, correlating to the observed decrease in mMCP1 levels in serum. This increase in SCFA levels was mainly explained by an increased presence of propionate. High fecal levels of SCFA during early life have been linked to lower incidence of atopic sensitization at one year of age [25], further substantiating the connection between SCFA and allergy prevention.

Some of the *in vitro* immunomodulatory effects of butyrate, 2'FL and 3FL could be observed in the murine study also. The reduced IL13 secretion by 3FL preincubation *in vitro* corresponds to the reduced percentage of Th2 cells observed *in vivo* in this chronic model for food sensitization. However, the presented *in vitro* model lacks multiple components present in the murine model. For example, this *in vitro* model only focusses on modulation of IEC and the effect of this modulation on subsequent crosstalk with moDC and T cells. While in the murine model, butyrate, 2'FL and 3FL may become systemically available and/or in direct contact with immune cells, therefore they may directly affect immune cell functioning. Future improvements of the *in vitro* model should allow interaction between butyrate, 2'FL and 3FL and moDC and/or T cells to provide a more complete overview of the immunomodulatory properties of these structures. Now these components were mainly exposed to the IEC. Furthermore, the *in vitro* model should be expanded in future experiments by including B cells and mast cells, to assess the complete immunological cascade involved in allergic sensitization and effector phase [52].

Conclusion

The present study investigated two common fucosylated human milk oligosaccharides, 2'FL and 3FL for their impact on allergy related responses in an *in vitro* mucosal immune model and an *in vivo* model for hen's egg (ovalbumin) allergy. *In vitro* butyrate exposure promotes the development of a downstream type 1 and regulatory response in the presence of 2'FL in homeostasis, but did not affect immunomodulatory effects of 3FL. 2'FL and 3FL differentially modulated ovalbumin induced mucosal inflammation independent of the presence of butyrate. Dietary supplementation with 3FL lowered Th2 frequency while enhancing Treg, but both 2'FL and 3FL diets did not affect food allergy symptoms or OVA-specific IgE within this murine model. 2'FL however improved the humoral immune response and lowered mucosal mast cell activation in association with increased fecal SCFA levels. Moreover, these results indicate that the interaction as present *in vivo* between SCFA and HMOs are important to understand immune development *in vivo*.

References

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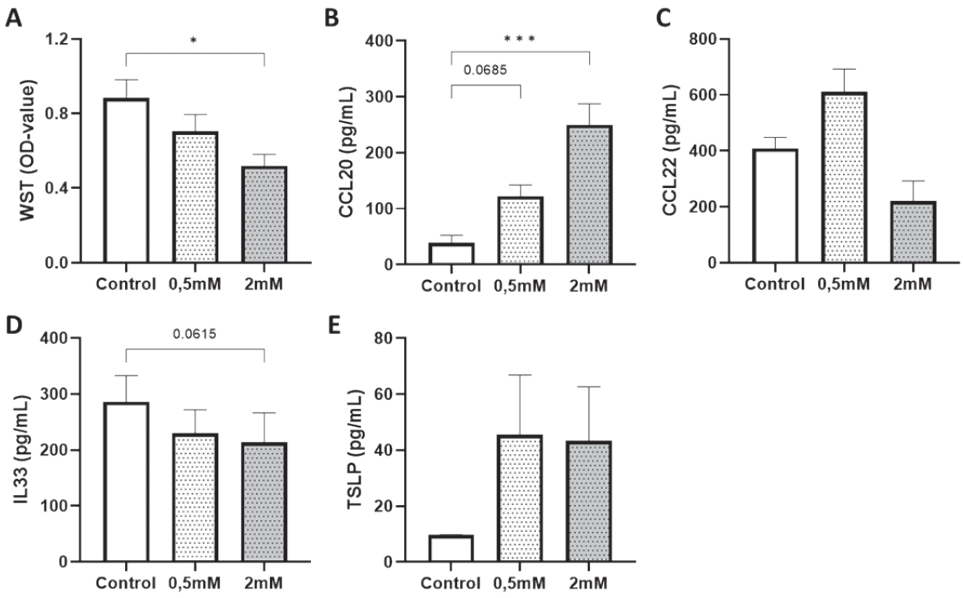
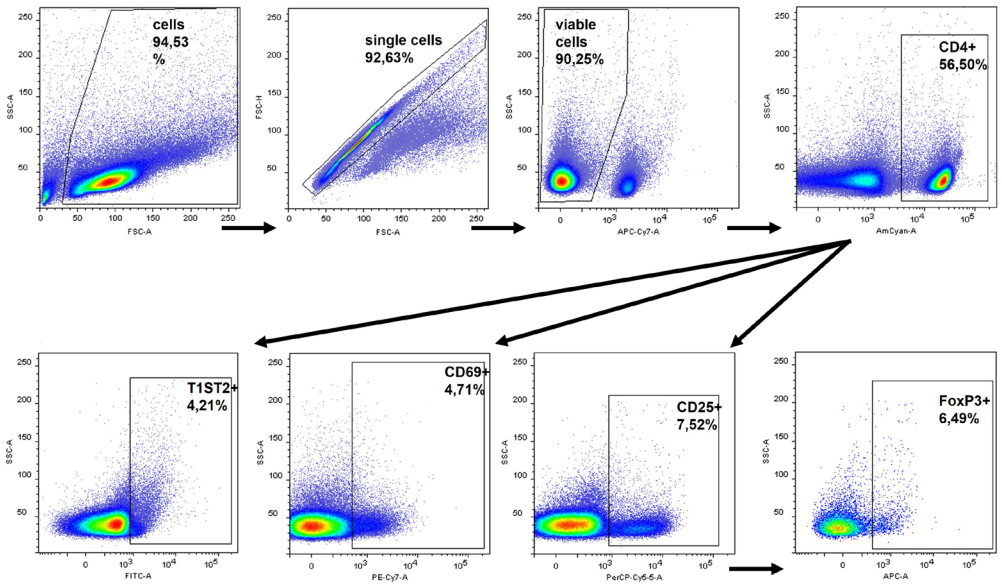
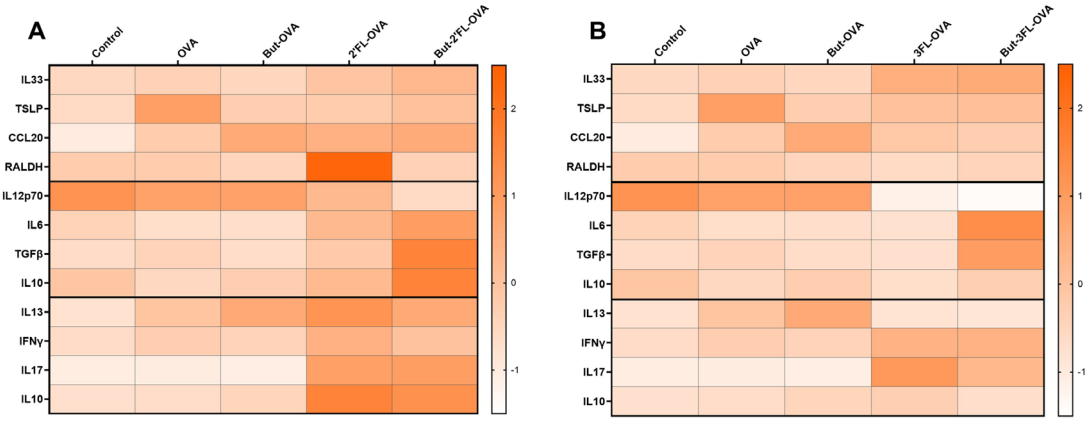
Supplements



Supplemental Figure 1. *Dose of butyrate for in vitro use was determined by exposing 5 different passages of IEC to butyrate in a 48 wells plate, Confluent HT29 cells were exposed to 0.5 or 2mM butyrate for 48h. After 48h, a) a WST-assay was performed to asses mitochondrial activity as a measure of viability, showing that 2mM butyrate significantly decreased cell viability. Secretion of the chemokines b) CCL20 and c) CCL22 as well as secretion of the alarmins d) IL33 and e) TSLP is was measured. . Data is analyzed by One-Way ANOVA followed by Dunett's post hoc test, n=5, mean ± SEM (*p < 0.5, **p < 0.01, ***p < 0.001).*



Supplemental Figure 2. *A representative gating strategy is shown to determine T cell subset populations in murine MLN samples. Appropriate FMO controls were used.*



Supplemental Figure 3 *Heatmap, based on z-scores, of the in vitro markers measured. Control and OVA exposed conditions as well as preincubations with butyrate and/or A) 2'FL or B) 3FL are presented.*

CHAPTER 8

2'FL and 3FL prevent house dust mite induced proinflammatory cytokine release *in vitro* and decrease house dust mite specific IgE production in a preclinical allergic asthma model

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Submitted

Abstract

Allergic asthma is characterized by sensitization to airborne allergens like house dust mite (HDM). After allergen uptake by dendritic cells (DCs), they instruct development of T helper 2 (Th2) cells, resulting in IgE production and both acute and chronic inflammation. Human milk oligosaccharides (HMOS) are linked to improved immune maturation and potentially alleviate allergy development. An *in vitro* model for crosstalk between bronchial epithelial cells (BECs), DCs and T cells during HDM exposure was established. Next, the preventive effects of HMOS were investigated in this *in vitro* model and subsequently in a house dust mite induced allergic asthma murine model. Calu-3 BECs were cocultured with monocyte-derived DCs (moDCs) during HDM exposure and subsequently cocultured with naïve Th cells. Immunomodulatory effects of the HMOS 2'-fucosyllactose (2'FL) and 3-fucosyllactose (3FL) were studied by 24h preincubation of BEC. 2'FL and 3FL supplemented diets were introduced to 6 week-old male Balb/c mice 14 days prior to and during HDM sensitization and subsequent challenges. *In vitro* exposure to 10 μg/mL HDM was sufficient to enhance secretion of interleukin (IL)33 and decrease barrier resistance in BECs. HDM exposure during BEC-DC coculture enhanced type 2 instructing TSLP, while reducing regulatory TGFβ secretion. Coculture of BEC-primed DCs with T cells enhanced allergy prone IL4 secretion. These effects were not observed upon HDM exposure of DCs in absence of BEC. Preincubation of BEC-DC with 2'FL or 3FL prior to HDM exposure, prevented HDM-induced TSLP and IL8 release. Furthermore, 0,01% 3FL preincubation enhanced TGFβ release from BEC-DC. Sequential coculture of 0,01% 3FL preincubated BEC-DC with T cells prevented an increase in IL4 secretion, while the percentage of IFNγ expressing cells was enhanced. HDM-allergic mice receiving a 1% 2'FL or 0,5% 3FL supplemented diet both had lower serum levels of HDM-specific IgE compared to mice fed control diet, and 1% 3FL lowered both IL13 and IFNγ in lung homogenates. An *in vitro* coculture model for HDM-induced BEC-DC activation and subsequent development of a type 2 Th cell response was established. Preincubation of BEC-DC with 2'FL or 3FL largely prevented HDM-induced activation and modified downstream T cell responses *in vitro*. Both HMOS reduced HDM-specific IgE in a murine model for HDM allergic asthma, but did not protect against airway inflammation. Indicating a translational capacity of the *in vitro* model to study impact on type 2 responses.

Keywords: advanced *in vitro* models, allergic asthma, human milk oligosaccharides, mucosal inflammation

Introduction

Allergic asthma is common in Westernized countries, with as many as 1 in 10 children and 1 in 12 adults affected [1]. A total of 300 million people worldwide is affected by the disease. Allergic asthma is characterized by sensitization to airborne allergens, which leads to T helper 2 cell (Th2) mediated airway inflammation and asthma symptoms [2]. The most common allergens in allergic asthma are derived from house dust mite (HDM), animal dander, cockroaches and fungi [3]. Sensitization to allergens mostly occurs in children within the first year of life, often accompanied by atopic eczema [1]. These children are generally more susceptible to develop food allergy, as well as allergic rhinitis and allergic asthma later in life [4]. There is no curative treatment available for allergic asthma and medication generally consists of a combination of inhaled corticosteroids and short- or long-acting β_2-adrenergic receptor agoinst. Allergic asthma might lead to chronic inflammation of the respiratory tract in which bronchial epithelial cells (BEC) and innate and adaptive immune cell activation contribute to type 2 inflammation, tissue remodeling and bronchial hyperreactivity (BHR) [1]. Symptoms include shortness of breath, wheezing, coughing, and chest tightness [5].

Allergic sensitization has been linked to decreased epithelial barrier function. Disruption of this epithelial barrier, can lead to increased sensitization as allergens can enter the lamina propria more easily, leading to increased exposure to immune cells [6], [7]. Thus, airway epithelial integrity is crucial in preventing sensitization and serves as a first line of defense against unwanted intruders. Besides being a physical barrier, the epithelium expresses pattern recognition receptors such as protease-activated receptors, Toll-like receptors (TLR), and C-type lectin receptors, which are involved in allergen binding. Furthermore, several allergens found in HDM, including Der p 1, have protease activity [8] disrupting epithelial tight junctions [9], [10]. Binding of allergens to epithelial receptors leads to activation of these epithelial cells and the release of alarmins, such as thymic stromal lymphopoietin (TSLP), interleukin (IL)25 and IL33 [11]. These alarmins act together with chemokines to activate and/or recruit dendritic cells (DCs), innate lymphoid cells type 2 (ILC2) or eosinophils, which promote the inflammatory response [1], [8], [12].

Uptake of allergens by antigen-presenting cells (APCs) occurs through sampling of the airway lumen by DCs or through allergens crossing the bronchial epithelium into the underlying lamina propria. Following uptake of the allergen and instruction via epithelial derived mediators, DCs travel to the mediastinal lymph nodes to present the captured allergen within their MHC class II receptor (MHCII) to the T cell receptor (TCR) on naïve T cells which may leads to differentiation into Th2 cells in the case of allergic sensitization [12]. Allergen-specific Th2 cells activate allergen-specific B cells, which leads to class switching of the B cells towards immunoglobulin E (IgE) producing plasma cells. Secreted allergen-specific IgE binds to the high affinity receptor FcεRI present on mast cells. At a second encounter with the allergen, allergen-specific IgE on the surface of mast cells crosslinks by binding to the allergen, leading to mast cell degranulation [13], [14]. Degranulation involves the release of pro-inflammatory mediators, which induce vasodilation, bronchoconstriction, and eosinophilic airway inflammation which becomes chronic when being uncontrolled [15].

As treatment of allergic asthma currently mainly consists of symptom suppression, preventing the development of allergic asthma has acquired scientific interest. The World Health Organisation

recommends exclusive breastfeeding for the first 6 months of life as it has overwhelming benefits for both mother and infant [16], including potential protection from allergic asthma development [17]. Human milk oligosaccharides (HMOS) are the third largest solid component of breastmilk after lipids and lactose, present in concentrations of up to 25 g/L in colostrum and 5-15 g/L in mature milk [18]–[20] and are thought to benefit immune maturation and protect against infections [14], [21]. HMOS are resistant to low gastric pH and cannot be digested by humans. However, they are substrates for bacterial fermentation in the large intestine [22], [23]. In addition, a small amount of ingested HMOS can be traced back in the blood and urine of the suckling infant [24]–[26]. HMOS directly bind to pathogens, promote growth of beneficial bacteria, can be used to produce bioactive metabolites like short chain fatty acids (SCFA) and act directly on receptors from epithelial cells and immune cells [14]. Therefore, differential effects from specific HMOS can be postulated in preventing the development of allergic asthma.

All mammalian milk contains oligosaccharides, but this is generally found in a concentration 10-100 fold lower than in humans [27]. Over 150 different HMOS structures have been identified, and are present in human milk [19], [28]. Variety in the produced HMOS substantially differs per individual. Factors such as period of lactation or polymorphism of the Lewis and Secretor genes can influence the variety and quantity of HMOS in breastmilk [19]. Neutral HMOS account for 75% of the total HMOS in human milk. All women can secrete the neutral fucosylated 3-fucosyllactose (3FL), independent of the expression of the Secretor or Lewis gene [29], [30]. In women who express an active form of the Secretor gene, the most abundant HMOS is 2'-fucosyllactose (2'FL). Although 2'FL and 3FL are structurally alike, affinities for different receptors have been described [14] and their effects on the neonatal development may be distinguishable.

Currently it is unknown whether these HMOS may protect against allergic sensitization. Therefore, the aim of this study was to develop a human *in vitro* bronchial epithelial mucosal immune model to study the crosstalk between BEC, DCs and T cells after exposure to HDM. Furthermore, the potential immunomodulatory effects of the commonly expressed 2'FL and 3FL were investigated in this *in vitro* model. Additionally, an *in vivo* murine HDM-induced acute allergic asthma model was used to study the allergy preventive effects of a dietary intervention with 2'FL and 3FL.

Materials & methods

In vitro culture and isolation of BEC, moDC and T cells

Calu-3 cell culture

Human lung adenocarcinoma Calu-3 cells (American Type Culture Collection, USA, passage 29-32) were used as a model for BEC. Cells were grown in minimal essential medium (MEM) (Gibco, USA) supplemented with 10% foetal bovine serum (FBS, Gibco), 1% penicillin, 1% streptomycin, 1% non-essential amino acids (Gibco), and 1% natrium pyruvate (Gibco) in a 75 cm^2 culture flask (Greiner, Germany) until approximately 75% confluency was reached. After trypsinization, cells were seeded on inserts (0.4 µm pores) of a 24-wells transwell plate (Corning Incorporated, USA) in 200 µL medium. 500µL medium was added to the basolateral compartment. The cells were incubated in 5% CO_2 at 37 °C. After 24 hours all medium was

removed and only 300 µL basolateral medium was added to start air-liquid interface (ALI) culture. 200 µL was added to the apical compartment and 500µL to the basolateral compartment for submerged culture. Medium was refreshed every 3-4 days. Cells were cultured for two weeks until 100% confluency, differentiation of the cells was assessed by measuring trans-epithelial electrical resistance (TEER) using the Locsense Artemis (Locsense, The Netherlands) or the Millicell ERS-2 Volt-ohm meter (Merck Millipore, USA).

PBMC isolation

Buffy-coats were obtained from healthy donor blood from the Dutch Blood Bank. Human peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation (1000 x g, 13 min) using Leucosep tubes (Greiner Bio-One, the Netherlands). The pellet was washed three times with PBS containing 2% FBS. To remove the remaining erythrocytes, lysis buffer was added (8.3 mg NH_4HCl, 1 g $KHCO_3$, 37.2 mg EDTA in 1 L demi water, sterile filtered) for 5 minutes. Cells were resuspended in 10 mL RPMI 1640 (Sigma-Aldrich, USA) supplemented with 10% FBS, 1% penicillin and 1% streptomycin. Obtained PBMCs were counted using the Z1 Coulter Particle Counter (Beckman Coulter, The Netherlands).

Monocyte and naïve T cell isolation

The PBMC suspension was centrifuged (300 x g, 10 min), the supernatant was discarded and the pellet resuspended in isolation buffer (2.5 g BSA, 2 mM EDTA in 500 mL PBS). Monocytes were isolated via negative selection using a QuadroMACS separator, LS columns and Monocyte Isolation kit II or naïve T cells via a naïve CD4+ T cell isolation kit (Miltenyi Biotec, Germany) according to manufacturer's instruction. Collected flow through contained the enriched cell fraction and was centrifuged (300 x g, 10 min). Monocyte containing pellets were resuspended in RPMI 1640 supplemented with 10% FBS, 1% penicillin, and 1 % streptomycin. Naïve T cell containing pellets were resuspended in IMDM supplemented with 5% FBS, 1% penicillin, 1% streptomycin, 20µg/mL apo-transferrin, and 50µM β-mercaptoethanol. Cells were counted using the Z1 Coulter Particle Counter (Beckman Coulter). Isolated naïve T cells were stored in 90% FBS and 10% DMSO in liquid nitrogen until further use.

Monocyte derived dendritic cell (moDC) culture

Monocytes were cultured in a 6 well plate (Greiner Bio-One) in RPMI 1640 supplemented with 10% FBS, 1% penicillin, and 1% streptomycin. Cytokines were added to a final concentration of 100 ng/mL IL4 and 60 ng/mL GM-CSF (ProSpec Bio, Israel) to induce differentiation into dendritic cells. Half of the medium and cytokines were refreshed every other day. Cells were cultured for 6 days before experimental use. After 6 days, moDCs were counted and diluted to a concentration of 1.5 x 10^6 cells/mL for use in coculture experiments.

HDM dose response in BEC

After 14 days of culture in transwell, Calu-3 cells were apically exposed for 72h to increasing doses of HDM extract (10-250µg/mL, Greer Laboratories, USA). TEER was measured during this exposure period (t=0h, 1h, 6h, 12h, 24h, 72h) and basolateral supernatants were collected after 24h to measure cytokine secretion.

Airway epithelial mucosal immune coculture model

Calu-3/moDC coculture

500 µL of moDC suspension ($1.5x10^6$ cells/mL) was added to the wells of a 24-wells plate (Greiner Bio-One), if appropriate inserts containing confluent ALI-cultured Calu-3 cells were added. MoDCs were (basolateral) exposed to 0,01% or 0,05% 2'FL or 3FL (Carbosynth, UK) and incubated for 24 hours at 37°C, 5% CO_2. Next, epithelial cells or moDC were apically exposed to 10 µg/mL HDM (in 200 µl) and incubated for 24 hours at 37°C, 5% CO_2. Afterwards, basolateral supernatant was collected and stored at -20°C for cytokine measurement, moDCs were collected for analysis by flow cytometry or subsequent coculture with naïve T cells.

MoDC/Naïve T cell coculture

5 x 10^4 MoDCs were collected for subsequent coculture with naïve T cells and were transferred to a 48-well culture plate (Greiner Bio-One) in 100 µL coculture medium (IMDM supplemented with 5% FBS, 1% penicillin, 1% streptomycin, 20 µg/mL apo-transferrin, and 50 µM β-mercaptoethanol). Isolated naïve T cells were thawed and diluted to a concentration of 1.25 x 10^6 cells/mL in coculture medium. 400µL allogenic naïve T cell suspension was added to the moDC suspension in the wells (1:10 DC:T cell ratio). Activation of naïve T cells was aided by adding 5 ng/mL IL2 (ProSpec Bio) and 150 ng/mL anti-CD3 (BD Biosciences, USA) to the culture. The coculture was incubated for 5 days without medium refreshments. Cells were collected for flow cytometric analysis and supernatants were stored at -20°C for cytokine analysis.

