Shaping mucosal and systemic immunity by non-digestible oligosaccharides and postbiotics

GROWING ROOTS WITH FOOD

Veronica Ayechu Muruzabal

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Shaping mucosal and systemic immunity by non-digestible oligosaccharides and postbiotics

GROWING ROOTS WITH FOOD

Opbouw van mucosale en systemische immuniteit door niet-verteerbare oligosachariden en postbiotica

(met een samenvatting in het Nederlands)

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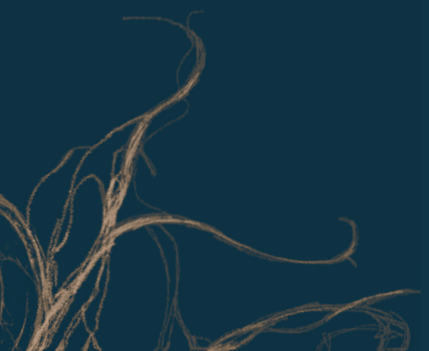
CHAPTER I 1

General introduction



Development of the gastro-intestinal tract and immune system in early life

When infants are born they undergo a rapid physiological adaptation process to prepare them for the hostile extra-uterine environment where many antigenic encounters occur [1, 2]. The intestinal mucosa and the immune system need to adapt rapidly and significantly to accommodate to the extra-uterine environment [1–3]. In the gastrointestinal tract (GIT), a rapid growth and development of the intestines is observed as seen by changes in its structure and function [3–5]. In addition, changes in the expression of specific enzymes and transporters occur [4] as well as the establishment of the gut microbiota [2, 6]. All these changes result in an improved nutrient absorption and development of the intestinal mucosa [4, 5].

Infectious diseases are among the top causes of disease burden in infants aged 0-9 years [7]. At birth, the immune system of the newborn differs in nature to that of the adult, due to its ineffective antigen presentation, less robust response upon TLR ligation and low Th1-polarizing immune capacity [1, 3, 8–10] leaving the infant vulnerable to infections [11]. An impaired response to vaccination is also characteristic of this stage [9] in which infants show a reduced ability for pathogen clearance as their immune system is focused to ensure vital functions such as intestinal development and avoiding tissue damage [12]. An age-dependent maturation of the innate and adaptive immune system occur while the infant encounters diverse antigens in early life; as seen by a gradual maturation of Th1-polarizing cytokine production and maturation of T- and B-cell compartments [1, 8]. The development of Th1 immune polarizing capacity is also of interest to avoid the risk of developing allergies, which are also highly prevalent in early life [13]. Early life exposure to microorganisms and various antigens play a key role in the maturation of the immune system, thereby supporting the protection against infections or allergy [2, 14, 15].

A proper development of the GIT as well as the immune system in early life is crucial in order to promote immune maturation to defend against harmful antigens or invasive organisms, while promoting tolerogenic responses towards non-harmful antigens such as food proteins or commensal bacteria. In particular, nutrition in early life is a factor directly influencing the growth and development of the newborn. Although some protective effects have already been described, knowledge regarding the specific mechanisms of action and interactions between bioactive components in human milk and components in the GIT is missing, as well as how these contribute to immune maturation. In this thesis, we take a closer look into the specific effects of human milk oligosaccharides (HMOS) and postbiotics in the interaction between intestinal epithelial cells (IEC) and immune cells, with the aim to better understand the complex interactions underlying mucosal immune homeostasis.

Intestinal epithelial cells: regulators of immune responses in the intestinal mucosa

IEC are key elements of the GIT contributing to the absorption of nutrients and provide a selective barrier against invading pathogens or harmful antigens [16]. A single layer of polarized IEC constitutes the intestinal epithelial layer containing mainly absorptive enterocytes, but also specialized cells such as Paneth cells, goblet cells or enteroendocrine cells, with relevant functions in the response against threats that reach the lumen, and maintaining intestinal immune homeostasis by secreting mucus or antimicrobial peptides and secretory IgA [16, 17]. The IEC are renewed constantly from stem cells located in the crypts thus, IEC proliferation and differentiation occur frequently to prevent tissue damage and control tissue homeostasis. Furthermore, IEC are implicated in the regulation of innate as well as adaptive immune responses [16].

IEC express specific pattern recognition receptors (PRR) such as Toll-like receptors (TLR) which act as sensors of luminal antigens that recognize possible threats. These receptors recognize antigens and pathogen associated molecular patterns (PAMP) such as bacterial DNA (TLR9 ligand) or viral RNA (TLR3 ligand) among others, making IEC active participants of mucosal immune responses [16]. The activation of such receptors promotes the secretion of specific cytokines and epithelial mediators that signal to surrounding immune cells and IEC to promote appropriate immune responses, emphasizing the unique regulatory role of IEC in supporting tissue homeostasis. In addition to specific receptors, soluble lectins such as galectins are also expressed and secreted by IEC and can act as PRR by binding to glycans in the surface of pathogens, and thus participate in their recognition and the modulation of immune responses downstream [18, 19]. IEC secrete mediators like IL-10, retinoic acid or TGFβ that condition dendritic cells (DC) to instruct differentiation of regulatory T-cells [20, 21] resulting in immunological tolerance. However, these DC can also instruct the development of effector T-cells (Th1, Th2 and/or Th17) in order to fight pathogens. The secretion of epithelial mediators such as galectin-9 was shown to skew the immune response towards Th1- and regulatory-type upon inflammation [22], which contributes to a regulatory Th1 response which may support intestinal homeostasis. In early life, Th1 and regulatory T-cell functions need to be established to skew away from the Th2-type phenotype, known to contribute to the development of allergies.

Organized lymph nodes are found in the intestines contributing to intestinal immune homeostasis. The gut-associated lymphoid tissue (GALT), including the lamina propria underneath IEC, as well as lymphoid follicles within the IEC layer, such as Peyer's patches or mesenteric lymph nodes (MLN), are key to maintain the homeostasis in

response to luminal antigens [20, 21]. Antigens and food proteins that reach the intestinal lumen are taken up by DC present in Peyer's patches and located underneath the intestinal epithelium [20, 21]. After recognition, activated-DC instruct the development of adaptive immune responses in the Peyer's patches or migrate towards MLN where antigens are presented to naïve $CD4^+$ or $CD8^+$ T-cells via MHC-II or -I molecules respectively. This presentation results in activation and development of naïve T-cells into regulatory- or effector T-cells, which migrate back to the lamina propria and promote specific effector immune responses that maintain intestinal homeostasis or promote an inflammatory response against potential harmful antigens [20, 21].

The control of homeostasis in the GIT requires complex interactions between IEC and immune cells which are not fully characterized yet. Besides, the ability of HMOS to be absorbed directly into the bloodstream emphasizes the relevance of understanding the protective roles of HMOS in the crosstalk of IEC and immune cells. Studying what are the mechanisms by which HMOS can modulate the interactions between IEC and immune cells underneath to support mucosal immune homeostasis will help to gain more insights regarding the protective effects of HMOS as well as providing better strategies to support mucosal immune homeostasis.

The role of human milk oligosaccharides in supporting mucosal immune development

The proper maturation of the intestine and immune system are of great relevance to develop appropriate immune responses against antigens encountered in early life, as well as promoting tolerance to harmless antigens and commensal bacteria colonizing the GIT. The development of the GIT and immune system, as well as the microbiome, are dependent on nutritional factors such as breastfeeding, and are known to greatly influence the growth of infants and the development of diseases in adulthood.

Breastfeeding is the preferred nutrition for infants due to its high nutritional value and it is associated with protection against infections [23–26] and disorders of the GIT, as well as reducing the incidence of developing obesity, diabetes or inflammatory bowel disease later in life [27, 28]. The composition of human milk is designed to meet the nutritional requirements of the infant and thereby, progressively changes to support the growth and health status of the newborn. Human milk is also a source of bacteria capable of colonizing the GIT of the newborn and provides IgA, which contributes to protection against pathogen infections [5]. Moreover, bioactive components present in human milk, and in particular HMOS, are known to contribute to the maturation and development of the mucosal immune system of the infants [2, 29]. In addition, the

presence of up to 1% HMOS in circulation [30] suggests that HMOS can promote direct effects on immune cells. Investigating the capacity of HMOS such as 2'-fucosyllactose (2'FL), a highly abundant HMOS in FUT2 secretor mothers [31], or 3'-galactosyllactose (3'GL), highly abundant in colostrum [32], in promoting immunomodulatory effects would be of interest. In particular, the identification of possible mechanisms by which specific HMOS might promote protection in the newborn are needed. The diversity of structures of HMOS and their ability in modifying IEC and immune cell function are further described in **Chapter 2**.

Non-digestible oligosaccharides as a strategy to support mucosal immune maturation

Without doubt, breastfeeding is the most optimal nutrition for newborns. That is why the WHO recommends exclusive breastfeeding for 6 months, which can be extended for up to two years or beyond next to complementary food introduction [33]. However, only 25% of children are exclusively breastfed between 0-6 months in Europe [34]. Due to the low percentage of newborns receiving exclusive breastfeeding and the low amounts of non-digestible oligosaccharides (NDO) present in cow's milk [35, 36] as well as the protective effects attributed to NDO naturally present in human milk [29, 37–39], alternative nutritional interventions to improve infant formulas are being studied. Based on the amount and structure diversity of NDO in human milk, a 9:1 mixture of short-chain galacto- and long-chain fructo-oligosaccharides (GOS/lcFOS), has been extensively studied and is currently used to supplement infant formulas. Multiple structures with degree of polymerization (DP) 2-8 are contained in GOS, which is obtained by elongation of galactose derived from the lactose present in milk, while lcFOS are derived from inulin containing plant sources, like chicory root, and are composed of DP size of 23 or longer [40].

In clinical studies, this 9:1 GOS/lcFOS mixture was shown to promote the growth of Bifidobacteria and Lactobacilli, which are commensal bacteria colonizing the GIT of breastfed infants [41, 42]. Additionally, a reduction in the incidence of atopic dermatitis was seen in infants at 6 months of age, fed a formula supplemented with GOS/lcFOS compared to control formula [43]. Furthermore, a reduction in allergic symptoms with increased galectin-9 secretion was observed upon a dietary intervention with GOS/lcFOS and *Bifidobacterium breve* M-16V, in a mouse model for food allergy [44]. *In vitro*, exposure of IEC and activated immune cells to GOS/lcFOS and DNA from *Bifidobacterium breve* M-16V or synthetic CpG oligodeoxynucleotides (ODN), which are synthetic single stranded DNA molecules containing unmethylated CpG dinucleotides

and known to act as TLR9 agonists, was shown to promote immunomodulation with increased galectin-9 secretion [22].

Benefits of postbiotic supplementation: a new approach

More recently, fermented-milk based formulas have been studied for their bioactive components and beneficial properties in the GIT. During the fermentation of a matrix using food-grade microorganisms, diverse bioactive components are produced including short-chain fatty acids, microbial cell fractions, functional proteins, extracellular proteins or cell lysates among others [45]. Evidence suggests that the ingestion of specific non-viable microbial components and the metabolites that are produced through fermentation can promote health benefits in the host [46, 47]. Thereby, the use of postbiotics to support infant nutrition is being studied. Although the concept of postbiotic is still a matter of debate, the International Scientific Association of Probiotics and Prebiotics (ISAPP) proposed a definition stating that postbiotics are "inanimate microorganisms and/or their components that confer a health benefit to the host" [48].

Fermentation of a milk matrix using *Streptococcus thermophilus* and *Bifidobacterium breve* C50 through a unique fermentation process denominated Lactofidus™, was shown to generate bioactive components naturally present in human milk such as 3'-galactosyllactose (3'GL) [49]. Clinical trials showed that the formula containing fermentation products (FP) from Lactofidus™ was safe to use and well tolerated by newborns and infants [50–54], and was shown to improve GIT and immune parameters such as reducing the severity of acute diarrhea episodes and increase fecal secretory IgA [50, 54]. More insight regarding the specific bioactive components present in FP as well as their specific effects in the GIT and on the immune system is of interest to better understand the beneficial effects of postbiotics.

Aim and outline of this thesis

This thesis aims to investigate the immunomodulatory effects of HMOS, NDO mixtures and specific postbiotics using diverse *in vitro* and *in vivo* models. Understanding specific features of HMOS, NDO and postbiotics that promote the maturation and development of the GIT and the immune system is needed to select appropriate dietary interventions that could be used to reinforce tissue homeostasis and contribute to the establishment of a balanced mucosal immune system. These results will contribute to unravel the mechanisms involved in the regulation of immune responses by HMOS, NDO and postbiotics and to implement strategies aimed to support mucosal immune homeostasis.

Chapter 2 outlines the diversity of HMOS structures found in human milk and the scientific knowledge regarding the diverse immunomodulatory properties of HMOS on intestinal epithelial cells as well as on immune cells, which support gut maturation and immune development in early life. The current knowledge regarding the mechanisms used by HMOS to promote mucosal immune development are described among which antimicrobial, prebiotic effects as well as effects in the immune function stand out.

IEC are constantly discriminating between harmful and harmless antigens and thus play a key regulatory role in supporting immune homeostasis. In **Chapter 3**, an *in vitro* model was established to study the regulatory role of IEC and immune cells in supporting the immune response against a viral trigger. We observed that IEC may support intestinal homeostasis by secreting mediators that regulate local viral defense. Additionally, to better understand the protective capacity of HMOS, the ability of 2'FL in supporting an immune response to a viral trigger was studied.

The main component of the specific NDO mixture designed to mimic the amount and structure diversity of HMOS in human milk are GOS. Over 40 different structures with degree of polymerization (DP) between 2-8 are contained in GOS which have already been characterized [55]. In **Chapter 4** we studied the immunomodulatory effects of CpG in combination with specific DP fractions of GOS using an *in vitro* co-culture model combining IEC and peripheral blood mononuclear cells (PBMC). A specific DP3-sized structure present in GOS and naturally found in human milk; β-3'GL was shown to support the CpG induced immunomodulatory effects.

To gain more insights regarding the capacity of HMOS such as 2'FL in supporting mucosal immune regulation, in **Chapter 5** we studied the specific immunomodulatory effects of 2'FL and CpG using *in vitro* co-culture models combining IEC and innate and adaptive immune cells. Furthermore, IEC-derived galectins were found to be key regulators of the immunomodulatory effects as observed in the IEC/PBMC co-culture model.

Due to the lack of knowledge regarding the ability of NDO and CpG to modulate IEC-derived galectins, we aimed to gain insights regarding the mechanisms used by IEC for galectin secretion. Therefore in **Chapter 6**, the immunomodulatory effects of a 1:1 mixture of GF and 2'FL were studied in combination with CpG, as well as the mechanism of secretion of galectins and their role in the immunomodulatory effects promoted by the NDO mixture and CpG. In addition, to expand our knowledge regarding the effects of HMOS in early life, a fetal intestinal primary cell line was used in the IEC/PBMC co-culture and exposed to NDO in combination with CpG.

Besides NDO, the use of postbiotics is being considered as a dietary strategy to support immune development in early life. Although the microorganisms are inactivated during the fermentation process, the presence of inactivated bacteria and their structural fragments which serve as PRR ligands as well as the derived metabolites, are thought to provide benefits for the host [46, 47]. In **Chapter 7**, the immunomodulatory effects of postbiotics were studied using an *in vitro* IEC/PBMC co-culture model. Furthermore, the ability of specific fermentation products containing postbiotics in supporting systemic immunity was studied *in vivo*. Therefore, a dietary intervention with specific fermentation products was studies using a murine influenza vaccination model and was shown to improve the influenza-specific DTH response, a primary marker measuring vaccination responsiveness.

The findings of this thesis are summarized and discussed in **Chapter 8** in respect to the current knowledge, and opportunities for future research are outlined.

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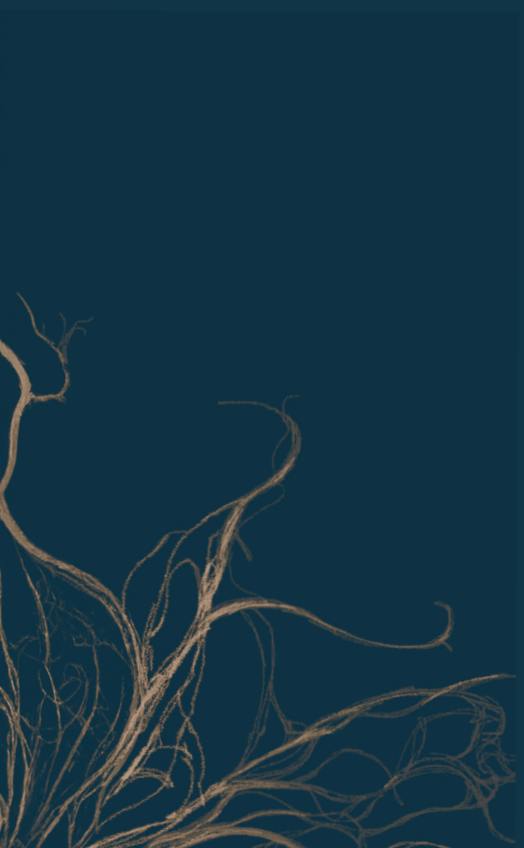
CHAPTER | 2

Diversity of human milk oligosaccharides and effects on early life immune development



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ABSTRACT

One of the well-known features of human milk, is the capacity to protect against the risk and impact of neonatal infections, as well as to influence the onset of allergic and metabolic disease manifestations. The major objective of this review is to provide a detailed overview regarding the role of human milk, more specifically the diversity in human milk oligosaccharides (HMOS), on early life immune development. Novel insights in immune modulatory effects of HMOS obtained by *in vitro* as well as *in vivo* studies, adds to the understanding on how early life nutrition may impact immune development. Extensive description and analysis of single HMOS contributing to the diversity within the composition provided during breastfeeding will be discussed with specific emphasis on immune development and the susceptibility to neonatal and childhood infections.

Keywords: human milk oligosaccharides, mucosal immunity, tolerogenic dendritic cells, infections, early life nutrition

INTRODUCTION

The protective effect of breastfeeding against infections

It has been long noted that breastfeeding protects newborns against infections. Infant formula has been developed over many decades into adequate nutrition for those infants who cannot receive human milk. However, even modern infant formulas lack many components tailor made by each mother for the immune imprinting of her baby, such as specific antibodies (based on the immunologic history of the mother) and human milk oligosaccharides (HMOS) (based on the mother's specific genetic makeup regarding e.g., Lewis (Le) blood group and secretor (Se) status). Exclusive breastfeeding until the age of 4 months followed by partial breastfeeding is associated with a reduction in respiratory and gastrointestinal infectious diseases [1]. For example, infants admitted to the hospital with Respiratory Syncytial Virus infection (RSV) are less likely to have been breastfed [2, 3]. Similarly, infants who are not exclusively breastfed at 6–8 weeks of age, have a higher risk of hospitalization in early life in relation to a wide range of common infections [2]. On the contrary, specific prebiotic oligosaccharides [like short chain galacto- and long chain fructo-oligosaccharides (GF)] are already added in a 9:1 ratio to plain infant formula and have been shown to reduce the development of atopic eczema and allergies as well as reduce the impact of pediatric infections [4, 5]. Therefore, further optimization of infant nutrition when breastfeeding is unavailable is a principal factor required to further support immune development in early life.

The protective effect of human milk is postulated to be achieved by several mechanisms including the provision of pathogen specific maternal antibodies. This will provide the infant with pathogen specific protection during the first months of life, in which infant's own B-cell development has not reached its full potential. Specifically, within the first years of life, the B-cell repertoire matures upon encounter of pathogens, eventually providing a full range of protection against the recurrent pathogens. The immunoglobulins in human milk possess a broad range of pathogen specificity, which mirrors the maternal antigenic state. In addition, the concentration of soluble IgA (sIgA) are remarkably high and variable, and correlate to levels of IgG and IgM detected within different regions [6]. At birth (for example via vaginal delivery as well as during breastfeeding) the neonate encounters a large variety of microorganisms which are determinant in the establishment of the microbiome in adult-life [7, 8]. The initial colonization follows successive steps and is altered through the first year of life by diverse factors including genetic as well as environmental factors such as, introduction of oral feeding. Human milk was shown to stimulate healthy intestinal microbial diversity which includes colonization of several Bifidobacteria and Lactobaccillus species, which in turn, will result in the development of a balanced metabolic response [8, 9].

In addition, human milk provides direct support to further development of the immune system in the neonate [10, 11].

The immune system of the neonates needs to adapt and respond to diverse stimuli encountered in early life. Immune homeostasis is determined by the crosstalk between exposure to the mucosal surfaces encompassing crosstalk between epithelial cells and underlying immune cells [12]. Human milk contains diverse factors like HMOS, milk epidermal growth factor or vitamin A, which contribute to the development of the neonatal mucosa and thus, to the promotion of the neonatal immune system by counterbalancing the deficiencies in early life. These variations are designated as poor IgA production, defective antimicrobial peptide secretion, lack of epithelial chemokine secretion as well as increased permeability among others [6, 10, 13].

Beyond functional components in human milk also the intestinal microbiota can help to further develop these aspects of the mucosal immune system [2]. This emphasizes the relevance of a healthy intestinal microbiome diversity for adequate immune development in the first years of life [9]. Next to the various classes of pathogen specific immunoglobulins in human milk, the presence of antimicrobial molecules, including specific nondigestible free carbohydrate structures and other molecules like glycoconjugates in breast milk have been shown to bind to pathogens [14]. The nutrient source in early life, in particular the non-digestible human milk oligosaccharides (HMOS) in case of breastfeeding are of importance for healthy neonatal microbial colonization [7, 8, 12], immune development [15–19], as well as B-cell development [20]. This review aims to reveal the current state of knowledge regarding the immunomodulatory properties of HMOS and its unique complexity with differences in short chain as well as long chain structures. Recently some of these structures have become available via manufacturing procedures, and it might be considered to apply these in future generations of infant milk formula [21].

Diversity of human milk oligosaccharide composition

HMOS are the third most abundant class of biomolecules found in human milk after lactose and lipids, reaching between 5 and 20 g/L in mature human milk [22]. Up to 1% of HMOS are absorbed in the gastrointestinal tract and found available in systemic circulation [23]. This diversity and abundance is unique in humans and not seen in other mammals [24]. The concentration of total HMOS is subjected to variations dependent on lactational stage [25], maternal nutrition [26], genetic predisposition [27] or even geographic localization and socioeconomic environment of milk donors [28]. Although HMOS are composed out of only 5 different monosaccharides, the structural complexity of HMOS encountered in human milk is unique [21]. The monosaccharides

which are used as building blocks for HMOS are glucose (Glc), galactose (Gal), N-Acetyl-Glucosamine (GlcNAc), fucose (Fuc), and sialic acid (Neu5Ac). These single monosaccharides are conjugated via several linkage types (i.e., glycosidic bonds). With only a few exceptions, HMOS structures do follow a strict building plan (**Figure 1**). Each HMOS structure starts with a lactose unit "Gal (β1-4) Glc" which results from formation of a β1-4 glycosidic linkage between galactose and glucose catalyzed by the lactose synthase protein complex [30]. Several tri-saccharides can be synthesized by appending either galactose or fucose to the reducing or non-reducing end of the lactose residue, which is performed through galactosyl- or fucosyl-transferase activity. Resulting components are e.g., 3′-galactosyllactose (Gal(β1-3)Gal(β1-4)Glc), 4′-galactosyllactose (Gal(β1-4)Gal(β1-4)Glc), 6′-galactosyllactose (Gal(β1-6)Gal(β1-4)Glc), 2′FL (Fuc(α1-2) Gal(β1-4)Glc), and 3-fucosyllactose (3-FL) (Gal(β1-4)[Fucα1-3]Glc). If sialic acids are connected to the non-reducing end of lactose via sialyl-transferases, 3′-sialyllactose (3′-SL; Neu5Ac(α 2-3)Gal(β1-4)Glc) and 6′-sialyllactose (6′-SL) (Neu5Ac(α2-6)Gal(β1-4) Glc) are formed. Further elongation of lactose via the free 3-OH group of galactose can occur by addition of Gal (β1-x)GlcNAc units of either type I (Gal(β1-3)GlcNAc, Lacto-N-biose) or type II (Gal(β1-4)GlcNAc, N-Acetyllactosamine). Up to now, 19 different human milk oligosaccharide core structures have been described. These core structures may be linear or branched and can be further decorated with fucoses or sialic acid residues. Which indicates a myriad of different HMOS structures produced in the human mammary gland. The cellular localization of HMOS synthesis in the mammary gland epithelium is believed to be the Golgi apparatus.

Among other early life factors, the individual maternal genetic disposition has a huge influence on the HMOS profile of human milk. More specifically, the individual expression pattern of Lewis (Le) and Secretor (Se) gene alleles codes for different fucosyltransferases (FUTs), as shown in **Table 1.** The activity of these FUTs can lead to fucosylation of lactose and various other human milk core structures as indicated.

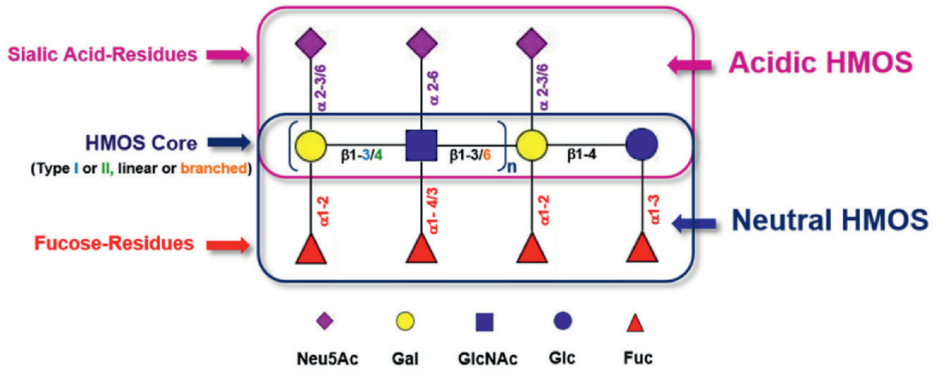

Figure 1. Generic building scheme of HMOS.
Lactose and Type I (Gal (β1-3)GlcNAc-R) or Type II (Gal (β1-4)GlcNAc-R) HMOS core structures can be further extended linearly by adding additional Gal-GlcNAc building blocks to terminal Galactoses via β1-3 glycosidic linkages or via β1-6 glycosidic linkages. In the latter case, branching of the HMOS structure occurs. The (elongated) HMOS core structures can be further decorated with Fucoses (Fuc) and/or Sialic Acid (Neu5Ac) residues following distinct rules. Symbolic representation of monosaccharides according to CFG guidelines [75].

Table 1. Relationship between maternal genotype and exemplified Le- or Se- related major HMOS expected to be present in milks of respective milk types.

Maternal genotype		Frequency in France/ Europe (31)	Prominent HMOS expected in milk group	Milk group
Secretor	Lewis			
Se/-	Le/-	69	2′FL, 3-FL, DFL, LNT, LNnT, LNFP I, LNFP II, LNFP III LNDFH I, LNDFHII, 3′-SL, 6′-SL	Type I
se/se	Le/-	20	3-FL, LNT, LNnT, LNFP II, LNFP III, LNDFH II, 3′-SL, 6′-SL	Type II
Se/-	le/le	9	2′-FL, 3-FL, DFL, LNT, LNnT, LNFP I, LNFP III, 3′-SL, 6′-SL	Type III
se/se	le/le	1	3-FL, LNT, LNnT, LNFP III, 3′-SL, 6′-SL	Type IV

An active Se gen codes for FUT2 which transfers fucose via an α 1-2 glycosidic linkage. Prominent HMOS resulting from FUT2 activity are e.g., 2′FL and LNFP I. Glycans like LNFP I which are carrying the reducing terminus Fuc (α1-2)Gal (β1-3) GlcNAc belong to the group of Le^d or H type 1 antigens. H type antigens link the Le/Se system with the blood group ABH system (31). In contrast, an active Le gene codes for FUT3 which in turn enables fucosylation via either α1-3 or α1-4 glycosidic linkage. FUT3 related structures are e.g., LNFP II and LNFP III. LNFP III is also an example

for an $Lewis^x$ (Le^x) structural motif, whereas LNFP II represents a $Lewis^a$ (Le^a) epitope. Le^a epitopes are characterized by the carbohydrate sequence Gal (β1-3)[Fuc (α1-4)] GlcNAc-R. Le^x-antigens contain type II structures with the following residue: Gal (β1-4) [Fuc (α1-3)]GlcNAc-R. If both, Se and Le genes are active, fucosylated HMOS structures bearing either one, two or all the possible types of fucosylation (i.e., via α 1-2, α 1-3, and α 1-4 glycosidic linkages) can occur. Lacto-N-difucohexaose I (LNDFH I) which also resembles a $Lewis^b$ epitope with the monosaccharide motif Fuc (α1-2)Gal(β1-3) [Fuc (α1-4)]GlcNAc-R, is a known metabolite of joined FUT2 and FUT3 activity. It is noteworthy to mention that also other, Le/Se-system independent fucosyl-transferases may contribute to formation of α1-3-fucosylated HMOS such as 3-FL or LNFP V.