In vivo house dust mite induced acute allergic mouse model

Diet preparation

Experimental diets (produced by ssniff-Spezialdiäten GmbH, Germany) were based on an AING93 diet. This diet was adapted by addition of methionine and cysteine, and soy protein was used instead of casein. Furthermore, diets were supplemented with or without 0,5% or 1% 2'FL or 3FL (Jennewein GmbH, Germany). Supplementation of 2'FL and 3FL was isocaloric compensated with cellulose. Animals had ad libitum access to food and water, which were fully refreshed weekly.

Animals

Six to seven week-old male BALB/cAnNCrl mice (Charles River, Germany) arrived at the animal facility of Utrecht University and were housed in individual ventilated cages with a 12h/12h light/dark cycle, controlled relative humidity (50-55%) and temperature (21 ± 2°C). Mice were randomly allocated to the experimental groups and housed with 3 animals per cage. Cage enrichment consisted of woodchipped bedding, wood curls (as nesting material) and a plastic shelter. This study was conducted in accordance with institutional guidelines for the care and use of laboratory animals of the Utrecht University, and all animal procedures were approved by the local Animal Welfare Body under an Ethical license provided by the national competent authority (Centrale Commissie Dierproeven, CCD), securing full compliance the European Directive 2010/63/EU for the use of animals for scientific purposes.

Animal procedures

A schematic overview of the experimental setup is shown in Figure 4A. After arrival mice immediately received the experimental diets. 14 days later, the mice were intranasally sensitized with 1 µg HDM (Greer Laboratories) in 40 µL PBS under isoflurane anesthesia. Mice were intranasally challenged on days 21-25 with 10 µg HDM in 40 µL PBS. 72h after the final challenge, airway hyperresponsiveness was measured. Subsequently, mice were sacrificed by intraperitoneal overdose of pentobarbital (Nembutal™, Ceva Santé Animale, The Netherlands) and samples were collected for further analysis.

Serum analysis

Mice were sacrificed and blood was collected via eyeball extraction in a Minicollect serum tube (Greiner Bio-One B.V., Netherlands). Collected blood was kept at room temperature for at least 30 minutes prior to centrifugation for 10 min at 14.000 rpm. Serum was collected and stored at -20°C for antibody measurement.

Bronchoalveolar lavage

After sacrificing, lungs were lavaged four times with 1 mL saline solution (0.9% NaCl, 37°C). Bronchoalveolar lavage (BAL) fluid was centrifuged (400 x g, 5 min) and the BAL cell-containing pellets were pooled. Total cell count was determined using a Bürker-Türk chamber (magnification 100x). Cytospin preparations were made and stained with Diff-Quick (Merz & Dade A.G., Switzerland) for differential BAL cell count. Cell counts were scored with light microscopy.

Preparation of lung homogenates

Collected lung tissue was homogenized using 1% Triton X100 (Sigma-Alrdrich) in PBS containing protease inhibitor (Complete Mini, Roche Diagnostics) with a Precellys Tissue Homogenizer and Precellys homogenizer tubes (Bertin, Rockville, MD, USA). Homogenates were centrifuged at for 10 min at 14,000 rpm. Supernatant was collected and stored at -20°C for cytokine analysis.

Preparation of lung single cell suspension

Lung tissue was collected after sacrifice and enzymatic digested using a buffer containing DNase I and Collagenase A (Roche Diagnostics, Switzerland). FBS was added to stop the digestion after 30 min. The lung tissue was passed through a 70µm filter to obtain single cell suspensions. Cell suspensions were incubated for 4 min on ice in red blood cell lysis buffer (4.14 g NH_4Cl, 0.5 g $KHCO_3$, 18.6 mg Na_2EDTA in 500 mL demi water, sterile filtered, pH 7.4). Lysis was stopped by adding FBS. Lung cells were washed with RPMI 1640 (Lonza, USA). After counting the cells with Z1 Coulter Particle Counter (Beckman Coulter Life Sciences), cells were stained for analysis by flow cytometry.

ELISAs

In vitro cytokine levels were determined in collected supernatants. IL4, IL8, IL10, IL13, IFNγ, TGFβ, TSLP (Invitrogen, USA), IL25 and IL33 (R&D systems, Minneapolis, MN, USA) were determined according to manufacturer's protocol. Optical density was measured at 450 nm.

To measure HDM-specific IgE levels in serum from mice, high binding 96 well plates

(Corning Costar) were coated with HDM (50mg/ml) and incubated overnight at 4°C. After blocking with 1% BSA in PBS, plates were washed and diluted serum samples were added to incubate for 2h. Plates were washed again and 1mg/ml biotin anti-mouse IgE (BD Biosciences) was added for 1.5h. Plates were incubated with streptavidin-HRP for 30 minutes, followed by addition of a substrate solution. Washing steps were performed in between. Reaction was stopped by addition of 2 M H_2SO_4 and absorbance was measured at 450nm.

Concentrations of IL13, IFNγ and IL10 (Invitrogen) in lung homogenates were measured according to the manufacturer's instructions. Levels of cytokines were calculated per mg of homogenized lung tissue.

Flow cytometry staining

Cells were stained with Fixable Viability Dye eFluor780 for 30 minutes. Nonspecific binding was blocked using human FC block (BD Biosciences) in PBS for *in vitro* samples and antiCD16/CD32 blocking buffer for murine samples. Subsequently samples were stained for 30 min at 4°C using titrated amounts of antibodies. To allow intranuclear staining of transcription factors, cells were fixated and permeabilized using FoxP3/Transcription Factor staining buffer set (eBioscience, USA) following the manufacturer's instructions. For intracellular staining, cells were fixated and permeabilized with Intracellular Fixation & Permeabilization Buffer Set (eBioscience) according to manufacturer's protocol. FACS Canto II (BD Biosciences) was used to measure stained samples and obtained data was analyzed using Flowlogic Software (Inivai Technologies, Australia).

Titrated amounts of the following antibodies were used to stain *in vitro* samples: CD11c-PerCP (3.9), HLA-DR-PE (LN3), CD80-FITC (2D10.4), CD86-PE/Cy7 (IT2.2) (All from eBioscience, USA), CD4-PerCP (OKTO4, eBioscience), CXCR3-Alexa Fluor 488 (1C6/CXCR3, BD Biosciences), CRTH2-APC (BM16, BD Biosciences), FoxP3-eFluor 660 (PCH101, Invitrogen), CD25-Alexa Fluor 488 (BC96, eBioscience), and IL13-PE (85BRD, eBioscience), and IFNγ-Amcyan (4S.B3, Biolegend). The gating strategy using representative samples is shown in Supplemental Figure 1.

Titrated amounts of the following antibodies were used to stain murine samples: CD4-BV510 (RM4-5, Biolegend), CD69-PE-Cy7 (H1.2F3, eBioscience), CXCR3-PE (CXCR3-173, eBioscience), T1ST2-FITC (DJ8, MD Bioproducts, USA), FoxP3-FITC (FJK-16s, Invitrogen), CD127-PE/Vio770 (A7R 34, Miltenyi Biotech) and CD25-PerCP/Cy5.5 (PC61.5, Invitrogen).

Statistical analysis

Statistical analyses were performed using Graphpad Prism (Version 9.4.1) software. TEER data was analyzed by Two-Way ANOVA followed by Bonferroni multiple comparisons test. After exposure to increasing HDM concentrations, cytokine levels were analyzed by One-Way ANOVA followed by Dunnett's multiple comparison test comparing all HDM concentration to the control condition. Control and HDM conditions (in presence or absence of BEC) were analyzed by paired t-test. Effects of HMOS preincubations were analyzed by One-Way ANOVA followed by Dunnett's multiple comparisons test comparing all conditions to the HDM-exposed condition. Murine data was analyzed using an unpaired t-test to compare Sham and HDM groups. All intervention groups were compared to the HDM group by One-Way ANOVA followed by Dunnett's multiple comparisons test. If data did not fit a normal distribution, logarithmic or

square root transformation was applied prior to further analysis. $p < 0.05$ is considered statistically significant, data is represented as mean ± SEM of 4-6 independent *in vitro* repeats or 6-12 animals per group.

Results

HDM exposure activates ALI cultured BEC and reduces barrier resistance

The initial step to develop an *in vitro* airway epithelial mucosal immune model, consisted of a comparison between ALI and submerged cultured Calu-3 cells. During a culture period of 14 days after seeding, development of barrier resistance was measured by means of TEER. Although a significant difference was found after 10 and 14 days of culture, Calu-3 cells developed proper barrier resistance in the ALI culture (Figure 1A). Therefore all experiments using Calu-3 cells were performed in ALI culture. Next, a dose-response (10-250μg/mL) HDM was added for 72h. 10μg/mL and 100μg/mL HDM significantly decreased barrier resistance as measured already 1h after exposure (Figure 1B), and a decreasing trend ($p=0.0503$) was observed using 25μg/mL HDM. Secreted cytokines were measured in basolateral supernatants collected 24h after the start of HDM exposure. IL33 secretion was enhanced using 10μg/mL HDM exposure (Figure 1C). Both 10μg/mL ($p=0.0847$) and 50μg/mL ($p=0.0855$) HDM exposure tended to increase IL25 levels, while 250μg/mL HDM significantly decreased the release of IL25 (Figure 1D). TSLP secretion was significantly increased using 25μg/mL HDM exposure (Figure 1E), whereas none of the HDM concentrations significantly enhanced IL8 secretion (Figure 1F). Based on these results, a dose of 10μg/mL HDM was chosen to be used for further *in vitro* experiments.

The TEER upon HDM exposure was automatically measured by the Locsense Artemis. In subsequent experiments this decrease was not observed, possibly due to manual measurements performed by a Millicell ERS-2 Volt-ohm meter.

Exposing BEC-DC to HDM induces a Th2 profile during coculture with naïve T cells

BECs cocultured with moDCs (BEC-DC) or moDCs alone were exposed for 24h to HDM (Figure 2A). moDC exposure to HDM in absence of BECs was found to increase IL33 and TGFβ secretion (Figure 2B, D) and the percentage of CD86 expressing DCs (Figure 2G), while the secretion of IL8 tended to be reduced ($p=0.0633$, Figure 2E). By contrast, when BEC were exposed to HDM while cocultured with moDC, secretion of TSLP (Figure 2C) and the percentage of CD80 expressing DC (Figure 2F) was enhanced, while secretion of TGFβ (Figure 2D) was decreased.

Subsequently, primed moDC were cocultured with allogenic naïve T cells to study the functional character of the HDM-DC or HDM-BEC-DC. Although development of Th subsets was unaffected by any of the conditions (Figure 2H,K,N), HDM-DC reduced the percentage of IL13 or IFNγ containing T cells (Figure 2I,L). By contrast, HDM-BEC-DC enhanced the release of IL4 (Figure 2J) after coculture with naïve CD4+ T cells (HDM-BEC-DC/T), while decreasing the percentage of IFNγ containing Th cells (Figure 2L). Although the secretion of IFNγ, IL10 (Figure 2M, O), IL13, IL5 and IL17 (Supplemental Figure 2A-C) remained unaffected, the ratio

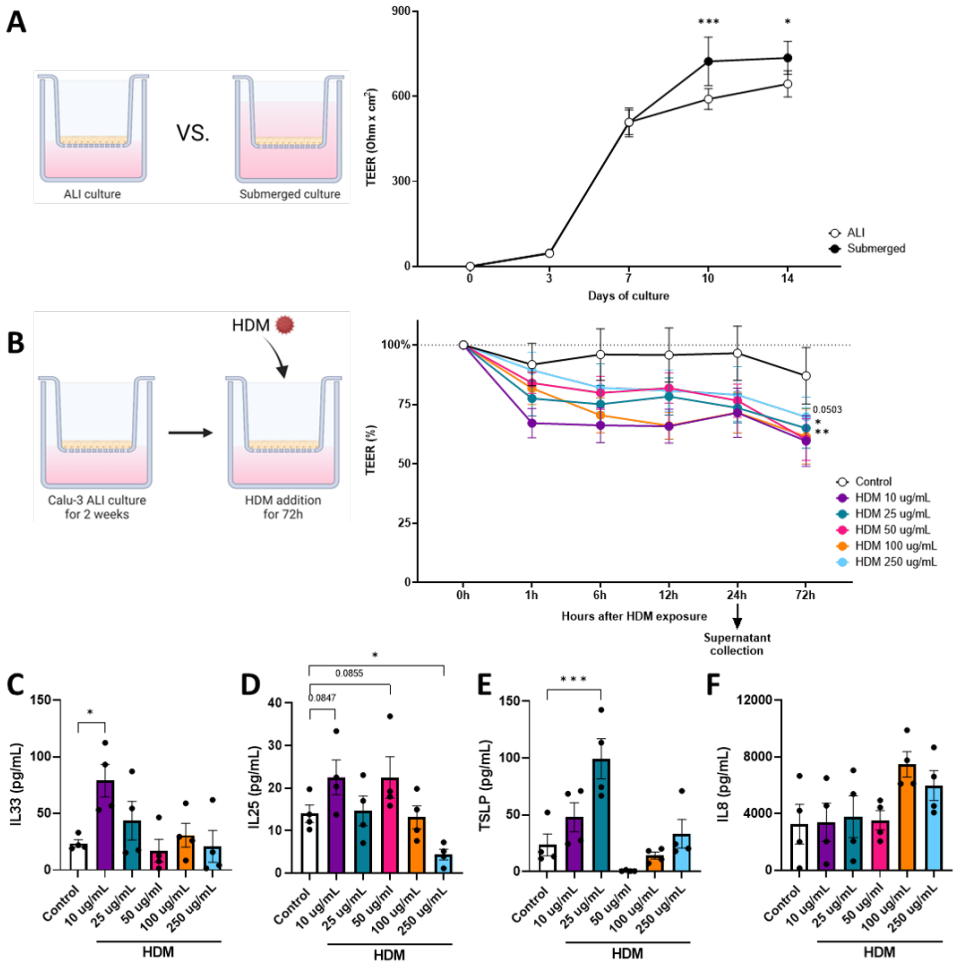

Figure 1. *In order to establish a physiological relevant in vitro model of bronchial epithelial cells, A) Calu-3 cells were cultured in air-liquid interface (ALI) or submerged conditions. The development of transepithelial electrical resistance (TEER) of these cells was followed 14 days post seeding. After confirming that ALI-cultured Calu-3 cells establish appropriate TEER, B) cells were exposed for 72h to increasing dosages of HDM 14 days after seeding. After 24h of HDM exposure, basolateral supernatant was collected to measure secretion of C) IL33, D) IL25, E) TSLP and F) IL8. Data is analyzed by One-Way or Two-Way ANOVA followed by a Dunnett's multiple comparisons test, n=4, mean ± SEM (* p<0.05, ** p< 0.01, *** p<0.001).*

of IL4 over IFNγ secretion was significantly increased for HDM-BEC-DC-T cells (Figure 2P), displaying the type 2 shifted immune character of HDM-BEC-DC-T cells. As the exposure to HDM via BEC resulted in a type 2 immune response, a relevant contribution of the BEC to the HDM-induced immune activation was hypothesized. Therefore, the latter model was chosen to study effects of specific HMOS.

Both 2'FL and 3FL reduce HDM mediated BEC-DC activation while consecutive BEC-DC/T cell responses are differentially modulated by these HMOS

BEC-DC were incubated basolateral with 2'FL and 3FL, while BEC were exposed to HDM. Next the BEC-DC were washed and cocultured with naïve T cells (Figure 3A). HDM induced TSLP and IL8 secretion (Figure 3C, E) was prevented when BEC-DC were incubated with 2'FL or 3FL. In addition, the HDM reduced TGFβ secretion was prevented by exposing the HDM-BEC-DC to 0,01% 3FL but not 2'FL (Figure 3D). The HDM induced IL33 levels or DC maturation was not affected by either 2'FL or 3FL (Figure 3B, F, G). In these cultures TEER was measured by means of the volt ohm meter, but HDM did not show a TEER reduction (data not shown).

When combining BEC-DC with T cells, the 0,01% 3FL incubated HDM-BEC-DC were found to reduce the secretion of IL4 (Figure 3J) and IL13 (Supplement Figure 2) from T cells. In addition, the percentage of Th1 cells, characterized by CXCR3 expression, (Figure 3K) was reduced but the IFNγ expression within the T cells was increased when comparing 0,01% 3FL preincubated HDM-BEC-DC-T cells to HDM-BEC-DC-T cells (Figure 3L). Although the secretion of IL5, IL17 (Supplemental Figure 2A-C) and IFNγ (Figure 3M) was not significantly affected by either 2'FL or 3FL preincubation, the ratio of IL4 over IFNγ was significantly reduced by 0,05% 2'FL as well as 3FL incubation of HDM-BEC-DC, indicating a shift in balance away from a type 2 immune response (Figure 3P). Furthermore, even though HDM did not affect the secretion of regulatory IL10, it was enhanced in T cells cultured with HDM-BEC-DC exposed to either 0,01% 2'FL or 0,05% 3FL (Figure 3O).

HDM sensitized and challenged mice receiving 1% 2'FL or 0,5% 3FL via diets have lower HDM-specific IgE levels in serum

To support the *in vitro* findings, a murine HDM-induced acute allergic asthma model was performed specifically to study the potential preventive effects of 2'FL and 3FL (Figure 4A). The influx of inflammatory cells into the lungs was increased in HDM-sensitized mice 72h after the final challenge (Figure 4B). In particular, the number of eosinophils (Figure 4C) and macrophages (Figure 4D) was enhanced in HDM sensitized and challenged mice as compared to control. Mice receiving an HMOS supplemented diet had similar levels of cell influx into the lungs. No significant increase in airway hyperresponsiveness was observed in HDM sensitized and allergic mice (Supplement Figure 3). As hallmark parameter of allergic sensitization, HDM-specific IgE levels were measured in serum. HDM sensitized and challenged mice had increased levels of HDM-specific IgE, which was reduced in mice who had received a 1% 2'FL or 0,5% 3FL diet (Figure 4E). Even though the influx of eosinophils and macrophages was not significantly altered in mice receiving an HMOS supplement diet, the number of both eosinophils and macrophages was positively correlated with the levels of HDM-specific IgE in serum (Figure 4F, G).

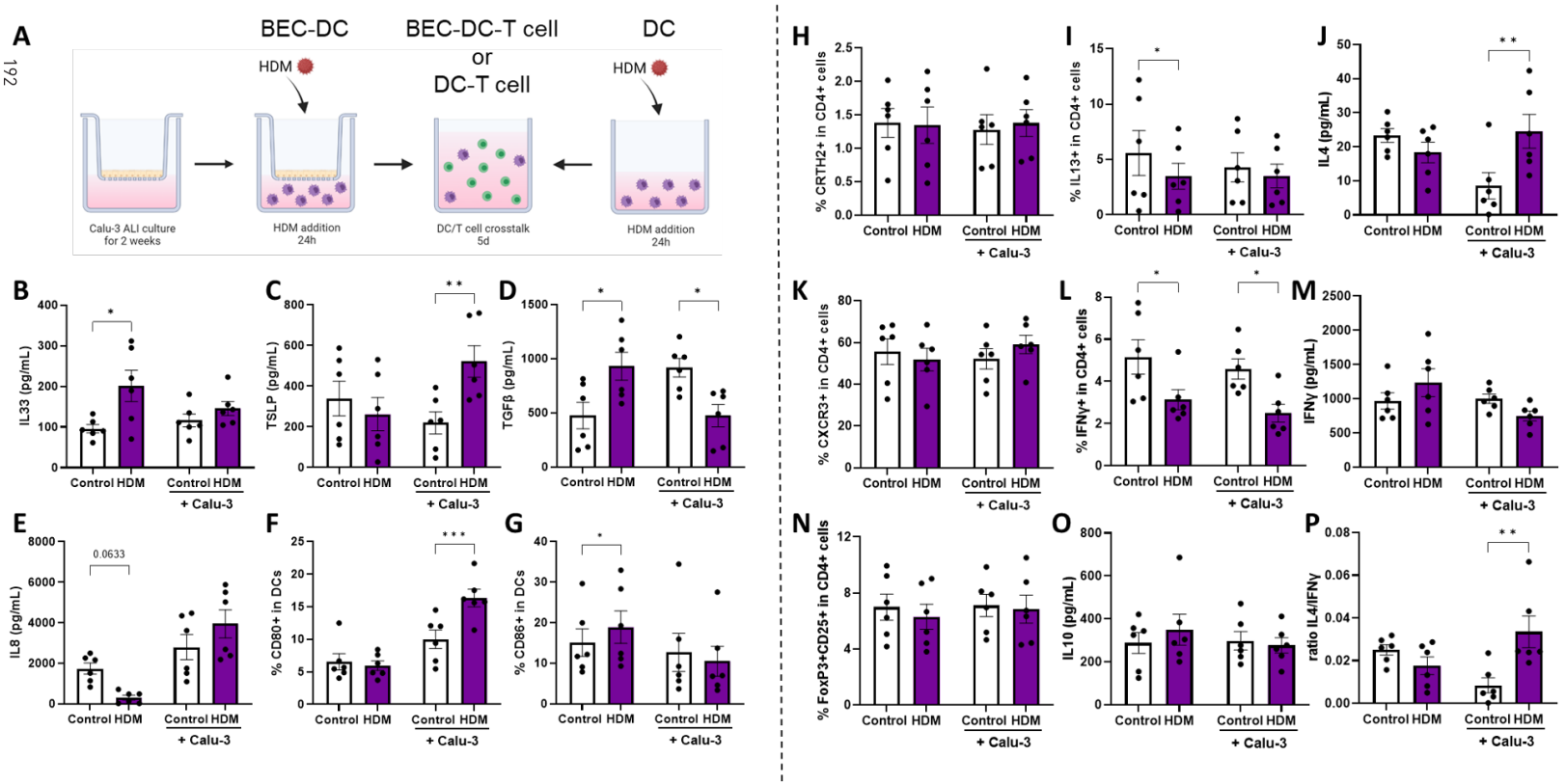

Figure 2. A) A schematic overview of the coculture steps in this human in vitro bronchial mucosal immune model. Calu-3 bronchial epithelial cells (BEC) were cultured for 14 days in air-liquid interface (ALI) prior to coculture with monocyte-derived dendritic cells (moDCs). BEC-DCs and DCs alone were apically exposed to 10μg/mL HDM for 24h. After 24h of exposure to HDM, primed DCs were collected for analysis and coculture with allogenic naïve T cells for 5 days. Upon 24h of HDM exposure, supernatants and moDCs were collected to measure secreted levels of B) IL33, C) TSLP, D) TGFβ, E) IL8 and determine the percentage of moDCs expressing the costimulatory markers F) CD80 and G) CD86. After subsequent coculture with of primed DCs with naïve T cells, supernatants and cells were collected to measure the percentage of H) CRTH2 and I) IL13 expressing cells and secreted J) IL4 as part of the T helper 2 cell response. In addition, the T helper 1 response was analyzed based on the percentage of K) CXCR3 and L) IFNγ expressing cells and secreted M) IFNγ. The regulatory T cell response was determined by identification of the percentage of dual expressing N) FoxP3 and CD25 cells and secretion of O) IL10. Finally, P) the ratio of type 2 IL4 and type 1 IFNγ secretion was calculated. Data is analyzed by paired t-tests, n=6, mean ± SEM (p<0.05, ** p< 0.01, *** p<0.001).*

Lower levels of pulmonary IL13 and IFNγ in HDM sensitized and challenged mice receiving 1% 3FL containing diets

Lung tissue from sacrificed mice were analyzed for the ratio of present Th cell subsets and cytokine levels. HDM sensitized and challenged mice tended to have a higher percentage of activated Th2 and Th1 cells present (based on expression of T1ST2 and CXCR3 respectively as well as CD69, Figure 5A, C, E), however these percentages were not altered in the mice receiving either 2'FL or 3FL in their diets. The percentage of regulatory T cells (Treg) remained the same in all HDM sensitized and challenged mice (Supplemental Figure 3B). However, levels of IL13, IFNγ (Figure 5B, D) and IL10 (Supplemental Figure 3C) were lowered in homogenized lung tissue from mice that had received a 1% 3FL enriched diet as compared to mice receiving a control diet.