The complexity and relative abundance of different HMOS contained in human milk can for instance be characterized by size exclusion chromatography (SEC) and coupled refractive index detection (RI). A resulting SEC-RI trace is shown in **Figure 2**. Even more detailed information about complexity and individual monosaccharide compositions of HMOS could be derived by a subsequent MALDI-MS [33] analysis of individual SEC HMOS fractions. The acidic sub-fraction adds a further dimension to the overall variety of HMOS. The total number of neutral and acidic HMOS structures based on the MALDI-MS analyses of total human milk carbohydrate SEC-fractions is estimated to exceed the number of 1.000 different structures [24]. Based on the Le/Se status of the mother and specifically the related fucosylated HMOS structures found in the respective human milks, milk group systems of 4 different milk types have been defined [27]. Therefore, it is possible to determine individual human milk types by probing presence of specific fucosylated HMOS like 2′FL, DFL, LNFP I, LNFP II, LNDFH I, and LNDFH II with suited analytical means. An overview of the relationship between maternal Le and Se genotype and some major HMOS structures present in the respective milk types is given in **Table 1**. A recent review has summarized most of the qualitative and quantitative approaches to characterize the diversity of HMOS structures present within human milk [21].

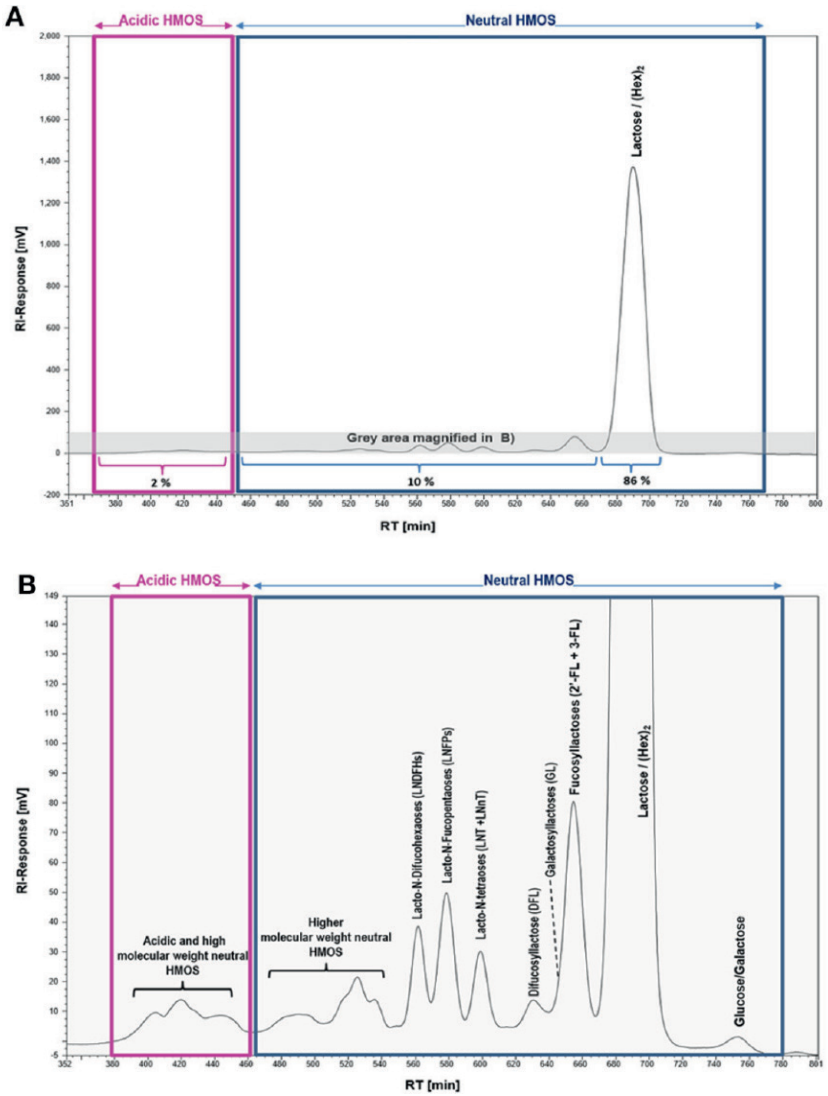

Figure 2. SEC-RI profiles of the total HMOS and mineral fraction from pooled human milk. (**A**) Full SEC-RI Profile, 86% of compounds detected by RI consist of Lactose/$(Hex)_2$, 10% of other neutral HMOS and 2% of acidic HMOS; (**B**) Magnified section of (**A**) zooming into acidic and neutral HMOS; HM sampling, pooling, isolation of the total HMOS fraction and SEC-RI analysis have been performed as described earlier [78].

Biological functions of the different HMOS

The presence of the unique diversity of HMOS, suggests different biological functions and mechanisms by which they may influence the infant's microbiome and

immune maturation and their susceptibility to infections as summarized below and shown in **Figure 3**. The topics exemplified in **Figure 3** are further substantiated point by point in the following section.

Antimicrobial and antiviral effects of HMOS

HMOS play a role in the prevention of infections in breastfed infants by direct blockage of viral and bacterial cellular pathogens and toxins infection by mimicking cell entry receptors [34–36]. The first mechanisms by which HMOS may exert their anti-infective properties are through the inhibition of virus binding to the host cells by mimicking viral receptors and/or by blocking virus entry into the cell, as well as intracellularly, by blocking viral replication. The anti-infective potential of HMOS has been demonstrated for both neutral as well as acidic HMOS, for example different strains of Norovirus have affinity for specific HMOS structures [35, 37]. In addition, both sialylated and fucosylated milk oligosaccharides reduced the infectivity of rotavirus [38]. Interestingly, HMOS with multiple Le^x epitopes were shown to inhibit HIV-1 transfer to $CD4^+$ T lymphocytes more efficiently than other HMOS structures [39]. HMOS may also block microbial pathogen entry, since HMOS from pooled human milk were shown to significantly reduce *Escherichia coli* attachment to cultured epithelial cells [40]. Likewise, it has been shown *in vitro* that LNT, or its fucosylated derivative LNFPI, both can inhibit the growth of Group B Streptococci [41]. Moreover, the presence of 3-FL within the complex mixture of HMOS structures has been inversely correlated with Group-B Streptococci abundancy in infants [42]. In addition, α(1-2)-fucosylated HMOS like 2′FL, or LNDFHI may reduce of early life diarrhea incidence and severity, via their ability to block specific diarrhea inducing pathogens [43].

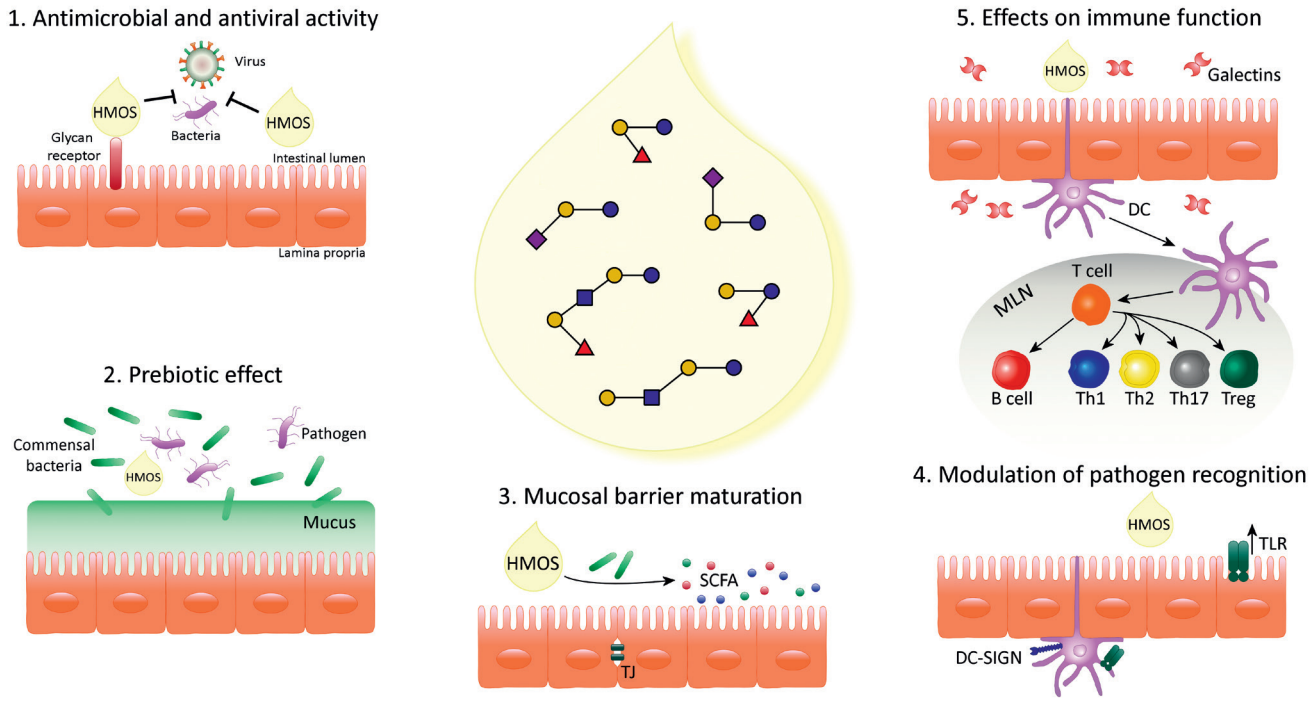

Figure 3. Schematic overview indicating the diversity in structure and function of HMOS.
HMOS are composed of a complex mixture of oligosaccharides. This diversity of structures results in various roles in the epithelial cell layer, surrounding mucosa and immune system composing the digestive tract of breastfed infants. (**1**) HMOS have shown antimicrobial and antiviral effects by binding to virus, bacteria, toxins and/or eukaryotes reaching the mucosal surfaces as well as by direct binding to epithelial surface receptors and blocking the access of pathogens. Thus, avoiding their replication and subsequent infection. (**2**) Commensal bacteria, illustrated as Bifidobacteria, metabolize HMOS and thus, their growth is promoted while pathogens less able to metabolize HMOS will experience growth suppression. (**3**) HMOS and Short Chain Fatty Acid (SCFA), metabolites of HMOS, were shown to influence intestinal epithelial cell (IEC) maturation by promoting differentiation while suppressing proliferation as well as tight junction development, required for proper intestinal barrier function. (**4**) Expression of receptors involved in pathogen recognition, such as TLR as well as their signal transduction was increased after HMOS exposure which in turn impacts the immune homeostasis. (**5**) DC in close proximity to the intestinal epithelial barrier are involved in the immunomodulatory effects described for HMOS. DCs exposed to HMOS play a role in the DC/T-cell interaction leading to T-cell differentiation and/or T-cell/B-cell interaction which may occur in secondary lymphoid organs, depicted as mesenteric lymph nodes (MLN), subsequently promoting immune homeostasis.

Prebiotic effect of HMOS

Development of selective bacterial strains is subjected to their capacity to metabolize HMOS [44]. The role of microbial modulation i.e., the prebiotic capacity of specific HMOS structures have in addition been subject of extensive studies. More specifically, secretor positivity of mothers, hence expressing FUT2 and therefore able to produce α(1-2)-glycosidic-fucosylated HMOS, have been shown to affect the gut bifidobacterial communities of breastfed infants [45]. Bifidobacteria and Bacteroides species are known to metabolize HMOS with high efficiency in contrast to other bacterial species such as *E. coli, Clostridia, Eubacteria, Enterococci* [44]. This appears strain specific and selective for specific HMOS structure [44, 46, 47]. For example, Bifidobacteria exhibited strong growth stimulation while expansion of *Clostridium perfringens and E. coli* were suppressed within cultures using specific HMOS (like 2′FL, 3-FL, and LDFT), whereas Enterobacteria could not grow on 2′FL or 6′-SL cultures [48]. In addition, utilization of fucosylated type human milk oligosaccharides by isolated human gut microbes was shown [49]. These data indicate selective and specific prebiotic capacities of different functional HMOS structures, showing growth of commensal bacteria such as Bifidobacteria at the expense of pathogens, as shown in **Figure 3**. Hence beyond directly blocking viral and bacterial entrance to the host also these prebiotic capacities of HMOS may help to reduce the susceptibility to infection of the host.

Mucosal barrier maturation by HMOS

HMOS interact with glycans present in the surface of intestinal epithelial cells (IEC) or with dendritic cells (DC) which protrude to the gut lumen from lamina propria. This results in direct support of epithelial barrier maturation or an indirect effect on barrier integrity via modulation of the microbiota and consequent short chain fatty acid (SCFA) production [50]. In this regard, beyond blocking pathogen invasion, HMOS may also promote mucosal barrier maturation by increasing the differentiation of IECs. Indeed, synthetic HMOS or HMOS isolated from human milk were shown to promote differentiation and reduce proliferation of various IEC cultures (HT-29 and Caco-2). Similarly, expression of mucosal maturation factors was promoted in fetal intestine cultures after exposure to HMOS isolated from colostrum. These findings suggest that some specific HMOS may be able to promote gut maturation and contribute to epithelial barrier integrity in the gastrointestinal tract of neonates [18, 50, 51].

Modulation of pathogen recognition by HMOS

Receptors involved in the recognition of microbes such as toll like receptors (TLR) are suggested to be modulated by HMOS. Subsequently the response of the host cell to pathogens is altered [17, 37]. *In vitro* studies to elucidate the receptors involved in HMOS effects have been performed mostly in cells isolated from adult individuals

which might not translate directly to the neonatal situation. Specific HMOS structures have been postulated to modulate bacterial and viral signaling on epithelial cells and/or DC [19]. For instance, 2′FL modulates CD14 expression in human enterocytes, thereby attenuating LPS induced inflammation *in vitro* [17]. On the contrary, HMOS such as sialyllactoses, human galactosyllactoses and/or LNFP III may be ligands for toll like receptors (TLR). For example, TLR3 signaling seems specifically inhibited by human milk 3′-galactosylactose [52]. Moreover, it has been shown that the addition of human milk as well as HMOS interacts directly with DCs, through DC-SIGN, Siglecs and related glycan binding proteins which are also essential in immune regulation [53–55]. DCs are key in directing the adaptive immune response toward effective immunity identification and clearance pathogens. Alpha-fucosylated HMOS (2′FL and 3-FL) showed specific binding to DC-SIGN [54]. Effects of GF were suggested to be mediated by TLR4 (56). Similarly, TLR4 as well as TLR3 have also been related to modulate the effects of HMOS. 3-FL, 2′FL were able to modulate TLR3 and elicit an anti-inflammatory effect, while exposure to 2′FL inhibited inflammation through TLR4 [52]. More specifically it has clearly been shown that the addition of GF ameliorates the microbial composition reducing the presence of clinically relevant pathogens [57]. Selectins were also suggested as possible receptors for binding of HMOS due to their ability to block P-selectin [58]. Several receptors are hypothesized to be involved in the recognition of HMOS. The diversity of HMOS structures present in human milk might determine HMOS-glycan receptor binding. HMOS target TLRs and C-type lectins which are vital in pathogen recognition, immune modulation and essential during development of the immune system in early life. Therefore, HMOS may contribute to the development of a balanced and effective immune response, hereby providing protection toward infections.

Effect on immune system development by HMOS

Specific HMOS, such as 2′FL, 3′-SL, 6′-SL, and LNT have been detected within the intestine as well as in systemic circulation of breastfed infants [23, 59, 60]. Increasing evidence collected during the past two decades suggesting a role of HMOS directly on immune cells. Despite all efforts, the effects described remain rather incomplete [19]. Nevertheless, it is suggested that HMOS play a role in supporting the developing mucosal and systemic immune system [13, 16]. HMOS derived from human colostrum can modulate intrinsic expression of inflammatory markers associated with cell trafficking and modulate signaling pathways related to maturation of lymphoid tissue and influence cytokine and chemokine networks that regulate Th1/Th2 lymphocyte balance. The anti-inflammatory effect of for instance 2′FL is known. 2′FL from pooled human milk showed the ability to dampen pro-inflammatory mediator IL-8 release from T84 IEC line after type 1 pili *E. coli* infection [17]. Similarly, reduced IL-8 expression was measured in fetal intestinal human tissue when exposed to 3′-, 4- and 6′-galactosyllactoses from

human milk colostrum [17, 52]. 2′FL was shown to inhibit the inflammatory mediators secreted after TNFα induced *in vitro*, possibly through the inhibition of NF-κβ activation [61]. Furthermore, *in vitro* data demonstrate 2′FL and LNFP I to be able to reduce monocyte activation and to modulate the release of IFNγ, IL-12, and IL-10 [62].

In addition, specific prebiotic oligosaccharides have been demonstrated to be immune modulatory [63–65]. Immunomodulatory effects have been demonstrated for 2′FL, suggesting an additional function of specific oligosaccharides [66–68]. However, if these effects also relate to improved infection susceptibility in infants remains to be established. From the *in vitro* based human milk immune cell interaction studies some specific anti-inflammatory effects have been identified.

Galectins are another class of lectins involved in the regulation of immune and inflammatory processes [55]. Interestingly, HMOS are reported to bind to various recombinant human galectins like hGal-1, -3, -4, -7, -8, and -9 in a very structure dependent and selective way. Human milk glycans with terminal type I sequences (Galβ1-3 GlcNAc) preferentially bind to hGal- 7, whereas hGal-2 did not bind to human milk glyco-types but to a human blood group A Type 2 determinant [55]. Beyond serving as a glycan receptor, galectins can also be secreted as soluble mediators and affect immune function. In this regard, IEC derived galectin-9 was increased after exposure of IEC to a mixture of GF in combination with a TLR9 ligand in an *in vitro* co-culture model of IEC and activated immune cells [69]. Galectin-9 played a key role in enhancing IFNγ and IL-10 production by immune cells underlying the IEC in this model [55]. Further research will reveal the specific role of galectins in immunomodulation after exposure to HMOS, as well as their similarities with the immunomodulatory properties seen by GF. However, it is important to realize that an efficient immune response remains to be mounted against the intruding pathogen. Providing efficient protection, in most cases, will go hand in hand with the induction of inflammation. If an anti-inflammatory response is beneficial in relation to the protection against pathogens, will be pathogen and host specific, and can only be elucidated *in vivo*.

Human milk oligosaccharides impact *in vivo*

It is the unique complexity of human milk oligosaccharides which leads to the speculation that these abundantly available structures in human milk play a key role in providing protection against infections in neonates. From the limited *in vivo* studies, we know that specific HMOS structures can reduce the interaction of specific pathogens like *Salmonella*, *Shigella*, *Vibrio cholerae*, *E. coli*, Polioviruses, Rotavirus and Respiratory Syncytial virus (RSV) with the host [11, 70]. Within some studies, levels of 2′-FL, lacto-N-difucohexaose (LNDFH I), (α 2-linked fucosyloligosaccharide) and ratios between

2-linked and 3-/4-linked oligosaccharide were associated, with presence of specific pathogens like *E. coli*, *Campylobacter* and *Norovirus* [35, 43]. Interestingly, the provision of secretory type related complex mixtures of HMOS, have been associated with a direct protection against specific infections [71]. Fucosyltransferase 2 non-secretor and low secretor status seems to associate with severe outcomes in premature infants. Meaning that within this study a low secretor phenotype was associated with the onset of NEC, and non-secretor genotype was associated with gram negative sepsis [71]. In addition, it has been suggested that FUT2, the regulator of Lewis and ABO(H) antigens in the intestinal mucosa, could be a host genotypic feature affecting susceptibility to ETEC infection [72]. Several intervention studies have reported the functional benefit of adding prebiotic oligosaccharides to infant formula. More specifically, specific prebiotic oligosaccharides have been shown to ameliorate the development of allergies as well as reduce the impact of pediatric infections [4, 5, 73–75]. In this regard, the immune modulating effect that seems to decrease the risk on developing atopy and allergy, also may lower the infection risk in neonates, which is suggestive for basic immune modulation early in life. The clinical consequences of specific individual HMOS structures however, remain to be further elucidated [76]. The first clinical safety studies are now reported on the use of specific HMOS combinations i.e., 2′FL and GOS [60] or the combination of two single oligosaccharides 2′FL and LNnT [68, 77]. Although growth and 2′-FL uptake were similar between formula receiving infants and as seen in breastfed infants, the possible functional benefits regarding immune development and/or infection susceptibility related to a single oligosaccharide are however not extractable from these studies. Therefore, the identification and understanding of protective elements in human breast milk decreasing infant's susceptibility to infection remains limited [75].

In conclusion, components of breast milk (including HMOS) play a key role in the development of the neonatal immune system by preventing pathogen replication, promoting healthy microbial diversity, inducing maturation of intestinal mucosa and by modulation of immune cells as well as pathogen recognition receptors. Currently, there is little understanding about the role of the diverse HMOS structures in optimally inducing microbiome and immune development and consequently how they may provide protection against infections. Therefore, we postulate that HMOS are involved in regulation of mucosal immune and barrier function in multiple ways, although the specific mechanisms remain poorly understood and may be a compilation of the biological functions of individual structures and their interactions. Further investigation into the components of breast milk and their roles in providing protection to infants is required, irrespective of the mechanism by which specific HMOS structures can provide protection toward certain pathogens.

Abbreviations: 2′-FL: 2′-Fucosyllactose; **3′-SL**: 3′-Sialyllactose; **6′-SL:** 6′- Sialyllactose; **APC**: Antigen-presenting cells; **DC**: Dendritic cell; **DC-SIGN**: Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin; **ETEC**: Enterotoxigenic E. coli; **FoxP3**: Forkhead box protein 3; **FUT2**: Fucosyltransferase 2; **HMOS**: Human Milk Oligosaccharides; **lcFOS**: Long Chain Fructo-oligosaccharides; **MHC-I (II)**: Major Histocompatibility Complex Class I (II) molecules; **NEC**: Necrotizing Enterocolitis; **SCFA**: Short Chain Fatty Acids; **scGOS**: Short Chain Galacto-oligosaccharides; **tDC**: tolerogenic dendritic cells; **Th**: T-helper cell; **TJ**: Tight-Junction; **TLR**: Toll-Like Receptors; **Treg**: Regulatory T-cells.

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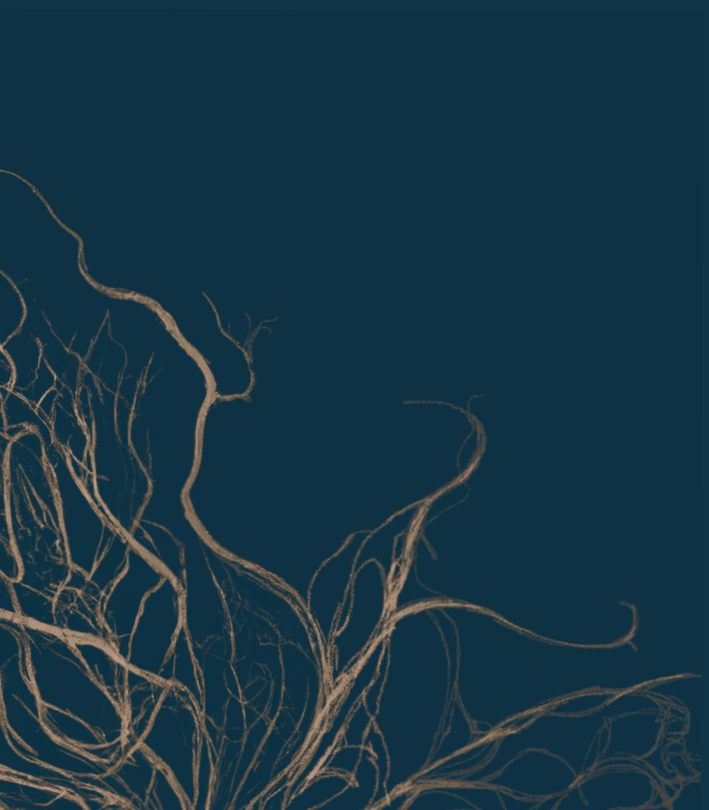
CHAPTER | 3

Human milk oligosaccharide 2’-fucosyllactose modulates local viral immune defense by supporting the regulatory functions of intestinal epithelial and immune cells



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ABSTRACT

Human milk contains bioactive components that provide protection against viral infections in early life. In particular, intestinal epithelial cells (IEC) have key regulatory roles in the prevention of enteric viral infections. Here we established an *in vitro* model to study the modulation of host responses against enteric viruses mimicked by poly I:C (pIC). The effects of 2'-fucosyllactose (2'FL), abundantly present in human milk, were studied on IEC and/or innate immune cells, and the subsequent functional response of the adaptive immune cells. IEC were pre-incubated with 2'FL and stimulated with naked or Lyovec™-complexed pIC (LV-pIC). Additionally, monocyte derived dendritic cells (moDC) alone or in co-culture with IEC were stimulated with LV-pIC. Then, conditioned-moDC were co-cultured with naïve $CD4^+$ T helper (Th)-cells. IEC stimulation with naked or LV-pIC promoted pro-inflammatory IL-8, CCL20, GROα and CXCL10 cytokine secretion. However, only exposure to LV-pIC additionally induced IFNβ, IFNλ1 and CCL5 secretion. Pre-incubation with 2'FL further increased naked pIC induced CCL20 secretion and LV-pIC induced CXCL10 secretion. LV-pIC exposed IEC/moDC and moDC cultures showed increased secretion of IL-8, GROα, IFNλ1 and CXCL10, and in the presence of 2'FL galectin-4 and -9 were increased. The LV-pIC exposed moDC showed a more pronounced secretion of CCL20, CXCL10 and CCL5. The moDC from IEC/moDC cultures did not drive T-cell development in moDC/T-cell cultures, while moDC directly exposed to LV-pIC secreted Th1 driving IL-12p70 and IL-15 and promoted IFNγ secretion by Th-cells. Hereby, a novel intestinal model was established to study mucosal host-defense upon a viral trigger. IEC may support intestinal homeostasis, regulating local viral defense which may be modulated by 2'FL. These results provide insights regarding the protective capacity of human milk components in early life.

Keywords: viral infection; poly I:C, 2'FL; host-defense; moDC; T-cell

INTRODUCTION

Enteric viral infections are one of the most common infectious diseases in humans [1] among which rotavirus (RV) infections are recognized as the leading cause of severe dehydrating diarrhea and mortality in children [2]. RV is a double-stranded RNA virus (dsRNA) which typically infects epithelial cells of the small intestine thereby disrupting the enterocyte morphology and absorptive functions, leading to diarrhea in early life [3].

Intestinal epithelial cells (IEC) provide a physical barrier to protect the host from pathogens as well as playing an important role in immuno-surveillance [4]. Specialized IEC such as mucus producing goblet cells, antimicrobial peptide secreting Paneth cells or enteroendocrine cells have developed strategies to support the protective function of IEC [5–7]. Furthermore, the gut-associated lymphoid tissue (GALT) and immune cells present in the lamina propria located underneath the epithelial barrier, have a unique role in initiating immune responses against pathogens that reach the intestinal lumen. In this regard, IEC recognize pathogen-associated molecular patterns (PAMP) such as viral RNA using pathogen recognition receptors (PRR) like cytosolic retinoic acid-inducible gene-I (RIG-I), melanoma differentiation-associated gene-5 (MDA-5) or endosomal TLR3 [3, 8–12]. The activation of these receptors subsequently leads to the transcription of pro-inflammatory genes. Downstream, the activation of PRR results in the secretion of pro-inflammatory cytokines such as IL-8, GROα, CXCL10, CCL20, CCL5 as well as type I and III IFNs, such as IFNβ and IFNλ [3, 7–9, 11–13]. These mediators are directly involved in viral defense by attracting and activating local innate (monocytes, neutrophils, eosinophils) and adaptive (helper and cytotoxic T-cells) immune cells since they promote the translocation of immune cells to the site of infection to support an adequate immune response against viral pathogens [8, 11, 12].

Dendritic cells (DC) play a crucial role in the induction of adaptive immune responses by presenting viral antigens and activating T- and B-cells [14, 15]. The PRR expressed by DC are also able to recognize PAMP which trigger DC maturation leading to upregulated co-stimulatory molecule and CCR7 expression, allowing DC migration to lymph nodes where naïve T-cells are located [14–16]. In addition, DC process the viral antigens for their presentation to $CD8^+$ or $CD4^+$ T-cells in the lymph nodes resulting in activation, cell division and differentiation of T-cell subsets [10, 14, 15]. The functions of DC in host defense against viral pathogens are not limited to antigen presentation but also the cytokines secreted by DC play a crucial role in the development of appropriate immune responses. Cytokines such as IL-12p70, IL-15 and type I IFNs are known to contribute to the development of Th1-type immunity and thereby might contribute to antiviral immune responses [11, 14, 17, 18]. In spite of the well-described roles of $CD8^+$

T-cells in viral clearance, $CD4^+$ T-cells are also key immune players in the fight against viral infections [19]. These $CD4^+$ helper T-cells (Th) contribute to viral clearance by promoting B-cell antibody production, improving $CD8^+$ cytotoxic T-cell (Tc) function by supporting cytokine and chemokine secretion from DC, regulating inflammatory responses as well as by directly mediating in viral clearance [18, 19].

Breastfeeding has been associated with lower risk of suffering intestinal and respiratory infections [20–22]. Besides, the WHO identified breastfeeding as a protective factor against diarrheal diseases [23]. Recently it was shown that longer duration of exclusive breastfeeding was associated with a later detection of enteric viral pathogens in the stool of infants [24]. Human milk is known to contain many bioactive factors such as immunoglobulins. In particular, secretory IgA as well as IgG are known to provide immunity to breastfed infants [25]. Besides immunoglobulins, human milk oligosaccharides (HMOS) are one of the main components in human milk for which antiviral properties have been described due to their ability to bind pathogens and thus, preventing infection of epithelial cells. Besides, HMOS were also shown to promote barrier integrity and supporting the development of balanced immune responses as reviewed previously [26]. Moreover, previous studies showed that HMOS isolated from pooled human milk supported the maturation of human monocyte-derived DC (moDC) and promoted regulatory T-cell differentiation [27].

One of the most abundant HMOS in human milk namely 2'-fucosyllactose (2'FL), was found to ameliorate the severity and incidence of RV-induced diarrhea possibly through the promotion of intestinal maturation and/or supporting neonatal immune response as shown in a neonatal rat model [28,29]. In addition, the duration of RV-induced diarrhea was shortened and increased immune cell populations were observed in piglets fed a formula containing 2'FL [30, 31]. However, although some studies suggested increased RV infectivity with 2'FL [32], other studies observed a reduced infectivity in MA104 cells *in vitro* [33]. Besides, 2'FL was shown to promote anti-inflammatory and immunomodulatory properties *in vitro* [34, 35], and *in vivo* a dietary intervention with 2'FL was shown to improve the vaccination immune response in an influenza-specific murine vaccination model associated with increased vaccine-specific IgG1 and IgG2a levels [36]. Therefore, 2'FL might be able to support the immune response against viral pathogens

Owing to the unique roles of IEC and regarding the key interactions with underlying immune cells in the fight against viral pathogens, we aimed to develop an *in vitro* model to study the role of IEC and moDC in coordinating the immune response upon a viral trigger. Therefore, IEC were stimulated with the TLR3 agonist and synthetic analog

of dsRNA polyinosinic-polycytidylic acid (pIC) directly or Lyovec™-complexed pIC (LV-pIC) to study if internalization into the cytosol via complexation of pIC affected the cytokine and chemokine secretion. In addition, to study the effect of viral exposed IEC on innate and adaptive immune responses LV-pIC exposed IEC were co-cultured with moDC. Alternatively, moDC were directly exposed to LV-pIC in the absence of IEC. Then, the ability of these moDC to instruct naïve $CD4^+$ Th-cells was studied. Furthermore, the capacity of 2'FL to modulate immune responses in these models was studied in order to shed some light on the effects of human milk oligosaccharide 2'FL in the immune development against a viral trigger.

MATERIAL AND METHODS

Intestinal epithelial cell culture

Human HT-29 cell line (ATCC, HTB-38) was used as IEC. The cells were cultured in 75 cm^2 flasks (Greiner Bio-One) using Mc Coy 5A medium (Gibco, Invitrogen) supplemented with 10% fetal calf serum (FCS), penicillin (100 U/mL) and streptomycin (100 µg/mL) (both from Sigma-Aldrich). IEC were kept in incubation at 37°C and 5% CO_2 and medium was refreshed every 2-3 days.

IEC model

IEC were seeded (50.000 cells/well) in 48-well plates (Costar Corning Incorporated) and grown until confluency in Mc Coy 5A medium. Medium was refreshed every 2-3 days. When IEC reached confluency, cells were pre-incubated with 0.5% (*w/v*; 5 mg/mL) 2'FL solutions (> 90% pure, produced by microbial fermentation) for 24 h. After the pre-incubation period, medium was removed and IEC were stimulated with 10 µg/mL high-molecular weight pIC (Invivogen) either naked or complexed with Lyovec™ (LV) (Invivogen) for 20 h after which, the supernatant was collected and stored for cytokine analysis.

Peripheral blood mononuclear cell isolation

Buffy coats from healthy donors (who had given informed consent) were used to isolate human peripheral blood mononuclear cells (PBMC) (Blood bank, Amsterdam, The Netherlands) by density gradient centrifugation using pre-filled Leucosep™ tubes (1.000 x g, 13 minutes, Greiner Bio-One). The isolated lymphocyte fraction was washed with PBS supplemented with 2% FCS and the remaining erythrocytes were lysed 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 = 7.4). The isolated PBMC fraction was resuspended in RPMI 1640 supplemented with 10% FCS, penicillin (100 U/mL) and streptomycin (100 µg/mL).

Monocyte isolation and culture

A negative selection MACS kit was used to isolate $CD14^+$ cells from PBMC following manufacturer's protocol (Miltenyi Biotec). RPMI 1640 supplemented with 10% FCS, IL-4 (100 ng/mL), GM-CSF (60 ng/mL, both from Prospec) penicillin (100 U/mL) and streptomycin (100 µg/mL) was used to culture isolated $CD14^+$ cells for 7 days. Medium was refreshed on days 3 and 6 of culture. On day 7, immature moDC were collected.

IEC/moDC co-culture model

One week before the experiments, IEC were seeded in 12-well transwell inserts diluted 8-10 times based on surface area (Corning). Medium was changed every 2-3 days (37°C, 5% CO_2). When confluency was achieved, IEC were pre-incubated with 0.5% 2'FL (*w/v*) for 24 h. After the pre-incubation, medium was removed and IEC/moDC co-culture was started. Therefore, immature moDC (0.5 x 10^6 cell) were cultured in the presence or absence of IEC. Apically, 10 µg/mL pIC complexed with Lyovec™ was added and 2'FL solution was refreshed and incubated for 48 h after which supernatant was collected. Additionally, conditioned moDC (ccDC) were collected and co-cultured with naïve T-cells.

DC/T-cell assay

A negative selection MACS kit was used to isolate $CD4^+CD45RA^+$ naïve T-cells from PBMC, following manufacturer's protocol (Miltenyi Biotec). IMDM medium supplemented with 10% FCS, 20 µg/mL apotransferrine (Sigma), β-mercaptoethanol (Sigma), penicillin (100 U/mL) and streptomycin (100 µg/mL) was used to co-culture isolated naïve T-cells (1 x 10^6 cell/well) with ccDC (0.1 x 10^6 cell/well) in 24 well flat-bottomed plates for 5 days. After incubation, supernatant was collected and stored for further analysis. Additionally, cells from DC/T-cell cultures were stained for flow cytometry analysis.

Enzyme-Linked Immunosorbent Assay (ELISA)

Supernatants from IEC, IEC/moDC and DC/T-cell assays were analyzed for cytokine and mediator secretion. Commercially available kits were used to determine IL-8, CXCL10, CCL5, GROα, IFNλ1, IFNβ, IL-22, galectin-3 (from R&D systems), IFNγ, IL-13, (from Thermo Fischer scientific), secretion according to manufacturer's protocol. Human galectin-4 and -9 were measured using antibody pairs (R&D systems). In short, high-binding Costar 9018 plates were incubated overnight at 4°C with 0.75 µg/mL human galectin-4 or -9 affinity purified polyclonal antibody. Non-specific binding was blocked with 1% bovine serum albumin (BSA, Roche Diagnostics) in PBS for 1 h after which plates were washed and streptavidin-HRP (R&D systems) was added and incubated for 40 minutes. After washing, tetramethylbenzidine was used as a substrate to develop the reaction (TMB, Thermo Fischer scientific), which was stopped with 1 M H_2SO_4. Optical density was measured at 450 nm.

Flow cytometry

After DC/T-cell assay immune cells were stained for flow cytometry analysis. Cells from DC/T-cell cultures were incubated with Fc receptor blocking solution (Biolegend) for 10 minutes on ice. Then, cells were washed in PBS supplemented with 1% BSA and incubated for 30 minutes on ice with the following antibodies: CD4-PerCp Cy5.5, CD69-eFluor 450, CXCR3-Alexa Fluor 488 and CCR6-APC (all from Thermofisher except CXCR3 from BD). Dead cells were excluded using Fixable Viability Dye eFluor® 780 (Thermofisher). Stained cells were measured by FACS Canto II (BD Biosciences) and analyzed using Flowlogic software version 7 (Inivai Technologies).

Statistical analysis

All statistical analysis were done using GraphPad Prism software version 8. Data were transformed prior to ANOVA analysis if they did not fit normal distribution. One-way repeated measures ANOVA followed by Bonferroni's post hoc test with selected pairs were used for statistical analysis. The conditions with naked pIC and LV-pIC as well as moDC cultures and IEC/moDC cultures were analyzed separately as represented by the dotted line. Probability values of $p < 0.05$ were considered significant.

RESULTS

Poly I:C stimulated IEC secrete viral defense related cytokines

IEC were grown in 48 well-plates and exposed to 10 µg/mL naked pIC or LV-pIC to study if the mode of delivery of pIC had an effect on the cytokine secretion. In addition, to study the effects of 2'FL, IEC were pre-incubated for 24 h with 2'FL (0.5% *w/v*) after which pIC stimulation was applied, the supernatant was collected and cytokine and chemokine secretion studied.

Exposure to naked pIC significantly increased IL-8, GROα, CCL20 and CXCL10 concentrations as compared to medium control, but did not affect IFNβ, IFNλ1 and CCL5 (**Figure 1**). Pre-incubation with 2'FL did not have an effect on IL-8, GROα, CCL20, CXCL10, IFNβ, IFNλ1 and CCL5 concentrations, but significantly increased CCL20 and tended to decrease GROα ($p = 0.06$) concentrations of naked pIC stimulated IEC as compared to naked pIC stimulation alone (**Figure 1B-C**).Stimulation of IEC with LV-pIC promoted significantly increased IL-8, GROα, CCL20, CXCL10 (**Figure 1A-D**). Also IFNβ, IFNλ1 and CCL5 concentrations were increased as compared to medium control (**Figure 1E-G**). IFNα secretion was under the detection limit. Pre-incubation with 2'FL further increased CCL20 and CXCL10 concentrations on top of LV-pIC stimulation (**Figure 1C-D**).

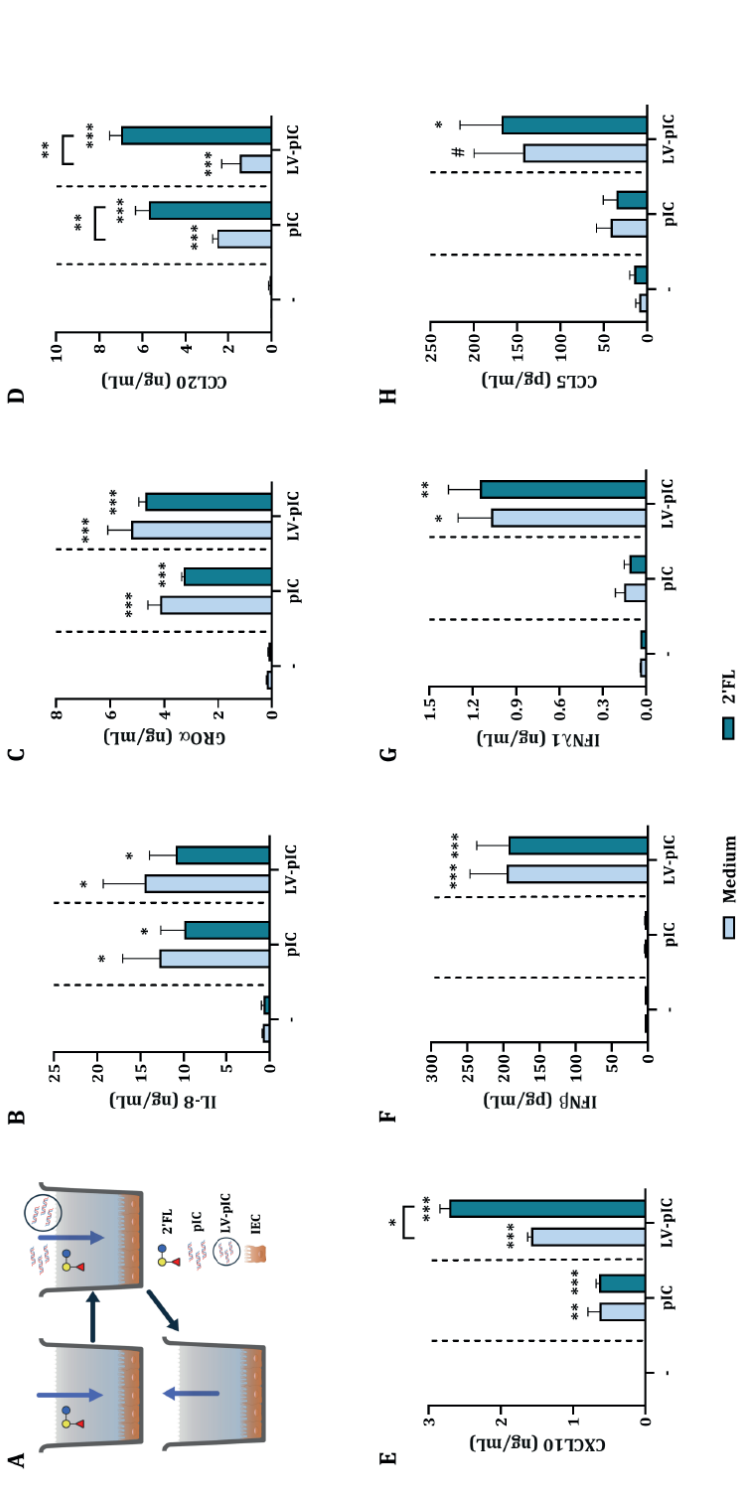

Figure 1. Cytokine and chemokine secretion from IEC upon stimulation with pIC. IEC were grown in 48-well plates until confluency and pre-incubated with 2'FL (0.5% *w/v*). After 24 h incubation, IEC were stimulated with naked or LV-pIC for 20 h after which the supernatant was collected and IL-8 (**B**), GROα (**C**), CCL20 (**D**), CXCL10 (**E**), IFNβ (**F**) IFNλ1 (**G**) and CCL5 (**H**) were measured. Results are represented as mean ± SEM of $n = 6$ independent experiments, except for CXCL10 $n = 4$ (# $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Exposure of IEC to pIC, either naked or LV-complexed, upregulated IL-8, GROα, CCL20, and CXCL10 concentrations (**Figure 1B-E**). Only upon exposure to LV-pIC IFNβ, IFNλ1 and CCL5 were upregulated (**Figure 1F-H**). Pre-incubation with 2'FL significantly increased CCL20 secretion of naked pIC exposed IEC. Meanwhile, pre-incubation with 2'FL significantly increased CCL20 and CXCL10 secretion of IEC stimulated with LV-pIC (**Figure 1D-E**). The LV-pIC conditions were chosen for further studies due to the increased secretion in type I and III IFNs.

IEC regulate the cytokine secretion from IEC/moDC cultures exposed to pIC

To study the regulatory function of IEC upon exposure to viral triggers, the cytokine secretion in IEC/moDC co-culture was assessed. In addition, the effects of 2'FL in IEC/moDC cultures was studied. Therefore, IEC were pre-incubated with 0.5% (*w/v*) 2'FL for 24 h, after which IEC were stimulated with 10 µg/mL LV-pIC in the presence of 2'FL. The stimulated IEC were then co-cultured with immature moDC for 48 h after which the secretion of cytokines and chemokines was measured in the basolateral supernatant. Alternatively, moDC were directly stimulated with LV-pIC in the presence of 2'FL.

There was no effect in the cytokine secretion in moDC or IEC/moDC cultures exposed to 2'FL in absence of LV-pIC (**Figure 2**). Exposure of immature moDC to LV-pIC alone or in combination with 2'FL significantly increased IL-8, CCL20, CXCL10, IFNλ1 and CCL5 concentrations as compared to medium control and/or 2'FL alone (**Figure 2B, D-E, G-H**). LV-pIC did not affect GROα and IFNβ concentrations (**Figure 2C, F**). Exposure to 2'FL did not further increase any of the cytokines and chemokines measured as compared to LV-pIC exposure, but reduced IFNβ as compared to 2'FL alone (**Figure 2F**).

When immature moDC were co-cultured with IEC, exposure to LV-pIC alone or in combination with 2'FL significantly increased IL-8, GROα, CXCL10 and IFNλ1 concentrations as compared to medium control (**Figure 2B-C, E, G**). There was no effect on CCL20, IFNβ and CCL5 concentrations upon exposure to LV-pIC alone (**Figure 2D, F, H**). However, in combination with 2'FL, increased concentrations of CCL20 and CCL5 were observed as compared to medium control or 2'FL alone (**Figure 2D, H**).

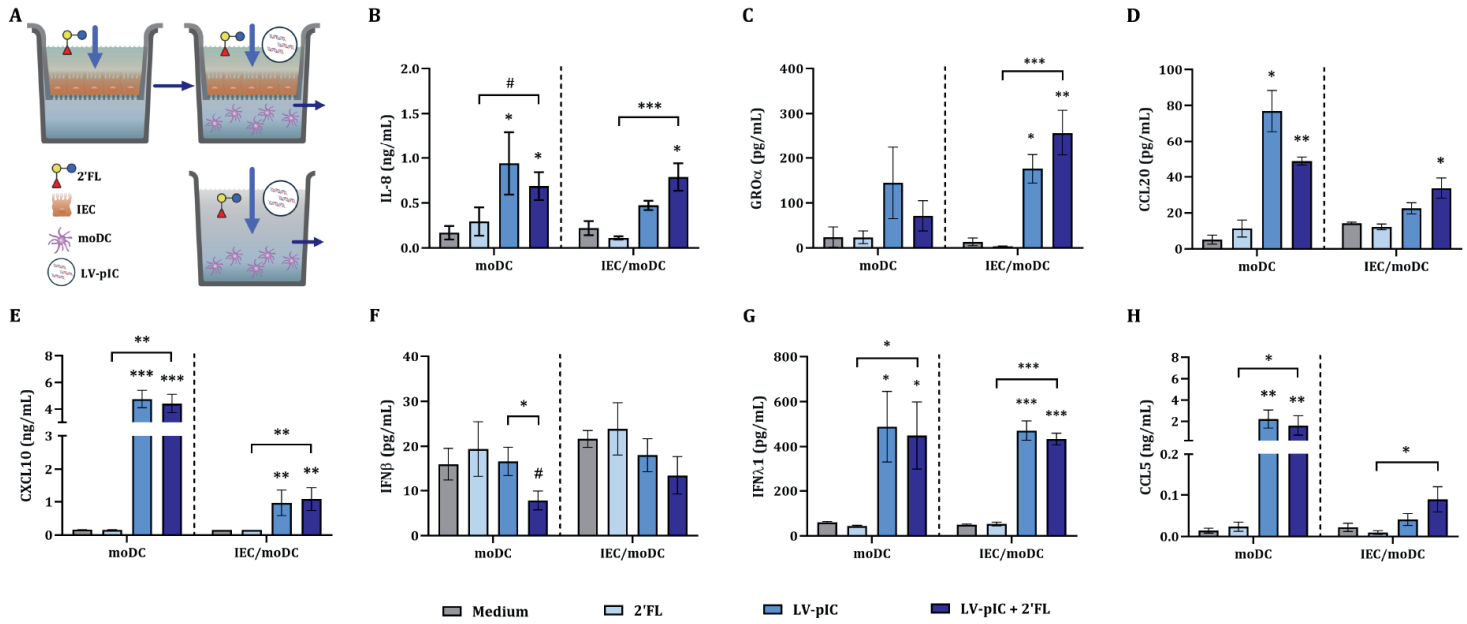

Figure 2. Cytokine and chemokine secretion upon stimulation with LV-pIC.
IEC were grown in 12-well transwell inserts until confluency and pre-incubated with 2'FL (0.5% *w/v*). After 24 h pre-incubation, IEC were stimulated with LV-pIC in fresh 2'FL and co-cultured with immature moDC for 48 h. Alternatively, moDC alone were stimulated with LV-pIC alone in the presence or absence of 2'FL for 48 h. After incubation, the basolateral supernatant was collected and IL-8 (**B**), GROα (**C**), CCL20 (**D**), CXCL10 (**E**), IFNβ (**F**), IFNλ1 (**G**) and CCL5 (**H**) were measured. Results are represented as mean ± SEM of $n = 6$ independent moDC donors except for CCL20 $n = 4$ (# $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

LV-pIC exposure in the presence or absence of IEC resulted in differential effects regarding the release of mediators involved in viral defense. IL-8 and IFNλ1 were increased in IEC/moDC and moDC exposed to LV-pIC, GROα was only increased in the presence of IEC. CXCL10 was increased in IEC/moDC and moDC, but the concentrations remained relatively low in the presence of IEC. CCL20 and CCL5 secretion only increased in moDC cultures in the absence of IEC, however 2'FL enhanced CCL20 and CCL5 secretion in IEC/moDC cultures.

Modulation of galectin secretion by LV-pIC and 2'FL

Galectins may play a role in viral defense and are known to be expressed by IEC and immune cells, therefore we studied the secretion of galectin-3, -4 and -9 in moDC alone or in IEC/moDC cultures. There was no galectin-4 secretion by moDC alone (**Figure 3C**). Exposure to 2'FL or LV-pIC alone did not have an effect on galectin-3 and -9 concentrations of moDC cultures (**Figure 3B, D**). However, combined exposure to LV-pIC and 2'FL significantly increased galectin-3 and -9 concentrations of moDC alone as compared to medium and/or 2'FL controls (**Figure 3B, D**).

In the IEC/moDC co-culture, exposure to 2'FL alone did not affect galectin-3 concentrations, but significantly increased galectin-9 and tended to increase galectin-4 concentrations ($p = 0.08$) as compared to medium control (**Figure 3**). Exposure to LV-pIC alone or in combination with 2'FL significantly decreased galectin-3 concentrations as compared to medium control and increased galectin-4 and -9 concentrations in the IEC/moDC co-culture (**Figure 3B-D**).

IEC/moDC exposed to LV-pIC increased galectin-4 secretion. Combined exposure to LV-pIC and 2'FL further increased galectin-4, only in the presence of IEC, and galectin-9 in moDC cultures exposed to LV-pIC.

Dendritic cell related cytokine secretion in IEC/moDC model by LV-pIC

To further study the effect on moDC, DC derived pro-inflammatory cytokine secretion was studied in the presence or absence of IEC. There was no effect on the IL-12p70, IL-15 and IL-6 concentrations in moDC or IEC/moDC cultures upon incubation with 2'FL (**Figure 4**). IL-12p70 and IL-6 concentrations were significantly increased in LV-pIC exposed moDC cultures in presence or absence of 2'FL, but IL-15 only increased in the presence of 2'FL (**Figure 4B-D**). 2'FL or LV-pIC alone did not affect IL-12p70, IL-15 and IL-6 concentrations (**Figure 4B-D**) in the IEC/moDC cultures except for significantly increased IL-6 concentrations upon combined exposure to 2'FL and LV-pIC as compared to 2'FL alone (**Figure 4D**).

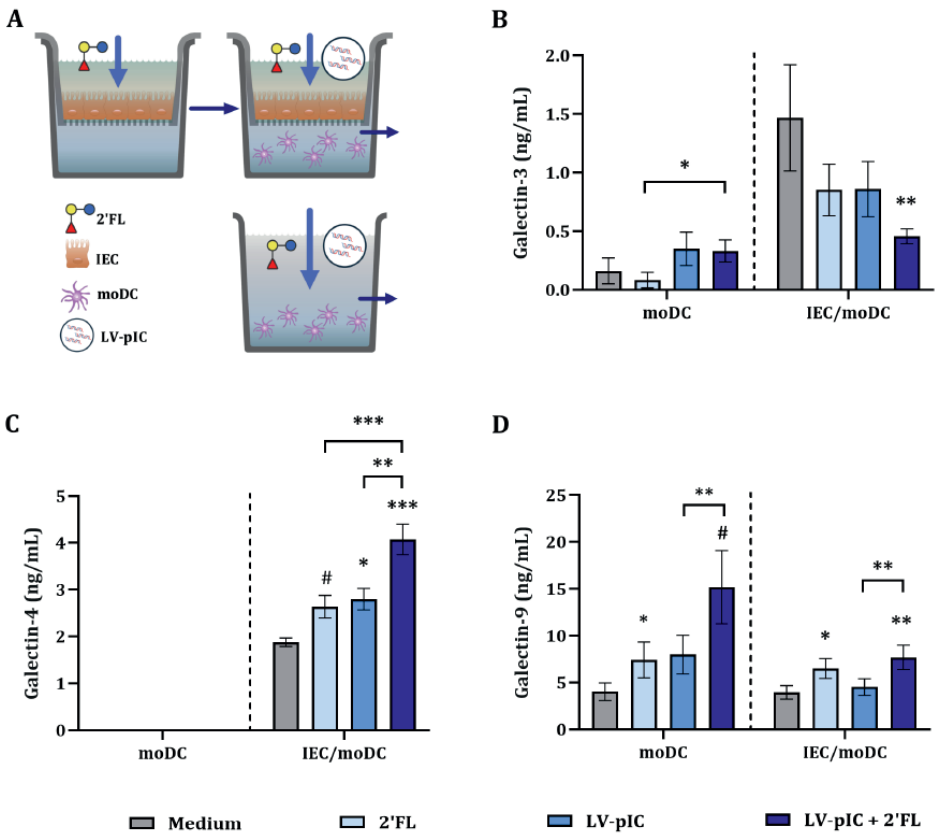

Figure 3. Galectin secretion upon stimulation with LV-pIC.
IEC were grown in 12-well transwell inserts until confluency and pre-incubated with 2'FL (0.5% *w/v*). After 24 h pre-incubation, IEC were stimulated with LV-pIC with fresh 2'FL and co-cultured with immature moDC for 48 h. Alternatively, moDC alone were stimulated with LV-pIC in the presence or absence of 2'FL for 48 h. After incubation the basolateral supernatant was collected and galectin-3 (**B**), -4 (**C**) and -9 (**D**) were measured. Results are represented as mean ± SEM of $n = 6$ independent moDC donors (# $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Increased pro-inflammatory IL-12p70, IL-15 and IL-6 secretion was observed in moDC cultures exposed to LV-pIC alone and/or in combination with 2'FL. This upregulation was not observed in IEC/moDC cultures except for a small increase in IL-6 upon combined exposure to 2'FL and LV-pIC.