Discussion

HMOS are the third most abundant solid component of human milk, they may act as prebiotics and are believed to have immunomodulatory properties. This may help to prevent allergic diseases such as type 2 asthma. Therefore, the immunomodulatory properties of two commonly expressed fucosylated HMOS, 2'FL and 3FL, were investigated in a novel developed *in vitro* bronchial epithelial HDM induced type 2 mucosal immune model and a murine model for HDM-induced acute allergic asthma.

To the best of our knowledge, this is the first study on the development and use of an *in vitro* bronchial epithelial mucosal immune model using HDM as the allergenic trigger. It is known that epithelial cells play a key role in initiating the allergic sensitization process by influencing the maturation and activation of DCs [31]. Therefore, Calu-3 cells were used as model for BEC. Although traditional transwell culture methods are fully submerged [32], [33], ciliated characteristics of the bronchial epithelium are diminished, resulting in less relevant physiological conditions. ALI culture is suggested to reflect a more representative condition as the cells are exposed to medium via the basolateral compartment and air via the apical compartment resembling the physiological airway conditions [34], [35]. Even though the TEER development of ALI cultured BEC was significantly lower compared to submerged cultured BEC, the ALI cultured BEC still acquired a high degree of barrier resistance and therefore this culture method was selected for the succeeding experiments.

It is known that HDM can disrupt bronchial epithelium integrity via proteolytic breakdown of tight junction proteins [36]–[39]. In the current study, we observed that the TEER was significantly decreased already at a relatively low apical HDM exposure (10μg/mL) within 1h after exposure. Exposure to the lowest HDM dose was also sufficient to enhance release of alarmins, known to drive a subsequent type 2 response via DCs. Next, the immunological crosstalk between BEC and DC was studied during HDM exposure and compared to HDM exposure of DC alone, in addition to the subsequent functional T cell response after coculture with HDM-DC or HDM-BEC-DC was investigated. In the absence of BEC, HDM exposed DC showed increased IL33 and TGFβ secretion together with enhanced CD86 expression, while IL8 secretion was reduced. IL33 is a type 2 instructing epithelial derived alarmin, which can be produced by DCs when stimulated via Dectin-1. From murine experiments, it is known that HDM binding to Dectin-1 is crucial for DC migration, Th2 development and allergic airway inflammation [40].

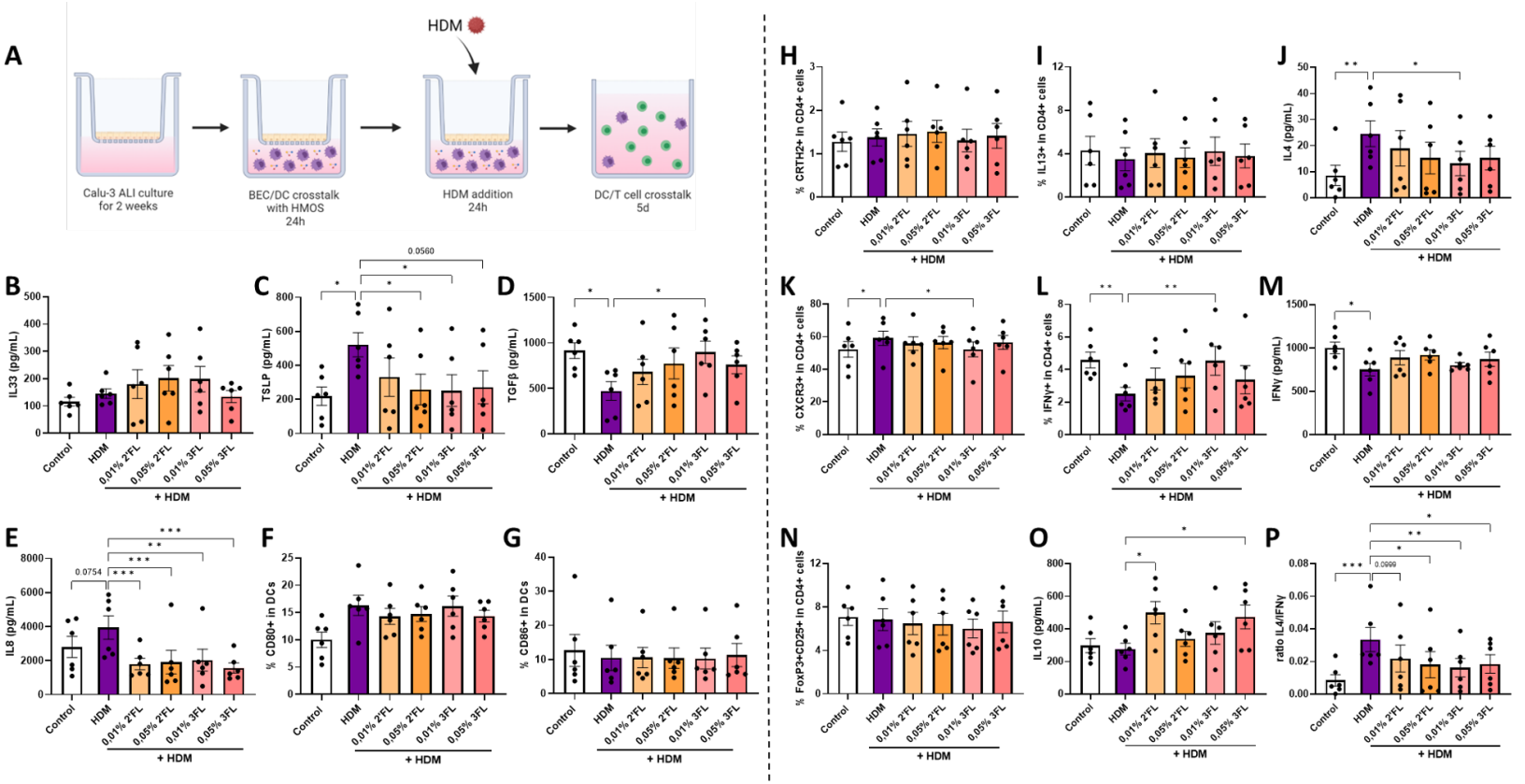

Figure 3. *The allergy preventive effects of HMOS were tested in this human in vitro bronchial mucosal immune model by preincubation of HMOS prior to HDM exposure. A) Calu-3 BECs were cultured in ALI for 14 days before coculture with moDCs and basolateral preincubation with 0,01-0,05% 2'FL or 3FL for 24h. Next, BEC/DC were apically exposed to 10μg/mL HDM for 24h. Followed by a coculture of primed moDCs with naïve T cells for 5 days. After HMOS preincubation and HDM exposure, primed DCs were collected for analysis and coculture with allogenic naïve T cells for 5 days. Upon HMOS preincubation and HDM exposure, supernatants and moDCs were collected to measure secreted levels of B) IL33, C) TSLP, D) TGFβ, E) IL8 and determine the percentage of moDCs expressing the costimulatory markers F) CD80 and G) CD86. After subsequent coculture with of primed DCs with naïve T cells, supernatants and cells were collected to measure the percentage of H) CRTH2 and I) IL13 expressing cells and secreted J) IL4 as part of the T helper 2 cell response. In addition, the T helper 1 response was analyzed based on the percentage of K) CXCR3 and L) IFNγ expressing cells and secreted M) IFNγ. The regulatory T cell response was determined by identification of the percentage of dual expressing N) FoxP3 and CD25 cells and secretion of O) IL10. Finally, P) the ratio of type 2 IL4 and type 1 IFNγ secretion was calculated. Data is analyzed by One-Way ANOVA followed by a Dunnett's multiple comparisons test, n=6, mean ± SEM (* p<0.05, ** p< 0.01, *** p<0.001).*

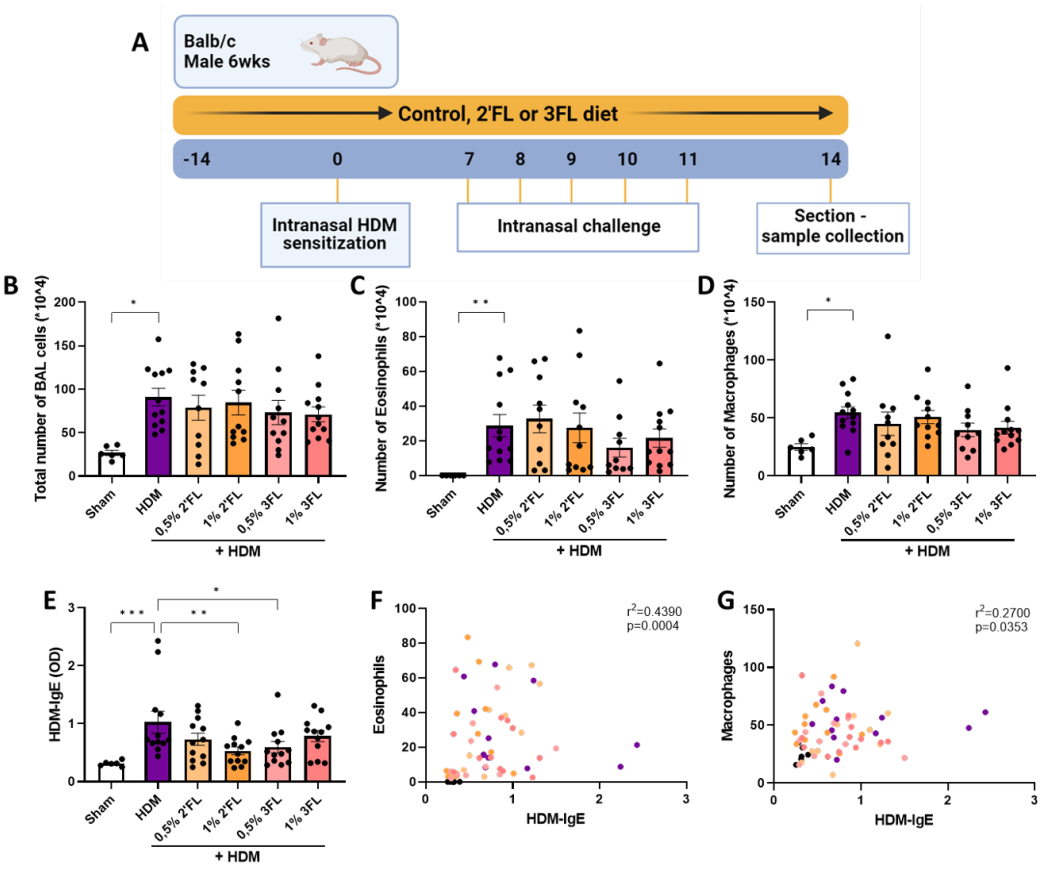

Figure 4. *A preclinical murine model for house dust mite induced acute allergic asthma was used to investigate the allergy preventive effects of dietary HMOS. A) 6 week old male Balb/cAnNCrl mice were fed a control or HMOS supplemented diet 2 weeks prior to intranasal sensitization with HDM. One week after sensitization, mice were intranasally challenged for 5 consecutive days. 72h after the final challenge, mice were sacrificed for sample collection. Lungs of sacrificed mice were lavaged to determine the influx of B) the total number of cells, C) eosinophils and D) macrophages into the lungs. E) HDM-specific IgE levels were determined in serum. The correlation between F) eosinophil influx into the lungs and HDM-specific IgE in serum as well as G) macrophage influx into the lungs and HDM-specific IgE in serum is displayed. Sham and HDM groups are analyzed by unpaired t-test, the intervention groups were compared to the HDM group by One-Way ANOVA followed by a Dunnett's multiple comparisons test, n=6-12, mean ± SEM (* p<0.05, ** p< 0.01, *** p<0.001).*

Although IL33 is mainly known for its proinflammatory, type 2 driving properties, this cytokine can induce the development of Treg cells as well [41]. Furthermore, increased expression of CD86 by DCs has been linked to promote the development of an allergic type 2 response [42], [43]. Yet in the presence of regulatory TGFβ this enhanced CD86 expression was linked to a tolerogenic phenotype in which IL8 secretion was decreased as well [44]. This may imply that HDM exposure to DC may instruct a more regulatory phenotype. However, subsequent coculture of HDM-DCs with naïve T cells had no significant effect on Treg cell development nor regulatory cytokine secretion (IL10). Yet the developed T cells did show lower intracellular expression of both IL13

as well as IFNγ, indicating some immunosuppressive effects which may relate to the phenotypic changes of HDM-DC. Previously, it was demonstrated that exposing DCs from healthy donors to HDM did not result in subsequent activation of naïve T cells, while DCs from allergic patients promoted Th2 cell polarization upon HDM exposure [45]. These differences in DC responses to HDM between allergic and healthy individuals may explain the observations in this study and highlights the relevance to further investigate the role of DCs in allergic diseases.

However, when BEC were exposed to HDM while cocultured with DC, secretion of TSLP and expression of CD80 on DC was enhanced, while TGFβ concentrations decreased. Although CD80 has been linked to a type 1 and Treg cell instruction [42], we hypothesize that due to the increase in TSLP and decrease in TGFβ the functional outcome of DC development was altered, leading to DCs that directed the consecutive T cell response towards a type 2 prone cytokine pattern. HDM-BEC-DC instructed T cells to increase the release of IL4, and the ratio of IL4 over IFNγ cytokine secretion, while a lower percentage of Th cells contained intracellular IFNγ when compared to medium controls. Epithelial-derived TSLP promotes DCs to induce Th2 differentiation resulting in IL4 secretion [46]–[48]. In addition, TGFβ stimulates the development of tolerogenic responses while inhibiting the production of proinflammatory cytokines [49]. Hence, the increased secretion of TSLP and reduced TGFβ secretion seemed sufficient to develop a type 2 directed DC response, affecting the subsequent coculture with naïve T cells, as generally observed in asthma allergic patients.

After the observation that HDM only induced a type 2 driven immune response in presence of BEC, the immunomodulatory effect of 2'FL and 3FL were investigated in the presence of BEC, thus using the BEC-DC-T model. Prior to HDM exposure, cocultured BEC and moDC were preincubated with a low dose of these HMOS in the basolateral compartment, mimicking the physiological concentrations that can become systemically available via the bloodstream after ingestion of HMOS in infants [24]–[26]. Both 2'FL as well as 3FL prevented the HDM-induced release of TSLP and IL8. IL8 secretion from DC can be triggered in the presence of TSLP [50], justifying the similar secretion patterns observed in these cytokines. However, in this setting the IL8 may also have been derived from BEC. The general decrease in HDM induced TSLP and IL8 secretion from BEC-DC after HMOS preincubation was associated with a reduction in HDM increased IL4 over IFNγ secretion by the T cells which were cocultured with the BEC-DC. Hence both HMOS not only suppressed HDM induced type 2 activation in BEC-DC cultures, it also prevented the instruction of type 2 prone cytokine secretion by T cells exposed to DC from these cultures. These data suggest that the HMOS are able to prevent HDM-induced TSLP secretion and hereby may be able to modify DC function to steer the subsequent T cell response away from a type 2 reaction, which is provoked by HDM when exposed to BEC [51], [52].

Interestingly, only 0,01% 3FL preincubation significantly restored the HDM-induced decrease of TGFβ secretion during BEC-DC coculture. During subsequent coculture of BEC-DC with T cells, 0,01% 3FL preincubation also significantly reduced HDM-BEC-DC instructed IL4 secretion by T cells, while preventing the decrease in percentage of IFNγ expressing cells as observed in the HDM-BEC-DC/T condition. TGFβ is known for its regulatory functions, such as promoting the development of Treg while suppressing Th1 and Th2 responses [53]. Here, 0,01% 3FL preincubation mediated enhanced secretion of TGFβ in BEC-DCs resulting in restoration of the HDM-induced type 1 and type 2 response in T cells, while Treg

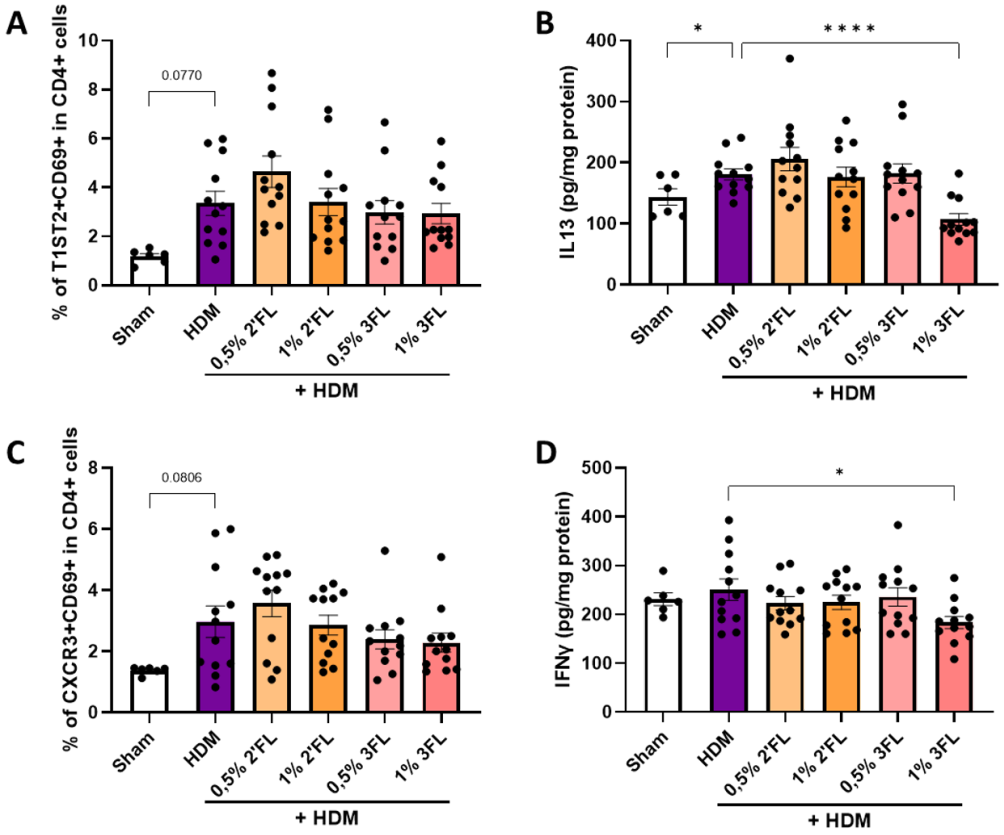

Figure 5. *Lung tissue of sacrificed mice was collected to determine the population of A) activated T helper 2 cells based on the expression of T1ST2 and CD69 and the concentration of B) IL13 in lung tissue as well as the population of C) activated T helper 1 cells based on the expression of CXCR3 and CD69 and the concentration of D) IFNγ in lung tissue. E) Representative flow cytometry samples from Sham and HDM mice. Sham and HDM groups are analyzed by unpaired t-test, the intervention groups were compared to the HDM group by One-Way ANOVA followed by a Dunnett's multiple comparisons test, n=6-12, mean ± SEM (* p<0.05, ** p< 0.01, *** p<0.001).*

differentiation remained unaffected. However, preincubation with 0,01% 2'FL and 0,05% 3FL of HDM-BEC-DC promoted subsequent regulatory IL10 release during coculture with T cells. Previously, we demonstrated that both 2'FL and 3FL preincubation of intestinal epithelial cells prior to ovalbumin exposure directed moDC to induce enhanced release of IL10 by T cells in an *in vitro* intestinal mucosal immune model for ovalbumin-induced type 2 inflammation [54]. Hence, both 2'FL and 3FL may be able to directly affect epithelial cells and/or immune cells and in this way suppress type 2 activation, while promoting regulatory cytokine secretion.

To further study the potential allergy preventive effects of 2'FL and 3FL, a murine model for HDM-induced acute allergic asthma was used. Mice received a control, 2'FL or 3FL containing diet 2 weeks prior to and during HDM sensitization and challenge. Previously, this murine model was used to study other prebiotic dietary interventions [55], [56]. It was demonstrated that diets containing 1% w/w galacto-oligosaccharides prevent the influx of inflammatory cells into

the lungs in HDM sensitized and challenged mice [55], [57], which was also demonstrated for a high-pectin fiber diet [58]. In the current study, HDM sensitized and challenged mice did not show signs of increased airway hyperresponsiveness (Supplemental Figure 3A). The influx of inflammatory cells was significantly increased in HDM-allergic mice, yet dietary intervention with 2'FL or 3FL did not decrease the influx of cells into the lungs significantly. However, the mice receiving 1% 2'FL or 0,5% 3FL containing diets had lower HDM-specific IgE levels in serum, indicative of reduced allergic sensitization towards HDM. Albeit this did not significantly protect against airway eosinophilia, as the levels of HDM-IgE were positively correlated with the number of eosinophils. Changes in dietary fiber or prebiotics intake indeed can affect allergy related humoral responses, as was previously demonstrated in HDM sensitized mice receiving a low-fiber diet that had higher levels of total IgE in serum, compared to HDM sensitized mice fed a high-fiber diet [58].

The 2'FL or 3FL containing diets did not affect the tendency of HDM to induce activation of Th1 and Th2 cells. Nonetheless, mice receiving the 1% 3FL containing diet had lower levels of both IL13 and IFNγ present in lung tissue, suggesting an additional anti-inflammatory capacity of 3FL compared to 2'FL which was also observed in the *in vitro* studies. Reduced cytokine levels were also observed in lung homogenates of HDM sensitized and challenged mice being fed GOS/lcFOS or scFOS/lcFOS plus *Bifidobacterium breve* M-16V supplemented diets compared to control diet [59]. Of note: the HMOS used in the *in vitro* studies were enzymatically derived from lactose. However, due to limitations in production capacity, the HMOS used in the *in vivo* studies were produced in genetically modified *E. coli*. Previously, we demonstrated that the source of HMOS (bacterial or enzymatic produced) affects the immunomodulatory effects in different types of *in vitro* immunoassays [60]. Future studies should take into account the possible translational difference between lactose derived versus E.coli derived HMOS on immunomodulatory efficacy. Even though single 2'FL or 3FL did not protect against HDM induced eosinophilic airway inflammation, the *in vivo* results do indicate 2'FL or 3FL to protect against HDM allergic sensitization as was predicted by the *in vitro* model.

Conclusion

In this study, we established an *in vitro* coculture model for HDM induced type 2 BEC-DC activation and subsequent development of a type 2 Th cell response, which resembles the *in vivo* situation. BEC necessary *in vitro* to induce type 2 immunity after HDM exposure via modulation of DC functionality. Preincubation of BEC-DC with 2'FL and 3FL largely prevented HDM-induced BEC-DC activation and suppressed downstream type 2 over type 1 T cell activation. Although both 2'FL and 3FL reduced HDM-specific IgE in a murine model for HDM allergic asthma, these mice were not protected against airway inflammation. Future studies should consider improvement of *in vitro* models from a 3R perspective to further investigate the potential allergy preventive effects of early life influencing factors.

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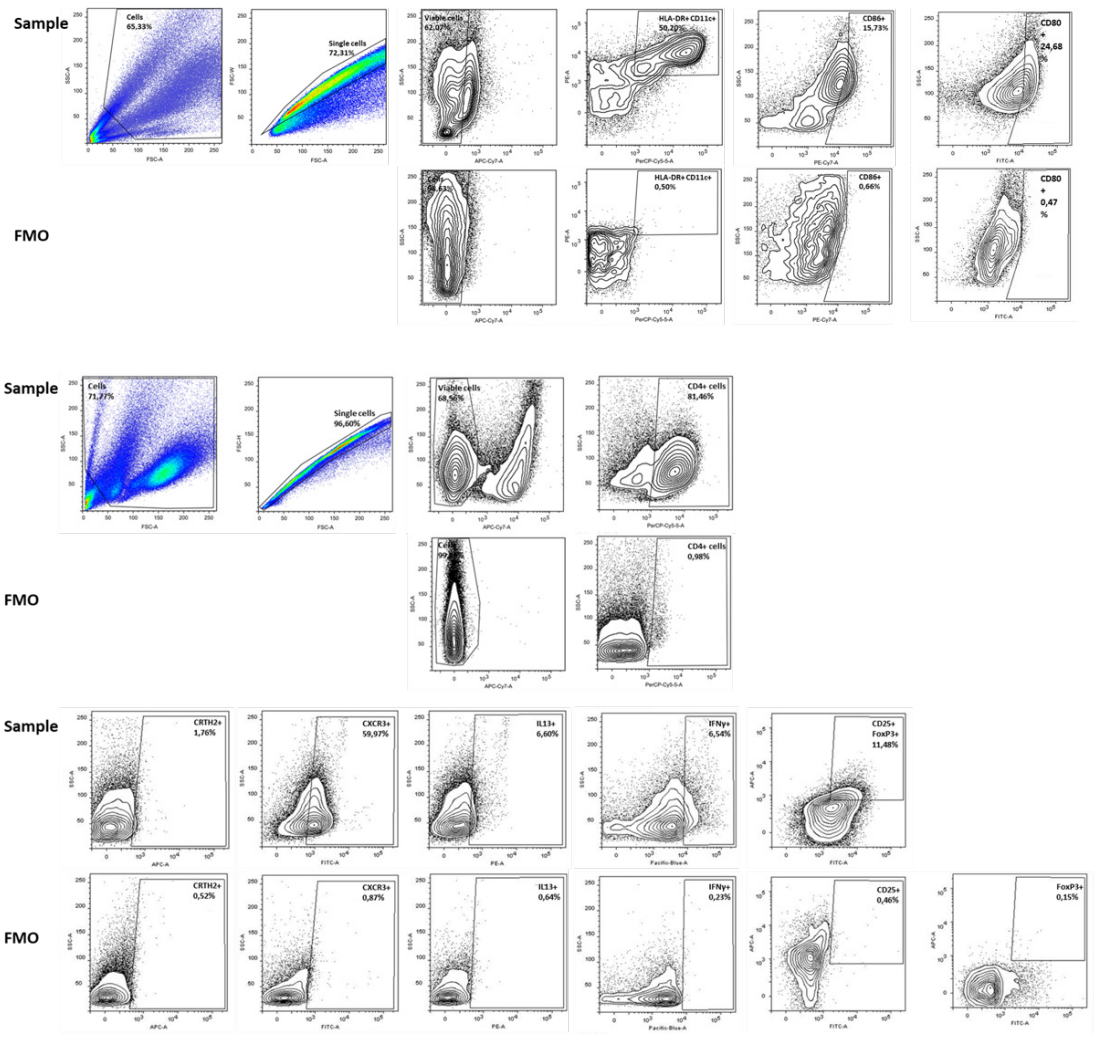
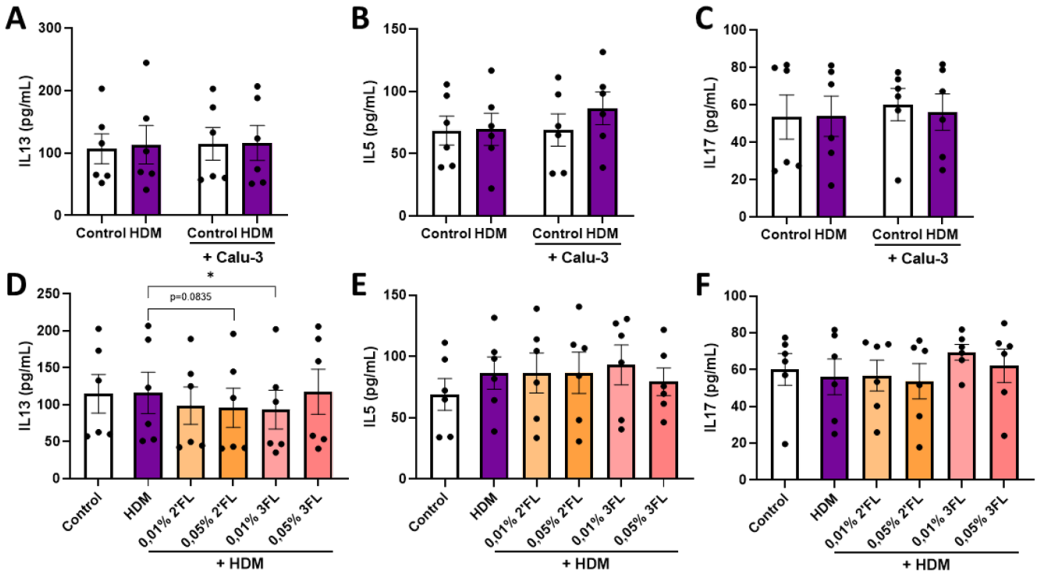
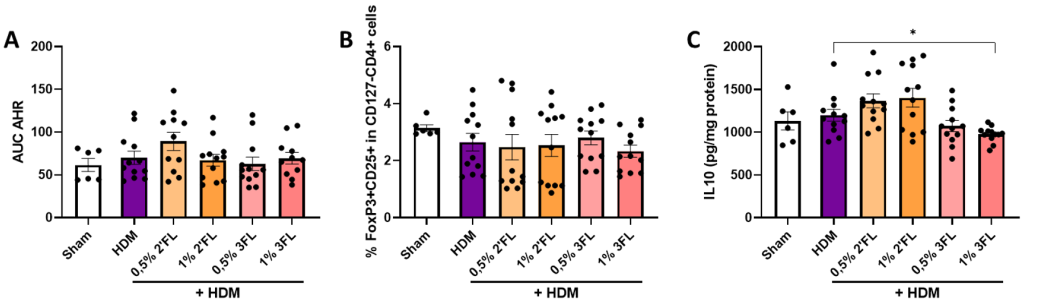
Supplemental figures



Supplemental figure 1. *FACS gating strategy of DCs and T cells from the human in vitro bronchial mucosal immune model.*



Supplemental figure 2. *Cytokine levels from supernatants collected after DC-T cell coculture. Concentrations of A,D) IL13, B,E) IL5 and C,F) IL17 were determined by ELISA.*



Supplemental figure 3. *72h after the final challenge, mice were terminally anesthetized to measure airway hyperresponsiveness upon methacholine exposure. A) Area under the curve of the increase in airway resistance after exposure to increasing dosages of methacholine (0,39-50mg/mL) compared to baseline was calculated. The population of regulatory T cells was determined in lung tissue based on B) expression of FoxP3 and CD25. C) Presence of IL10 in lung tissue was determined by ELISA.*

CHAPTER 9

3'SL and 6'SL lower airway hyperresponsiveness in a preclinical house dust mite induced allergic asthma model, while 3'SL also reduces sensitization and airway inflammation

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Abstract

Allergic asthma is increasingly common in Western societies, with over 300 million people affected worldwide. The underlying allergic response to airborne allergens, such as house dust mite (HDM) is characterized by a type 2 reaction, which includes eosinophilia, IgE production and symptoms such as bronchial hyperresponsiveness and wheezing. During infancy, breastfed children ingest high concentrations of human milk oligosaccharides (HMOS), which are known for their immunomodulatory effects and may prevent the onset of allergic diseases including asthma. Therefore the immunomodulatory effects of two common and structurally similar sialylated HMOS, 3'-sialyllactose (3'SL) and 6'-sialyllactose (6'SL), were investigated in a murine model for acute HDM induced allergic asthma. 6 week old male BALB/c mice were fed an AIN93G diet with or without 0,1% or 0,5% 3'SL or 6'SL from 2 weeks prior to intranasal HDM sensitization on day 0 and challenge on days 7-11. 72h after the final HDM challenge, airway hyperresponsiveness was measured, broncho-alveolar lavage fluid (BALF) and lung tissue were collected for analysis. Dietary intervention with 0,5% 3'SL or 0,1% 6'SL or 0,5% 6'SL prevented the airway hyperresponsiveness to methacholine in HDM challenged mice when compared to mice fed control diet. SCFA levels were elevated in mice receiving the 0,5% 3'SL diet. HDM induced influx of macrophages in the BALF was prevented in both the 3'SL and 6'SL groups when compared to control diet. The 3'SL diets lowered the total inflammatory cell influx as well. By contrast, the 0,5% 6'SL diet significantly increased the presence of eosinophils which was associated with an increase in IL33, TNFα and CCL5 in lung homogenate supernatants. Furthermore, the 0,5% 6'SL diet increased IFNγ, while reducing the presence of stable regulatory T cells in the lung. Both 3'SL diets prevented increase in HDM-specific IgE and mucosal mast cell derived mMCP1 levels in serum. Dietary 3'SL and 6'SL established dose dependent differential clinical and immunological outcomes in HDM sensitized mice. Both 0,5% 3'SL, 0,1% 6'SL and 0,5% 6'SL protected against airway hyperresponsiveness in HDM allergic mice. Although mice fed 0,5% 6'SL displayed improved airway resistance they exhibited increased eosinophilic airway inflammation. By contrast, 3'SL protected against HDM induced allergic sensitization and HDM allergic asthma development.

Keywords: allergic asthma, human milk oligosaccharides, immunomodulation, sialylated oligosaccharides

Introduction

Allergic asthma is increasingly common in Western societies, with over 300 million people affected worldwide [1]. The underlying allergic response is characterized by a type 2 immune response to airborne allergens, such as pollen, animal dander, fungi and house dust mite (HDM) [2]. Uptake of allergens by antigen-presenting cells (APCs) occurs through sampling of the airway lumen by dendritic cells (DCs) and/or through crossing of the allergen into the lamina propria. Following uptake of the allergen, DCs travel to secondary lymphoid tissue to present the allergen to naive Th cells which leads to differentiation into especially Th2 cells [3]. These Th2 cells activate B cells, which leads to class switching towards immunoglobulin E (IgE) producing plasma cells. After activation of B cells, excreted allergen-specific IgE binds to the high affinity receptor (FcεRI) present on mast cells (MCs). During a second encounter with the allergen, the challenge, crosslinking of the cell bound allergen-specific IgE occurs, which leads to mast cell degranulation [4], [5]. Degranulation involves the release of proinflammatory mediators present in mast cell granules, inducing vasodilation, bronchoconstriction, and increased capillary permeability, resulting in clinical symptoms [6].

The WHO recommends exclusive breastfeeding for the first 6 months of life, as it has various benefits for both mother and child [7]. Breastmilk consists of the necessary nutrients and bioactive compounds to provide appropriate nutrition for the infant [8], [9]. It is a dynamic fluid, whose composition changes depending e.g. on the stage of lactation, the mother's ethnicity, diet, age, and even the time of the day and the period since the last feed [10], [11]. Breastfed children ingest high concentrations of human milk oligosaccharides (HMOS), as HMOS are the third largest solid component of breastmilk. All mammalian milk contains oligosaccharides, however human milk contains a uniquely high concentration and complex structures [12]–[14]. Some of these have a prebiotic function, meaning that they can influence the microbiome which in turn can influence the resilience and maturation of the immune system. In addition to the microbiome dependent effects, some of the oligosaccharides can influence immune cells directly, as was observed e.g. by the immunomodulatory and tolerogenic effects observed after dendritic cells were *in vitro* exposed to isolated total HMOS [15]. The immunomodulatory effects induced by HMOS may therefore be involved in the prevention of the onset of allergic diseases such as allergic asthma [5].

Sialylated oligosaccharides are abundantly present in mammalian milk [16]. Sialylated HMOS, such as 3'-sialyllactose (3'SL) and 6'-sialyllactose (6'SL), are thought to affect several biological functions, including supporting the neonatal developing immune system [17]. Studies have shown that both 3'SL and 6'SL promote *in vitro* differentiation of intestinal epithelial cells [18]–[21] and decrease the incidence and severity of diarrhea in piglets [22]. These effects are partially explained by binding of 6'SL to G protein-coupled receptor 35 (GPR35) [23] as well as promoting growth of beneficial bacteria such as *Bifidobacteria* and *Bacteroides* strains by both 3'SL and 6'SL [24], [25]. The latter is reflected by increased production of short chain fatty acids (SCFAs), as utilization of 3'SL and 6'SL occurs in high levels by these bacterial species [26]. Increased levels of specific SCFAs are linked to improved allergic outcomes both in *in vivo* preclinical models as well as in children [27], [28]. In addition to the local effects in the intestines, sialylated HMOS are suggested to be systemic immunomodulators as well. For instance, 3'SL was

found to decrease leukocyte adhesion to endothelial cells [29], while binding of 3'SL to TLR4 on dendritic cells strengthened both Th1 and Th17 immunity [30].

Previously, it was demonstrated that dietary prebiotics, such as galacto- and fructo-oligosaccharides (GOS and FOS), and their fermentation products, like SCFAs, can positively influence the immunological outcomes in HDM-induced allergic asthma in murine models [27], [31]. Based on these findings, the immunomodulatory effects of two common and structurally similar sialylated HMOS, 3'-sialyllactose (3'SL) and 6'-sialyllactose (6'SL), were investigated in a murine model for acute house dust mite induced allergic asthma.

Materials & methods

Diet preparation

The HMOS 3'-sialyllactose (3'SL) and 6'-sialyllactose (6'SL) were purchased from Jennewein Biotechnlogie GmbH (Germany). Experimental diets were based on an AING93-G diet supplemented (sniff-Spezialdiëten GMBH, Germany). with or without 0,1% or 0,5% 3'SL or 6'SL. Supplementation with 3'SL or 6'SL was isocaloric compensated with cellulose. Animals had ad libitum access to food and water.

Animals

Six to seven week old male BALB/cAnNCrl mice (Charles River) arrived at the animal facility of Utrecht University and were housed in individually ventilated cages under a 12h/12h light/dark cycle, controlled relative humidity (50-55%) and controlled temperature (21 ± 2°C) conditions. Mice were randomly divided over the experimental groups immediately received the intervention diets upon arrival. Cage enrichment consisted of woodchipped bedding, wood-curls as nesting material and a plastic shelter. This study was conducted in accordance with institutional guidelines for the care and use of laboratory animals of the Utrecht University, and all animal procedures were approved by the local Animal Welfare Body under an Ethical license provided by the national competent authority (Centrale Commissie Dierproeven, CCD), securing full compliance the European Directive 2010/63/EU for the use of animals for scientific purposes.

Animal procedures

An overview of the experimental design is given in Figure 1A. 14 days after arrival and start of the experimental diets, mice were intranasally sensitized with 1 µg HDM (Greer Laboratories, USA) in 40 µL PBS under isoflurane anesthesia. From days 21 to 25, mice were daily challenged intranasally with 10 µg HDM in 40 µL PBS. 72h after the final challenge airway hyperresponsiveness to increasing doses of methacholine was measured under terminal anesthesia, subsequently mice were sacrificed by intraperitoneal overdose of pentobarbital (600 mg/kg, Nembutal™, Ceva Santé Animale, The Netherlands) and samples were collected for further analysis.

Bronchoalveolar lavage

After sacrifice, lungs were lavaged with 1 mL pyrogen-free saline (0,9% NaCl, 37°C) supplemented with protease inhibitor cocktail tablet (Complete Mini, Roche Diagnostics, Germany). The lungs were lavaged three more times with 1 mL saline solution (0,9% NaCl, 37°C). BALF cells were

centrifuged (400 x g, 5 min) and the pellets were collected. Total cell count was determined using a Bürker-Türk chamber (magnification 100x). Cytospin preparations were prepared and stained with Diff-Quick (Merz & Dade A.G., Switzerland) for differential BAL cell count. Cell counts were scored using light microscopy.

Serum analysis

Blood was collected after sacrifice via eyeball extraction in a Minicollect serum tube (Greiner Bio-One B.V., Netherlands). Collected blood was kept at room temperature and centrifuged for 10 min at 14.000 rpm. Serum was collected and stored at -20°C until further analysis.

Preparation of lung homogenates

Lungs were homogenized in 1% Triton X100 (Sigma-Alrdrich) in PBS containing protease inhibitor (Complete Mini, Roche Diganostics) using a Precellys Tissue Homogenizer and Precellys homogenizer tubes (Bertin, USA). Homogenates were centrifuged at 14.000 rpm for 10 minutes. Supernatant was collected and stored at -20°C for cytokine analysis.

Flow cytometric analysis of lung tissue

Lung tissue was collected after sacrifice and enzymatic digested using a buffer containing DNase I and Collagenase A (Roche Diagnostics, Switzerland). Fetal calf serum (FCS) was added to stop the digestion after 30 min. The lung tissue was passed through a 70µm filter to obtain single cell suspensions. Cell suspension were incubated for 4 min on ice in red blood cell lysis buffer (4.14 g NH_4Cl, 0.5 g $KHCO_3$, 18.6 mg Na_2EDTA in 500 mL demi water, sterile filtered, pH 7.4) Lysis was stopped by adding FCS. Lung cells were washed with RPMI 1640 (Lonza, USA).

$5x10^5$ cells were used for extracellular staining, $1x10^6$ cells were used for intracellular staining. Cells were stained with Fixable Viability Dye eFluor780 for 30 minutes. Nonspecific binding was blocked using anti-CD16/CD32 blocking buffer. Next, samples were stained for 30 min at 4°C using titrated amounts of the following antibodies: CD4-BV510, CD69-PE-Cy7, CXCR3-PE, T1ST2-FITC, CD25-PerCP Cy5.5, CD127-PE Vio770, FoxP3-FITC, RORγt-AF647, CCR6-PE, LAP-BV421. For intranuclear staining of transcription factors, cells were permeabilized using FoxP3/Transcription Factor staining buffer set (eBioscience, USA) following the manufacturer's instructions. FACS Canto II (BD Biosciences) was used to measure samples and obtained data was analyzed using Flowlogic Software (Inivai Technologies, Australia). The gating strategy using a representative sample is given in Supplemental Figure 1.

ELISA

To measure serum HDM-specific IgE levels, high binding 96 wells plates (Corning Costar) were coated with HDM (50 mg/ml) and incubated overnight at 4°C. After blocking with 1% BSA in PBS, plates were washed and serum samples were added to incubate for 2h. Serum samples had to be diluted prior to incubation, all samples were equally diluted and measured on the same plate. Plates were washed again and 1mg/ml biotin anti-mouse IgE (BD Biosciences) was added for 1.5h. Plates were incubated with streptavidin-HRP for 30 minutes, followed by addition of a substrate solution. Washing steps were performed in between. Reaction was stopped by addition of 2 M H_2SO_4, and absorption was measured at 450nm.

Serum mMCP1 concentrations, and lung homogenate supernatant concentrations of CCL5, CCL20, CCL22 (R&D systems, USA) IL5, IL13, IL33, IFNγ, IL17, IL10, TGFβ and TNFα (Invitrogen, USA) in were measured according to the manufacturer's instructions. Cytokine concentrations in lung homogenates were calculated per mg protein in the homogenate supernatant.

Short chain fatty acid levels in cecum content

Cecum contents were collected and stored in -80°C until further use. After thawing, samples were weighed and 5x diluted with ice cold PBS. 1.0mm glass beats (BioSpec, USA) were added and samples were vortexed for 90 seconds to allow homogenization. Homogenized samples were centrifuged for 10 minutes at 13.000 rpm at 4°C, supernatants were collected and stored at -80°C until further analysis. SCFAs were detected by gas chromatography as previously described [32].