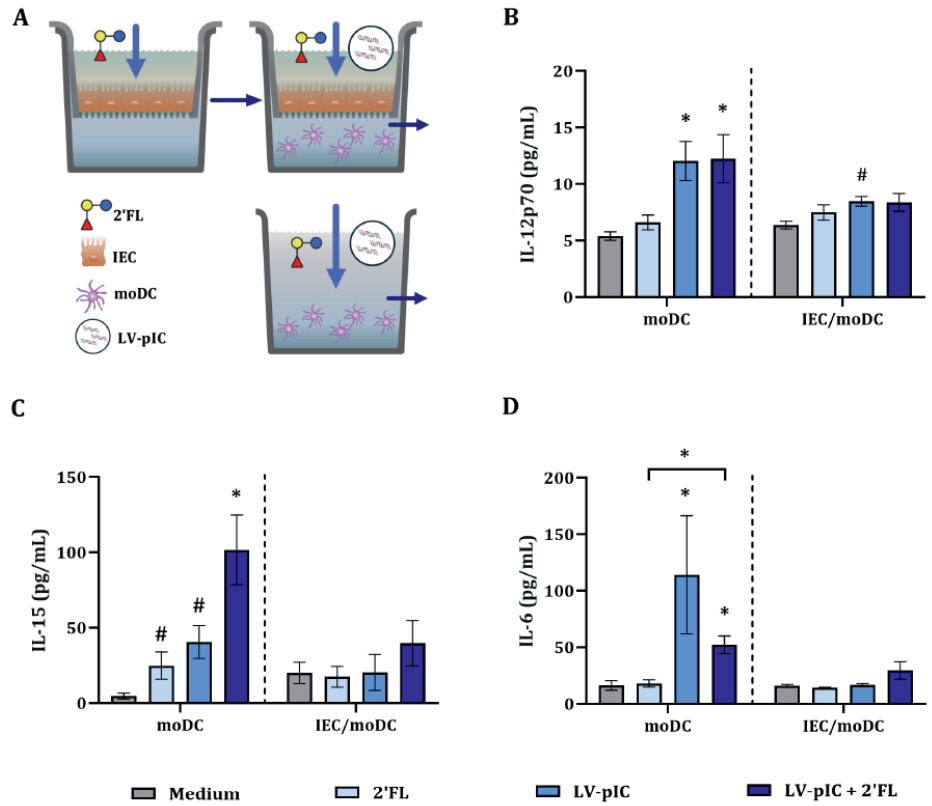

Figure 4. Cytokine secretion upon stimulation with LV-pIC.
IEC were grown in 12-well transwell inserts until confluency and pre-incubated with 2'FL (0.5% *w/v*). After 24 h pre-incubation, IEC were stimulated with LV-pIC with fresh 2'FL and co-cultured with immature moDC for 48 h. Alternatively, moDC alone were stimulated with LV-pIC in the presence or absence of 2'FL for 48 h. After incubation, the basolateral supernatant was collected and IL-12p70 (**B**), IL-15 (**C**) and IL-6 (**D**) were measured. Results are represented as mean ± SEM of $n = 6$ independent moDC donors (# $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Cytokine secretion in DC/T-cell assay

In an allogeneic DC/T-cell assay we studied if ccDC (moDC derived from IEC/moDC or moDC cultures exposed to LV-pIC with or without 2'FL) were able to instruct T-cells. Therefore, ccDC were incubated with naïve T-cells for 5 days after which the cytokine secretion was studied (**Figure 5**).

There was no effect on the IFNγ, IL-22 and IL-13 concentrations in the supernatant of DC/T-cells of ccDC that had been exposed to 2'FL (**Figure 5**). ccDC from LV-pIC moDC cultures significantly increased IFNγ and IL-13 concentrations in DC/T-cell

cultures, as compared to medium controls. Increased IL-13 secretion was observed in DC/T-cell cultures from LV-pIC and 2'FL exposed to moDC cultures, while IL-22 tended to increase ($p = 0.08$) as compared to medium control (**Figure 5B-D**).

In DC/T-cell supernatants of ccDC derived from IEC/moDC cultures incubated with 2'FL, no effect was observed on IFNγ and IL-22 concentrations (**Figure 5A-B**). However, in DC/T-cell supernatants from ccDC derived from IEC/moDC cultures exposed to LV-pIC alone or in combination with 2'FL, significantly decreased IL-13 concentrations were observed, while IL-22 tended to decrease ($p = 0.07$) (**Figure 5C-D**). IL-10, IL-17A and IL-23 concentrations remained under the detection limit. Additionally, the phenotype of the T-cells was studied after DC/T-cell assay. There was no effect on the percentages of $CD4^+$, $CD69^+$ or $CD69^+CXCR3^+$ cell populations (**Figure S1**).

DC/T-cell cultures from moDC exposed to LV-pIC increased Th1-type IFNγ and Th2-type IL-13. Meanwhile, in DC/T-cell cultures from ccDC derived from IEC/moDC conditions IFNγ secretion was not affected, but decreased IL-13 concentrations were observed.

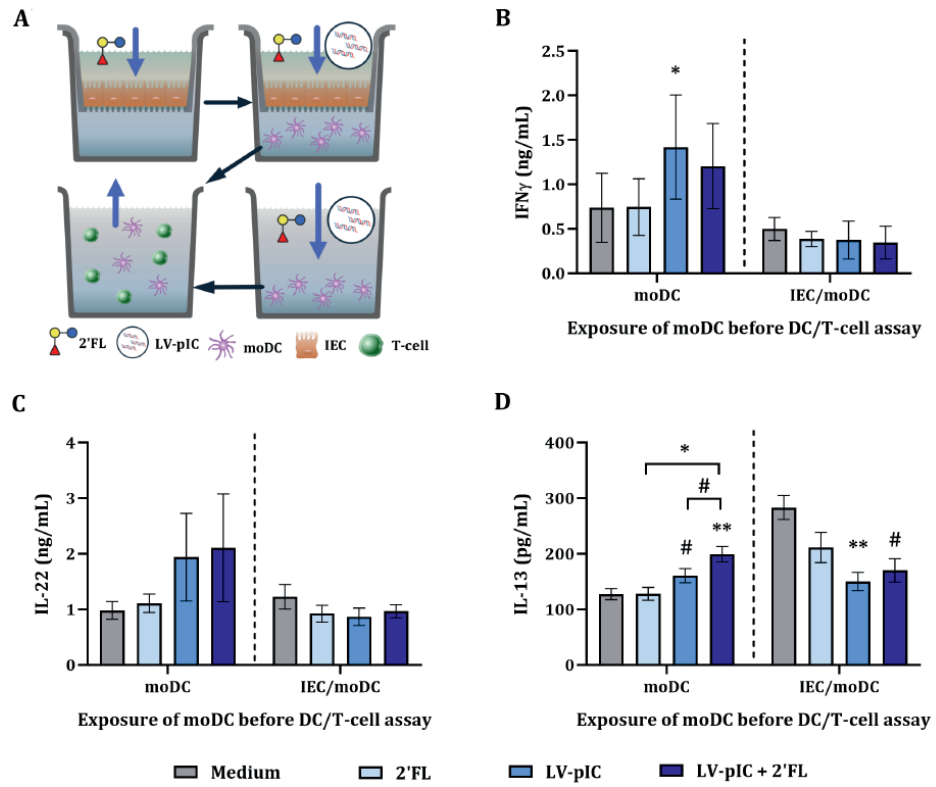

Figure 5. Cytokine secretion in DC/T-cell assay.
Conditioned moDC previously exposed to LV-pIC conditioned IEC or to LV-complexed pIC directly, were incubated with naïve T-cells for 5 days after which the supernatant was collected. After incubation, the basolateral supernatant was collected and IFNγ (**B**), IL-22 (**C**) and IL-13 (**D**) were measured. Results are represented as mean ± SEM of $n = 6$ independent moDC donors (# $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

DISCUSSION

Besides providing a barrier, IEC contribute to host defense by regulating PRR expression, crosstalk with underlying immune cells as well as regulating cytokine and chemokine secretion [4, 11]. Here, we study the involvement of IEC in the development of immune responses against a viral trigger and the modulation by 2'FL, an HMOS abundant in human milk.

To setup an enteric viral infection model, a synthetic analog of dsRNA, namely pIC was used. Therefore, pIC was added directly on top of the IEC monolayer (naked pIC) and compared to LV-complexed pIC (LV-pIC), which supports the passage of pIC

through the cellular membrane into the cytosol [37]. Widely used to mimic a viral trigger, pIC is known to activate TLR3 and was found to promote a significant increase in IL-8, CXCL10 and CCL20 secretion from stimulated IEC cells [38–40]. These results are in line with our observations as shown in this manuscript, where upregulated IL-8, CXCL10 and CCL20 release was observed upon either naked or LV-pIC stimulation. Furthermore, pIC stimulated IEC also secreted significantly increased GROα levels independent of LV-complexation, which to our knowledge has not been previously shown. However, in HT-29 cells infected with an enteric virus like RV, upregulated IL-8 and GROα concentrations were observed [41], suggesting that stimulation of IEC with either naked or LV-pIC might mimic to some extent an enteric viral infection similar to RV.

In addition, upon infection of IEC with RV, increased IFNβ secretion was observed which was suggested to be mediated by RIG-I and MDA-5 signaling [12, 42–44]. Besides TLR3, pIC was shown to activate RIG-I and MDA-5 [45]. Here, IFNβ was not detectable upon exposure to naked pIC. Only exposure to LV-pIC, promoted IFNβ secretion from IEC cells, suggesting that naked pIC might not be able to fully stimulate cytosolic RIG-I and MDA-5 signaling and thus, failed to promote IFNβ secretion, as opposed to LV-pIC. These results suggest that the mode of delivery of pIC results in the activation of different signaling pathways and that only upon LV-complexation of pIC, a strong activation of RIG-I and MDA-5 is obtained leading to IFNβ secretion in HT-29 cells.

Not only type I IFNβ secretion was increased by LV-pIC. An upregulation of type III IFNλ1 and CCL5 secretion was also observed upon stimulation of IEC with LV-pIC. Both type I IFNβ and type III IFNλ1 secretion by infected cells is known to signal in an autocrine and paracrine manner to surrounding cells which by activating IFN-stimulated genes (ISG) induce a local antiviral state [11–13]. Similarly, this autocrine and paracrine activation loop might result in further stimulation of CCL5 secretion by amplification of IFN production and induction of the activation of other ISG [46]. Type I IFNs are key factors contributing to modulate the antiviral immune response by suppressing viral replication, while type III IFNs are particularly important for innate immune responses at mucosal barriers such as the gut barrier [47, 48]. Interestingly, previous studies observed increased CCL5 secretion in RV-infected HT-29 cells [41]. These results support the idea that LV-pIC stimulation of IEC might mimic to some extent the immune responses observed upon an enteric viral infection.

Dendritic cells act as a bridge between the innate and adaptive immune responses and as such, their interaction and crosstalk with IEC is key to maintain immune homeostasis in the fight against pathogens such as viruses [4, 7, 11]. In this regard, the contribution

of IEC in supporting moDC activity in response to a viral trigger was studied. Upon stimulation of moDC with LV-pIC, significantly increased CCL20, CXCL10 and CCL5 secretion was observed, as opposed to IEC/moDC cultures where secretion of such chemokines was limited. On the contrary, GROα was only increased in IEC/moDC cultures while IL-8, IFNβ and IFNλ1 were induced equally in IEC/moDC and moDC cultures. GROα is known to function in an autocrine regulatory manner leading to the promotion of cell survival and immunomodulation in IEC [49] which might have led to its increase secretion in IEC/moDC cultures. In line with our results, an improved antiviral state was observed by increased CXCL10 and IFNβ as well as decreased IL-8 in a co-culture model using porcine intestinal cells and immune cells upon exposure to probiotics [8,50]. Our results indicate that IEC might selectively regulate CCL20, CXCL10 and CCL5 in order to restrict the propagation of the viral infection and the subsequent tissue damage thus, promoting local clearance of the virus and facilitating re-establishment of tissue homeostasis [4, 51]. However, when the virus crosses the epithelial lining and DC are fully exposed to the virus without the regulatory action of epithelial cells, a much more strong immune activation is generated with the induction of systemic adaptive immune response to the viral trigger.

Due to the benefits of breastfeeding in the protection against infections [20–22], we studied the ability of HMOS in modulating the cytokine and chemokine secretion in IEC and IEC/moDC cultures exposed to LV-pIC. In particular one of the most abundant HMOS, namely 2'FL, showed anti-inflammatory and immunomodulatory properties *in vitro* [34, 35] as well as a reduction in the incidence and severity of RV-induced diarrhea *in vivo* [28–31]. Here, we observed that pre-incubation of IEC with 2'FL and subsequent stimulation with pIC significantly increased CCL20 and/or CXCL10 concentrations compared to activation with pIC alone. Besides, in the IEC/moDC or moDC cultures, no effect was observed upon 2'FL exposure, except for a slight increase of CCL20 and CCL5 secretion in IEC/moDC cultures when combined with LV-pIC. These chemokines are known to be involved in DC, monocyte and T-cell (Th and Tc) recruitment or NK cell activation and might be beneficial to induce a proper anti-viral state [8, 11, 12, 52–55] since these can promote an appropriate immune response against the encountered threat. 2'FL was found to selectively enhance the secretion of chemokines in LV-pIC exposed IEC or IEC/moDC cultures suggesting possible immunomodulatory roles, which may be beneficial in viral defense. Further studies are needed to confirm the ability of 2'FL to enhance mucosal defense and protect against specific enteric viruses using viral proteins and/or inactivated viruses.

Beyond the cytokine and chemokine functions on the immune response against viruses, galectins have also shown to be key regulators of many immune processes.

In the current study, galectin-4 and -9 secretion was further increased by 2'FL in LV-pIC stimulated IEC/moDC cultures, while in moDC cultures galectin-9 and -3 were increased. These results point towards the involvement of galectins in the regulation of immune responses against pIC and the ability of 2'FL in modulating galectin secretion in response to a viral trigger. Galectins were shown to bind viral glycans and PRR extracellularly and interact with viral and cytosolic components present in the cytoplasm and thereby, might be able to modulate immune responses [56–58]. Furthermore, increased galectin levels, and in particular galectin-9 levels, were observed upon viral infections [59] to dengue [60], influenza [61], HIV [62], hepatitis B and C [63, 64] as well as COVID-19 [65] as compared to healthy controls. Besides, recent studies have suggested the use of plasma concentrations of galectins as biomarkers for disease prognosis since elevated plasma galectins were linked to higher viral load or more severe infection, since galectins are probably produced as protective factors with regulatory functions that can boost immunity and thereby, promote host defense [60, 66–68].

Although the exact mechanism is still unknown, it is hypothesized that galectins might promote the induction of cytokines and immune cells to support viral immune defense. In particular galectin-9 was shown to act as damage associated molecular patterns (DAMP) and to induce immunomodulation of various immune cells [69]. However, galectin-9 plasma levels also correlated with pro-inflammatory mediator secretion (IL-6, TNFα, CXCL10) in dengue and COVID-19 infected patients [68, 70]. In addition, galectins are known to exert biological functions that could contribute to host defense such as cell adhesion and migration [70], Treg cell differentiation and function [71], suppressing $CD4^+$ Th and $CD8^+$ Tc [72] or controlling apoptosis to reduce tissue damage [72–74] as well as affecting DC maturation [75]. Furthermore, circulating galectins were shown to be associated with enhanced vaccine-specific immune responses in a murine influenza vaccination model [76]. Galectins, are associated with various immune processes and as such, can interact with many innate immune cells to support host defense against viruses. Innate immune cells are able to secrete galectins which can result in the modulation of innate and adaptive immune cells at the site of infection. Here they may regulate immune responses and thereby contribute to the resolution of the viral infection, as well as keeping local tissue homeostasis by regulating and limiting exaggerated immune responses which could lead to tissue damage.

The secretion of pro-inflammatory cytokines IL-12p70 and IL-6 was induced in moDC exposed to LV-pIC, and also IL-15 was secreted but only in the presence of LV-pIC and 2'FL, as opposed to IEC/moDC cultures. This supports the regulatory role of IEC in promoting local viral clearance and tissue homeostasis while downregulating excessive immune activation. Both IL-6 and IL-12 are produced upon DC activation

and contribute to DC/T-cell communication and immune cell recruitment to the site of infection or inflammation [77]. Meanwhile IL-15 is known to induce DC differentiation and thereby promoting Th1-type immune responses in the intestine [17]. Particularly, IL-12p70 secretion and increased DC maturation was observed in immature DC stimulated with TLR3 ligand pIC as shown by an increase in the expression of CD80, CD86 and MHC-II markers [78]. In activated T-cells, IL-15 promoted IFNγ production as well as synergizing with IL-12 to upregulate IFNγ production [79]. Meanwhile, the differentiation of naïve $CD4^+$ Th-cells and the production of $CD8^+$ Tc-cells were induced by IL-6 and IL-15 respectively [17, 79–82]. Furthermore, IL-6 and IL-15 are known to contribute to tissue protection [17, 80]. In this manuscript, we observed that when DC were co-cultured with naïve T-cells, these LV-pIC exposed moDC, were capable of activating $CD4^+$ Th-cells as shown by increased Th1-type IFNγ, Th2-type IL-13 as well as an increasing tendency in IL-22 secretion in the presence of 2'FL. DC producing high amounts of IL-12p70 and IL-15 are known to drive Th1-type immune responses [14, 17, 79, 81]. Thereby, we suggest that the increased IFNγ secretion observed in DC/T-cell cultures from LV-pIC-stimulated moDC might be associated with the Th1-promoting cytokine secretion seen in moDC cultures. Contrarily, no increase in T-cell activation was observed in DC/T-cell cultures from IEC/moDC conditions, indicating a possible regulatory role of IEC in suppressing the instruction of DC towards promoting a general systemic inflammatory response.

Overall, LV-pIC was found to induce a full repertoire of mediator release by IEC which are involved in viral defense, of which CCL20 and CXCL10 were further enhanced by 2'FL. LV-pIC exposure to moDC also induced inflammatory mediator release associated with Th1 cell activation, as opposed to IEC/moDC cultures. In the latter, 2'FL was found to enhance galectin-4 and -9 secretions during LV-pIC exposure, which may help to control local immune activation and maintain tissue homeostasis during viral defense. In this novel *in vitro* mucosal viral defense model using viral RNA, we observed a unique role of IEC in regulating immune responses against a viral trigger. This suggests a tight control of local intestinal viral defense which may be supported by dietary components such as 2'FL.

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SUPPLEMENTARY FIGURES

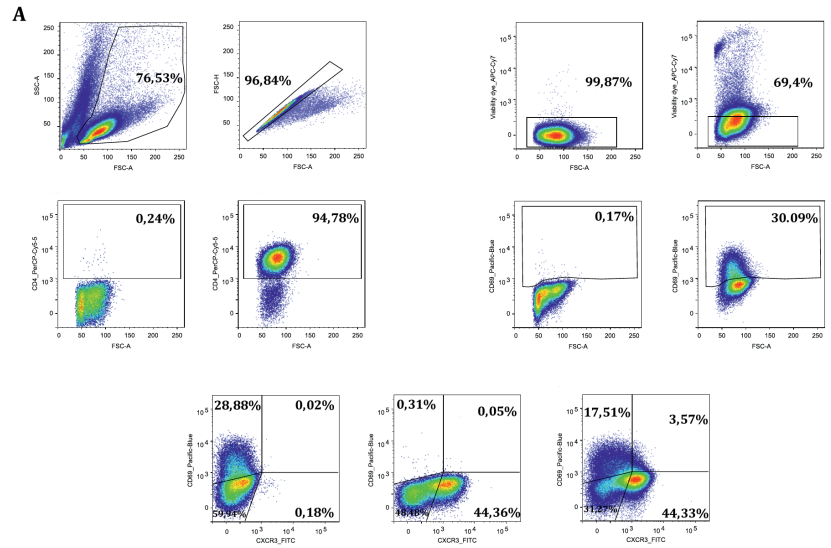

Figure S1. Phenotype of T-cells after DC/T-cell assay.
Conditioned moDC previously exposed to LV-pIC conditioned IEC or to LV-complexed pIC directly, were incubated with naïve T-cells for 5 days after which the phenotype was studied. Representative plots showing the used gating strategy are shown. The percentage of $CD4^+$ (**A**), $CD69^+$ (**B**) and $CD69^+CXCR3^+$ (**C**) are shown. Data are represented as mean ± SEM of $n = 6$ independent moDC donors.

CHAPTER | 4

Modulation of the epithelial-immune cell crosstalk and related galectin secretion by DP3-5 galacto-oligosaccharides and β-3'galactosyllactose

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ABSTRACT

Prebiotic galacto-oligosaccharides (GOS) were shown to support mucosal immune development by enhancing regulatory-type Th1 immune polarization induced by synthetic CpG oligodeoxynucleotides (TLR9 agonist mimicking a bacterial DNA trigger). Epithelial-derived galectin-9 was associated with these immunomodulatory effects. We aimed to identify the most active fractions within GOS based on the degree of polymerization (DP), and to study the immunomodulatory capacities of DP3-sized β-3'galactosyllactose (β-3'GL) using a transwell co-culture model of human intestinal epithelial cells (IEC) and activated peripheral blood mononuclear cells (PBMC). IEC were apically exposed to different DP fractions of GOS or β-3'GL in the presence of CpG, and basolaterally co-cultured with αCD3/CD28-activated PBMC, washed, and incubated in fresh medium for IEC-derived galectin analysis. Only DP3-5 in the presence of CpG enhanced galectin-9 secretion. DP3-sized β-3'GL promoted a regulatory-type Th1 response by increasing IFNγ and IL-10 or galectin-9 concentrations as compared to CpG alone. In addition, IEC-derived galectin-3, -4, and -9 secretion was increased by β-3'GL when combined with CpG. Therefore, the GOS DP3-5 and most effectively DP3-sized β-3'GL supported the immunomodulatory properties induced by CpG by enhancing epithelial-derived galectin secretion, which, in turn, could support mucosal immunity.

Keywords: galacto-oligosaccharides; galectins; intestinal epithelial cells; β-3'galactosyllactose; immunomodulation; mucosal immunity

INTRODUCTION

Non-digestible oligosaccharides (NDO) are the third major component in human milk [1]. Based on the amount and structure diversity of NDO in human milk, a 9:1 mixture of short-chain galacto- and long-chain fructo-oligosaccharides (GF) was studied for its effects on the microbiota and the intestinal mucosa [2]. Various clinical studies have shown that this GF mixture promoted the growth of commensal bacteria, induced stool softening, reduced the incidence of infections and the incidence of atopic dermatitis, as well as modulated the antibody profile in infants at high risk of allergy [3–8]. Furthermore, when combined with *Bifidobacterium breve* M-16V, it effectively lowered allergic symptoms in a murine model for cow's milk or hen's egg allergy in association with increased intestinal and/or serum galectin-9 levels [9–12]. In addition, in children affected with atopic dermatitis, this synbiotic mixture was found to enhance serum galectin-9 levels after 12 weeks of intervention, in association with reduced atopic dermatitis symptom scores and lower risks of developing asthma [10, 13, 14].

The major component of the GF (9:1) mixture is GOS, which is composed of galactose units coupled to a terminal glucose with a degree of polymerization (DP) ranging between 2 and 8 [13, 14]. Upon ingestion, GOS reach the lower parts of the gastrointestinal tract intact where fermentation by the gut microbiota occurs. Consumption of GOS promotes the growth of beneficial commensal bacteria and provides health benefits to the host [15, 16]. Besides the microbiota-dependent effects, GOS was also shown to have direct effects on the epithelial barrier. Regarding the effect of GOS on intestinal epithelial cells (IEC), it was shown that GOS can inhibit the adherence of pathogenic bacteria to IEC [17, 18], enhance the barrier function by preventing the disruption of gut barrier integrity [19], and promote goblet cell function [20]. Furthermore, GOS supported the absorption of minerals such as iron and calcium in young infants [21, 22], as well as lowered the incidence and severity of travelers' diarrhea in humans travelling to high-risk countries [23].

In addition to the effects on the microbiota and the IEC, NDO such as GOS have been shown to promote direct immunomodulatory effects. GOS was shown to interact with T-cells and dendritic cells, and to selectively promote the release of regulatory IL-10 *in vitro* [24, 25]. The increased IL-10 was also observed in a study performed in suckling piglets [26]. Furthermore, in a double-blind, placebo-controlled study performed in healthy elderly, GOS supplementation positively influenced immune parameters by increasing the production of IL-10, increasing NK cell activity, and reducing pro-inflammatory IL-6 and IL-1β measured in peripheral blood mononuclear cells (PBMC) [27, 28].

Although previous studies have shown effects of GOS either on epithelial or on immune cells, we have identified immunomodulatory properties of the NDO mixture GF in a transwell model developed to study the crosstalk between IECs and immune cells [29–32]. The crosstalk between IEC and underlying immune cells is key to maintain the intestinal mucosal homeostasis and to develop appropriate immune responses [33]. Previous studies using this well-established *in vitro* IEC/PBMC co-culture model reported regulatory-type Th1 responses upon exposure to NDO in association with bacterial DNA or synthetic CpG oligodeoxynucleotides, known to be TLR9 ligands. The immunomodulatory effects observed upon exposure to NDO and CpG in the IEC/PBMC model could support mucosal immune development and were shown to be mediated by epithelial-derived galectin-9, which was found to be a key mediator contributing to the effects observed *in vitro* [29–31, 34, 35]. These studies also showed that the immunomodulatory properties of CpG and NDO occurred only when the PBMC underlying the IEC were activated, mimicking inflammatory conditions [29].

GOS as the main component in the GF mixture contains multiple oligomers (DP2-8), out of which the active immunomodulatory component has not yet been identified. Due to the variety of structures present in GOS, this study aimed to investigate the most active oligomers within GOS by investigating their immunomodulatory capacity using a transwell co-culture model combining IEC and activated PBMC. Therefore, specific GOS DP fractions were isolated by size-exclusion chromatography and exposed to IEC in the presence of CpG oligodeoxynucleotides to study the crosstalk with the underlying immune cells and their effect on IEC-derived galectins. Additionally, we studied the immunomodulatory effects of a specific NDO present in the GOS mixture and found in human milk, namely β-3'galactosyllactose (β-3'GL) [14, 36], using the IEC/PBMC coculture model. Studying the immunomodulatory properties of specific NDO structures will provide further insights regarding their potential role in mucosal immune development.

MATERIALS AND METHODS

GOS DP separation by size exclusion chromatography

Vivinal GOS syrup (derived from lactose, 45% pure) produced by the elongation of galactose catalyzed by β-galactosidases (Friesland Campina) was diluted in Milli-Q water (1:1) and fractionated using a Bio-Gel P-2 column. Milli-Q water was used as eluent. The flow rate used was 0.2 mL/min. The fractions collected (6–12 mL) were freeze-dried (Christ) and fractions containing DP4-7, DP3-7, or DP3-5 were pooled upon analysis by electrospray ionization-mass spectrometry (ESI-MS) (microTOF-Q-II Bruker) using a HILIC column (X-Bridge™ HILIC, Waters). An amount of 0.1% ammonia was used in the running buffer (acetonitrile:water; gradient of acetonitrile going from 5% to 50% aqueous solution in 10 min) (**Figure 1**). Additionally, the DP3-5 fraction was further separated into DP3, DP4, and DP5 (**Figure 2**) using the same experimental conditions as described above.