Statistical analysis

Statistical analyses were performed using Graphpad Prism (Version 9.4.1) software. Data was analyzed using an unpaired t-test to compare the Sham and OVA groups. All intervention groups were compared to the OVA group using One-way ANOVA followed by Dunnett's multiple comparisons test. If data did not fit a normal distribution, a logarithmic transformation was applied prior to further analysis. $p < 0.05$ is considered statistically significant, data is represented as mean ± SEM. The Sham group contained n=6 and the HDM sensitized and challenged groups had n=12 animals per group.

Results

Dietary 3'SL and 6'SL prevent the development of airway hyperresponsiveness

To study the effects of dietary 3'SL or 6'SL in HDM sensitized and challenged mice, airway hyperresponsiveness (increased airway resistance) in response to inclining concentrations of methacholine was assessed (Figure 1B). The area under the curve (AUC) was calculated (Figure 1C). The AUC of increased airway resistance as measured in response to methacholine was significantly higher in HDM sensitized and challenged mice compared to PBS sensitized and saline challenged mice (Sham). AUC of airway hyperresponsiveness in mice fed 0,5% 3'SL, 0,1% 6'SL or 0,5% 6'SL diets was significantly lower compared to the HDM sensitized and challenged mice fed a control diet (Figure 1C). At baseline (Saline, Figure 1D) no significant differences were observed between the groups. Airway resistance was significantly lower in HDM sensitized and challenged mice fed the 0,5% 3'SL or 0,1% 6'SL or 0,5% 6'SL diets compared to HDM sensitized and challenged mice fed control diet when exposed to 3.13 and 6.25mg/mL methacholine (Figure 1D). These data show that dietary intervention of 3'SL and 6'SL prevent the development of airway hyperresponsiveness.

Dietary 0,5% 3'SL increases SCFA levels in cecum content

SCFAs are produced upon utilization and fermentation of prebiotic fibers in the cecum of the mice and were therefore measured in cecum content. Total SCFA levels (acetate, propionate,

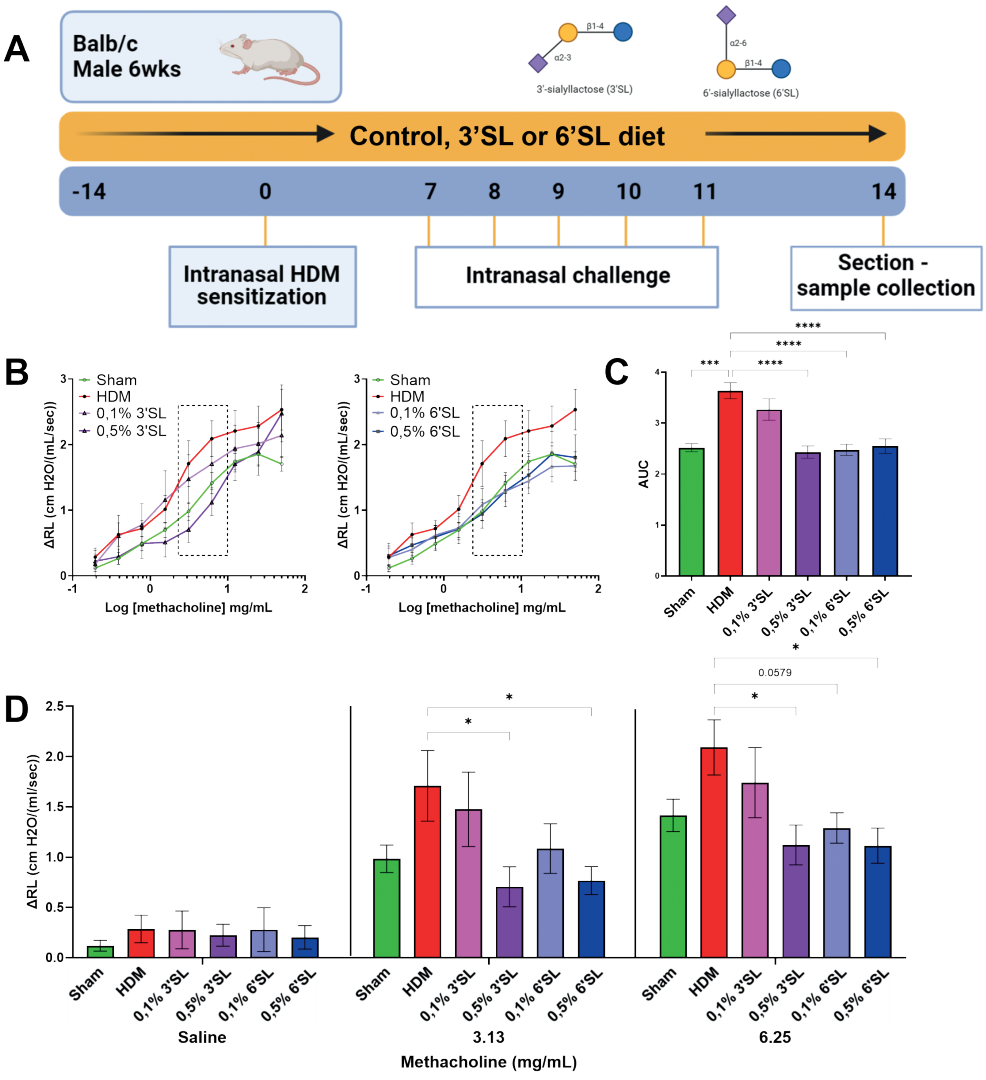

Figure 1. *A) A schematic overview of the experimental design, including dietary intervention with 3'SL and 6'SL supplemented diets fed from 14 days prior to and during intranasal sensitization and challenge with HDM. 72h hours after the final challenge, mice were sacrificed and samples for analysis were collected. B) The increase in airway resistance in response to progressive doses of methacholine was measured in ventilated anesthetized mice. The values inside the dotted boxes are separately displayed in D). C) The area under the curve was calculated of the increased airway resistance was calculated and D) the airway resistance in response to saline, 3,13mg/mL and 6,25mg/mL methacholine are shown. Data is presented as mean ± SEM of 6-12 animals per group. Sham and HDM-allergic groups were statistically compared using an unpaired t-test. Dietary intervention groups were compared to the HDM-allergic group by One-Way ANOVA followed by a Dunnett's post hoc test (* p< 0.05, ** p< 0.01, *** p,< 0.001, **** p< 0.0001).*

butyrate, iso-butyrate, valerate and iso-valerate) in cecum content were elevated in mice fed with 0,5% 3'SL diet (Figure 2A). This was explained by a rise in acetate (Figure 2B), propionate (Figure 2C) and butyrate (Figure 2D), which was not observed for the other dietary intervention groups. Levels of iso-butyrate, valerate and iso-valerate in cecum content were below detection limit and are therefore not individually displayed.

Dietary 3'SL limits HDM-induced pulmonary influx of immune cells

BALF cell counts were determined to assess influx of inflammatory cells into the airways 72h after the final HDM challenge. The total number of BALF cells was increased in the HDM sensitized mice compared to Sham (Figure 3A). Both 3'SL supplemented diets largely prevented a rise in BALF cell influx in HDM sensitized and challenged mice compared to mice receiving the control diet. The HDM sensitized and challenged mice did not show a significant increase in eosinophil influx compared to Sham mice (Figure 2B), but HDM sensitized mice fed the 0,5% 6'SL had a significantly higher number of eosinophils present in BALF compared to HDM sensitized and challenged mice on control diet. The number of lymphocytes was not significantly changed in any of the groups (Figure 2C). Yet, HDM sensitized and challenged mice fed control diet had an increased influx of macrophages into the airways compared to Sham mice. All dietary intervention groups showed a reduced macrophage influx in the BALF (Figure 2D). Influx of immune cells in inflamed tissue is a consequence of chemoattraction upon increased secretion of proinflammatory cytokines and chemokines, such as IL33, TNFα and CCL5. Levels of IL33 and TNFα (Figure 3E and 3F) in lung homogenate supernatants were found to be further increased in HDM sensitized and challenged mice receiving a 0,5% 6'SL supplemented diet. These data show that dietary 3'SL, but not 6'SL, limits immune cell influx, even though both dietary interventions reduced airway hyperresponsiveness.

Dietary 6'SL decreases the percentage of regulatory T cell subsets in lung tissue

To further assess the immunomodulatory effects of dietary 3'SL and 6'SL on HDM-induced allergic asthma development, the ratio of regulatory T cell subsets and regulatory cytokines in lung tissue was assessed. The percentage of regulatory T (Treg, FoxP3+ in CD25+CD127-CD4+) cells was not altered in any of the groups (Figure 4A). Although HDM sensitized and challenged mice had a similar percentage of stable Treg (FoxP3+RORγt+ in CD25+CD127-CD4+) cells compared to Sham mice (Figure 4B), HDM sensitized and challenged mice receiving a 6'SL supplemented diet had a significantly lower proportion of stable Tregs present in lung tissue compared to HDM sensitized and challenged mice receiving control diet. In addition, the population of regulatory type Th3 (LAP+ in FoxP3-CD25-CD4+) cells was determined and was found to be more abundant in HDM sensitized and challenged mice compared to Sham mice (Figure 5C). The presence of these regulatory Th3 cells was, however, also lower in HDM sensitized and challenged mice receiving the 0,5% 6'SL diet. Effector functions of these regulatory cells depend in part on the secretion of the regulatory cytokines IL10 and TGFβ (Figure 5D and 5E). No significant differences were found in the presence of pulmonary IL10. TGFβ was increased in HDM sensitized and challenged mice fed control diet, while TGFβ was normalized in HDM sensitized and challenged mice receiving 0,1% 3'SL diet. These data indicate that dietary 6'SL, but not 3'SL, lowers the abundance of several pulmonary Treg cell subsets.

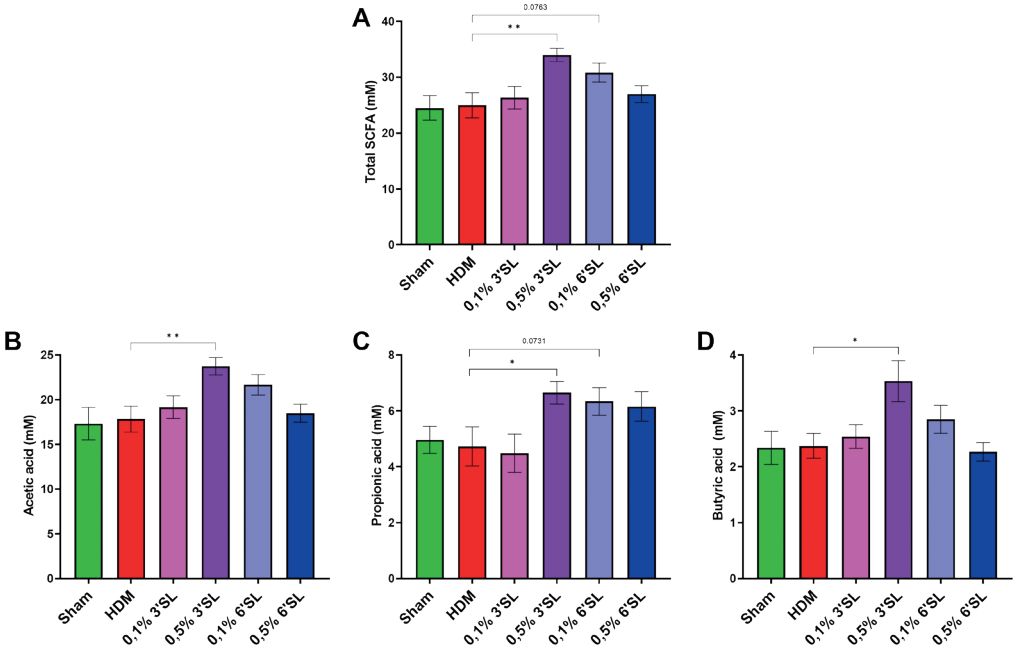

Figure 2. *SCFA concentrations were determined in cecum content. A) Total SCFA, B) acetate, C) propionate and D) butyrate are displayed. Data is presented as mean ± SEM of 6-12 animals per group. Sham and HDM-allergic groups were statistically compared using an unpaired t-test. Dietary intervention groups were compared to the HDM-allergic group by One-Way ANOVA followed by a Dunnett's post hoc test (* p< 0.05, ** p< 0.01).*

Dietary 3'SL and 6'SL affect pulmonary levels of IL13 and IFNγ

To further phenotype the allergic T cell response in the lung tissue, the presence of Th1 and Th2 type cytokines and percentage of activated T helper cell subsets was examined. Total Th1 and Th2 populations are shown in Supplemental Figure 2B and 2C. An increased percentage of activated Th2 (T1ST2+ in CD69+CD4+) cells and elevated levels of IL13 were observed in HDM sensitized and challenged mice (Figure 5A and 5D). HDM sensitized and challenged mice fed a 0,1% 3'SL had reduced levels of IL13 in long homogenate supernatants compared to HDM sensitized and challenged mice fed control diet. However, a decrease in the percentage of activated Th2 cells was not observed. Furthermore, presence of activated Th1 (CXCR3+ in CD69+CD4+) cells was not altered upon HDM sensitization and challenge, shifting the Th2/Th1 cell balance in favor of the Th2 cells in HDM sensitized and challenged mice receiving control diet (Figure 5B and 5C). None of the groups receiving an HMOS containing diet had an altered percentage of activated Th1 cells or change in the Th2/Th1 cell ratio. However, HDM sensitized and challenged mice receiving 6'SL containing diets had a significant increase in IFNγ present in lung homogenate supernatants (Figure 5E). The ratio of IL13/IFNγ present in lung tissue was significantly lower in groups receiving 0,1% 3'SL, 0,1% 6'SL or 0,5% 6'SL diets compared to HDM sensitized and challenged mice fed control diet (Figure 5F). Therefore, these data show immunomodulation by dietary 3'SL and 6'SL on the levels of cytokine secretion in the lungs.

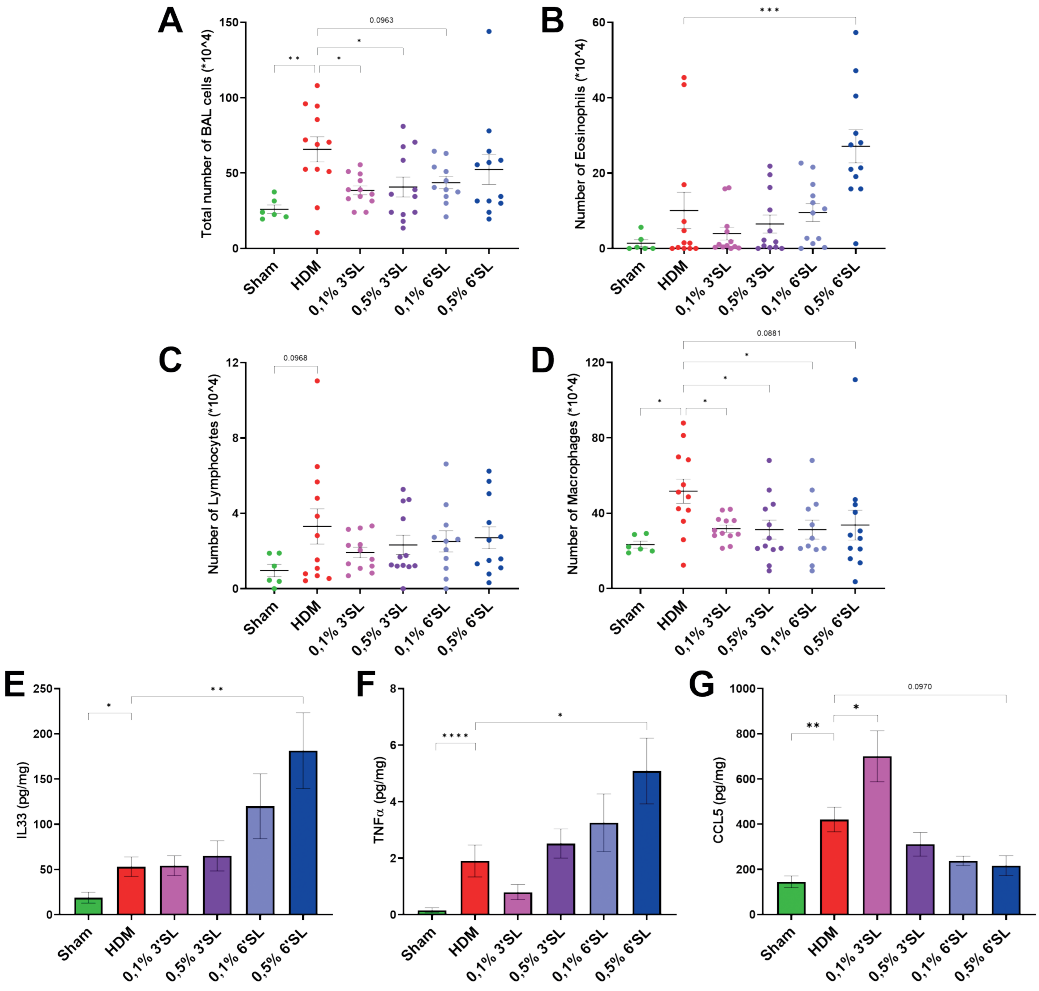

Figure 3. *Influx of inflammatory cells in the BALF of mice. A) The total cell count in BAL fluid, B) eosinophils, C) lymphocytes and D) macrophages are displayed. Furthermore, presence of proinflammatory cytokines and chemokines E) IL33, F) TNFα and G) CCL5 in lung tissue homogenate supernatants were quantified. Data is presented as mean ± SEM of n=6 (sham) or n=12 (allergic) animals per group. Sham and HDM-allergic groups were statistically compared using an unpaired t-test. Dietary intervention groups were compared to the HDM-allergic group by One-Way ANOVA followed by a Dunnett's post hoc test (* p< 0.05, ** p< 0.01, *** p,< 0.001).*

Dietary 3'SL reduces HDM-IgE serum levels and mucosal mast cell degranulation

During allergen sensitization, allergen-specific IgE is produced which can be measured in serum. HDM sensitized and challenged mice receiving control diet had higher levels of HDM-specific IgE present in serum (Figure 6A). Dietary intervention with 3'SL largely prevented this increase in HDM-IgE levels. This was not observed in mice receiving a 6'SL containing diet. During allergen challenge, mucosal mast cells degranulate upon IgE crosslinking and proinflammatory mediators are released. One of these mediators, murine mast cell protease 1 (mMCP1), was measured in serum to reflect mucosal mast cell degranulation (Figure 6B). mMCP1 was elevated in HDM sensitized and challenged mice compared to Sham mice. HDM sensitized and challenged mice fed a 0,1% 3'SL diet had a significantly lower level of mMCP1 in serum compared to HDM sensitized and challenged mice on control diet; this was not observed for the other dietary intervention groups. Altogether, these data indicate that sialylated HMOS can modulate immune response *in vivo* on multiple levels within a model for developing HDM-induced allergic asthma.

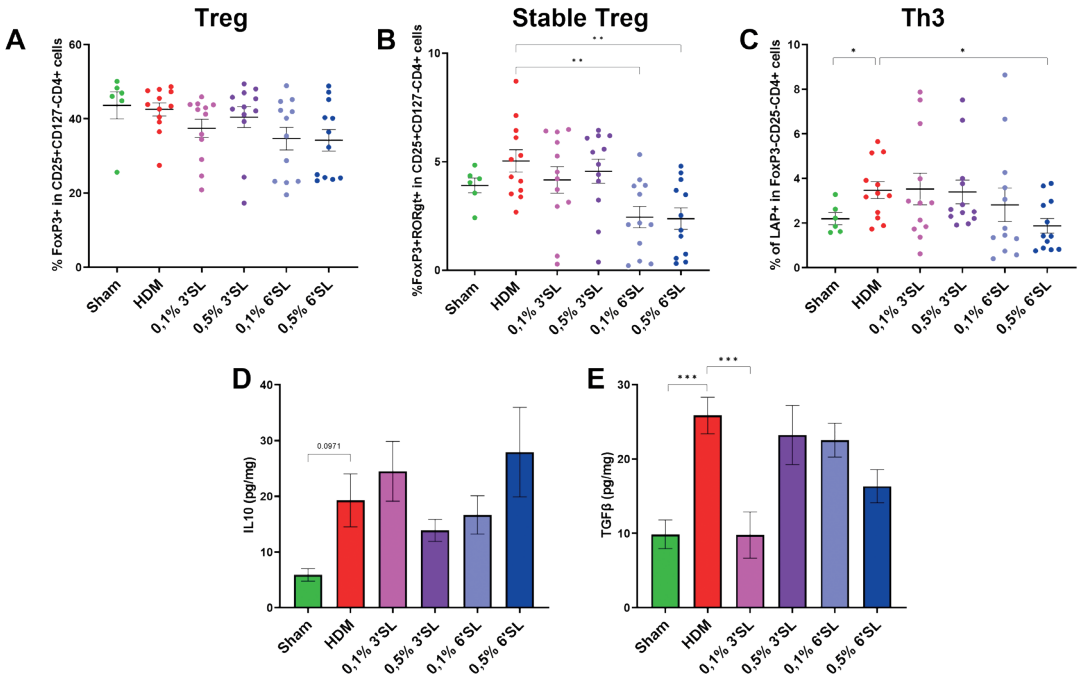

Figure 4. *Populations of A) Treg, B) stable Treg and C) Th3 cells were determined by flow cytometric analysis of single cell suspensions obtained from collected lung tissue. Normalized levels of D) IL10 and E) TGFβ were determined in lung homogenate supernatants. Data is presented as mean ± SEM of n=6 (sham) or n=12 (allergic) animals per group. Sham and HDM-allergic groups were statistically compared using an unpaired t-test. Dietary intervention groups were compared to the HDM-allergic group by One-Way ANOVA followed by a Dunnett's post hoc test (* p< 0.05, ** p< 0.01, *** p,< 0.001).*

Discussion

Sialylated HMOS are found in abundance in human milk and are therefore thought to fulfill biological functions during neonatal development [16], [17]. These sialylated HMOS are described to possess immunomodulatory effects [29], [33] and modulate immune function in allergic settings [34], [35]. These effects may be partially explained by the presence of sialic acid groups. Previously dietary supplementation with several prebiotic structures like short chain (sc) GOS and long chain (lc)FOS, which do not contain sialic acid groups, and their metabolites improved asthma allergic outcomes in mice [27], [31], [36]–[38]. Therefore, the aim of this study was to investigate the effects of 3'SL and 6'SL dietary supplementation on the development of HDM-induced acute allergic asthma in mice. Airway hyperresponsiveness and immunological parameters were determined to assess the severity of the allergic airway inflammation.