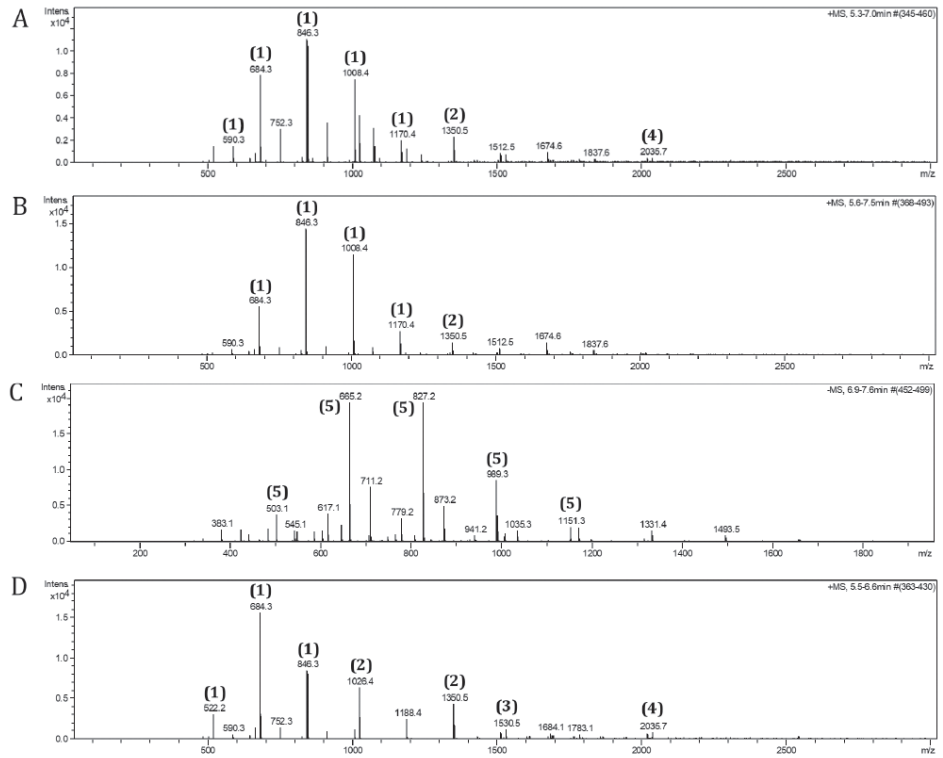

Figure 1. Electrospray ionization-mass spectrometry (ESI-MS) spectra of DP fractions. GOS DP oligomers were separated by size-exclusion chromatography from GOS mixture. The profiles of GOS (**A**), GOS DP4-7 (**B**), GOS DP3-7 (**C**), and GOS DP3-5 (**D**) are shown as ammonia adducts (1) = $[M+NH4]^+$, (2) = $[2M+NH4]^+$, (3) = $[3M+NH4]^+$, (4) = $[M+NH4]^+$, and (5) = [M, no ammonia adduct].

Culture of IEC

The human colon adenocarcinoma HT-29 cell line (ATCC, HTB-38) was used as a model for IEC. The HT-29 cell line was cultured in McCoy 5A medium (Gibco, Invitrogen) supplemented with 10% fetal calf serum (FCS), penicillin (100 U/mL), and streptomycin (100 µg/mL) (Sigma-Aldrich) IEC were grown in 75 cm^2 flasks (Greiner Bio-One) and maintained at 37 °C, 5% CO_2. Medium was refreshed every 2–3 days. One week before the experiments, IEC were diluted 8–10 times based on surface area and

seeded in 12-well transwell inserts (Costar Corning Incorporated). When confluency was reached, the IEC monolayers were used to perform co-culture experiments.

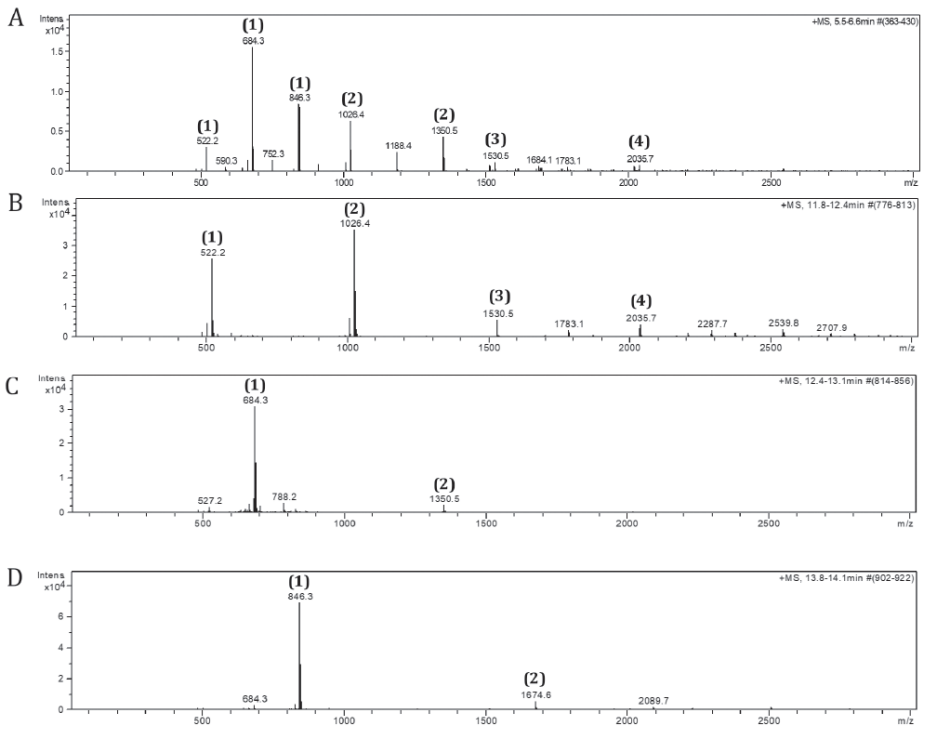

Figure 2. Electrospray ionization-mass spectrometry (ESI-MS) spectra of DP fractions. GOS DP oligomers were separated by size-exclusion chromatography from GOS mixture. The profiles of GOS DP3-5 (A), GOS DP3 (B), GOS DP4 (C), and GOS DP5 (D) are shown as ammonia adducts (1) = $[M+NH4]^+$, (2) = $[2M+NH4]^+$, (3) = $[3M+NH4]^+$, and (4) = $[M+NH4]^+$.

Isolation of PBMC

Buffy coats from healthy donors were purchased (Blood bank, Amsterdam, The Netherlands) and used to isolate human PBMC. Buffy coats were diluted (1:1) using PBS supplemented with 2% FCS. PBMC were isolated by density gradient centrifugation (1,000 x g, 13 min) using Leucosep™ tubes (20 mL per tube) (Greiner Bio-One). After washing, the remaining red blood cells were lysed (4.14 g of NH_4Cl, 0.5 g of $KHCO_3$, 18.6 mg of Na_2EDTA in 500 mL of demineralized water, sterile-filtered, pH = 7.4). Isolated PBMC were resuspended in RPMI 1640 supplemented with 2.5% FCS, penicillin (100 U/mL), and streptomycin (100 µg/mL).

IEC/PBMC co-culture model

IEC grown in transwell filters were apically exposed to 0.1 or 0.5% (*w/v*) GOS DP fractions or β-3'GL (Carbosynth) in the presence or absence of synthetic CpG oligodeoxynucleotides (ODN M362, 0.1, 0.5 or 5.0 µM) (Invivogen). In the basolateral compartment, αCD3 and αCD28-activated PBMC were added (2 x 10^6 cells/mL) (0.15 µg/mL and 0.2 µg/mL, respectively, from Sanquin or BD Biosciences, San Jose, CA, USA) and incubated for 24 h (37 °C, 5% CO_2), after which the basolateral supernatant was collected and stored at -20 °C for cytokine analysis. Subsequent to IEC/PBMC co-culture, transwell inserts containing IEC monolayers were collected for quantitative Polymerase Chain Reaction (qPCR) analysis or transferred into a new plate separated from the PBMC and washed with PBS. Then, fresh medium was added and IEC were incubated in fresh medium for an additional 24 h (total 48 h; 24 h in IEC/PBMC co-culture and 24 h in culture of IEC in fresh medium) to determine the IEC-derived basolateral mediator release. In addition, IEC were collected and stored for qPCR analysis.

Enzyme-linked immunosorbent assay (ELISA)

The basolateral supernatants were used to analyze the cytokine and galectin-9 secretion in the IEC/PBMC co-culture, as well as the IEC-derived galectin-3, -4, and -9. Commercially available kits were used to determine IFNγ, TNFα, IL-13 (all from Thermo Fisher Scientific), IL-10 (U-Cytech), and galectin-3 (R&D systems) following the manufacturer's protocol. Human galectin-4 and galectin-9 were measured using antibody pairs (both from R&D), as described before [29].

Gene expression analysis by qPCR

RNA was isolated from IEC samples using the Nucleospin® RNA Plus kit (Macherey- Nagel). Contaminating DNA was removed by incubating with DNAse for 15 min on ice (Qiagen). Complementary DNA (cDNA) was obtained using the iScript™ cDNA synthesis kit (Bio-Rad) following the manufacturer's protocol. The IQ SYBR Green Supermix and CFX96 real-time PCR detection system (both from Bio-Rad) were used for the quantification of gene expression. Commercially available primers for galectin-3, -4, and -9 were used and compared to RPS13, as a reference gene (all from Qiagen). Relative mRNA expression was calculated as 100 x $2^{(Ct\ reference\ -\ Ct\ gene\ of\ interest)}$ [37].

Statistical analysis

All statistical analyses were performed using GraphPad Prism 8 software. When the data did not fit a normal distribution, transformation was applied prior to ANOVA analysis. One-way repeated measures ANOVA followed by Bonferroni's post hoc test with selected pairs were used for the statistical analysis. The conditions with and without

CpG were analyzed separately as represented by the dotted line. Within the analysis of CpG-exposed conditions, a comparison between the medium control group and CpG alone was included. Probability values of $p < 0.05$ were considered significant.

RESULTS

Immunomodulatory effects of GOS DP fractions in IEC/PBMC co-culture model

The immunomodulatory effects of DP fractions isolated from GOS were studied using a transwell IEC/PBMC co-culture model used to investigate the crosstalk between epithelial cells and innate, as well as adaptive, immune cells. IEC were apically exposed to the GOS DP fractions DP4-7, DP3-7, and DP3-5 (0.5% *w/v*) in combination with 5 µM CpG. In the basolateral compartment, αCD3/CD28-activated PBMC were added and incubated for 24 h.

Exposure of GOS DP4-7, DP3-7, or DP3-5 alone did not affect IFNγ, IL-10, or galectin-9 secretion, but CpG alone enhanced IL-10 secretion by activated PBMC in the IEC/PBMC co-culture (**Figure 3A–C**). Only combined exposure to GOS DP4-7 and CpG resulted in significantly increased IFNγ concentrations as compared to CpG alone (for DP3-7 and DP3-5; $p = 0.053$) (**Figure 3A**). Upon combined exposure to DP3-5 and CpG, significantly increased galectin-9 concentrations were observed as compared to CpG alone. Meanwhile for IL-10, this did not reach significance ($p = 0.06$) (**Figure 3B, C**).

Th1-type IFNγ secretion was increased by GOS DP4-7 when combined with CpG as compared to CpG alone. However, as GOS DP3-5 and CpG enhanced galectin-9 secretion, while showing a similar pattern for IFNγ and IL-10 secretion, the following studies were performed using GOS DP3-5.

Additionally, the GOS DP3-5 fraction was further separated into DP3, DP4, and DP5 fractions to study the most active oligomer/s within GOS DP3-5. Therefore, IEC were apically exposed to 0.5% (*w/v*) DP3, DP4, and DP5 in the presence or absence of 5 µM CpG. In the basolateral compartment, αCD3/CD28-activated PBMC were added and incubated for 24 h.

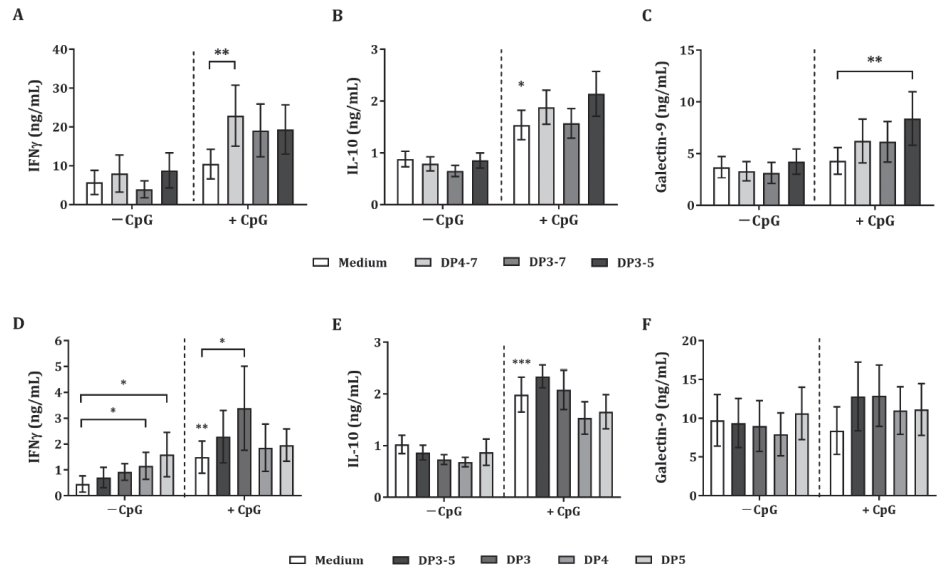

Figure 3. Cytokine and galectin-9 secretion in IEC/PBMC co-culture.
IECs were apically exposed to GOS DP4-7, DP3-7, and DP3-5 in combination with 5 µM CpG and basolaterally to αCD3/CD28- activated PBMC. Additionally, GOS DP3-5 was further separated into DP3, DP4, and DP5 fractions. IEC were apically exposed to GOS DP3, DP4, and DP5 in combination with 5 µM CpG and basolaterally to αCD3/CD28-activated PBMC. After 24 h of incubation, IFNγ (**A, D**), IL-10 (**B, E**), and galectin-9 (**C, F**) concentrations were measured in the basolateral supernatant. Data represent mean ± SEM of $n = 6$ independent PBMC donors, except for $n = 4$ in (**A**) and $n = 5$ in (**C, D**). Statistical analysis was performed separately for conditions with and without CpG (represented as dotted line). However, a comparison between the medium control and CpG-exposed condition was included (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

There was no effect on IFNγ, IL-10, and galectin-9 concentrations upon exposure to GOS DP3-5, DP3, DP4, and DP5 in the absence of CpG, except for DP4 and DP5, which significantly increased IFNγ concentrations (**Figure 3D–F**). CpG alone significantly increased IFNγ and IL-10 concentrations but did not affect galectin-9 secretion (**Figure 3D–F**). Only exposure to DP3 in combination with CpG further increased IFNγ concentrations compared to CpG alone. DP3-5 did not enhance IFNγ but, similar to DP3, showed a similar pattern in IL-10 and galectin-9 concentrations, although these did not reach significance (**Figure 3D–F**). No effect was observed on IFNγ, IL-10, and galectin-9 concentrations upon exposure to DP4 and DP5 in combination with CpG (**Figure 3D–F**).

Galectin-9 mRNA expression and IEC-Derived galectin-9 secretion by apical exposure to GOS DP3-5 and CpG

To study the involvement of galectin-9 secretion resulting from apical GOS DP3-5 and 5 μM CpG exposure, IEC were collected after IEC/PBMC co-culture. In addition to this, IEC were washed and incubated in fresh medium for up to 48 h (24 h in IEC/PBMC co-culture and an additional 24 h in culture of IEC in fresh medium), after which galectin-9 mRNA expression and IEC-derived galectin-9 secretion were studied.

In IEC, the relative galectin-9 mRNA abundance was not significantly affected by GOS DP3-5, CpG, or the combination after 24 h in IEC/PBMC co-culture (**Figure 4A**). However, after 48 h (24 h in IEC/PBMC co-culture and an additional 24 h in IEC culture in fresh medium) of exposure to CpG alone in the presence or absence of GOS DP3-5, galectin-9 mRNA expression was significantly increased as compared to the medium control and/or GOS DP3-5 exposure (**Figure 4A**).

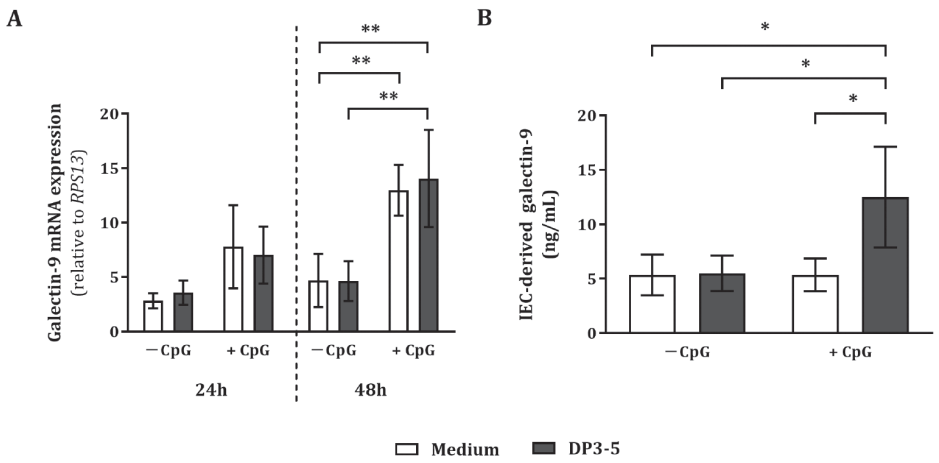

Figure 4. Galectin-9 mRNA expression and IEC-derived galectin-9 release.
After IEC/PBMC co-culture, IEC were collected and galectin-9 mRNA expression was measured (**A**). Alternatively, IEC were washed and incubated in fresh medium for an additional 24 h (48 h in total; 24 h in IEC/PBMC co-culture and an additional 24 h in IEC culture in fresh medium), after which IEC and the basolateral supernatant were collected. The relative galectin-9 mRNA abundance at 24 h and 48 h (**A**) and the IEC-derived galectin-9 secretion at 48 h (**B**) were analyzed. Data represent mean ± SEM of $n = 3$ and $n = 6$ independent PBMC donors for (**A, B**), respectively (* $p < 0.05$, ** $p < 0.01$).

IEC-derived galectin-9 secretion at 48 h was not affected by GOS DP3-5 or CpG alone (**Figure 4B**). Combined exposure to GOS DP3-5 and CpG significantly increased IEC-derived galectin-9 as compared to the medium, GOS DP3-5, and CpG alone

(**Figure 4B**). Galectin-9 mRNA expression, but not IEC-derived galectin-9 secretion, was upregulated by CpG. Combined exposure to GOS DP3-5 and CpG upregulated both galectin-9 mRNA expression, as well as IEC-derived galectin-9 secretion.

Increased Th1-type IFNγ and regulatory galectin-9 by β-3'GL and CpG in the IEC/PBMC co-culture model

GOS DP3-5 and GOS DP3 showed immunomodulatory capacities when combined with CpG. Therefore, we aimed to study a specific DP3-sized NDO present in the GOS mixture and found in human milk, namely β-3'GL. IEC were apically exposed to β-3'GL (0.1 and 0.5% *w/v*) alone or in combination with 0.1 μM CpG and basolaterally to activated PBMC after which IFNγ, IL-10, and galectin-9 secretion was studied. Additionally, IEC-derived galectins were measured after an additional 24 h of incubation of IEC with fresh medium (48 h in total). Instead of 5 μM CpG, we used 0.1 μM in the following experiments in order to better identify the additional effects of NDO on top of the CpG effect, as was shown for GOS DP3-5 in **Figure S1**.

Exposure to β-3'GL, in either concentration, or GOS DP3-5 alone did not have an effect on IFNγ, IL-10, and galectin-9 concentrations (**Figure 5A–C**). Exposure to CpG alone significantly increased IL-10 concentrations as compared to the medium control but did not affect IFNγ and galectin-9 concentrations (**Figure 5A–C**). There was no effect on the cytokine and galectin secretion upon combined exposure to CpG and 0.1% β-3'GL or DP3-5 (**Figure 5A–C**). When IEC were exposed to 0.5% β-3'GL and CpG, significantly increased IFNγ and galectin-9 concentrations were observed, as compared to CpG alone or to 0.1% β-3'GL and CpG (**Figure 5A, C**). CpG-induced IL-10 concentrations were not further enhanced by 0.5% β-3'GL or GOS DP3-5 (**Figure 5B**).

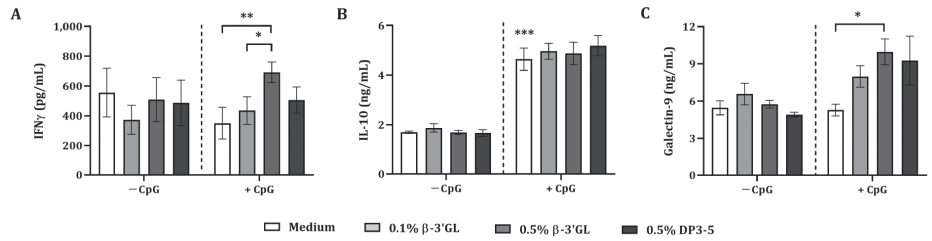

Figure 5. Cytokine and galectin-9 secretion by β-3'GL and CpG in the IEC/PBMC co-culture model.
IEC were apically exposed to β-3'GL (0.1% and 0.5% *w/v*) or GOS DP3-5 (0.5% *w/v*) in combination with 0.1 μM CpG and basolaterally to αCD3/CD28-activated PBMC. After 24 h of incubation, IFNγ (**A**), IL-10 (**B**), and galectin-9 (**C**) were measured in the basolateral supernatant. The data shown are represented as mean ± SEM of $n = 6$ independent PBMC donors (except for IFNγ n = 5) (* $p < 0.05$. ** $p < 0.01$, *** $p < 0.001$).

Apical exposure of IEC to GOS DP3-5 or β-3'GL alone did not have an effect on the cytokine secretion in the IEC/PBMC co-culture model. Exposure to 0.5% β-3'GL and CpG more strongly promoted the secretion of Th1-type IFNγ and regulatory IL-10 compared to 0.1% β-3'GL or GOS DP3-5 and CpG. For the following studies, 0.5% β-3'GL was used.

Increased epithelial-derived galectin secretion by β-3'GL and CpG

To further study the immunomodulatory effects of β-3'GL and CpG and the involvement of galectins in the IEC/PBMC co-culture model, IEC were exposed to GOS DP3-5 or β-3'GL (0.5% *w/v*) and 0.1 μM CpG.

Exposure to CpG alone did not affect IFNγ or galectin-9 concentrations but significantly increased IL-10 concentrations compared to medium control levels (**Figure 6A–C**). Combined exposure to CpG and β-3'GL or GOS DP3-5 significantly increased IL-10 concentrations as compared to the medium control and/or CpG alone, but no effect was observed on galectin-9 concentrations (**Figure 6B-C**). Increased IFNγ concentrations were observed upon β-3'GL and CpG as compared to CpG alone (**Figure 6A**). Significantly decreased IL-13 and TNFα concentrations were observed upon exposure to CpG alone as compared to the medium control (**Figure S2**). These remained reduced and were not further affected by combined exposure to CpG and GOS DP3-5 or β-3'GL.

To study the involvement of epithelial-derived mediators in the immunomodulatory effects promoted by NDO and CpG, IEC-derived galectin-3, -4, and -9 secretion was measured after 48 h (24 h in IEC/PBMC co-culture and an additional 24 h in IEC culture in fresh medium) and correlated to the cytokine secretion in the IEC/PBMC co-culture. Additionally, galectin-3, -4, and -9 mRNA expression was measured at 48 h.

There was no effect on the IEC-derived galectin-3 and -4 secretion by CpG alone, but increased IEC-derived galectin-9 was observed as compared to the medium control (**Figure 6D–F**). The increased IEC-derived galectin-9 was also observed upon exposure to GOS DP3-5 or β-3'GL in combination with CpG as compared to the medium control (**Figure 6F**). Only upon combined exposure to β-3'GL and CpG significant increases were observed in IEC-derived galectin-3 and -4 concentrations as compared to the medium control, CpG alone, and/or GOS DP3-5 and CpG (**Figure 6D-E**).

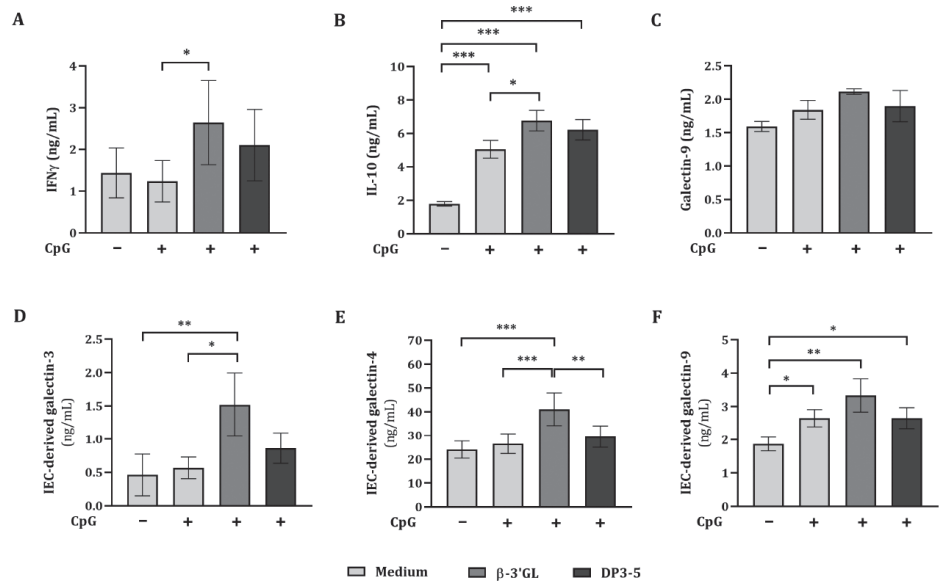

Figure 6. Cytokine and galectin-9 secretion in IEC/PBMC co-culture and IEC-derived galectin secretion at 48 h.
IEC were apically exposed to GOS DP3-5 or β-3'GL (0.5% *w/v*) in combination with 0.1 μM CpG, and basolaterally to αCD3/CD28-activated PBMC. After 24 h of incubation, IFNγ (**A**), IL-10 (**B**), and galectin-9 (**C**) were measured in the basolateral compartment. Additionally, after IEC/PBMC co-culture, IEC were washed and incubated in fresh medium for an additional 24 h (48 h in total; 24 h in IEC/PBMC culture and an additional 24 h in IEC culture in medium), after which IEC-derived galectin-3 (**D**), -4 (**E**), and -9 (**F**) were measured in the basolateral supernatant. The data shown are represented as mean ± SEM of $n = 6$ independent PBMC donors (except for galectin-9 $n = 4$) (* $p < 0.05$. ** $p < 0.01$, *** $p < 0.001$).

In order to link the IEC-derived galectins with the outcome of the immune response, correlations were calculated. These results are summarized in **Table 1**. A positive correlation was observed between IFNγ and IEC-derived galectin-3, -4, and -9. There was no correlation between IL-10 and IEC-derived galectin-3 and -4. However, a positive correlation was observed between IL-10 and IEC-derived galectin-9. Only IEC-derived galectin-9 was positively correlated to galectin-9, but not to IEC-derived galectin-3 or -4. A negative correlation was observed between IL-13 and IEC-derived galectin-3 and -9, but not with galectin-4 (**Figure S2**). Meanwhile, TNFα concentrations were not correlated to epithelial-derived galectins (**Figure S2**).

Table 1. Correlations of cytokines and galectin-9 in the IEC/PBMC co-culture and IEC-derived galectins. Cytokine and galectin-9 secretions measured in the IEC/PBMC co-culture were correlated to IEC-derived galectin concentrations (48 h) using Pearson correlations.

	IEC-derived galectin-3	IEC-derived galectin-4	IEC-derived galectin-9
IFNγ	+ r = 0.65 $p < 0.0001$	+ r = 0.49 $p = 0.003$	+ r = 0.38 $p = 0.02$
IL-10	n.s. r = 0.06 $p = 0.7$	n.s. r = 0.07 $p = 0.7$	+ r = 0.37 $p = 0.03$
Galectin-9	n.s. r = 0.02 $p = 0.9$	n.s. r = 0.3 $p = 0.1$	+ r = 0.44 $p = 0.003$

+ Positive correlation; n.s. non-significant correlation.

IEC-derived galectin-3, -4, and -9 were upregulated upon the exposure of IEC to β-3'GL and CpG in the IEC/PBMC co-culture model. IEC-derived galectin-9 secretion was correlated positively to IFNγ, IL-10, and galectin-9 secretion and negatively to IL-13 in the IEC/PBMC co-culture. However, IEC-derived galectin-3 was correlated positively to IFNγ and negatively to IL-13, while IEC-derived galectin-4 was correlated only to IFNγ concentrations.

Additionally, the galectin gene expression was measured at 48 h. There was no effect on the galectin-3, -4, and -9 mRNA expression upon exposure to 0.1 μM CpG alone or in the presence of DP3-5 (**Figure S3**). Combined exposure to β-3'GL and CpG significantly decreased galectin-4 mRNA expression as compared to the medium control or GOS DP3-5 in combination with CpG (**Figure S3**). Galectin-9 mRNA expression was significantly increased by exposure to β-3'GL and CpG as compared to GOS DP3-5 and CpG (**Figure S3**).

DISCUSSION

Human milk is highly abundant in NDO for which diverse immune regulatory functions have been described [1]. GOS comprise the main component in the prebiotic mixture resembling the amount and structure diversity of NDO in human milk [2, 4] and are composed of a complex variety of NDO with DPs ranging between 2 and 8 [14]. The purpose of this study was to evaluate the most active oligomers within the GOS mixture regarding the immunomodulatory capacity using the IEC/PBMC co-culture model in which the crosstalk between IEC and immune cells was studied.

Up to 60% of GOS DP structures have a DP size of DP2 or DP3 [13, 14]. Within the DP2 fraction, various NDO structures have been characterized also including lactose residues [14]. Previous studies did not observe immunomodulatory effects upon exposure to lactose in the IEC/PBMC co-culture model [35]. However, due to the presence of lactose and the inability to separate this from other possible active NDO within the DP2 fraction, this study focused on analyzing GOS fractions with size of DP3 and longer fractions, namely DP4-7, DP3-7, and DP3-5 fractions, which were isolated by size-exclusion chromatography.

In the current study, Th1-type IFNγ concentrations were increased upon exposure to GOS DP4-7 in association with CpG. However, only GOS DP3-5 showed increased CpG-induced regulatory-type galectin-9 secretion with a similar Th1 secretion, suggesting a pattern of Th1 and regulatory cytokine secretion. Therefore, GOS DP3-5 was used for further studies, based on previous findings identifying galectin-9 as a key mediator in driving a regulatory-type Th1 response [31, 33, 36]. The current study showed the presence of structures with immunomodulatory properties within the GOS DP3-5 fraction, and, also within this fraction, GOS DP3 was identified for being able to upregulate IFNγ concentrations only when combined with CpG. This suggests that the most active oligomers in terms of Th1-type regulatory immunomodulation might be DP3-sized.

Previous studies investigated the involvement of galectins in general and galectin-9 in particular in supporting the regulatory-type Th1 immunomodulatory effects by the blocking of galectins, which resulted in the suppression of regulatory-type Th1 immune effects [31]. Furthermore, stimulation of αCD3/CD28-activated PBMC with recombinant galectin-9 enhanced IFNγ and IL-10 secretion and increased the percentage of Th1 and regulatory T-cells [10], which reinforces the role of galectin-9 as a key immune regulator.

The TLR9 agonist CpG used to mimic a bacterial trigger (bacterial DNA) in the IEC/PBMC co-culture model was required to support the immunomodulatory effects of NDO. Although TLR9 is mostly described as an endosomal receptor, previous studies have observed a surface expression of TLR9 in IEC [32,38–40]. Even though combined exposure to CpG and GOS DP3-5 resulted in increased galectin-9 secretion, the addition of GOS DP3-5 did not result in the further upregulation in galectin-9 mRNA in the IEC at 24 h or at 48 h after apical CpG exposure. This suggests that CpG can regulate galectin-9 expression at the level of gene transcription, while the oligosaccharides facilitate the basolateral release of galectin-9, and thereby support mucosal immunomodulation and/or development.

As GOS DP3-5 showed the most potent immunomodulatory activity out of the DP fractions tested, and this effect was mimicked to some extent by GOS DP3, we further investigated the capacity of a DP3-sized NDO, namely β-3'GL, in promoting immunomodulatory effects. A recent study determined the presence of low concentrations of β-3'GL in human milk samples [36]. Furthermore, β-3'GL is present in the GOS DP3 mixture (**Figure 7A**) [14]. Thus, we further studied the structure-specific effects of β-3'GL compared to GOS DP3-5 in combination with CpG in the IEC/PBMC model. Increasing the concentration of β-3'GL to 0.5% (*w/v*) supported a Th1-type regulatory immune response, as shown by increased IFNγ and galectin-9 or IL-10 secretion on top of CpG alone, which is in line with previous studies describing the immunomodulatory effects of other NDO [29–31, 34]. In those studies, similar immune polarization profiles for GF and 2'-fucosyllactose (2'FL), a NDO abundantly present in human milk, were also shown in the presence of CpG [29, 31], suggesting that this type of immunomodulation may be relevant for immune development [1]. Similar to 2'FL, β-3'GL may be an important NDO structure present in human milk, capable of supporting mucosal immune development driven by microbial signals (such as bacterial CpG DNA) in early life [29].

Due to the influence of IEC-derived galectins in supporting the immunomodulatory effects boosted by NDO and CpG described in previous studies [29–31, 34], we studied IEC-derived galectin secretion. Only β-3'GL in the presence of CpG boosted the secretion of IEC-derived galectin-3, -4, and -9. However, only IEC-derived galectin-9 secretion was upregulated by DP3-5 and CpG. Besides, only IEC-derived galectin-9, but not IEC-derived galectin-3 or -4, correlated to IL-10 and galectin-9 secretion in the IEC/PBMC co-culture, which indicates that IEC-derived galectin-9 supports the regulatory-type immunity. Other studies have confirmed that incubation of activated PBMC with recombinant human galectin-9 was able to enhance not only IFNγ secretion, but also regulatory IL-10 release [10]. However, beyond galectin-9, epithelial-derived galectin-3 and -4 were also found to significantly correlate positively with IFNγ concentrations. This suggests that all IEC-derived galectins might have been involved in increasing the IFNγ release by the activated PBMC under β-3'GL and CpG-exposed conditions. These results emphasize the need to better understand the complexity involved within the mucosal interactions between IEC and immune cells, as well as the role of galectins in these processes.

The IEC/PBMC model used was set-up to mimic the epithelial cell and immune cell crosstalk representing the intestinal mucosa. The model makes use of PBMC instead of lamina propria mononuclear cells (LPMC), because LPMC isolation requires access to clinical bowel samples and a laborious isolation procedure [41]. LPMC do show

similarities as well as differences in the composition and function when compared to PBMC [42, 43]. However, mitogen stimulation or activation via the T-cell receptor in both PBMC and LPMC results in induced levels of IFNγ, IL-10, and TNFα [41–43]. Furthermore, the results obtained from the IEC/PBMC model used were validated using *in vivo* animal models for food allergy [10, 11], and in clinical samples of a NDO dietary intervention study, serum galectin-9 levels were shown to be enhanced [10].

The high dose of CpG was found to enhance the transcription of galectin-9; however, this was not further modified by GOS DP3-5, while it was capable of enhancing galectin-9 secretion. In addition, the effects of β-3'GL on epithelial galectin release could not be explained by increased galectin mRNA expression. Little is known about the factors inducing the transcription of galectins, as well as their intracellular storage. Galectins are widely known for their ability to recognize and bind extracellular carbohydrates with high affinity for β-galactoside structures such as NDO. Several studies have described the affinity of specific NDO structures to galectins according to their chemical structure and observed how chemical modifications such as fucosylation or galactosylation, among others, can improve the binding affinity of galectins to specific NDO structures. The increased binding affinity might, in turn, result in improved biological functions of NDO such as raft formation or attachment to pathogenic bacteria [44–47]. Although further research is needed, recent studies have proposed that changes in O-GlcNAcylation might be involved in regulating galectin expression [48].

To summarize, GOS DP3-5 in the presence of CpG, a TLR9 agonist mimicking a bacterial trigger, was able to promote immunomodulatory effects by enhancing immune responses in association with modified epithelial-derived galectin secretion. Moreover, exposure to DP3-sized β-3'GL, a NDO present in human milk, was most effective in enhancing CpG-induced galectin-9 release, while also enhancing galectin-3 and -4 secretion, which correlated with the instruction of a regulatory-type Th1 response. The most relevant findings described in this manuscript are summarized in **Figure 7B**. The use of PBMC might not fully resemble the immune cell populations present in the lamina propria and, therefore, the immune responses might differ from those shown in this manuscript. However, previous studies have shown the predictive value of the IEC/PBMC model *in vivo* in a murine model for food allergy, by identifying dietary interventions with immunomodulatory properties in which galectin-9 had a key role [10]. This emphasizes the translational value of the IEC/PBMC co-culture model. Our aim is to use the IEC/PBMC co-culture model as a first step to identify relevant bioactive components that should later be studied using more complex models. In addition to this, by the use of these models combining epithelial and immune cells, we aim to better

understand the complex interactions occurring between these types of cells, which further contribute to our understanding.

In conclusion, epithelial-derived galectins were demonstrated to be key players in the mucosal immune development supported by GOS of which DP3-size β-3'GL showed relevant immunomodulatory properties.

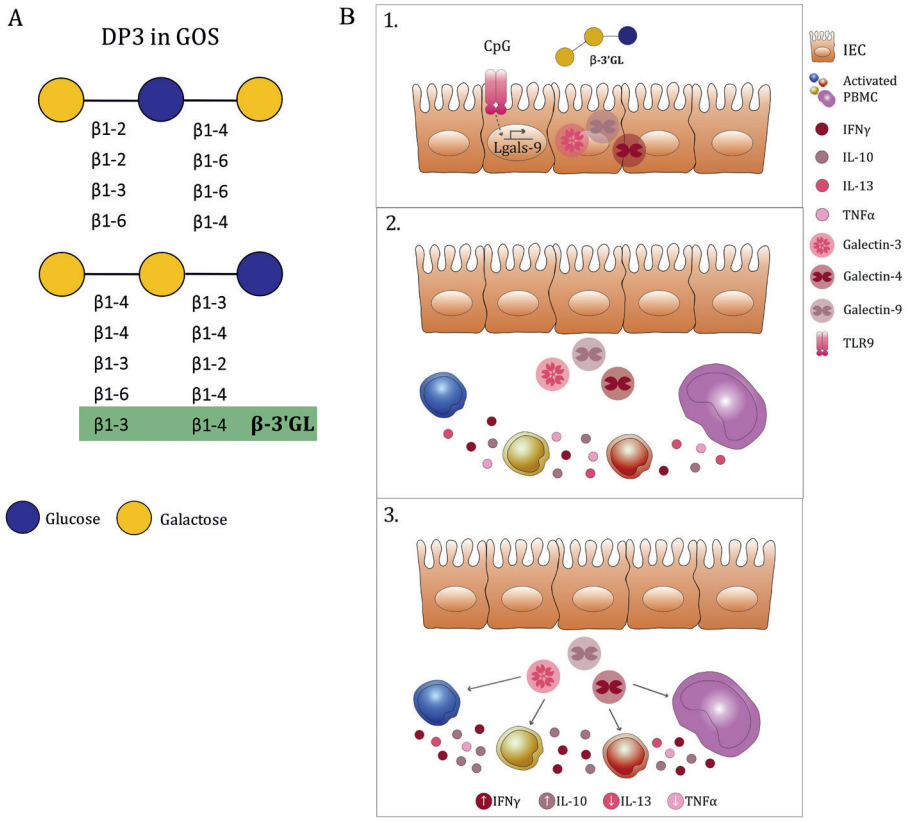

Figure 7. DP3-sized oligomers in GOS and description of the effects by β-3'GL and CpG in the IEC/PBMC co-culture model.
DP3-sized structures present in GOS including β-3'GL are shown in (**A**). A summary of the effects observed in the IEC/PBMC co-culture model upon combined exposure to β-3'GL and CpG is shown in (**B**). Galectins are synthesized in the cytosol of IEC upon exposure to β-3'GL and CpG. Additionally, an upregulation of galectin-9 mRNA expression might be promoted by CpG (1). Galectins are then pushed out from the IEC toward the underlying immune compartment (2). Upon inflammatory conditions, defined by the activation of PBMC using αCD3/CD28, the immune cells in the basolateral compartment representing the lamina propria produce cytokines (2). Combined exposure to β-3'GL and CpG enhances IEC-derived galectin-3, -4, and -9 secretion. The IEC-derived galectins modulate the cytokine secretion by upregulating IFNγ and IL-10 while downregulating IL-13 and TNFα secretion (3).

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Abbreviations: β-3'GL: β-3'galactosyllactose; **2'FL**: 2'-fucosyllactose; **cDNA**: complementary DNA, **ESI-MS**: electrospray ionization-mass spectrometry; **ELISA**: enzyme-linked immunosorbent assay; **FCS**: fetal calf serum; **FOS**: fructo-oligosaccharides; **GOS**: galacto-oligosaccharides; **IEC**: intestinal epithelial cells; **LPMC**: lamina propria mononuclear cells; **NDO**: non-digestible oligosaccharides; **ODN**: oligodeoxynucleotides; **PBMC**: peripheral blood mononuclear cells; **qPCR**: quantitative Polymerase Chain Reaction.

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SUPPLEMENTARY FIGURES

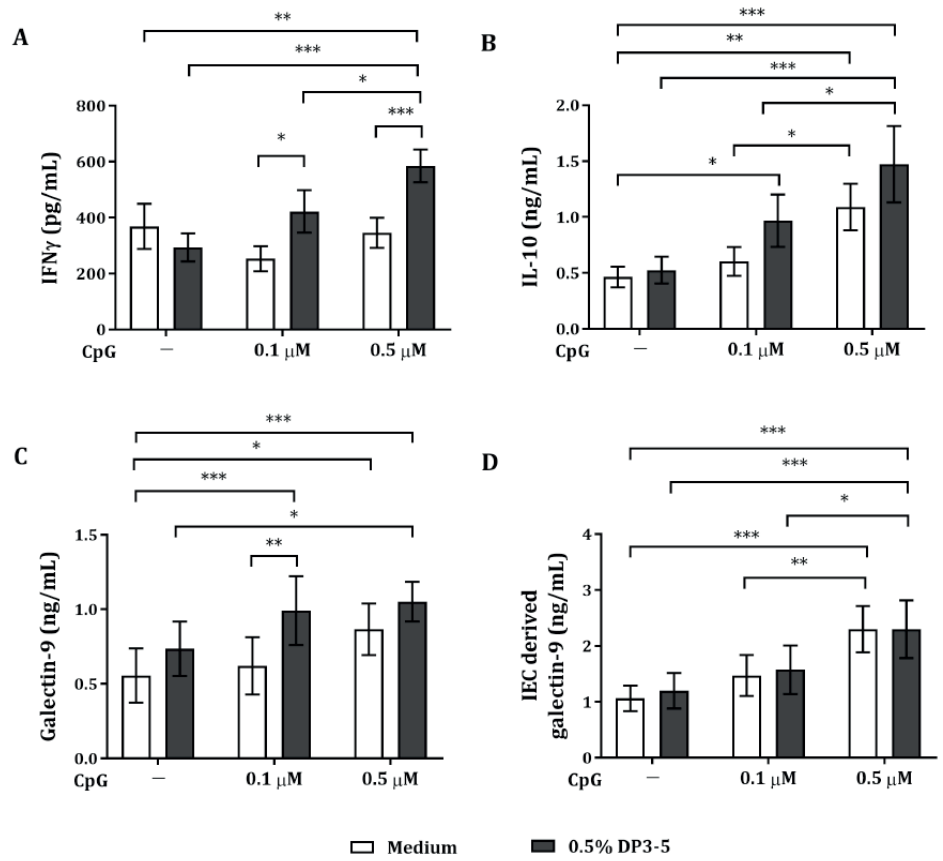
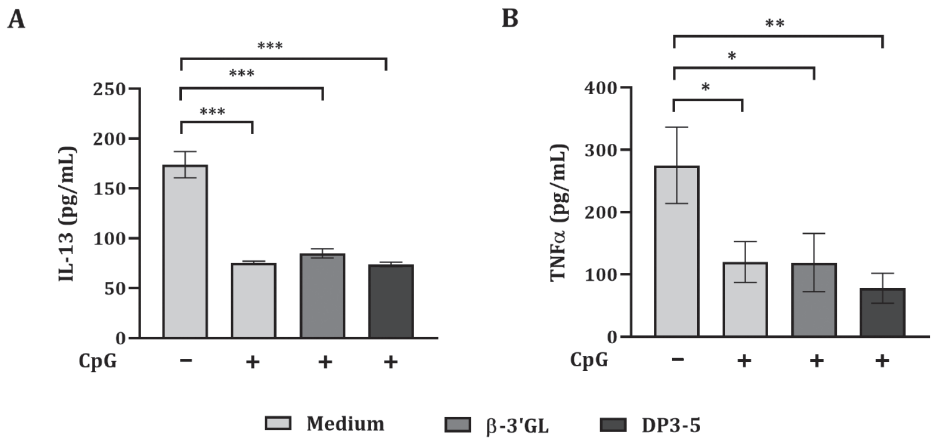


Figure S1. Lower CpG concentrations effectively supported immunomodulatory effects by GOS DP3-5.
IEC were apically exposed to 0.5% GOS DP3-5 (*w/v*) in combination with 0.1 or 0.5 μM CpG and basolaterally to αCD3/CD28-activated PBMC. After 24 h incubation, IFNγ (**A**), IL-10 (**B**) and galectin-9 (**C**) were measured in the basolateral supernatant. After IEC/PBMC co-culture, IEC were washed and incubated in fresh medium for additional 24 h after which IEC-derived galectin-9 (**D**) was measured. Data represents mean ± SEM of $n = 6$ independent PBMC donors ($n = 5$ for IFNγ) (* $p < 0.05$. ** $p < 0.01$, *** $p < 0.001$).



C

	IEC-derived galectin-3	IEC-derived galectin-4	IEC-derived galectin-9
TNFα	n.s. r = 0.32 p = 0.06	n.s. r = 0.32 p = 0.06	n.s. r = - 0.009 p = 0.96
IL-13	- r = - 0.42 p = 0.04	n.s. r = - 0.34 p = 0.1	- r = - 0.54 p = 0.007

(-) Negative correlation; (n.s.) non-significant correlation

Figure S2. Cytokine secretion in IEC/PBMC co-culture model.
IEC were apically exposed to GOS DP3-5 or β-3'GL (0.5% *w/v*) in combination with 0.1 μM CpG, and basolaterally to αCD3/CD28-activated PBMC. After 24 h incubation, TNFα (**A**) and IL-13 (**B**) were measured in the basolateral compartment. The data shown are represented as mean ± SEM of $n = 4$ independent PBMC donors for IL-13 and $n = 6$ for TNFα. Additionally, IL-13 and TNFα secretion was correlated using Pearson correlation to IEC-derived galectins as shown in table (**C**) (* $p < 0.05$. ** $p < 0.01$, *** $p < 0.001$). Negative correlations are represented as (-) and (n.s.) represents non-significant correlations.

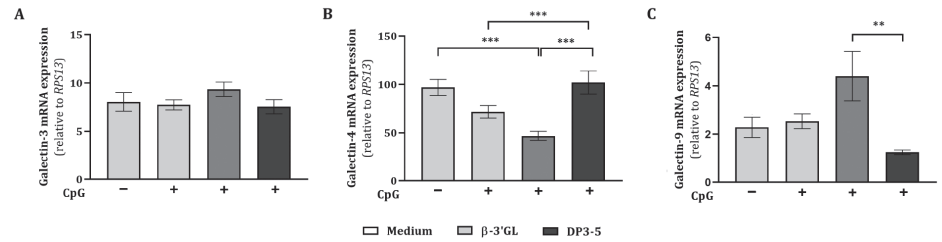


Figure S3. Galectin mRNA expression.
After IEC/PBMC co-culture, IEC were separated from the PBMC fraction, washed and cultured in fresh medium for 24 h after which IEC were collected and the relative mRNA expression of galectin-3 (**A**), -4 (**B**) and -9 (**C**) was measured. Data represent mean ± SEM of $n = 6$ independent PBMC donors (** $p < 0.01$, *** $p < 0.001$).

CHAPTER | 5

Exposure of intestinal epithelial cells to 2’-fucosyllactose and CpG enhances galectin release and instructs dendritic cells to drive Th1 and regulatory-type immune development

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ABSTRACT

Intestinal epithelial cells (IEC) release immunomodulatory galectins upon exposure to CpG DNA (mimicking bacterial triggers) and short-chain galacto- and long-chain fructo-oligosaccharides (GF). This study aims to investigate the immunomodulatory properties of 2′-fucosyllactose (2′FL), a non-digestible oligosaccharide (NDO) abundantly present in human milk, using a co-culture model developed to study the crosstalk between IEC and innate and adaptive immune cells. IECs, co-cultured with αCD3/CD28-activated peripheral blood mononuclear cells (PBMC), were apically exposed to NDOs and CpG, washed and co-cultured with immature monocyte-derived dendritic cells (moDC). Subsequently, moDC were co-cultured with naïve $CD4^+$ T-cells. In the presence of CpG, both 2′FL or GF-exposed IEC enhanced Th1-type IFNγ and regulatory IL-10 secretion of PBMCs, compared to CpG alone, while Th2-type IL-13 was reduced. Both NDOs increased IEC-derived galectin-3, -4, -9 and TGFβ1 of CpG-exposed IEC. Only galectin-9 correlated with all modified immune parameters and TGFβ1 secretion. MoDCs exposed to 2′FL and CpG-conditioned IEC instructed IFNγ and IL-10 secretion by $CD4^+$ T-cells, suggesting the development of a regulatory Th1 response. These results reveal that 2′FL and GF could contribute to the mucosal immune development by supporting the effect of microbial CpG DNA associated with the modulation of epithelial galectin and TGFβ1 secretion.

Keywords: 2′-fucosyllactose; non-digestible oligosaccharides; galectins; intestinal epithelial cells; dendritic cells; mucosal immunity

INTRODUCTION

Non-digestible oligosaccharides (NDO) are abundantly present in human milk and consist of complex and diverse structures which vary during the course of lactation [1]. Over 160 different NDOs have been characterized so far, out of which 2′-fucosyllactose (2′FL) is the most abundant in human milk of secretor-positive lactating women [2–4]. Up to 1% of NDO are absorbed and found in the systemic circulation [5] indicating that NDOs are able to interact with the immune cells present in circulation and thus, promote systemic effects. Infants fed formula supplemented with galacto-oligosaccharides and 2′FL showed a lower inflammatory cytokine profile in serum, similar to the profile seen in breastfed infants [6]. Furthermore, 2′FL was shown to support the maturation of intestinal epithelial cells (IEC) [7, 8] as well as promoting immunomodulation through the interaction with immune cells [9–12].

The crosstalk between IEC, migratory dendritic cells (DC) and the resident immune cells is key to maintain the intestinal mucosal homeostasis and develop appropriate immune responses [13]. The migratory DC take up available antigens and travel to the mesenteric lymph nodes, where they can instruct naïve T-cells to develop into regulatory T-cells or effector T-cells which home back to the lamina propria via the bloodstream [14, 15]. The function of DC can be modified by epithelial cell-derived mediators such as galectins or TGFβ [16–18]. Galectins are immunomodulatory glycan binding proteins highly expressed and secreted by epithelial cells [16, 19] and thought to play a key role in infant immunity due to their ability to bind NDOs present in human milk [3]. Meanwhile, TGFβ is known for its contribution in sustaining immune homeostasis and mucosal protection [20, 21], and can act in conjunction with galectins [22–25]. Epithelial release of these mediators may therefore affect both innate and adaptive mucosal immune functions. Hence, using dietary interventions to target IEC might be of interest to instruct immune development in the gastrointestinal tract.

Specific NDOs derived from milk or plant sources such as a 9:1 mixture of short-chain galacto- and long-chain fructo-oligosaccharides (GF) and *Bifidobacterium breve* M-16V were shown to reduce the development of allergic symptoms in mice by increasing galectin-9 levels locally, in the gastrointestinal tract, as well as systemically [26]. In addition, combined exposure to GF and synthetic CpG DNA or the CpG DNA derived from *Bifidobacterium breve* M-16V (TLR9 agonists), resulted in increased IFNγ and IL-10 secretion in an IEC/peripheral blood mononuclear cell (PBMC) *in vitro* co-culture model [27, 28]. These studies corroborate the ability of NDO in enhancing CpG induced immunomodulation as well as revealing the involvement of galectins in promoting such effects.

The aim of this study was to investigate the immunomodulatory effects elicited by 2′FL and CpG-exposed IEC in a transwell IEC/PBMC co-culture model. Additionally, the crosstalk between IEC and monocyte-derived DC (moDC) was studied, followed by additional *in vitro* models to investigate the functional interaction of IEC-imprinted moDC with naïve $CD4^+$ T-cells. Particularly, the association of epithelial-derived galectins and TGFβ1 secretion was analyzed regarding its contribution in the immune development.

MATERIALS AND METHODS

Culture of intestinal epithelial cells

Human colon adenocarcinoma HT-29 cell line (ATCC, HTB-38) was used as IEC. The cells were cultured in 75 cm^2 culture flasks (Greiner Bio-One) using McCoy 5A medium (Gibco) supplemented with 10% heat inactivated fetal calf serum (FCS), penicillin (100 U/mL) and streptomycin (100 µg/mL) (Sigma-Aldrich). IEC were kept incubating at 37°C and 5% CO_2. The medium was refreshed every 2–3 days.

Peripheral blood mononuclear cell purification

Human PBMCs were isolated from buffy coats from healthy donors (Sanquin, Amsterdam, The Netherlands) by density gradient centrifugation (1,000 x g, 13 min), and washed with PBS (Lonza) supplemented with 2% FCS. Red blood cell lysis buffer was used to lyse the remaining erythrocytes (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). The isolated PBMC were resuspended in RPMI 1640 supplemented with 2.5% FCS, penicillin (100 U/mL) and streptomycin (100 µg/mL).

Culture of immature monocyte-derived dendritic cells

$CD14^+$ cells were isolated from PBMC according to the manufacturer's protocol by cell separation using a negative selection MACS kit (Miltenyi Biotec). $CD14^+$ cells were cultured for 7 days in RPMI 1640 medium supplemented with 10% FCS, penicillin (100 U/mL) and streptomycin (100 µg/mL) (Sigma-Aldrich), IL-4 (30–100 ng/mL) and GM-CSF (15–60 ng/mL) (both from Prospec). The medium was refreshed on days 2, 3 and 6 of culture. At day 7, immature moDC were collected.

IEC/PBMC and IEC/moDC co-culture model description

IEC were diluted 5 to 10 times based on surface area and seeded in 12-well transwell inserts (Costar Corning Incorporated) one week prior to the experiments. IEC were incubated at 37 °C, 5% CO_2 and the medium was refreshed every 2–3 days. Confluent IEC monolayers were used to perform co-culture experiments.

IEC/PBMC model description

IEC were basolaterally exposed to PBMC from healthy donors (2 x 10^6 cells/mL) either activated with αCD3 and αCD28 (clone CLB-T3/2 and clone CLB-CD28 respectively, both 1:10.000, Sanquin) or non-activated. 2'FL or GF solutions (0.25-1% *w/v*; 2.5–10 mg/mL) either alone or in combination with CpG (0.5 µM CpG oligodeoxynucleotide M362 type C, Invivogen) were added apically (**Figure 1A**). After 24 h IEC/PBMC incubation, basolateral supernatant was collected and stored at -20°C for cytokine secretion analysis.

2'FL produced by microbial fermentation with >90% purity may contain traces of glucose, fucose, lactose, 3-FL, difucosyllactose, and water. GF is composed of a 9:1 mixture of short-chain galacto- and long-chain fructo-oligosaccharides. Galacto-oligosaccharides are obtained from lactose by enzymatic transglycosylation, while fructo-oligosaccharides are obtained from plant sources derived from inulin-type fructans. In order to evaluate the purity of 2'FL and GF an endotoxin level assessment was performed by loading 25 µL of 1% NDO solution into an Endosafe® cartridge, which was measured using an Endosafe® Portable Test System (PTS) (Charles River Laboratories). Endotoxin levels from NDO were compared to a known LPS concentration (*E. coli* O111:B4, Invivogen). A concentration of 0.1 µg/mL LPS showed an equivalent of 0.76 EU/mL measured using the Endosafe® test, while GF and 2'FL gave 0.44 EU/mL and 0.88 EU/mL, respectively.

IEC/moDC co-culture model description

Subsequent to IEC/PBMC co-culture, IEC cell monolayers were washed with PBS (Lonza) and set apart in a new plate in the absence of PBMC for an additional 24 h (**Figure 1B**). After that, IEC-derived galectin-3, -4, -9 and TGFβ1 secretion was analyzed in the basolateral compartment. Alternatively, after IEC/PBMC co-culture the IEC cell monolayers were washed with PBS and co-cultured with immature moDC for 48 h in RPMI 1640 (Lonza) supplemented with 10% FCS, penicillin (100 U/mL) and streptomycin (100 µg/mL) (**Figure 1C**). After 48 h, the conditioned immature moDC (ccDC) were collected and their phenotype was studied. Additionally, the supernatant was collected and stored at -20°C for cytokine secretion analysis.