In this study, HDM sensitized and challenged mice receiving the 0,5% 3'SL, 0,1% 6'SL or 0,5% 6'SL containing diets were protected from an increased airway hyperresponsiveness which is a general disease feature in allergic asthma patients. Previously, it was shown that prebiotic fibers improved HDM-allergic outcomes in a similar preclinical HDM-induced acute allergic asthma model [31]. Prebiotic fibers are fermented into SCFAs by intestinal microbes. SCFAs are potent immunomodulators, known to affect e.g. different types of leukocytes promoting homeostasis [39]. Administration of the SCFA propionate was previously found to improve HDM allergic asthma outcomes, including airway hyperresponsiveness, in mice [27]. In the current study, mice receiving the 0,5% 3'SL supplemented diets contained higher concentrations of SCFAs, including propionate in the cecum content, and demonstrated decreased airway hyperresponsiveness. On the other side, mice receiving 6'SL supplemented diets demonstrated a reduction in airway hyperresponsiveness. Although, the SCFAs levels in these mice were not elevated significantly, the 0,1% 6'SL diet did tend to increase propionate levels compared to control diet fed HDM allergic mice ($p=0.0731$). This indicates that bacterial metabolites, such as propionate, may have contributed to the protection against airway hyperresponsiveness in HMOS fed HDM allergic mice [40].

A lower influx of leukocytes, especially macrophages, into the lungs was observed in mice receiving the 3'SL containing diet. Prebiotic dietary interventions using scGOS led to a reduction in total influx of cells into the lungs [31], [37], which was observed in this study as well. Mice fed GOS containing diets had lower numbers of eosinophils present in broncho alveolar lavage [31], [37]. However, in the current study, the airway eosinophilia was not significantly increased in the HDM allergic mice. The decrease in total cell influx could mainly be explained by lower numbers of macrophages, which was observed in both the 3'SL intervention groups, but also in the 0,1% 6SL group. The rise in BALF number of monocytes/macrophages during allergic inflammation may be a reflection of proliferation of local alveolar and interstitial macrophages and/or an increase in newly attracted bone marrow derived monocytes which further differentiate into macrophages in the lung tissue [41]. 3'SL or 6'SL may suppress local tissue macrophage proliferation and M2 phenotype development for example by affecting the HIF1alpha/VEGF axis [42]. Furthermore, inhibition of the β-catenin/CBP pathway may have resulted in selective lowering of macrophage type inflammation [43]. Future studies should focus on mechanisms underlying the reduced monocyte/macrophage inflammation in the 3'SL or 6'SL fed HDM sensitized and challenged mice.

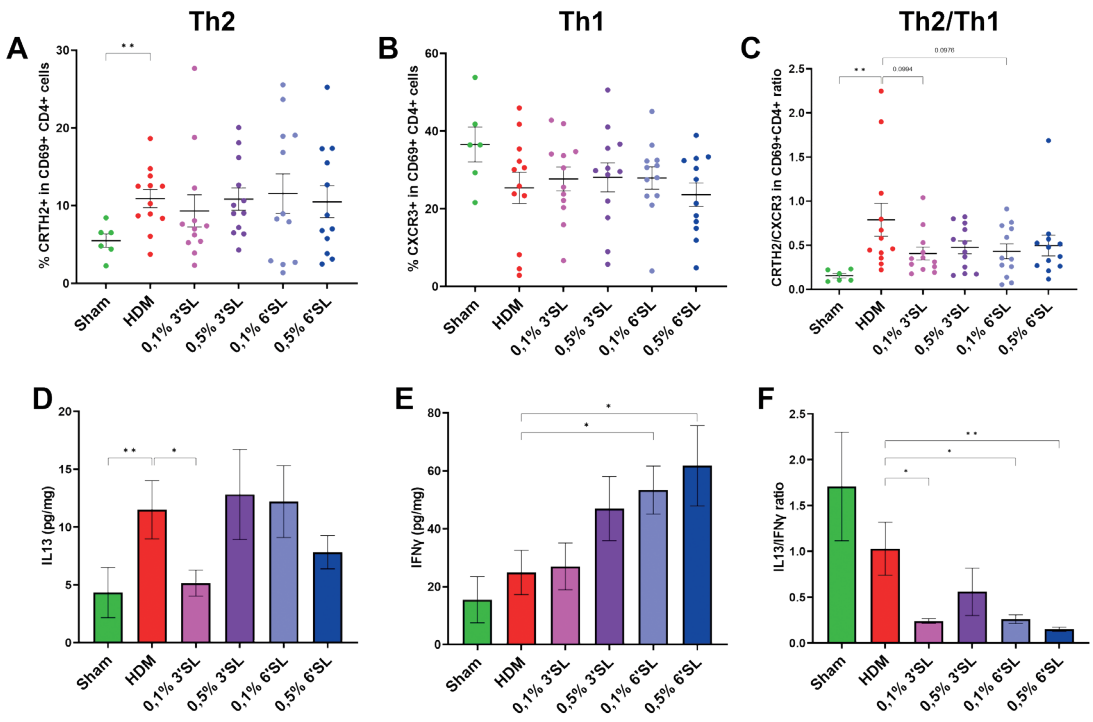

Figure 5. *Single cell suspensions were obtained from collected lung tissue for flow cytometric analysis. Populations of A) activated Th2 cells, B) activated Th1 cells and C) the ratio of Th2/Th1 cells were determined. Levels of D) IL13, E) IFNγ and F) IL13/IFNγ ratio were determined in lung homogenates. Concentrations were normalized per mg of lung tissue. Data is presented as mean ± SEM of n=6 (sham) or n=12 (allergic) animals per group. Sham and HDM-allergic groups were statistically compared using an unpaired t-test. Dietary intervention groups were compared to the HDM-allergic group by One-Way ANOVA followed by a Dunnett's post hoc test (* p< 0.05, ** p< 0.01).*

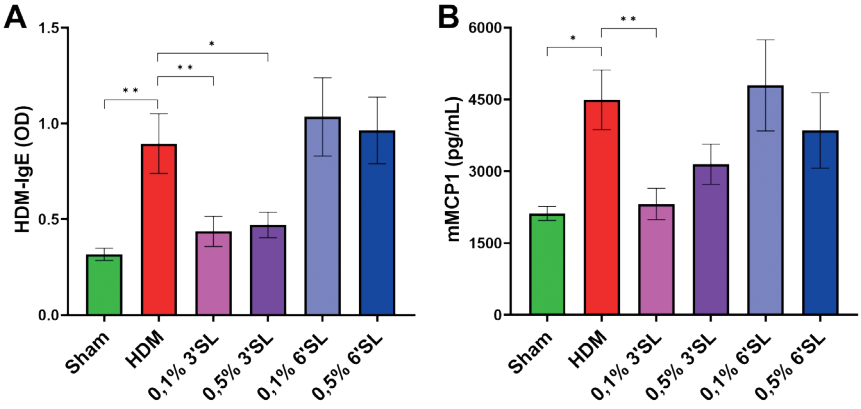

Figure 6. *A) Serum levels of HDM-specific IgE and B) mMCP1 were measured. Data is presented as mean ± SEM of n=6 (sham) or n=12 (allergic) animals per group. Sham and HDM-allergic groups were statistically compared using an unpaired t-test. Dietary intervention groups were compared to the HDM-allergic group by One-Way ANOVA followed by a Dunnett's post hoc test (* p< 0.05, ** p< 0.01).*

Even though mice receiving the 0,5% 6'SL diet showed improved clinical outcomes, an increase in eosinophil influx into the lungs was observed. Secretion of IL33 by airway epithelial cells is a key phenomenon in allergic asthma [44]. Neutralization of IL33 was found to reduce the influx of eosinophils into the lungs of mice [45], revealing a link between IL33 and eosinophilic inflammation in the lungs dependent on systemic IL5 secretion [46]. Here, the increased eosinophilic influx in the lungs was associated with elevated IL33 levels, which occurred in the absence of increased IL5 in the lungs (Supplemental Figure 2). The presence of IL33 promotes the release of TNFα from macrophages and local mast cells [44], and TNFα in turn promotes airway epithelial cells to secrete IL33 [47]. Dietary 0,5% 6'SL therefore may have induced IL33 and/or TNFα secretion by local tissue inflammatory cells. Sialylated HMOS are known to possess binding affinity to the TLR4 receptor [30]. HDM allergens are known to activate bronchial epithelium via direct binding to TLR4 or proteolytic activity indirectly activating TLR4 [48], [49]. Furthermore, HDM allergens can cross the epithelial barrier via disruption of tight junctions [50] and become systemically available for immune cells. Activation of T cells via TLR4 induces TNFα secretion, which is reduced by Th3 cells [51]. In neonatal mice, 6'SL supplemented formula milk reduced TNFα gene expression partially via direct binding to and inhibition of TLR4 [52]. Yet, in a murine model for colitis, 3'SL supplementation promoted dendritic cell function in a TLR4-dependent manner [30]. 3'SL or 6'SL may become available systemically, which is known for specific HMOS structures, and occupy the TLR4 receptor in this way hampering the HDM-induced activation cascade. While both 3'SL and 6'SL indeed are known to modify mucosal immunity via TLR4 interaction, this present study clearly demonstrated differences in immunological outcomes in mice fed a 3'SL or 6'SL containing diet in this HDM-induced acute allergic asthma model. The mice receiving dietary 3'SL had lower airway inflammation, while dietary 0,5% 6'SL enhanced eosinophilic airway inflammation. Differential TLR4 binding and signaling features of 3'SL versus 6'SL may play a role in the dose dependent differences of effect observed between these HMOS.

CCL5 (also known as RANTES) is a chemokine secreted by bronchial epithelial cells [53] and fibroblast from asthmatic individuals [54] and is known to attract several immune cells, including T cells and eosinophils [53]. Yet, this chemokine can drive a Th2 response towards a Th1 response [55] and the presence of CCL5 is linked to a decreased airway hyperresponsiveness during repeated allergen exposure [56]. However CCL5 has been linked to the development of fibrosis as well [57]. Furthermore, TGFβ is considered a regulatory cytokine, known to generally dampen immune responses. On the contrary, this growth factor is also involved in fibrosis during asthma [58]. High levels of TGFβ in the lungs are therefore associated with markers of increased airway remodeling [59], [60]. In the current study the HDM sensitized and challenged mice suffered from airway hyperresponsiveness in response to methacholine in the presence of elevated TGFβ and CCL5 which may be indicative for ongoing tissue remodeling. HDM sensitized and challenged mice fed a 0,1% 3'SL diet had decreased levels of TGFβ, while CCL5 presence was further elevated in lung tissue. By contrast, in the other dietary intervention groups, pulmonary TGFβ remained higher while CCL5 levels remained low in association with reduced the airway hyperresponsiveness. Thus, beyond the previously discussed role for SCFAs in protection against airway hyperresponsiveness, 3'SL or 6'SL may also differentially affect the balance between TGFβ and CCL5 hereby affecting the development of airway hyperresponsiveness.

In addition to the aforementioned effects, 3'SL and 6'SL may have affected the development of Treg cells. The population of both TGFβ producing pulmonary stable Treg and Th3 cells were lower only in mice fed the 0,5% 6'SL diet, but not affected in the other dietary interventions groups. Therefore, the populations of Treg cells do not provide an explanation for the improved clinical outcomes. In addition, the clinical improvement cannot be explained by the ratio of Th2 and Th1 cells present in lung tissue either, even though this balance in the mice fed the 0,1% 3'SL or 0,1% 6'SL diet tended to shift in favor of the Th1 cells. These effects became more pronounced when focusing on Th2 and Th1 cytokines present in the lung tissue. In all HMOS diet groups the balance was shifted towards type 1 (based on IFNγ) and away from type 2 (based on IL13) when compared to HDM sensitized and challenged mice receiving the control diet. Mice receiving the 0,1% 3'SL diets had significantly lower levels of IL13 in lung tissue. Yet, the mice fed a 6'SL supplemented diet were found to have increased levels of IFNγ present in lung tissue, shifting the balance towards Th1 dominant cytokine production, which may have counteracted the effect of type 2 cytokines in chronic inflammation and airway hyperresponsiveness [61]

Furthermore, mice receiving 0,1% or 0,5% 3'SL, but not 6'SL, containing diets had lower levels of HDM-specific IgE and mMCP1 in serum. These data indicate prevention of sensitization and local mucosal mast cell activation. Even though mast cells enhance mMCP1 gene expression in the presence of TGFβ [62], the level of mMCP1 found in serum does not fully correlate with TGFβ in lung tissue. Activation of mast cells can be prevented in the presence of SCFAs [63], however, these effects were only enhanced in mice fed the 0,5% 3'SL diet and local tissue levels may not be sufficient to reduce mast cell activation. Therefore, it can be hypothesized that 3'SL may have acted upstream in the sensitization cascade, suppressing the HDM-induced activation of bronchial epithelial cells and/or dendritic cells and macrophages [64]. These findings indicate that future studies should elaborate on the exact mechanisms and pathways involved in interaction between the immunomodulatory effects of sialylated HMOS and the development of allergic asthma.

Conclusion

In conclusion, this study demonstrated that dietary 3'SL and 6'SL resulted in differential clinical and immunological outcomes in HDM sensitized and challenged mice. Mice receiving the 0,5% 3'SL diet showed increased cecal SCFA levels and were largely protected from HDM sensitization, an increase in airway hyperresponsiveness and allergic airway inflammation. Mice receiving 6'SL containing diets also showed protection from increased airway resistance, but the airway inflammation was enhanced in the 0,5% 6'SL fed mice. Both 3'SL and 6'SL were found to lower BALF macrophage counts. Future studies should focus on elucidating the underlying mechanism via which sialylated HMOS exert their preventive effect in the airway allergic response.

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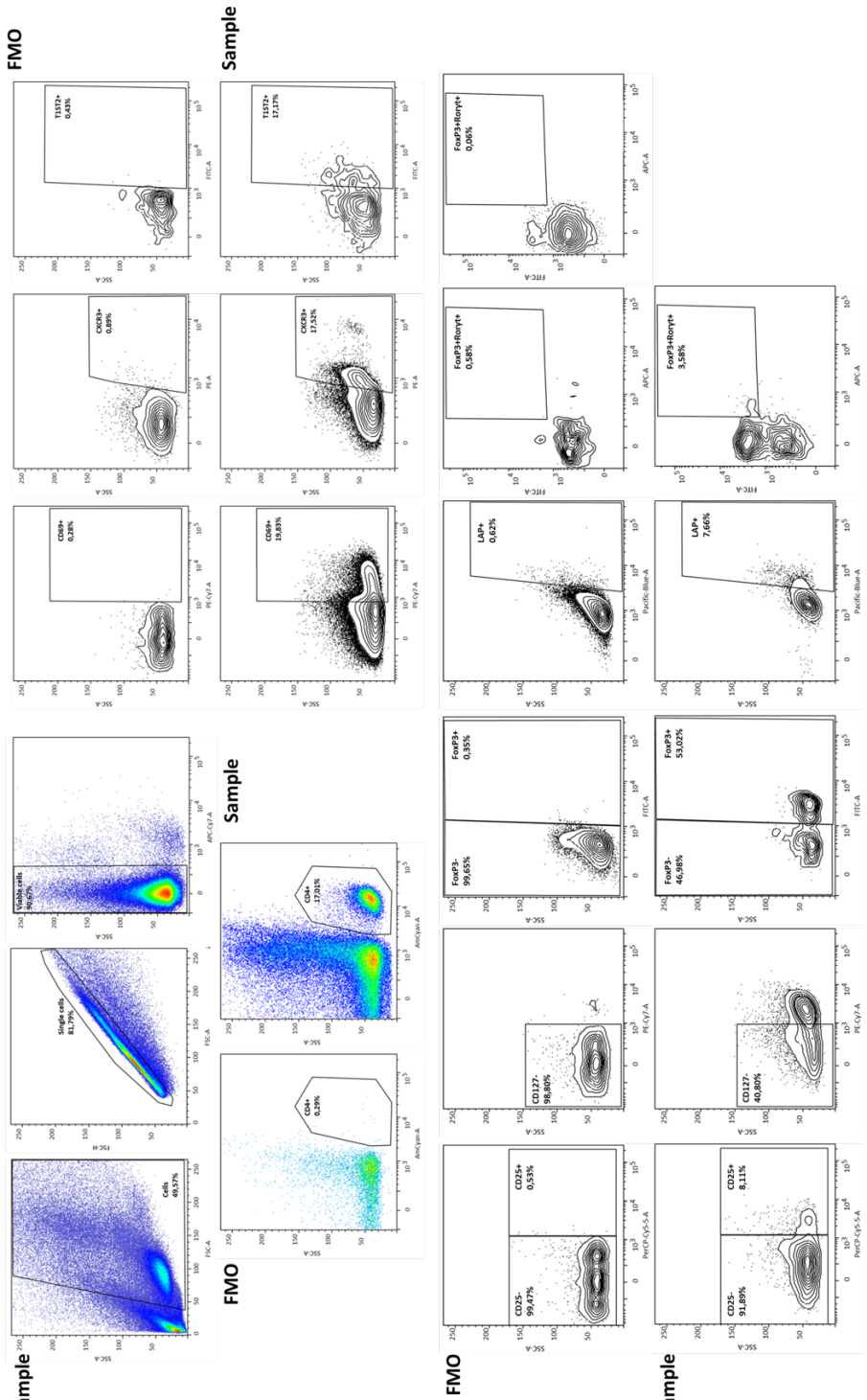
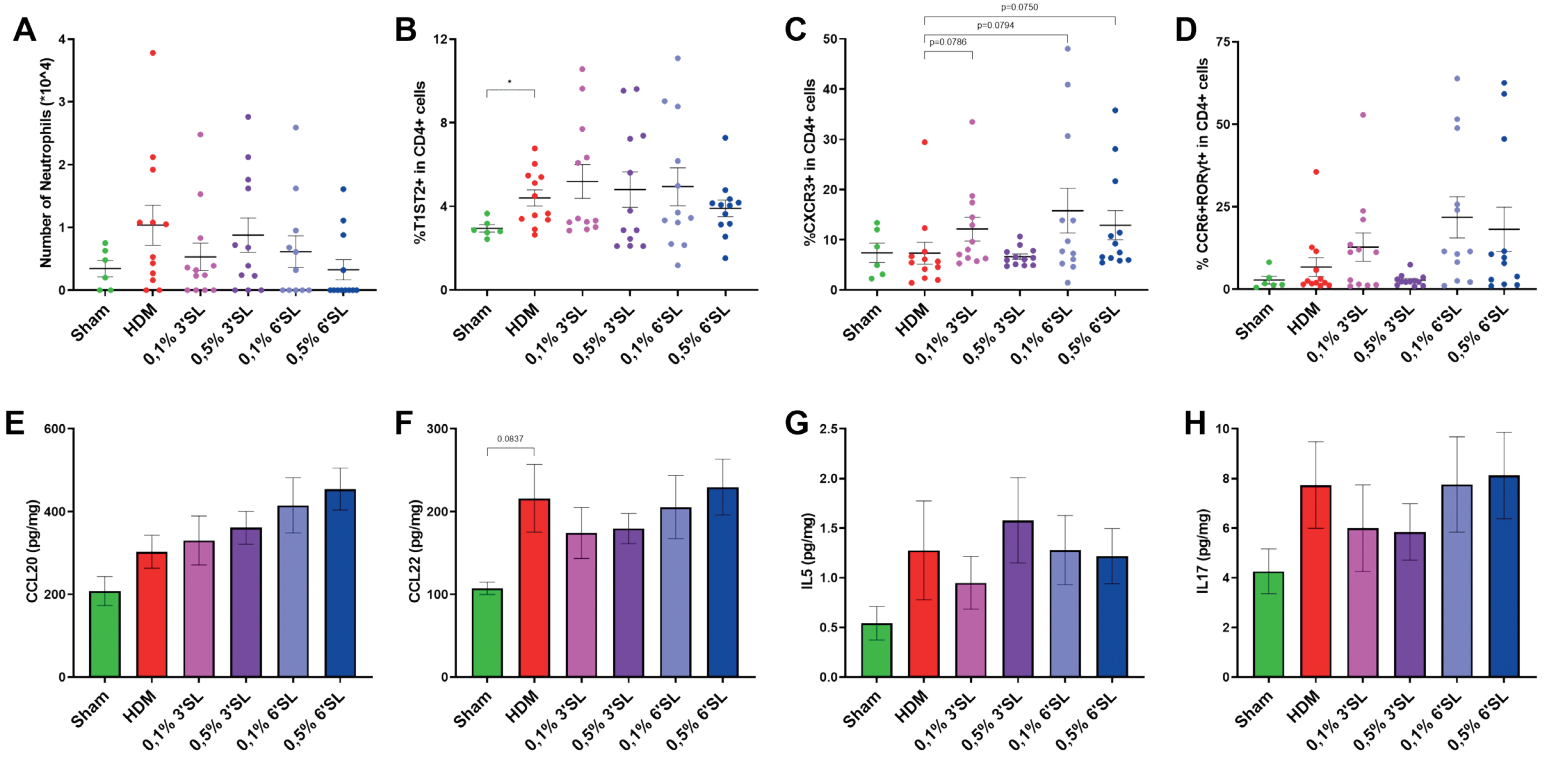
Supplemental figures



Supplemental figure 1. *Representative gating strategy, including corresponding FMOs for flow cytometric analysis of single cells suspensions obtained from lung tissue.*



Supplemental figure 2. *A) Influx of neutrophils was quantified in BALF. Percentages of T helper subsets present in lung tissue were determined by flow cytometry. Populations of B) Th2 (T1ST2+ in CD4+) cells, C) Th1 (CXCR3+ in CD4+) cells and D) Th17 (CCR6+RORγt+ in CD4+) cells were determined. Furthermore, levels of E) CCL20, F) CCL22, G) IL5 and H) IL17 per mg lung tissue were measured by ELISA. Data is presented as mean ± SEM of n=6 (sham) or n=12 (allergic) animals per group. Sham and HDM-allergic groups were statistically compared using an unpaired t-test. Dietary intervention groups were compared to the HDM-allergic group by One-Way ANOVA followed by a Dunnett's post hoc test (* p< 0.05, ** p< 0.01, *** p,< 0.001, **** p< 0.0001).*

CHAPTER 10

Summarizing Discussion

After birth there is a direct need for adaptation to the various environmental triggers, which makes neonates vulnerable. Mucosal tissues and the immune system are at the interface with the outside world and need to undergo rapid maturation and adaptation in early life in order to respond adequately to these environmental triggers. This period of maturation provides a unique window of opportunity during which adverse events may result in later life immune disfunction, such as the development of allergic diseases [1], [2]. This period is also a window of opportunity to promote and protect healthy growth and development of neonates. Supportive strategies, including dietary approaches, should be studied in detail aiming at the development and understanding of dietary intervention protocols, including human milk components, during early life.

Almost a century ago, the first protective association between breastfeeding and allergy development was described [3], suggesting that breastfed children less frequently develop allergic diseases in later life compared to formula-fed children [4]–[9]. Human milk, as the gold standard of infant nutrition, consists of many biological active nutrients which promote a healthy development of the infant's microbiome, brain, gastro-intestinal tract and immune system [10]. Therefore, studying the exact composition of human milk is of utmost importance to understand the mechanisms underlying its health promoting effects.

This thesis aims to promote the understanding of the immunomodulatory effects of specific human milk oligosaccharides (HMOS) as they are a major immune active component of human milk and in that respect very different from the oligosaccharides present in e.g. cow's milk [11]. Specific HMOS structures are studied in the intestine and lungs, focusing on preventing type 2 immune responses and allergic sensitization. Therefore, novel advanced *in vitro* human mucosal immune models of the gut and lung were developed, to study immune activation during allergen exposure. These types of research models are essential to study the immunomodulatory effects of e.g. dietary components such as oligosaccharides, polyunsaturated fatty acids, peptides, etc.. These improved insights into dietary approaches, potentially contribute to the reduction of animal studies and eventually will lead to increased understanding of the added value for these specific nutrients in dietary compositions for infants early in life.

To tolerate or not?

Directly after birth an infant's immune system is skewed towards T helper 2 (Th2) responses as a result from tolerating (non-self) maternal antigens in utero [12], and needs to gain a proper Th1 response via adequate and controlled antigen exposure during the first period of life. Interestingly neonatal naïve T cells are biased towards differentiating into regulatory T (Treg) cells as well [13]. These characteristics create a unique environment, in which the infant's immune system can produce protective tolerogenic responses. With regards to allergen exposure, a tolerogenic response should be developed in order to prevent the onset of allergies. If the development of this immune tolerance is disrupted, allergic sensitization can occur, characterized by a Th2 directed inflammation and high levels of specific IgE antibodies (i.e. sensitization). Upon subsequent exposure of sensitized individuals to the specific allergen, the IgE molecules on mast cells will crosslink, leading to degranulation and subsequent allergic symptoms.

Early life tolerance vs. sensitization

Establishment of oral tolerance is achieved early in life via the gastrointestinal mucosal immune system upon oral exposure to harmless food proteins. The induction of this peripheral tolerance allows the body to balance the response against exogenous antigens and maintain immune homeostasis [14]. Although the exact mechanisms underlying the development of oral tolerance are not fully understood, a central role for Treg cell development has been identified [15]. A sufficient development of Treg cells (numbers and activity) ensures a peripheral tolerogenic response upon a subsequent allergen encounter, irrespective of the route of allergen exposure. Preclinical animal studies have demonstrated the tolerogenic effect of oral administration of antigens in models of both autoimmunity and allergy [16], [17]. Even though first results from human trials indicate that similar principles are in play, its importance and relevance in humans needs further studies and better understanding [18].

In early life, environmental factors and genetic predispositions may impair the skin barrier, mostly visible as atopic dermatitis or eczema, allowing enhanced allergen penetration [19]. Exposure to food and aero allergens via the (impaired) skin, may result in priming of an allergen-specific Th2 cell response and IgE producing B cells due to tissue-specific features [20], [21]. Once the individual is sensitized, exposure to this specific allergen via a different route, e.g. oral exposure to a food allergen, results in acute inflammation and allergic symptoms. The increased susceptibility to sensitization via the skin may be explained at least in part by difference in the expression of homing factors specific for the gut and skin on Treg cells. In neonates, the majority of peripheral Treg cells are programmed to migrate to lymphoid tissues and the gut, while a relatively small number of Treg cells will migrate to the skin, to counterbalance a proinflammatory response [22], [23].

Atopic dermatitis and potential subsequent epicutaneous sensitization are the first steps in the 'Atopic March', after which other allergic diseases might develop such as food allergy and allergic asthma [24]. Children with atopic dermatitis are approximately at a six times higher risk of developing food allergy, than children without atopic dermatitis [25]. Early onset and severity of atopic dermatitis in young children is associated with a higher chance of developing allergic asthma from the age of 3 years as well [25]–[28]. Even though epicutaneous sensitization may play an important role in the development of early life allergic sensitization, sensitization against an allergenic protein in the intestines or airways occurs as well and can be triggered by many different environmental factors, such as pathogens and mycotoxins [29]–[33].

What makes an allergen sensitizing

While an increasing proportion of the population is affected by one or more allergic diseases, only a small number of proteins will act as allergens. Allergens share structural and physicochemical characteristics contributing to their potential allergenicity [34]. However, due to the diversity in physicochemical properties in allergenic proteins, it is challenging to establish general directions for predicting allergenicity based on these properties. Therefore, the focus has been shifted to predict potential allergenicity based on the capacity to induce intrinsic mucosal immune activation, via e.g. protease activity resulting in permeabilization of the intestinal epithelial barrier and activation of these epithelial cells [35], [36]. Allergens, such as Act d 1 (kiwi), Ara h2 (peanut) and Der p 1 (house dust mite, HDM), affect tight junctions and barrier integrity via

protease activity [37]–[40]. This increased permeability facilitates allergen uptake, but concurrent activation of Toll-like receptor 4 (TLR4) has been directly linked to allergen exposure and a subsequent allergic immune response [41], [42]. Furthermore, binding of allergens to epithelial receptors (e.g. C-type lectin receptors, Toll-like receptors and protease-activated-receptors) leads to epithelial activation and secretion of chemokines such as IL8 and CCL20 as well as the alarmins IL33, TSLP and IL25. These mediators are known to drive subsequent a Th2 polarization via priming of type 2 instructing DCs to migrate to local lymph nodes and interact with naïve T cells [43]–[48].

Proteins such as hen's egg derived ovalbumin (OVA) and tropomyosins (TM) are common allergens and are therefore expected to trigger an intestinal mucosal immune response. In this thesis it was aimed to develop an *in vitro* model mimicking key components of the intestinal mucosa with the capacity to distinguish between low and high allergenic proteins. Therefore, in **Chapter 4**, we compared a low and high allergenic tropomyosin in several *in vitro* models of the intestinal barrier and mucosa. The allergenic TM Pen m 1 from black tiger shrimp (shrimp TM) decreased intestinal epithelial resistance, while promoting the release of chemokines and alarmins involved in allergic sensitization. OVA was found to induce a release of chemokines and alarmins by epithelial cells also, but had no effect on barrier resistance. Furthermore, epithelial release of type 2 driving alarmins was increased after shrimp TM exposure. Tropomyosins are an interesting group of proteins, as the invertebrate variants are highly allergenic, while their vertebrate homologous are considered non-allergenic despite a high degree of structural similarity [49]. This is in line with our findings comparing shrimp TM to a chicken tropomyosin (chicken TM), as shrimp TM exposure resulted in a significant epithelial and subsequent immune activation, which was not observed for the chicken TM, indicating that structural homology indeed does not provide an explanation of allergenic properties. The mechanisms underlying this differential immune activating capacity has not yet been studied in detail before, but would provide important insights in understanding potential factors determining the allergenic capacity of proteins. The *in vitro* models presented in this thesis can be used to promote the understanding of the sensitizing allergenicity of proteins.

Epithelial cells as key players in allergic sensitization

Epithelial cells are the first cells, and thus first line of defense, to come into contact with potential allergens and are therefore seen as initiators and directors of a subsequent immune response. In **Chapter 5**, dendritic cells (DCs) were, either directly or indirectly via intestinal epithelial cells (IECs), exposed to OVA to identify the role of IECs in allergic inflammation. The presence of IECs upon coculture with DCs was sufficient to prevent the subsequent Th2 response (in DC-T cell cultures) or enhanced mast cell degranulation (upon T-B cell supernatant exposure). Yet, direct exposure of OVA to DCs resulted in a Th2 dominant response and increased mast cell degranulation after coculture. These findings substantiate the relevance of IECs in immune homeostasis [50]. On the other hand, exposing IEC to OVA did promote activation of IEC as demonstrated by enhanced release of chemokines and alarmins. However, when OVA-primed IEC (24h pre-exposure) were cocultured with DCs after the OVA was washed away, the DCs cocultured with these OVA-IEC directed a T cell activation towards Th2 polarization as described

in **Chapter 6**. Even though immune activation was observed, DCs did not mature based on the expression of costimulatory markers. Yet, the secretion of Th1 driving cytokines was decreased, potentially resulting in a Th2 dominant response. In order to trigger maturation of DCs properly to allow polarization of naïve T cells, uptake of an antigen by the DC is a prerequisite [51]. The *in vitro* model used in **Chapter 6** does not allow the DCs to come into direct contact with and internalize the OVA. Therefore, incomplete DC maturation can be expected, resulting in weak T cell stimulation [51]. These data indicate that the crosstalk between IEC and DCs promotes a homeostatic response to OVA, while exposing these cells separately to OVA appears to promote a type 2 response.

In addition to the development of an *in vitro* intestinal mucosal immune model, a similar approach was used to develop an *in vitro* coculture based model for the airways. Allergic asthma is a growing health problem, affecting ~10% of children. A significant percentage of the asthma allergic patients are sensitized to HDM [47], [52]. To advance the understanding of the crosstalk between cells involved in asthma allergic sensitization, we aimed in **Chapter 8** to establish an *in vitro* model to study the bronchial epithelial response to aero allergens, using HDM as model allergen, and subsequent mucosal immune response via cocultures with monocyte-derived DCs and naïve T cells. Interestingly, we demonstrate that the presence of the bronchial epithelial cells (BECs) is required to induce a subsequent Th2 shifted immune response upon HDM exposure. Even though HDM allergens are known to bind receptors on both epithelial and DCs [34], immune activation involved interaction with BECs as a less pronounced response to HDM was observed when DCs were directly exposed. These differences in allergen induced immune activation promoted or inhibited by epithelial cells could be a result of variation in epithelial function at distinctive sites of the human body. These variations should be confirmed by using primary cells from these epithelial sites, or differences in allergen induced immune activating mechanism to gain further insight in the location specific mechanisms of allergic sensitization.

DCs appear to use separate processing mechanisms when exposed to different allergens [53]. When human plasmacytoid DCs were exposed to OVA or Der p 1 (from HDM), a higher percentage of cells internalized OVA, compared to Der p 1 exposure [53]. Both the mannose receptor and DC-SIGN are involved in the uptake of Der p 1 and OVA by DCs [54]–[56]. However, this receptor mediated endocytosis pathway generally leads to cross-presentation via MHC class I, while uptake of OVA via micropinocytosis (non-specific bulk endocytosis) leads to presentation of the allergen via MHC class II [57]. The underlying mechanisms involved in processing of different allergens by DCs and subsequent T cell activation is poorly understood and needs further investigations.

Development of advanced *in vitro* human mucosal immune models

To further investigate the development of allergic diseases and crosstalk between different immune cells involved in this complex disease, the improvement of advanced *in vitro* models is essential. Steps can be taken into account to represent the individual immune cells and interactions as present the *in vivo* situation. In the future these advanced human *in vitro* models can improve the translational value compared to e.g. existing assays and preclinical animal models.

Although animal models, especially murine models, are mostly used to study allergic diseases, these models do not completely mimic the human pathophysiology. Yet, the use of preclinical models has contributed significantly to gain understanding in the sensitization and effector phase of allergic inflammation [58], [59]. Many murine models have been developed to study food allergic diseases, however the application of these models is based on the use of well-known allergens and are not directly suitable to study the allergenicity of novel food proteins [60]. The induction of food allergic sensitization to purified proteins often relies on the use of adjuvants, since otherwise oral tolerance would arise for the food proteins. In addition, the route of sensitization can vary from intraperitoneal and intradermal injection to intragastric administration [59], while both the use of adjuvants and the administration route may affect the subsequent sensitization status. Similar limitations are found in the use of murine models for allergic asthma studies. Initial models were developed based on the use of OVA as allergen via intraperitoneal injections, which has limited clinical relevance for human asthma. Furthermore, modelling chronic asthma in mice was found to be challenging due to anatomical differences as compared to human lungs [61]. Despite the limitations in the use of animal models, they have provided an enormous improvement in our understanding of allergic diseases. Yet, in light of the current trends towards minimizing the use of experimental animals and improving translational value for human purposes, advanced *in vitro* models may complement and reduce the use of preclinical models.

In **Chapter 5** we aimed to develop a first of its kind human *in vitro* sequential coculture model, mimicking all relevant key events involved in food allergic sensitization resulting in effector cell degranulation. This model combines the crosstalk between all major cells involved in both phases of an allergic immune response. Similarly, in **Chapter 8**, an *in vitro* model combining the crosstalk between BECs, DCs and subsequently naïve T cells has been developed to represent the initial steps in airway allergic sensitization and inflammation. These types of models can be valuable tools to study the potential allergenicity of novel foods or airborne particles as well as interactions of the allergens and other environmental triggers. The use of human cells increases the potential translational value when investigating human allergic diseases. However, the use of these models is also subject to several limitations. The epithelial cells used in these models are cancer cell lines and therefore do not fully mimic the function of healthy primary epithelial cells, even though they display similar characteristics [62], [63]. Making use of primary epithelial cells would be the next step in refining these models. Further development of such models can help e.g. identifying the risks of food allergy development against novel proteins as part of safety testing prior to entering the market. In addition, such models can help to further understand why some proteins have the intrinsic capacity to induce allergic sensitization or which environmental pollutants may trigger potential sensitization. Further improvement of these models should involve validation of the selectivity of sensitizing capacity by investigating the responses to multiple low and high allergenic proteins, as model development was now performed using OVA and selectivity only tested using a low and high allergenic tropomyosin. Furthermore, as the currently presented models involve antigen-independent responses, focusing on allergen specific responses by using cells derived from allergic patients and methods to connect different mucosal sites, to study e.g. epicutaneous sensitization to food and aero allergens and effector responses in the intestines or airways are to be explored in future studies. Novel technologies

aimed at development of organs on a chip models and linking different type of organs on a chip may further promote the development of improved predictive assays for testing of allergenicity of novel food proteins [64].

HMOS structure-function relationship in the allergic setting

The rise in prevalence of allergic diseases, especially in western communities, is a major public health concern. Current treatments do not provide curative options, but rather offer an acute relieve of symptoms. Therefore, preventive strategies to avoid the development of allergic diseases are of importance and need research priority. Breastfeeding is the gold standard in early life nutrition, packed with numerous bio-active nutrients, promoting adequate growth and development of the infant including its immune system. This development includes supporting microbial colonization and maturation of the immune system. Human milk oligosaccharides (HMOS) account for up to 15-20g/L of the milk composition [65] and these abundantly present structures are highly variable [66]. Unique for the HMOS, compared to other mammalian oligosaccharides, are the copious functional groups present. These functional groups consist of terminal fucose and sialic acid groups, decorating the core oligosaccharide structure. These functional groups are thought to play an important role in the immunomodulatory effects of HMOS (described in **Chapter 2**) as these fucose and sialic acid groups interact with specific receptors on e.g. immune cells and epithelial cells conveying their effects.

In **Chapter 3**, five simple HMOS structures have been studied to demonstrate the differential immunomodulatory effects of 2'FL, 3FL, 3'SL, 6'SL and LNnT. The most potent immunomodulatory effects in three different *in vitro* models for the intestinal epithelium and their cross talk with immune cells were observed for both 2'FL and 3FL. Although comparable immunomodulatory effects by exposure to 2'FL and 3FL could be hypothesized due to their structural similarity, we demonstrated that they exert different functions in an *in vitro* model for the intestinal mucosa both in resting and activated status. The effects induced by the presence of HMOS were partially mediated by the presence of IEC, resulting in altered cytokine secretion and a difference in the functional capacity of underlying DC to drive Th2 and Treg formation differed between 2'FL or 3FL exposure. IEC are also known to be involved in mediating the immunomodulatory effects of other oligosaccharides such as short chain galacto-oligosaccharides (scGOS)/long-chain fructo-oligosaccharides (lcFOS) as described in earlier studies [67], substantiating a key role for IEC in conveying immune balancing effects of non-digestible oligosaccharides. Previously, the direct immunomodulatory effects of 3'SL and 6'SL were questioned [68], [69], despite their effects on intestinal epithelial maturation [70]. In **Chapter 3**, indeed we observed minimal effects of exposure to 3'SL, 6'SL as well as LNnT on cytokine secretion and barrier function in the used *in vitro* models. However, these data do not exclude other potential indirect mechanisms via which specific HMOS can interact with the immune system including other types of immune cells. A key feature of HMOS is the known prebiotic capacity. By promoting the growth of beneficial bacteria and enhanced production of their metabolites like short chain fatty acids (SCFAs), additional immune developmental benefits can be promoted by specific HMOS structures within an *in vivo* situation.

Studies into the function of HMOS in relation to infant's development are relatively new and due to the large number of HMOS structures and high levels in human milk, it is challenging to extract specific structure function relationships from large cohort studies. However, some clinical studies have been performed and described interesting findings already. Chen et al. observed that the breastfed children developing eczema and/or food allergy at 24 months old, received milk with higher concentrations of 2'FL at one month old compared to infants that did not develop eczema and/or food allergy [71]. However, Siziba *et al*. described that higher levels of LNnT or 3'SL in human milk are associated with eczema at one year of age [72]. The Melbourne Atopy Cohort Study investigated the long term effects of HMOS profile consumption via breastmilk and allergy development until the age of 18 years [73]. In this study, consumption of milk high in sialylated HMOS reduced the risk of food sensitization at 12 years. While, similar to the study by Chen *et al*. [71], a positive association was described between 2'FL levels and eczema at 24 months. Interestingly, despite the increased risk of eczema at early age upon high 2'FL consumption, the infants who had a high hereditary risk to develop allergic diseases, were born via cesarian section and consumed human milk containing high levels of 2'FL were protected against eczema at 24 months [74]. These contradicting findings demonstrate the need to gain further understanding in the interaction between specific HMOS and immune development.

As large-scale commercial production of simple HMOS structures has become available, clinical trials with the focus on safety and growth have been and will be performed to study the effects of adding specific HMOS to infant formula. Even though these studies are not powered to support a solid functional benefit, interesting associations to health can be suggested. Addition of 2'FL to infant formula shifts the infant's intestinal microbiome to a composition resembling more the microbiome of breastfed infants [75]–[78]. This shift in microbiome was also associated with higher concentrations of fecal SCFAs [79]. Furthermore, infants receiving 2'FL, in combination with GOS, containing formula had lower concentrations of proinflammatory cytokines, such as TNFα and IFNγ, present in their plasma [75]. Addition of a mixture of five HMOS (2'FL, DFL, LNT, 3'SL and 6'SL) to infant formula resulted in increased fecal IgA levels in infants receiving the HMOS supplemented formula [80]. A small group of infants receiving a 2'FL and LNnT supplemented formula were reported to suffer less frequently from upper and lower respiratory tract infections compared to infants receiving standard formula [81], [82]. With regards to the development of allergic diseases, allergic symptoms were significantly reduced in cow's milk allergic infants receiving hydrolyzed formula containing 2'FL and LNnT compared to infants receiving formula without these HMOS [82]. Although these studies are preliminary, and only limited studies have investigated the potential prevention of allergic diseases by addition of specific HMOS to infant formula, the currently available data indicate that addition of specific HMOS to infant formula may to some extent contribute to the development of adequate local and systemic immune function [83]. Although studies using scGOS/lcFOS supplemented infant formula have reported similar beneficial effects for immune development [84], [85], supplementation of specific HMOS may provide further developmental benefits. Additional properly powered, studies with dedicated primary end-points are required to elucidate the exact contributions of specific HMOS in neonatal immune maturation and development of allergic diseases and to understand the conflicting data as presented earlier.

The *in vitro* studies in **Chapter 6** were performed to investigate the specific immunomodulatory

effects of 2'FL and 3FL in OVA-induced allergic inflammation. Epithelial 2'FL exposure boosted general T cell cytokine production during OVA inflammation, while 3FL prevented Th2 type cytokine release. Indicating that 3FL may be more capable of steering away from a type 2 skewed immune response than 2'FL, which promotes inflammatory and regulatory T cell cytokine release in general. As some ingested HMOS structures are fermented by the present microbial community into SCFAs, **Chapter 7** describes the potential interaction between 2'FL and 3FL and the most potent immunomodulatory SCFA, butyrate [86], *in vitro*. While butyrate alone had no effect in the used *in vitro* setting, immunomodulation by 2'FL and 3FL was altered in presence of butyrate. In OVA-induced inflammation, butyrate alone had no effects on the type 2 inflammatory response, while 2'FL and 3FL modified OVA-induced type 2 inflammation independent of the presence of butyrate. These findings indicate that butyrate may only have minimal effects on mucosal immunity, which was previously described by others [87]–[89], yet may support immunomodulatory effects of HMOS.