DC/T-cell co-culture model description to study the moDC function

$CD4^+CD45RA^+$ naïve T-cells were isolated from PBMC by negative selection using MACS separation kit, following the manufacturer's protocol (Miltenyi Biotec), and resuspended in IMDM medium supplemented with 10% FCS, 20 µg/mL apotransferrine (Sigma-Aldrich), 50 µM β-mercaptoethanol (Sigma-Aldrich), penicillin (100 U/mL) and streptomycin (100 µg/mL). Naïve T-cells (1 x 10^6) were co-cultured with ccDC (0.1 x 10^6) from IEC/moDC culture, after IEC/PBMC exposure, in 24 well flat-bottom plates for 5

to 6 days in the presence of 1 ng/mL TGFβ (Prospec) (**Figure 1D**). After incubation, the supernatant was collected and stored at -20 °C for cytokine analysis. After the ccDC/T-cell co-culture, over 90% of the $CD4^+$ T-cell were viable. The viability was not affected by exposure to NDO and/or CpG.

Enzyme-linked immunosorbent assay (ELISA)

Supernatants from IEC/PBMC, IEC/moDC and ccDC/T-cell co-cultures were analyzed for cytokine and mediator secretion. Commercially available kits were used to determine IFNγ, IL-13, IL-17A (Thermo Fisher scientific), IL-10 (U-Cytech), galectin-3, -4, -9 (R&D systems) and IL-5 (Biolegend) secretion according to the manufacturer's protocol. Human galectin-4 or -9 were measured using antibody pairs (R&D systems). In short, high-binding Costar 9018 plates were incubated overnight at 4 °C with 0.75 µg/mL human galectin-4 or -9 affinity-purified polyclonal antibody. Non-specific binding was blocked with 1% BSA in PBS for 1 h, after which samples were incubated for 2 h at room temperature. After washing, biotinylated galectin-4 or -9 affinity-purified polyclonal antibodies (0.75 µg/mL) were added and incubated for 1 h. Then, plates were washed and streptavidin-HRP (R&D systems) was added and incubated for 1 h. After washing, tetramethylbenzidine was used as a substrate to develop the reaction (TMB, Thermo Fisher scientific), which was stopped with 1 M H_2SO_4. Optical density was measured at 450 nm.

Flow cytometry analysis

After IEC/moDC co-culture, ccDC were collected and stained for flow cytometry analysis using CD11c-PerCP eFluor 710 (clone 3.9), CD14-APC (clone 61D3), HLA-DR-PE (clone LN3), CD80-FITC (clone 2D10.4) and CD86-PE Cyanine 7 (clone IT2.2) (all from eBioscience). Viability was determined using Fixable Viability Dye 780-APC Cyanine 7 (eBioscience). Non-specific binding sites were blocked using PBS supplemented with 5% FCS before extracellular antibody staining. Flow cytometry measurements were done using BD FACS Canto II (Becton Dickinson) and data were analyzed using Flowlogic software version 7 (Inivai Technologies).

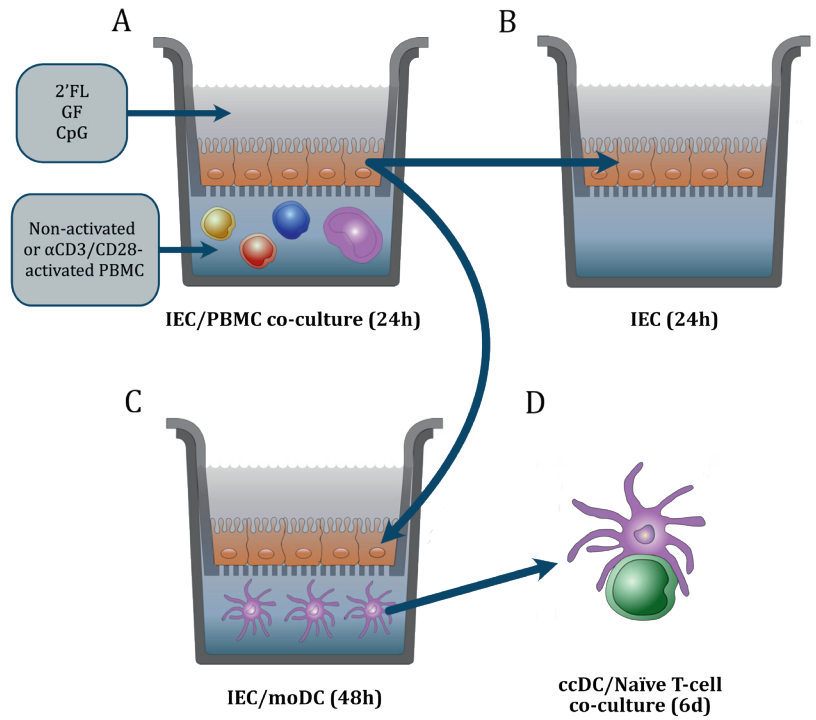

Figure 1. Co-culture model description.
IEC were grown in 12-well transwell inserts until confluency and basolaterally exposed to either non-activated or αCD3/CD28-activated PBMC. Apically, IEC were conditioned with 2'FL or GF in the presence or absence of CpG, a TLR9 agonist mimicking a bacterial trigger (**A**). After 24 h incubation, basolateral supernatant was collected to analyze the T-cell mediator release. The IEC were set apart and washed with PBS. Then, fresh medium was added and IEC were kept in incubation for an additional 24 h to study the IEC-derived mediator release (**B**). Alternatively, IEC were washed with PBS and co-cultured with immature moDC for 48 h (**C**). Then, the basolateral supernatant was collected where the mediator release was studied. Additionally, the phenotype of moDC after IEC/moDC co-culture was analyzed. Subsequently, conditioned moDC (ccDC) were exposed to naïve T-cells in an allogeneic DC/T-cell assay (**D**). After 5–6 days incubation, the cytokine release was measured in the supernatant.

Statistical analysis

Data were analyzed using Graphpad Prism 7 software. Data were analyzed using one-way or two-way ANOVA followed by Bonferroni's multiple comparison post hoc test on selected pairs. When data were not normally distributed, square root or logarithm transformation was applied prior to ANOVA analysis. In order to determine the strength of the association between specific mediators released, the Spearman's rank correlation was applied. Data are represented as mean ± SEM of 6 to 12 independent PBMC donors. *p* values below 0.05 were considered of statistical significance.

RESULTS

2'FL enhances the cytokine release of activated-PBMC when apically exposed to IEC, in the presence of CpG

To investigate the immunomodulatory effects of 2'FL and GF in the presence or absence of CpG under homeostatic or inflammatory conditions, IEC were apically exposed to NDO and basolaterally co-cultured with non-activated or αCD3/CD28-activated PBMC for 24 h (**Figure 1A**). Culture of IEC with non-activated PBMC in the presence or absence of 2'FL, GF and/or CpG did not promote the release of cytokines (**Figure 2**). Meanwhile, activation of PBMC with αCD3/CD28 resulted in increased IFNγ, IL-10 and IL-13 cytokine concentrations in the IEC/PBMC co-culture. These cytokines were not affected by exposure of IEC to 2'FL, GF or CpG alone. However, upon apical exposure to 2'FL or GF combined with CpG, IFNγ and IL-10 cytokine release was increased and IL-13 decreased, as compared to the medium control (**Figure 2**).

These results indicate that the immunomodulatory effects of the NDO described are exclusively elicited in the presence of an inflammatory milieu and upon availability of CpG, a TLR9 ligand representing bacterial CpG DNA. Hence, NDOs act synergistically with CpG to promote the immunomodulatory effects. Therefore, the following studies were performed using only αCD3/CD28-activated condition in IEC/PBMC co-culture.

Dose-dependent Th1 and regulatory-type immune polarizing effects of NDOs and CpG in the IEC/PBMC co-culture

Using the IEC/PBMC model (**Figure 1A**) we further studied whether 2'FL has similar immunomodulatory properties as GF and the optimal dose at which these effects are elicited was established. Hence, dose-response studies were performed using CpG in combination with 0.25%, 0.5% and 1% NDO apically in the IEC/PBMC co-culture with αCD3/CD28-activated PBMC.

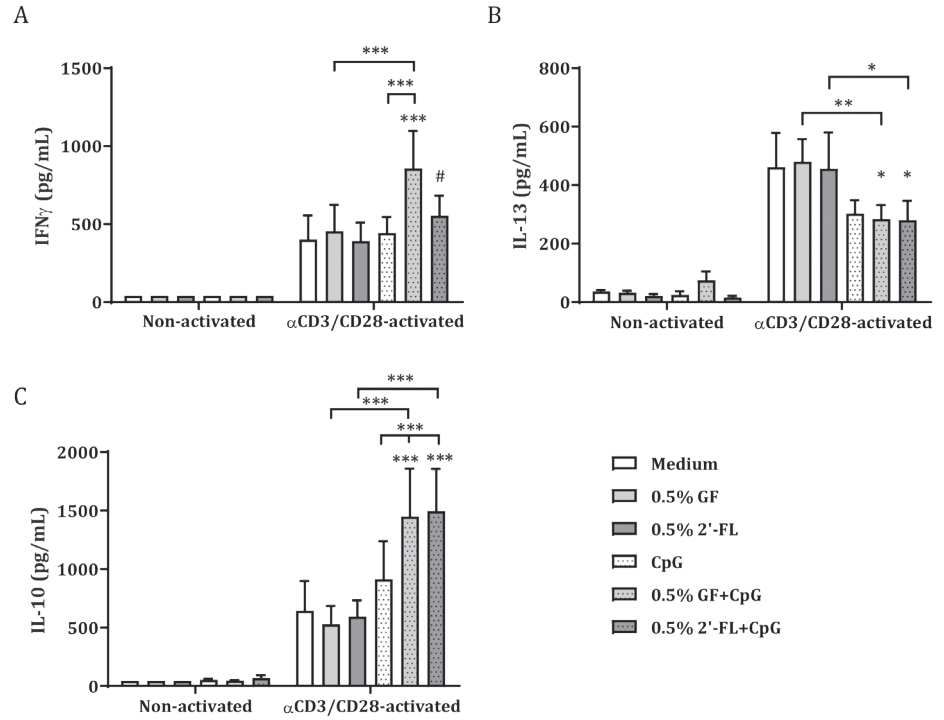

Figure 2. Cytokine secretion in IEC/PBMC co-culture after exposure to non-activated or αCD3/CD28-activated PBMC.
IEC were basolaterally co-cultured with either αCD3/CD28-activated or non-activated PBMC for 24 h. Apically, IEC were exposed to 2'FL or GF alone or in combination with CpG, a TLR9 agonist mimicking a bacterial trigger (**Figure 1A**). IFNγ (**A**), IL-13 (**B**) and IL-10 (**C**) concentrations were measured in the basolateral supernatant after IEC/PBMC co-culture. Data are represented as mean ± SEM of $n = 6$ independent PBMC donors. Two-way ANOVA and Bonferroni's post-hoc tests were used to analyze statistical differences. Square root transformation was performed when data did not fit normal distribution (# $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Upon exposure of IEC to CpG alone, IL-13 concentrations were decreased, and IL-10 concentrations were increased as compared to medium control. IFNγ and galectin-9 concentrations were not affected (**Figure 3**). Combined exposure to 0.5% or higher concentrations of 2'FL or GF with CpG resulted in significantly increased IFNγ concentrations compared to CpG alone, while the 0.25% dose did not show this effect. NDO, at a concentration of 0.25%, was however able to further increase IL-10 and tended to further reduce IL-13 concentrations ($p = 0.08$) compared to CpG alone, in IEC/PBMC co-culture (**Figure 3**). Only 1% 2'FL, but not GF, combined with CpG increased galectin-9 concentrations in IEC/PBMC as compared to the medium. Exposure to 1% 2'FL resulted in significantly increased galectin-9 concentrations compared to

CpG alone and compared to the combination of CpG with lower 2'FL concentrations. Galectin-9 release was positively correlated with IFNγ (r = 0.52, $p < 0.0001$) release and negatively with IL-10 (r = -0.38, $p = 0.002$) and IL-13 (r = -0.28, $p = 0.036$) secretion in IEC/PBMC co-culture (**Figure S1**). IL-5, a Th2 cytokine, showed similar results as IL-13, while no differences were found for IL-17A or TNFα concentrations (**Figure S1**).

Galectins and TGFβ1 release by IEC obtained from IEC/PBMC co-culture

To study the contribution of IEC to the immunomodulatory effects described in the IEC/PBMC model, conditioned epithelial cells derived from the co-culture model were washed and incubated with fresh medium for an additional 24 h (**Figure 1B**). Galectin-3, -4, -9 and TGFβ1 were measured in the basolateral compartment to study how 2'FL and GF influence epithelial cell mediator release of CpG-exposed IEC in IEC/PBMC co-culture.

CpG did not affect IEC-derived galectin-3, -4 or -9 release but lowered TGFβ1 concentrations (**Figure 4**). In the presence of CpG, the highest dose of both NDOs (1% *w/v*) significantly increased galectin-3, -4 as well as -9 release from IEC, compared to CpG-exposed IEC (1% 2'FL showed only a trend for galectin-3 release, $p = 0.08$). Exposure to 0.5% 2'FL or GF in combination with CpG also resulted in a significant increase in TGFβ1 release compared to CpG alone. Galectin-3, -4 and/or TGFβ1 concentrations were increased after combined exposure to CpG and 1% 2'FL or GF as compared to 0.5% or 0.25% NDO (**Figure 4**). IEC-derived galectin-9 but not TGFβ1, correlated with galectin-3 (r = 0.4, $p = 0.004$) as well as with galectin-4 (r = 0.5, $p = 0.0003$) (**Figure S2**).

IEC-derived galectin-9 was positively correlated to IL-10 (r = 0.6, $p < 0.0001$), IFNγ (r = 0.3, $p = 0.03$) and IEC-derived TGFβ1 (r = 0.5, $p < 0.0001$), while being negatively correlated to IL-13 (r= -0.5, $p = 0.0007$), measured in the IEC/PBMC co-culture (**Figure 5**).

Beyond galectin-9, other epithelial-derived galectins were also found to correlate with the immune mediator production in the IEC/PBMC co-culture. Galectin-3 showed a strong positive correlation to IFNγ (r = 0.6, $p < 0.0001$) and a less strong negative correlation to IL-13 (r = –0.4, $p = 0.01$), while no correlation was found with IL-10 (r = 0.08, $p = 0.6$) (**Figure S3**). Meanwhile, galectin-4 concentrations were significantly correlated to IL-10 (r = 0.3, $p = 0.02$) but not to IFNγ (r = 0.1, $p = 0.3$) or IL-13 (r = –0.04, $p = 0.8$) (**Figure S3**).

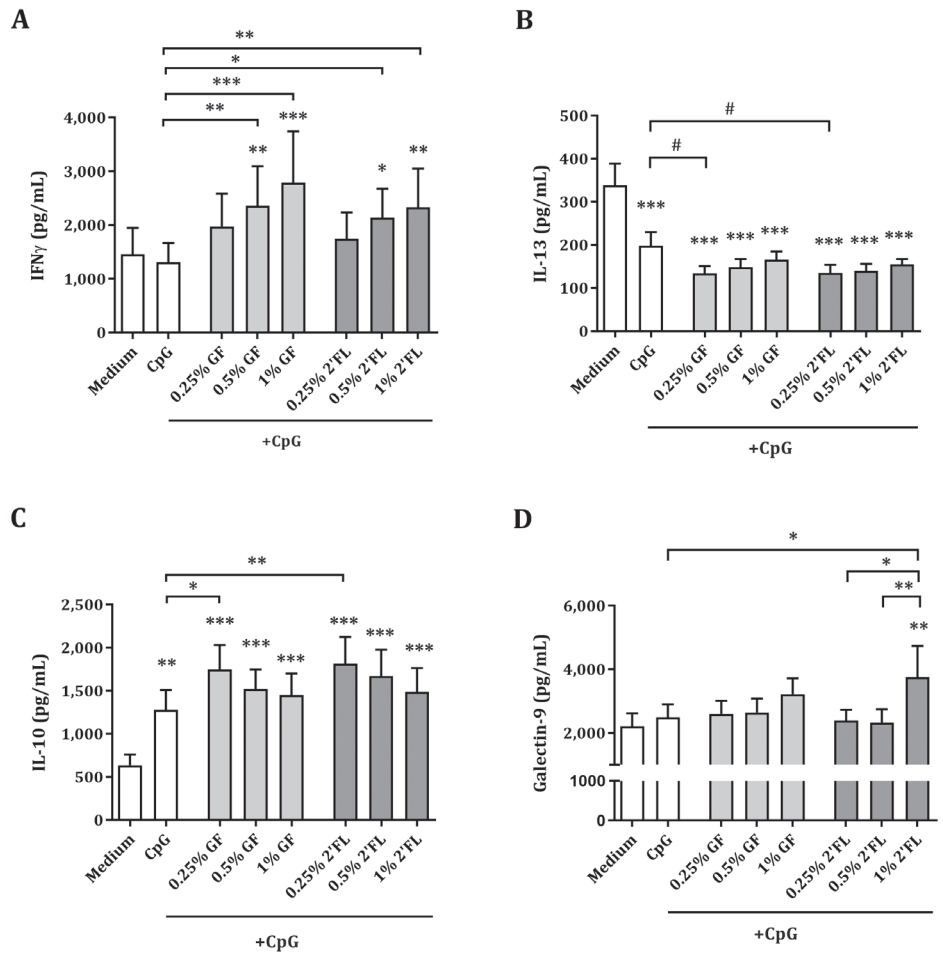

Figure 3. Cytokine and mediator secretion in IEC/PBMC co-culture.
IEC were basolaterally exposed to αCD3/CD28-activated PBMC and apically to 0.25–1% NDO (2'FL or GF) in combination with CpG (**Figure 1A**). After 24 h incubation, IFNγ (**A**), IL-13 (**B**), IL-10 (**C**) and galectin-9 (**D**) concentrations were measured in the basolateral supernatant. Data are represented as mean ± SEM of 7–8 independent PBMC donors (# $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

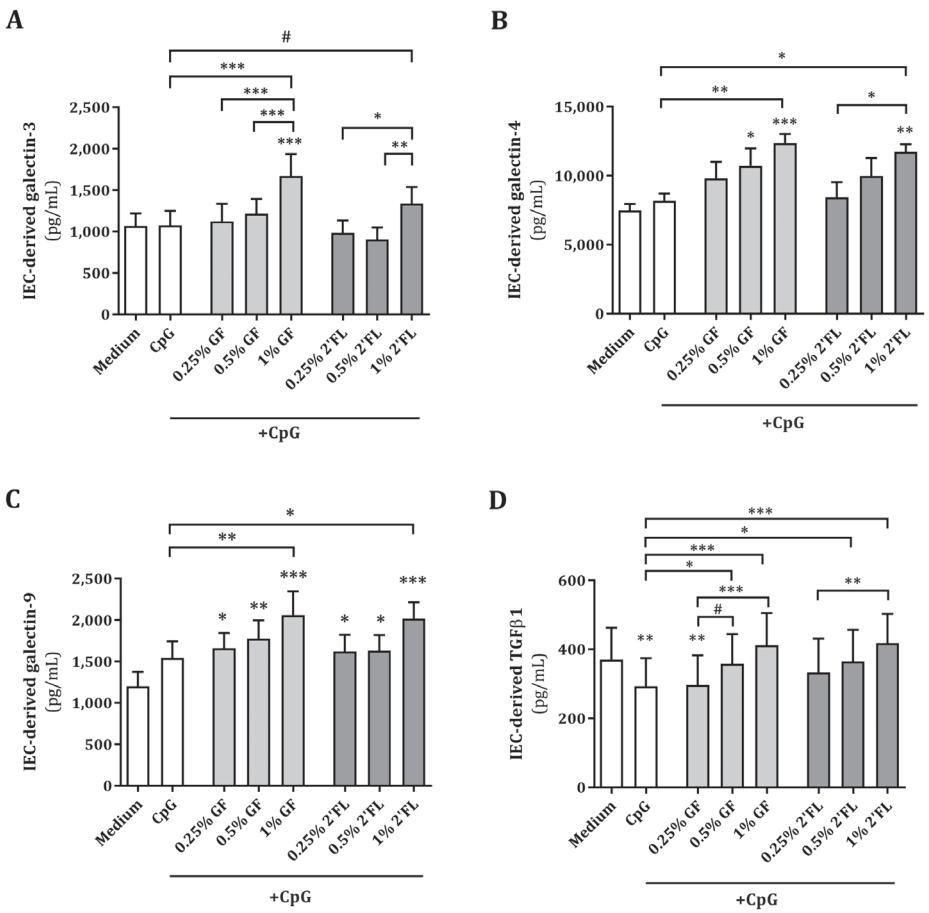

Figure 4. IEC-derived galectins and TGFβ1 secretion.
IEC were washed after IEC/PBMC co-culture and with fresh medium for an additional 24 h (**Figure 1B**). After the incubation period, IEC-derived galectin-3 (**A**), galectin-4 (**B**), galectin-9 (**C**) and TGFβ1 (**D**) were measured in supernatant. Data are represented as mean ± SEM of 6–8 independent PBMC donors (# $p < 0.1$,* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Variations in the expression of CD80 in moDC after co-culture with conditioned IEC

Subsequent to IEC/PBMC co-culture, the αCD3/CD28-activated PBMC were removed, and the conditioned IEC were washed and co-cultured with immature moDC in fresh medium for 48 h (**Figure 1C**). After this incubation, the phenotype of the ccDC was studied (**Figure 6A**). No significant differences were observed in the percentage of live cells, $CD11c^+HLA\text{-}DR^+$ cell populations or $CD86^+$ expression by IEC-conditioned moDC from the IEC/moDC co-culture (**Figure 6B**). ccDC conditioned with IEC that

were exposed to the combination of GF and CpG in the IEC/PBMC co-culture showed significantly decreased expression of $CD80^+$ as compared to both CpG alone or medium control (**Figure 6C**).

Additionally, the release of galectin-3, -4, -9 and TGFβ1 was measured in the supernatant after IEC/moDC culture (**Figure S4**). No significant differences were observed in galectin-3 and TGFβ1 release. However, IEC-derived from IEC/PBMC co-cultures exposed to 0.5% GF alone or in combination with CpG resulted in a significant increase in galectin-4 release in the IEC/moDC co-culture. IEC from IEC/PBMC co-cultures exposed to CpG tended to increase galectin-9 concentrations in IEC/moDC co-culture which was not further affected by 2'FL or GF (**Figure S4**).

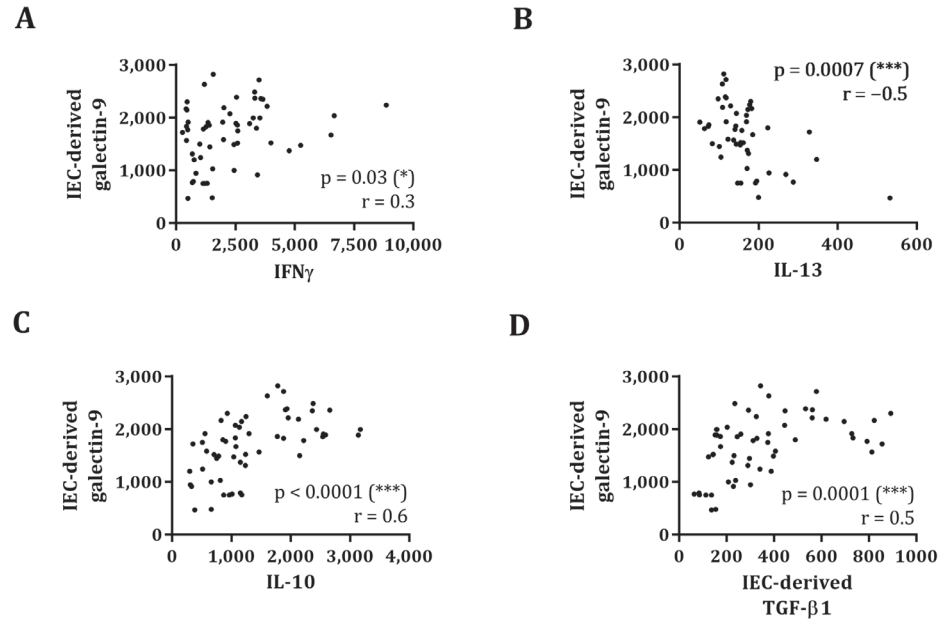

Figure 5. IEC-derived galectin-9 secretion correlates with IEC/PBMC co-culture cytokine release and IEC-derived TGFβ1.
After 24 h IEC/PBMC co-culture, IFNγ, IL-13 and IL-10 concentrations were measured in the basolateral supernatant (**Figures 1A and 3**). Thereafter, IEC were washed with PBS, the medium was refreshed and IEC incubated for additional 24 h (**Figure 1B**). After the incubation, the basolateral supernatant was collected and IEC-derived galectin-9 and TGFβ1 were measured (**Figure 4**). The correlation between IEC-derived galectin-9 and IFNγ (**A**), IL-13 (**B**), IL-10 (**C**) and TGFβ1 (**D**) release was tested using Spearman's test (* $p < 0.05$, *** $p < 0.001$).

ccDC derived from IEC/moDC co-cultures after conditioning of IEC with 2'FL and CpG in IEC/PBMC co-cultures instruct IFNγ and IL-10 production by allogeneic CD4$^+$ T-cells

Conditioned moDC (ccDC) were then incubated with naïve T-cells for a maximum of 6 days in an allogeneic DC/T-cell assay (**Figure 1D**). IFNγ, IL-13 and IL-10 release was measured in the supernatant of the DC/T-cell culture (**Figure 7**). ccDC conditioned with IEC from IEC/PBMC co-cultures exposed to 2'FL, GF or CpG alone did not show any effects in any of the cytokines measured. However IEC from IEC/PBMC co-cultures exposed to 2'FL in combination with CpG, showed increased IFNγ and IL-10 production in the ccDC/T-cell assay, compared to ccDC conditioned with IEC exposed to 2'FL and/or CpG alone (**Figure 7A,C**). In addition, IFNγ production by T-cells was further increased when ccDC were conditioned to 2'FL and CpG-exposed IEC from the IEC/PBMC as compared to GF and CpG-exposed conditions (**Figure 7A**). Meanwhile, IL-13 secretion was not affected (**Figure 7B**).