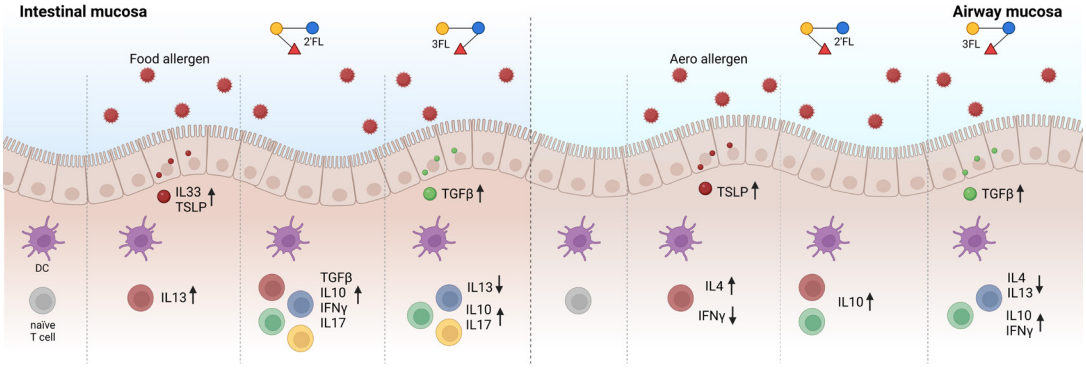


Figure 1. *Overview of in vitro mucosal immune models mimicking allergen exposure and impact of 2'FL and 3FL in the intestine and airways. Created with BioRender.com*

In addition to investigating the effects of HMOS on the intestinal epithelium and in a food allergy in *in vitro* and *in vivo* settings, HMOS were in parallel applied in models for the airway epithelium and in allergic asthma *in vitro* and *in vivo* studies. Similar immunomodulatory patterns of 2'FL and 3FL were observed in an *in vitro* airway mucosal model in **Chapter 8**. Both 2'FL and 3FL prevented HDM-induced proinflammatory IL8 and TSLP release from bronchial epithelial cells (BEC) and moDCs, while specifically 3FL promoted a regulatory response by restoring TGFβ secretion as well. This resulted in minimal modulation of the HDM-induced Th2 skewing by 2'FL, whereas 3FL preincubation restored the type 1 and type 2 T cell responses. In HDM sensitized and challenged mice, both dietary 2'FL and 3FL reduced IgE serum levels, however these mice were not protected from airway inflammation. Previously, also no clear link between HMOS profile and the development of airway sensitization was observed in children up to 18 years old [73], although some associations between children receiving milk higher in 3FL and an increased risk of allergic asthma development was described. However, the exact relation between HMOS profile and allergic asthma are poorly understood. Also the underlying

mechanisms are not yet understood, yet these findings provide a basis to further investigate the differential immunomodulatory effects of specific fucosylated HMOS in the development of allergic asthma.

Less data is available describing the immunomodulatory effects of sialylated HMOS on allergy development. *In vitro* and/or preclinical studies demonstrated that the most abundant sialylated HMOS, 3'SL and 6'SL, promote differentiation of IECs, decrease the incidence and severity of diarrhea, and bind to GPR35 and TLR4 present on e.g. epithelial cells and DCs [90]–[94]. Furthermore, fermentation of sialylated HMOS by beneficial bacteria into SCFA, is linked to improved allergy outcomes in mice and children [95], [96]. In **Chapter 9**, dietary 3'SL and 6'SL were found to beneficially affect the development of HDM-induced allergic asthma in mice. Especially 3'SL was found to improve both clinical and immunological parameters, whereas 6'SL lowered airway hyperresponsiveness but not the allergic inflammation. 3'SL prevented both sensitization and local mast cell activation, while despite structural similarity, this was not observed for dietary 6'SL. HDM-induced allergic asthma can be prevented in mice via exposure to the TLR4 ligand LPS [97]. 3'SL exerts immunomodulatory effects via interaction with TLR4 on DCs, which was not observed for 6'SL [98]. Altogether, it can therefore be hypothesized that the preventive effects against HDM-induced allergic asthma that were observed, may occur via interaction with TLR4 expressing cells, such as BECs, immature DCs and macrophages [99]. Alternatively, sialylated HMOS are known to interact with several Siglec receptors, which have been associated with allergic asthma immune responses [100], [101]. The exact contributions of these interactions in preventing the development of allergic asthma should be further studied, to elucidate the underlying mechanisms involved in the allergy preventive effects.

Conclusions

This thesis describes the development of advanced *in vitro* human mucosal immune models to improve our understanding of allergic sensitization upon allergen exposure and predict sensitizing allergenicity risk. So far, we have demonstrated that these models allow for differentiation between high and low allergenic tropomyosins. However, these types of models also provide a platform to study the interaction between allergen exposure and environmental factors. In this thesis, these models were used to reveal the ability of specific fucosylated and sialylated HMOS to affect crosstalk between epithelial and immune cells during allergic inflammation in intestinal and airway mucosal settings.

The capacity of 2'FL and 3FL in modulating innate and adaptive responses *in vitro* and in preclinical models to potentially prevent allergic inflammation is described. Both 2'FL and 3FL were found to differentially modulate cytokine secretion by boosting general cytokine secretion or promoting Th1 and regulatory cytokines respectively *in vitro*. These findings are supported by preclinical studies, demonstrating significant immunomodulatory effects from dietary 2'FL or 3FL in partial prevention of food allergy or allergic asthma in mice. Furthermore, initial studies were performed to investigate the immunomodulatory effects of the sialylated HMOS 3'SL or 6'SL administered via the diet in the prevention of allergic asthma in a murine model. In this model 3'SL showed effective mitigation of both airway inflammation as well as protection against airway hyperresponsiveness.

In conclusion, this thesis provides advanced tools and insights into our understanding of allergic sensitization upon allergen exposure aiming to predict sensitizing allergenicity risk also when different environmental factors are included. Furthermore, we demonstrated that the structurally similar HMOS 2'FL and 3FL as well as 3'SL and 6'SL differentially support mucosal immune responses during allergic inflammation. Future studies should clarify the underlying mechanisms via which these and other HMOS exert their immunomodulatory effects, as this would provide a basis to further develop supportive strategies for neonatal immune maturation in order to reduce allergy risk in infants that cannot be breast fed. This will also promote understanding the unique immunomodulatory effects of HMOS in breastfed babies.

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Addendum

Nederlandse samenvatting

Het immuunsysteem van een pasgeborene is kwetsbaar voor infecties en het ontwikkelen van allergie (sensibilisatie), dit komt doordat het immuunsysteem nog moet uitrijpen na de geboorte. Deze periode van ontwikkeling is cruciaal en verstoringen kunnen leiden tot een suboptimale werking van het immuunsysteem later in het leven. Ondersteuning van een optimale ontwikkeling van het immuunsysteem, zoals via dieetinterventies, kan bijdragen aan het voorkomen van onder andere allergische aandoeningen.

Uit onderzoek is gebleken dat moedermelk het risico op het ontwikkelen van een allergie bij een pasgeborene kan verkleinen. Moedermelk wordt ook beschouwd als de meest optimale voeding voor een pasgeborene, aangezien het naast belangrijke voedingsstoffen om te groeien ook veel biologisch actieve componenten bevat die een gezonde ontwikkeling van het maagdarmkanaal, de hersenen en het immuunsysteem bevorderd. Dit proefschrift tracht de effecten van een specifiek bestandsdeel van moedermelk, namelijk de humane melk oligosachariden, op de ontwikkeling van het immuunsysteem te bestuderen in de context van allergie ontwikkeling in de darm en longen. Voor dit onderzoek zijn nieuwe *in vitro* modellen opgezet die gebruik maken van humane epitheelcellen afkomstig uit de darm of longen, samen met cellen van het aangeboren en adaptieve immuunsysteem om immuun activatie tijdens blootstelling aan een allergeen te bestuderen. Deze modellen zijn toegepast om te begrijpen op welke manier biologisch actieve componenten, zoals de humane melk oligosachariden, de uitrijping van het immuunsysteem kunnen bevorderen en daarbij ondersteuning kunnen bieden aan een optimale ontwikkeling tijdens het vroege leven.

Direct na de geboorte is het immuunsysteem van een pasgeborene gevoeliger voor het ontwikkelen van een allergie als overblijfsel van de unieke situatie tijdens de zwangerschap. Het is in deze periode van cruciaal belang dat het immuunsysteem leert om wel een afweerreactie op te bouwen tegenschadelijke virussen en bacteriën, terwijl onschadelijke eiwitten uit voeding geen immuun activatie moet opwekken (orale tolerantie). Bij sommige voedingseiwitten is er een verhoogd risico op sensibilisatie. Dit zijn allergenen die bijvoorbeeld voorkomen in melk, pinda of ei. Wanneer de blootstelling aan een allergeen bij een pasgeborene niet adequaat verloopt, bijvoorbeeld door een eerste contact via beschadigde huid of als orale tolerantie inductie in de darm niet goed gaat, kan er een allergeen-specifieke Th2 gemedieerde reactie van het immuunsysteem ontstaan. Tijdens deze reactie worden er ook B cellen geactiveerd die allergeen-specifieke IgE produceren. Bij een volgende blootstelling aan het allergeen kan deze binden aan allergeen-specifieke IgE op effector cellen, deze degranuleren en daardoor ontstaan er acute allergische symptomen, die mogelijk levensbedreigend kunnen zijn.

In potentie kunnen alle eiwitten een allergische sensibilisatie uitlokken. Echter is er slechts een kleine groep eiwitten die verantwoordelijk is voor het merendeel van de allergische reacties. Ondanks de structurele en chemische eigenschappen die gelijk kunnen zijn tussen de verschillende allergenen, is het voorspellen van potentiële capaciteit om allergische sensibilisatie te induceren lastig op basis van deze eigenschappen. Om dit te kunnen bestuderen zijn modellen nodig die onderscheid kunnen maken tussen eiwitten die wel of geen verhoogde capaciteit hebben op het induceren van allergische sensibilisatie. In dit proefschrift is daarom een *in vitro* model opgezet voor de mucosa van de darm. Dit model is gebruikt om de capaciteit van een eiwit om bepaalde cellen van het mucosale immuunsysteem (epitheelcellen, antigen presenterende

cellen en adaptieve immuuncellen) te activeren en te bestuderen. Allergenen zoals ovalbumine uit kippenei of tropomyosines uit garnaal kunnen het mucosale immuunsysteem activeren, door bijvoorbeeld het verstoren van de darmepitheel barrière. Ook kunnen ze specifieke receptoren op epitheelcellen binden en deze cellen activeren. Een soortgelijk tropomyosine uit kip, dat qua structuur veel overeenkomsten heeft met het tropomyosine uit garnaal, is echter veel minder in staat om het mucosale immuunsysteem te activeren. In **Hoofdstuk 4** hebben wij de effecten van deze laag en hoog allergene eiwitten bestudeerd in modellen van de darmepitheel barrière. In het model van het mucosale immuunsysteem in de darm, dat door ons is opgezet, kan de potentiële capaciteit van een voedingseiwit om het mucosale immuunsysteem te activeren opgepikt worden. Zo hebben we gevonden dat tropomyosine uit garnaal zowel de darmepitheelcellen als antigen presenterende cellen kan activeren, terwijl tropomyosine uit de kip dit veel minder doet. Deze type modellen kunnen in de toekomst bijdragen aan onderzoek naar de immunologische mechanismen die betrokken zijn bij allergische sensibilisatie.

Epitheelcellen zijn de eerste cellen in het lichaam die in contact komen met allergenen. Zij spelen daardoor een belangrijke rol in de aard van de immuunreactie die daarop volgt. In **Hoofdstuk 5** hebben wij de rol van darmepitheelcellen tijdens ovalbumine blootstelling bestudeerd in een model dat de communicatie tussen dendritische cellen (antigeen presenterende cellen), T cellen, B cellen en mestcellen (effector cel) nabootst. De aanwezigheid van darmepitheelcellen bleek voldoende om een Th2 reactie en daaropvolgende mestceldegranulatie te verminderen. Deze bevindingen dragen bij aan de hypothese dat epitheelcellen betrokken zijn bij het bewaren van immuun homeostase. In **Hoofdstuk 6**, hebben wij de effecten van ovalbumine blootstelling op epitheel bestudeerd. Deze blootstelling resulteerde in verhoogde secretie van cytokines die Th2 reacties bevorderen. Tijdens daaropvolgende coculturen van ovalbumine blootgesteld epitheel met dendritische cellen en naïeve T cellen, observeerden wij slechts zwakke activatie van deze immuuncellen. Activatie van dendritische cellen en daaropvolgende stimulatie van T cellen is afhankelijk van de opname van een eiwit door de dendritische cel. In deze experimentele opzet is het onwaarschijnlijk dat de dendritische cellen in contact zijn gekomen met ovalbumine, waardoor een zwakkere T cell stimulatie naar verwachting is.

Naast de mucosale immuun modellen die wij hebben ontwikkeld voor de darm, is er een vergelijkbare benadering gekozen voor het opzetten van mucosale immuun modellen relevant voor de longen. Ongeveer 10% van de kinderen lijdt aan allergische astma, bij het merendeel van deze patiënten wordt de allergische reactie getriggerd door blootstelling aan huisstofmijt. **Hoofdstuk 8** beschrijft de ontwikkeling van een mucosaal immuun model waarbij gebruik is gemaakt van huisstofmijt blootstelling om de reactie van bronchiale epitheelcellen en daaropvolgende reacties van dendritische cellen en T cellen te bestuderen. De aanwezigheid van bronchiale epitheelcellen bleek noodzakelijk om een Th2 dominante reactie tegen huisstofmijt op te wekken, terwijl directe blootstelling van dendritische cellen een minder sterke reactie veroorzaakte. Deze verschillen in allergeen geïnduceerde immuun activatie die worden versterkt of geremd door de aanwezigheid van epitheelcellen, worden mogelijk veroorzaakt door de verschillende functies die epitheelcellen in separate delen van het lichaam uitoefenen of de unieke eigenschappen van de bestudeerde allergenen.

De hierboven beschreven ontwikkeling van complexe modellen van het mucosale immuunsysteem in de darm en longen draagt bij aan het verder bestuderen van de immunologische

mechanismes die betrokken zijn bij allergische ziektes en het bestuderen van effecten van mogelijke interventies. Ze kunnen ook bijdragen aan het verminderen van het gebruik van diermodellen voor het bestuderen van allergische aandoeningen. Echter is verdere ontwikkeling van de modellen beschreven in dit proefschrift noodzakelijk om de translationele waarde naar de humane situatie te kunnen bepalen en verbeteren. Dit zou bijvoorbeeld gedaan kunnen worden door gebruik te maken van primaire epitheelcellen in plaats van cellijnen met een carcinogene achtergrond. Bovendien kunnen de beschreven modellen verbeterd worden door gebruik te maken van cellen afkomstig van allergische patiënten om ook allergeen-specifieke reacties te kunnen bestuderen, aangezien de huidige modellen op basis van allergeen-onafhankelijke communicatie tussen de immuuncellen functioneren. Nieuwere technieken die zicht richten op orgaan-op-een-chip modellen kunnen de ontwikkeling van voorspellende testen die de potentiële capaciteit van een eiwit om allergische sensibilisatie te induceren in de toekomst verder bevorderen.

Aangezien curatieve behandelingen van allergische ziektes niet of beperkt beschikbaar zijn, worden preventieve strategieën bestudeerd. Moedermelk, als optimale voeding voor een pasgeborene, draagt bij aan een adequate ontwikkeling van het immuunsysteem. De humane melk oligosachariden zijn een belangrijk bestandsdeel van moedermelk en kunnen een interactie aan gaan met cellen van het immuunsysteem, dit is beschreven in **Hoofdstuk 2**.

De immuunmodulerende effecten van vijf losse humane melk oligosacharide structuren zijn door ons bestudeerd in drie verschillende modellen van het darmepitheel en onderliggende immuuncellen in **Hoofdstuk 3**. De meest potente immuunmodulerende effecten hebben wij geobserveerd voor 2'FL en 3FL. Ondanks de grote overeenkomsten in hun structuur, beïnvloeden ze type 2 en regulatoire cytokine secretie van immuuncellen op verschillende manieren, dit bleek gedeeltelijk afhankelijk van de aanwezigheid van epitheelcellen. In de gebruikte modellen hebben wij slechts minimale immuunmodulerende effecten van 3'SL, 6'SL en LNnT gevonden. Echter dit sluit mogelijke positieve effecten op andere immuuncellen of de ontwikkeling van een pasgeborene via bijvoorbeeld beïnvloeding van het microbioom in de darmen niet uit.

In **Hoofdstuk 6**, zijn de immuunmodulerende effecten van 2'FL en 3FL op ovalbumine geïnduceerde allergische inflammatie onderzocht. Blootstelling van darmepitheel aan 2'FL resulteerde in algemene versterking van cytokine uitscheiding in T cellen, terwijl 3FL blootstelling de Th2-type IL13 uitscheiding verminderde. Aangezien humane melk oligosachariden in de darm gefermenteerd kunnen worden tot korteketenvetzuren door het aanwezige microbioom, hebben wij de potentiële interactie op het immuunmodulerende effect door 2'FL of 3FL met een belangrijk korteketenvetzuur, butyraat, bestudeerd in **Hoofdstuk 7**. Blootstelling aan butyraat had geen effecten op de type 2 inflammatoire reactie, daarentegen werden de immuunmodulerende effecten van 2'FL en 3FL beïnvloed door de aanwezigheid van butyraat. Deze resultaten laten zien dat butyraat mogelijk de werking van humane melk oligosachariden kan ondersteunen.

Als aanvulling op de effecten van 2'FL en 3FL in de darm, zijn deze componenten in **Hoofdstuk 8** bestudeerd in mucosale immuun modellen met luchtwegepitheel en allergische astma. Zowel 2'FL als 3FL waren in staat om de secretie van proinflammatoire IL8 and TSLP veroorzaakt door blootstelling van het epitheel aan huisstofmijt te voorkomen. Bovendien, 3FL stimuleerde de uitscheiding van TGFκ, een cytokine dat betrokken is bij het behoud van homeostase. Tijdens daaropvolgende coculturen ging eerdere blootstelling van de luchtwegepitheelcellen en dendritische cellen aan 3FL in aanwezigheid van huisstofmijt, de activatie van een Th1 en Th2

reactie tegen. In een muizenmodel van acute allergische astma, was de IgE concentratie in serum verlaagd maar luchtweginflammatie onverminderd bij verrijking van de diëten met 2'FL of 3FL. Wel zorgde met name 3FL voor een verminderde productie van type 1 en type 2 cytokines na blootstelling aan huisstofmijt.

Er is minder bekend over de immuunmodulerende effecten van gesialyleerde humane melk oligosacharides zoals 3'SL en 6'SL. Daarom hebben wij in **Hoofdstuk 9** de effecten van deze structuren via het dieet ook bestudeerd in een muizenmodel van acute allergische astma. 3'SL verbeterde zowel klinische als immunologische parameters, terwijl 6'SL de luchtweghyperreactiviteit verminderde zonder effect te hebben op allergische inflammatie. Verder bleek dieetinterventie met 3'SL sensibilisatie en lokale mestcelactivatie te verminderen, deze effecten zijn niet gevonden voor 6'SL.

Tot slot zijn in **Hoofdstuk 10** alle bevindingen en conclusies van dit proefschrift beschreven die nieuwe inzichten verschaffen in het begrip van allergische sensibilisatie, met als doel methodes te ontwikkelen die allergische sensibilisatie kunnen voorspellen. Bovendien draagt dit proefschrift bij aan de ontwikkeling van dieetstrategieën op basis van specifieke humane melk oligosachariden ter ondersteuning van de uitrijping van het immuunsysteem van een pasgeborene die mogelijk kunnen leiden tot een lager risico op het ontwikkelen van allergie.

List of publications

1. **Zuurveld, M.**, Van Witzenburg, N. P., Garssen, J., Folkerts, G., Stahl, B., van't Land, B., & Willemsen, L. E. M. (2020). Immunomodulation by human milk oligosaccharides: the potential role in prevention of allergic diseases. *Frontiers in Immunology, 11*, 801.
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About the author

Marit Zuurveld was born on February 26th 1995 in Deventer, the Netherlands. After graduating from high school (Gymnasium, S.G. de Waerdenborch) in 2013, she studied Biology at Wageningen University and Research. After completing the bachelor program with a thesis on immunomodulation of allergic diseases by food, she continued with a master in Biology specializing in health and disease biology. During her masters she performed an internship at Intravacc, Bilthoven focusing on a method for marker free genetic modifications in *Neisseria meningitidis*. In 2017, she moved to Stockholm, Sweden to join the group of Prof. dr. Eva Sverremark Ekström for her final graduation project under the supervision of Dr. Marieke van der Heijden. In this project she studied the differences in T helper cell polarization profiles in children and adults.

In 2019, Marit started as a PhD candidate in the division of Pharmacology of the Utrecht Institute for Pharmaceutical Sciences at Utrecht University. This PhD project was part of the strategic alliance between the division of Pharmacology and Danone Nutricia Research B.V. under the supervision of Prof. dr. Johan Garssen, Prof. dr. Gert Folkerts, Dr. Linette E.M. Willemsen and Dr. Belinda van't Land. Her PhD project focused on elucidating the role of human milk oligosaccharides in the context of preventing allergic sensitization. The results of this work are described and discussed in this thesis.

Marit presented her research during several (inter)national conferences and received a travel grant to attend the EAACI Allergy Prevention School in Cork, Ireland in 2022. She was invited to design the cover image of the international journal *Biomolecules,* Volume 13, Issue 2. Besides her research activities, she was involved as a teacher in the bachelor program Pharmacy at Utrecht University, supervised numerous bachelor and master students and obtained her University Teaching Qualification in 2023. As a member of the Future Medicine Fellows at Utrecht University, she organized a summer school on Future Medicines: Gene and Cell Therapies in 2020. Since September 2023, Marit is appointed as a postdoc at the division of Pharmacology at Utrecht University in a collaborative project with Nutricia Danone Research B.V. on further development of an advanced *in vitro* model to study food allergic sensitization.

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Are you still here…? Fine, stay… Now try to find your name somewhere. BUT! Assuming that I will be forgetting to mention many people, even if your name is not mentioned here, please know that I'm eternally grateful for your incredible support.

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This summer we found out that we met over 10 years ago, while we were starting our studies in Wageningen, and it has been such a great journey with you since then. Although we always made our own and often quite different choices, we ended up in very similar spaces. Dear **Marion,** thank you for all the great times we had in Wageningen and here in Utrecht. Let's keep this up for at least another 10 years.

Dan natuurlijk nog de mensen thuis. Lieve **pa** en **ma**, haha daar staat ze dan. Kleine meisjes worden groot, of nou ja… In lengte is het in ieder geval niet gelukt 😉. Maar mede dankzij jullie ben ik wel op dit punt gekomen. Urenlang kon ik aan de telefoon of als we met Chris (, Zazou en Hayley) wandelden tegen jullie kletsen over alles wat mee of tegen zat op het lab. Eindelijk krijgen jullie nu ook een idee van de mensen met wie ik de afgelopen jaren heb samen mogen werken, waar jullie al zoveel verhalen over hebben moeten aanhoren.

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ADDENDUM

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