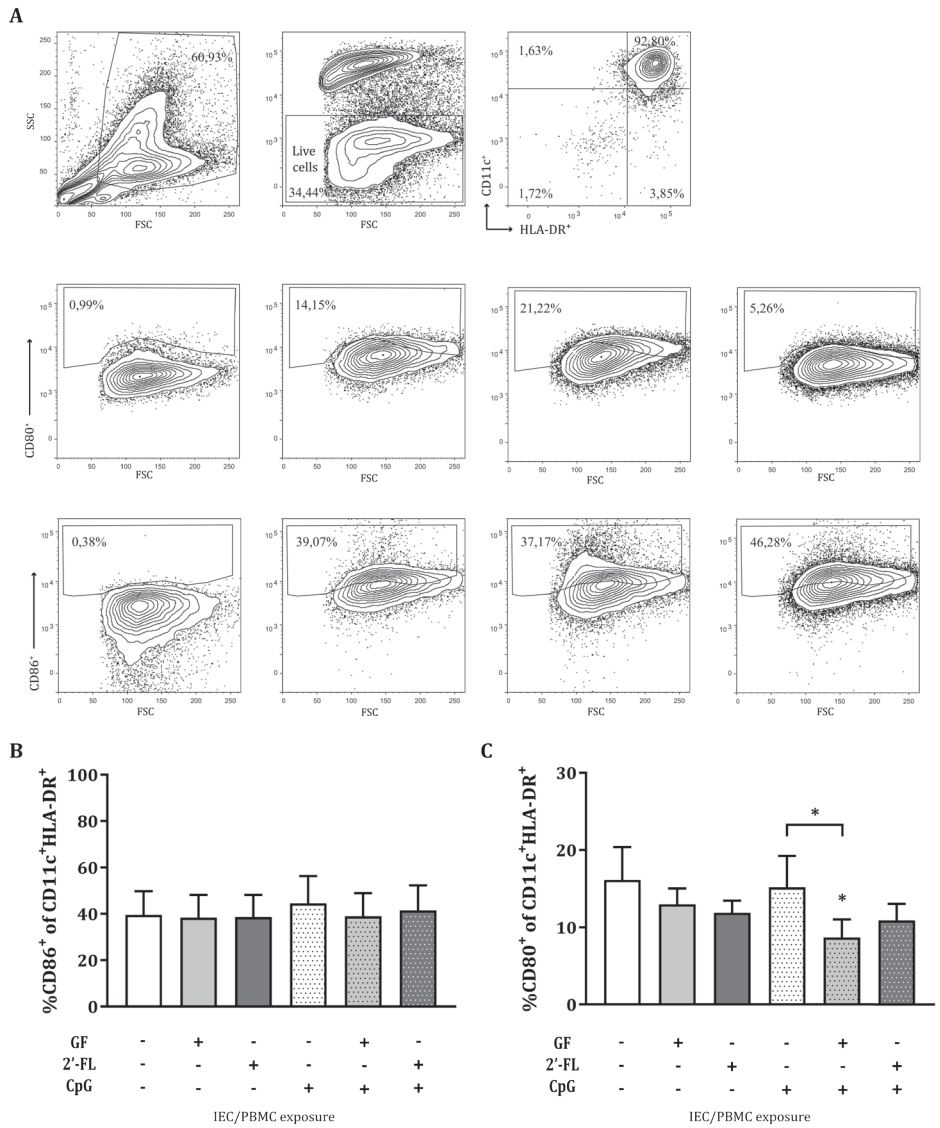

Figure 6. Phenotype of moDC after IEC/moDC co-culture.
After exposure of IEC to 0.5% 2'FL or GF, in the absence or presence of CpG, and co-culture with αCD3/CD28-activated PBMC, IEC were washed and co-cultured with immature moDC for 48 h (**Figure 1C**). The phenotype of moDC was studied after co-culture. Representative FACS plots are shown in (**A**). Expression of CD86$^+$ (**B**) and CD80$^+$ (**C**) was determined in the CD11c$^+$HLA-DR$^+$ population. Data are represented as mean ± SEM of $n = 8$ independent moDC donors (* $p < 0.05$).

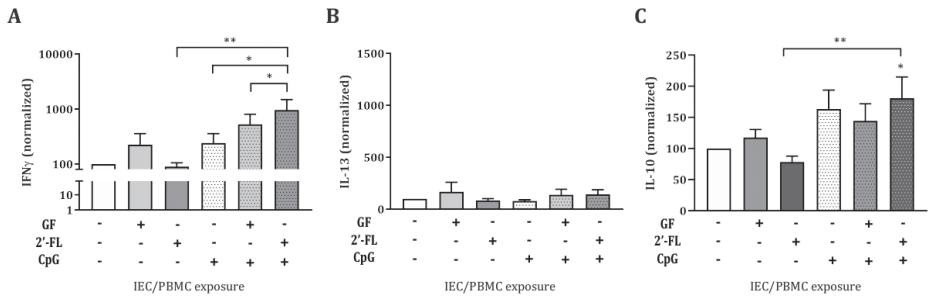

Figure 7. Cytokine secretion in ccDC/T-cell assay after moDC co-culture with conditioned IEC. Conditioned moDC (ccDC), previously exposed to conditioned IEC, were incubated with naïve T-cells for 5-6 days in an allogeneic ccDC/T-cell assay (**Figure 1D**). Afterwards, IFNγ (**A**), IL-13 (**B**) and IL-10 (**C**) were measured. Data were normalized per donor and represented as mean ± SEM from 5-12 independent PBMC donors (* $p < 0.05$, ** $p < 0.01$).

DISCUSSION

NDO in human milk are thought to modulate innate and adaptive immune properties and thereby promote the development of the mucosal immune system [29]. Direct immunomodulatory functions were previously described *in vitro* for 2'FL through the interaction with IEC [7, 8, 10, 12] as well as immune cells [6, 7, 11]. *In vivo*, a dietary intervention with 2'FL resulted in an improved immune response to vaccination [9].

Previous studies have also described the mechanisms by which direct immunomodulatory effects of NDO such as GF could contribute to bacterial or synthetic CpG DNA in immune development in an *in vitro* IEC/PBMC co-culture model combining intestinal epithelial and immune cells [27, 28, 30–32]. These studies identified IEC-derived galectin-9 as an important factor contributing to immune development, which was confirmed *in vivo* by dietary intervention studies for food allergy prevention in mice [18, 26]. In the present study, it was evaluated whether 2'FL, one of the most abundant NDO present in human milk, could promote immunomodulatory effects under inflammatory conditions, using an *in vitro* IEC/PBMC co-culture model [27, 28, 30–32]. Moreover, the relation between IEC-derived galectin release and the immunomodulatory effects was addressed.

The current study shows that when PBMC are activated by means of αCD3/CD28, mimicking an inflammatory milieu, the secretion of immunomodulatory cytokines is boosted, as opposed to the non-activated condition. This was also seen in previous studies using the same model [30, 32]. Apical exposure of IEC to 2'FL or GF in combination with CpG was able to further increase Th1-type cytokine IFNγ as well as regulatory-

type cytokine IL-10 secretion, while suppressing Th2-type cytokine IL-13 (**Figure 3**). This suggests that 2'FL, as well as GF, can promote immunomodulatory effects in the IEC/PBMC model, which indicates that exposure to specific NDOs and CpG might contribute to the promotion of immune development. However, only exposure to high concentrations of 2'FL resulted in increased galectin-9 concentrations already at 24 h after co-culture, as opposed to GF-exposed conditions, which did not show this effect (**Figure 3**). Following the dose-dependent studies in the IEC/PBMC co-culture experiments, the dose of 0.5% NDO was chosen for the subsequent studies, since this dose, when combined with CpG, increased Th1-type IFNγ and regulatory cytokine IL-10, while reducing the concentration of Th2-type IL-13.

2'FL is composed of a fucose moiety linked to galactose and glucose while GF is a NDO mixture mainly composed of glucose bound to multiple galactose or multiple fructose residues, respectively. In addition, 2'FL has a lower degree of polymerization as compared to GF. Previous studies showed that the cytokine profile can be affected by the type [33] as well as the chain length [34] of specific NDOs. Longer oligosaccharide chains might be able to interact with more than one receptor (or receptors located more distantly), forming a cluster, while shorter chains might interact only with receptors located around them. The differences in degree of polymerization, and thus chain length, of the studied NDOs, may be responsible for the distinct galectin-9 secretion found in the IEC/PBMC model.

Galectins are thought to play a key role in infant immunity due to their ability to bind NDOs present in human milk [3]. In particular, the ability of 2'FL to bind galectin-9 and galectin-3 was previously described by Hirabayashi et al. using frontal affinity chromatography [35]. Galectins are soluble lectins that can also be secreted and consequently function as innate and adaptive immune modulators by binding several receptors on immune cells such as TIM-3 and CD44 in addition to binding specific glycosylation patterns on immune cells [16, 36]. Galectins are secreted by several cell types, among which IEC are known to be a rich source [19]. In this study, exposure to both 2'FL and GF, in combination with CpG, showed enhanced IEC-derived galectin-9 release (**Figure 4**). This was opposed to galectin-9 secretion 24 h after IEC/PBMC co-culture, which was not increased by exposure to GF and CpG, but only by 2'FL and CpG (**Figure 3**). Although knowledge regarding the mechanism of action or receptors used by NDO in eliciting the direct immunomodulatory effects remains to be further developed, IEC-derived galectin-9 concentrations were correlated with IFNγ, IL-10 and IL-13 concentrations in the IEC/PBMC model (**Figure 5**). This indicates a role of galectin-9 in the immunomodulatory effects elicited by 2'FL and GF. These results are in line with previous studies where the blocking of IEC-derived galectin-9 resulted in a reduction of IFNγ and IL-10 secretion and/or increase in IL-13 [27, 28].

Beyond galectin-9, other galectins are also known to participate in diverse immune processes. Galectin-3 and -4 have shown anti-inflammatory properties by inhibiting mucosal inflammation in a colitis model [37, 38]. The current study shows that IEC-derived galectin-3 and -4 were increased after exposure to 2'FL or GF in combination with CpG. Galectin-3 concentrations were correlated to IFNγ and IL-13 but not to IL-10 secretion in the IEC/PBMC co-culture. Meanwhile, galectin-4 release was correlated only to IL-10 production.

The secretion of IEC-derived galectin-9 was correlated with IEC-derived galectin-3 and -4 as well as with the cytokines secreted in the IEC/PBMC model, which might strengthen the idea that not only galectin-9, but also other galectins, might have contributed to the immunomodulatory effects seen in the IEC/PBMC model. Nevertheless, although the secretion of galectin-3 and -4 was increased after NDO and CpG exposure, epithelial-derived galectin-9 was the only galectin found to be correlated with the modulated IFNγ, IL-10, IL-13 secretion by PBMC as well as epithelial-derived TGFβ1, which reinforces the role of galectin-9 as a key factor in immunomodulation and thereby in immune development. The ability of galectin-9 in promoting immune regulation has also been described before, which substantiates the contribution of galectins in the regulation of immune homeostasis [23, 24].

Furthermore, IEC-derived galectin-9 secretion was found to be strongly positively correlated with IEC-derived TGFβ1. This supports the idea that both mediators act synergistically in the promotion of the differentiation of regulatory T-cells [22–24]. Conversely, TGFβ1 concentrations did not correlate to IEC-derived galectin-3 and -4. In line with our results, the relation between epithelial galectin-9 and TGFβ1 mediator release, and the immunomodulatory effects elicited by combined exposure to GF and CpG in the IEC/PBMC model, has been previously described [25, 27]. The formation of galectin-glycan lattices is known to influence cell signaling processes. All three galectins were found to be involved in cell signaling processes such as lipid raft stabilization and apical targeting of glycoproteins [39]. As a result of NDO and CpG exposure, IEC might have been able to increase signal transduction and thus promote mucosal immune homeostasis, maybe through the formation of galectin-glycan lattices. In agreement with the *in vitro* studies showing the involvement of epithelial-derived galectin-9 in promoting immunomodulation [25, 27], *in vivo* NDO and *Bifidobacterium breve* were also shown to effectively induce galectin-9 concentrations, associated with mucosal immune regulation [26]. This shows the translational value of this type of co-culture models.

In addition to the immunomodulatory effects studied, we were interested in understanding whether the imprinted IEC, derived from the IEC/PBMC co-culture

and exposed to medium only, had the ability to instruct DC to promote specific immune responses. Upon antigen exposure, IEC release several mediators that are able to activate migratory DC and their migration to the mesenteric lymph nodes, where they can promote adaptive immune responses by interacting with T-cells [14, 15]. Due to the proximity of the migratory DC with the epithelial layer, their phenotype can also be affected upon epithelial mediator release, even in the absence of direct contact. As a result of the ability of 2'FL and GF to modify epithelial mediator release in combination with CpG, the moDC phenotype was studied after conditioned-IEC/moDC co-culture. Subsequently, the functionality of the conditioned moDC was assessed by co-incubation with $CD4^+$ T-cells in an allogeneic DC/T-cell model. Interestingly, this study shows that in the presence of CpG, exposure of IEC to GF, but not 2'FL, resulted in a decreased CD80 marker expression of conditioned DC in the IEC/moDC model, pointing towards a lower activation status. The decreased expression in CD80 should be considered with caution due to the high background signal present in the staining. Prospective studies should be done using an Fc block to control for this technical issue. Moreover, IEC imprinted by exposure to 2'FL and CpG, in the IEC/PBMC model, instructed moDC to promote increased IFNγ and IL-10 production in the allogeneic DC/T-cell model, as compared to CpG alone. Meanwhile, GF-exposed IEC did not give rise to this effector Th-cell response. These results indicate the ability of 2'FL and CpG-exposed IEC to instruct moDC to drive the development of naïve T-cells into Th1 and regulatory-type effector cells.

This study supports the idea that exposure to NDO in early life might promote immune development under conditions such as inflammation and emphasizes the ability of IEC to educate DC in strengthening mucosal immune function.

By developing the models described in this manuscript, it was aimed to highlight the relevance of the crosstalk between epithelial cells and immune cells in immunomodulation. The HT-29 intestinal epithelial cell line used in these studies, however, is a colon adenocarcinoma cell line. Although they are unable to completely mimic the structural and functional complexity of the *in vivo* situation, they serve as useful tools to study intestinal processes to some extent, which in future studies needs to be confirmed using primary human epithelial cell models. Great efforts have been made in order to develop primary epithelial cell 3D and 2D organoid models which provide regional-specific properties and more closely resemble the physiology of the gastrointestinal tract. Both pluripotent stem cell-derived organoids and *ex vivo* intestinal enteroid and colonoid cultures are powerful tools to study the heterogeneity and multicellular organization of intestinal epithelial cells in the gastrointestinal tract [40, 41]. Beyond providing a physical barrier, IEC actively participate in diverse functions involving, among others, the immune system. The interactions between IEC and immune

cells are key processes in immune homeostasis. The next step would therefore be to combine these primary epithelial cultures with immune cells as was already conducted with macrophages [42]. Beyond macrophages, the lamina propria consists of many other innate and adaptive immune cells which, preferably, could be isolated from intestinal tissues as well [43].

Despite its restrictions, the HT-29 transwell co-culture model was previously shown to have predictive value as the intervention with NDO not only identified an immunomodulatory role for galectin-9 in this *in vitro* model, but this was confirmed in murine models for food allergy. Furthermore, in infants with atopic dermatitis, formula milk containing NDO was capable of enhancing serum galectin-9 levels in association with symptom reduction [26], which supports the translational value of the HT-29 transwell co-culture model to the human situation. Our aim was to use the HT-29 transwell co-culture model as a first step of a sequence of models to illustrate the relevance of studying the interaction between structural cells and immune cells, thereby confirming the contribution of epithelial cells to modify innate and adaptive immune responses. The HT-29 transwell co-culture model could serve as a complementary model for future studies using primary 2D cultured enteroids.

CONCLUSIONS

This study reveals that both 2'FL and GF can promote immunomodulatory effects under inflammatory conditions upon combined exposure with bacterial CpG DNA through the modulation of IEC function. These immunomodulatory effects were associated with the release of galectins and TGFβ1 by IEC. Thus, our study emphasizes the importance of understanding epithelial mediator release, such as galectins and TGFβ1, and their role in the mucosal immune development.

Additionally, we describe a possible role of 2'FL and CpG-exposed IEC in instructing DC to drive naïve T-cell development. Future research should be directed towards further understanding of the mechanism of action by which these effects occur.

Prospective studies using 2D cultured human organoids will be needed to further validate the results discussed in this manuscript.

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SUPPLEMENTARY FIGURES

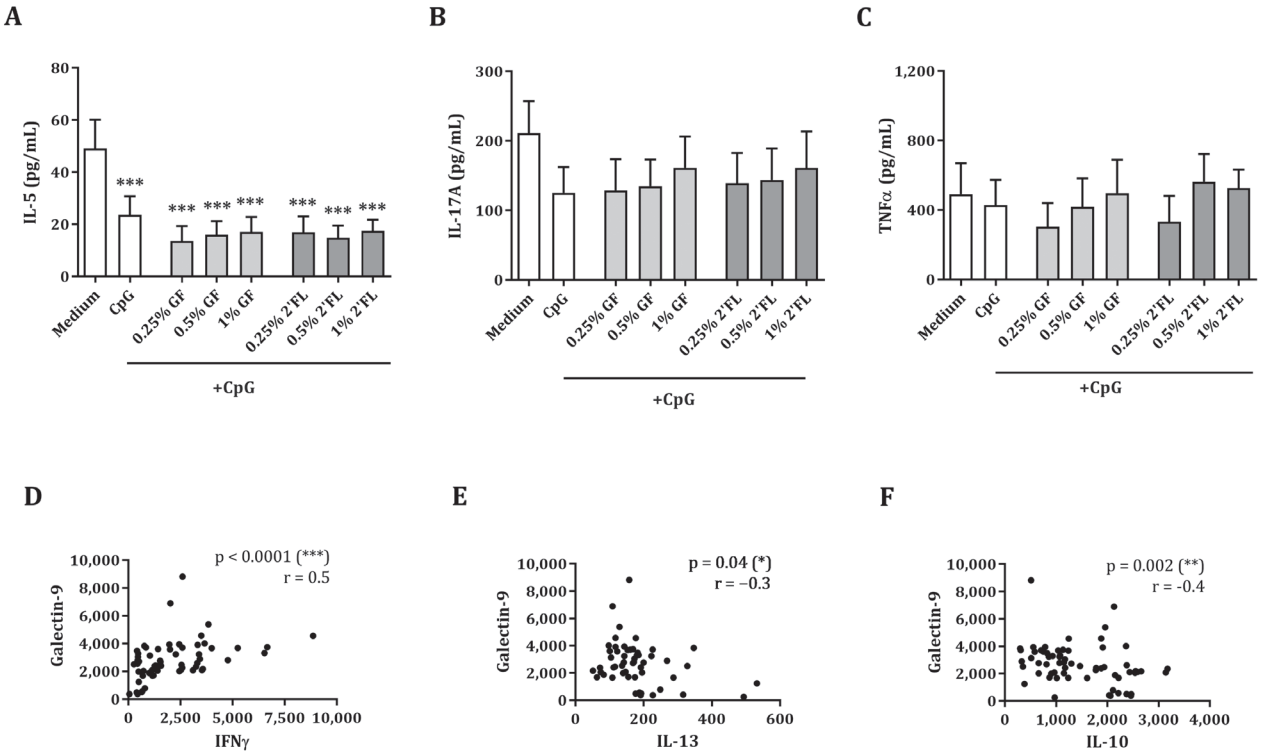

Figure S1. Cytokine release and correlations in IEC/PBMC co-culture.
IEC were basolaterally exposed to αCD3/CD28-activated PBMC and apically to 0.25-1% NDO (2'FL or GF) in combination with CpG (**Figure 1A**). After 24 h incubation, IL-5 (**A**), IL-17A (**B**) and TNFα (**C**) concentrations were measured in the basolateral supernatant. Additionally, the correlations between galectin-9 and IFNγ (**D**), IL-13 (**E**) or IL-10 (**F**) concentrations were studied in the IEC/PBMC co-culture. Data are represented as mean ± SEM of n = 6 independent PBMC donors (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

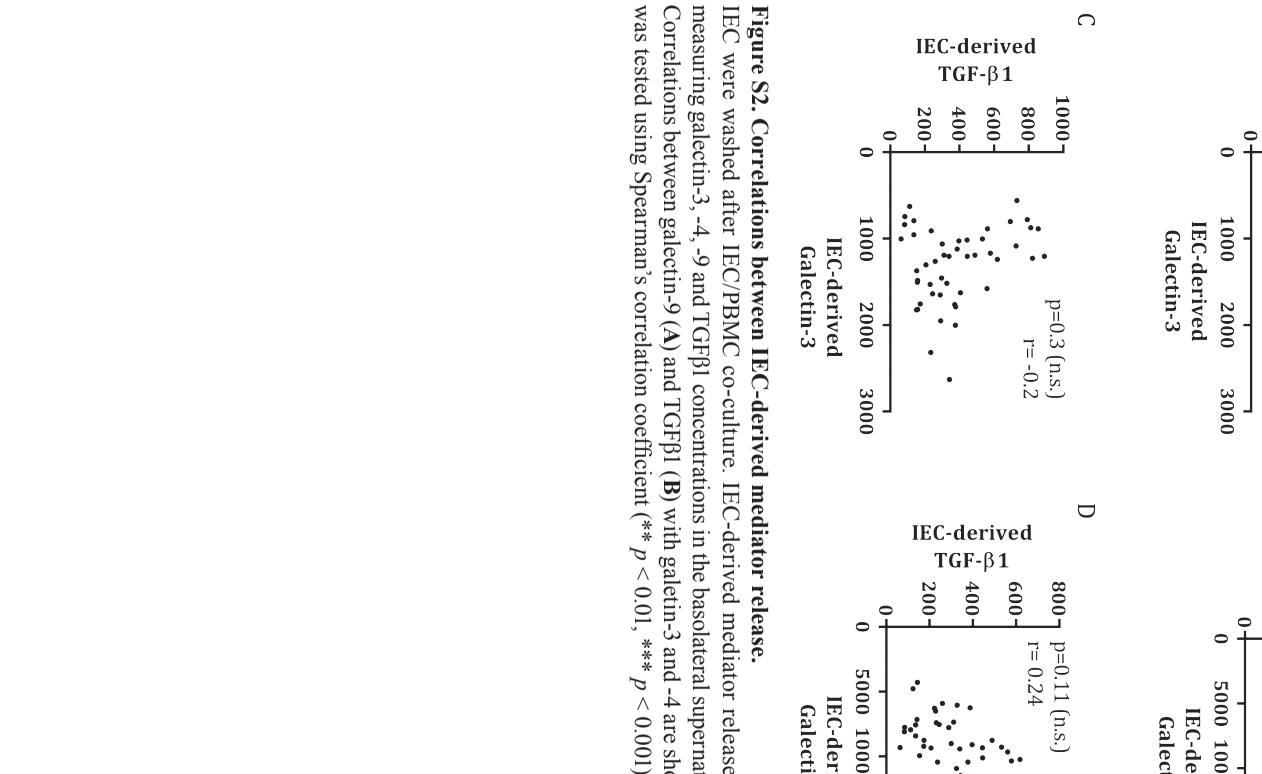

Figure S2. Correlations between IEC-derived mediator release.
IEC were washed after IEC/PBMC co-culture. IEC-derived mediator release was studied by measuring galectin-3, -4, -9 and TGFβ1 concentrations in the basolateral supernatant (**Figure 1B**). Correlations between galectin-9 (**A**) and TGFβ1 (**B**) with galetin-3 and -4 are shown. Correlation was tested using Spearman's correlation coefficient (** $p < 0.01$, *** $p < 0.001$).

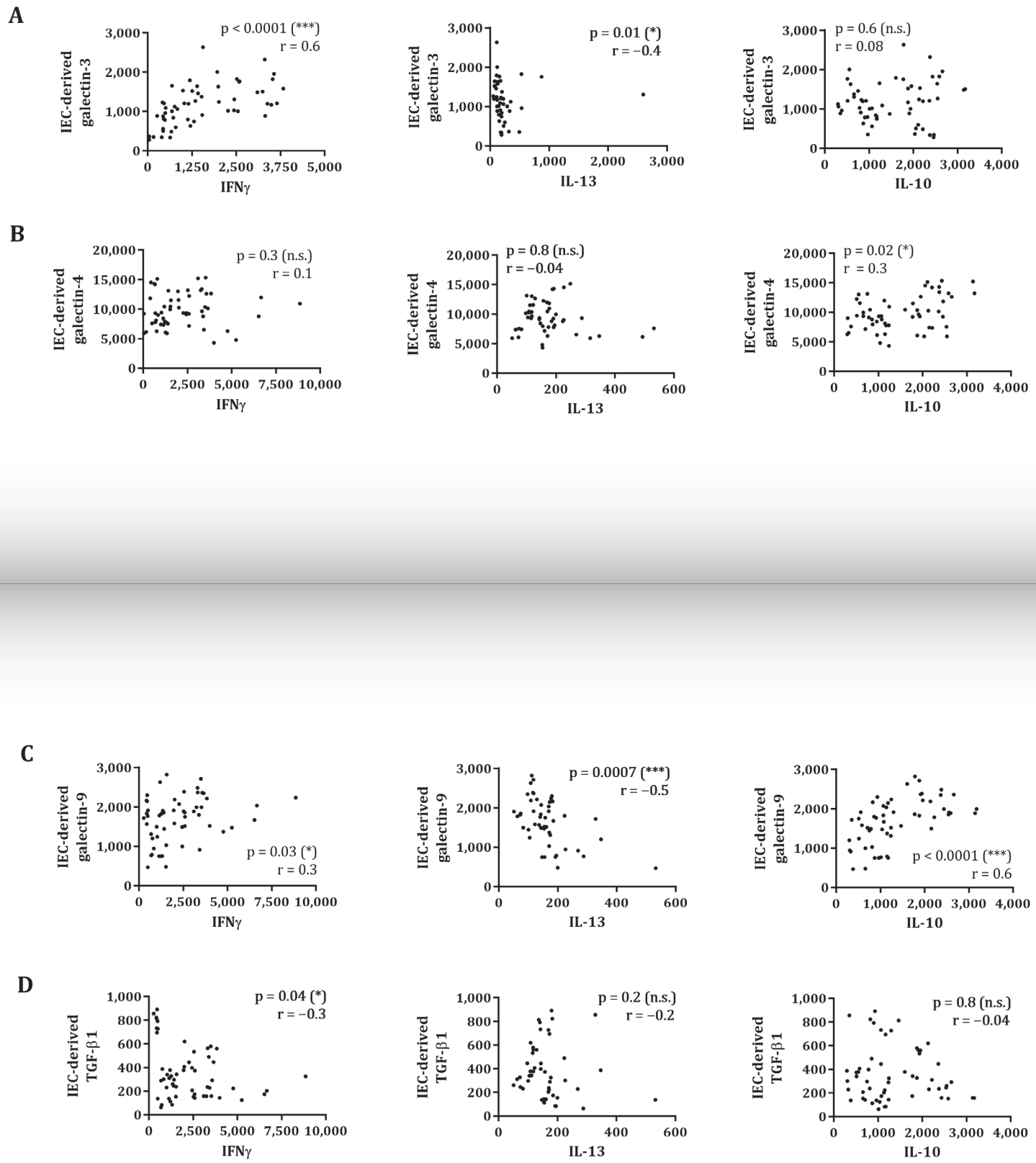

Figure S3. Correlations of IEC-derived mediator and cytokine release in the IEC/PBMC co-culture.
IEC-derived mediator release galectin-3 (**A**), galectin-4 (**B**), galectin-9 (**C**) and TGFβ1 (**D**) were correlated to the concentrations of cytokines in IEC/PBMC (IFNγ, IL-13 and IL-10). Correlations were tested using Spearman's correlation coefficient (* $p < 0.05$, *** $p < 0.001$).

CHAPTER | 6

Epithelial-derived galectin-9 containing exosomes contribute to the immunomodulatory effects promoted by 2'-fucosyllactose and short-chain galacto- and long-chain fructo-oligosaccharides

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Submitted

ABSTRACT

Introduction: Early life exposure to non-digestible oligosaccharides (NDO) present in human milk or microbial components are known to affect immune development. NDO in combination with a TLR9 agonist mimicking a bacterial trigger (CpG), promoted the secretion of galectins in a co-culture model combining intestinal epithelial cells (IEC) and immune cells. In the present study, we investigated the involvement of galectins in the immunomodulatory activity of a mixture of NDO containing 2'-fucosyllactose (2'FL) in combination with short-chain galacto- and long-chain fructo-oligosaccharides (GF). Exosomes regulate intercellular communication and are known to contain galectins but their secretion pathway is still unknown. Therefore we aimed to inhibit exosome biogenesis to study the contribution of exosomes in epithelial galectin secretion and subsequent immunoregulation.

Methods: Human IEC (FHs 74 Int or HT-29) were apically exposed to 2'FL and GF, alone or in combination with CpG. Basolaterally, non-activated or αCD3/CD28-activated peripheral blood mononuclear cells (PBMC) were added. After 24 h incubation, IEC were washed and incubated in fresh medium to analyze the epithelial-derived galectin secretion. Additionally, 1 h before exposure to NDO and CpG, IEC were exposed to GW4869 to inhibit exosome biogenesis. After 24 h of incubation, IEC were washed and incubated for additional 24 h, in the presence of GW4869 after which epithelial-derived galectin secretion was studied. Also, epithelial-derived exosomes were isolated to study the presence of galectins within the exosomes.

Results: Compared to CpG alone, exposure to 2'FL/GF mixture and CpG, significantly enhanced Th1-type IFNγ and regulatory IL-10 and decreased Th2-type IL-13 secretion, while increasing IEC-derived galectin-9 secretion in the HT-29/PBMC model. In the FHs 74 Int/PBMC co-culture, similar 2'FL/GF induced immunomodulatory effects were already observed in the absence of CpG. Interestingly, galectin-9 and -4 were present in CD63 expressing exosomes isolated from HT-29 supernatants after IEC/PBMC co-culture. Exposure to GW4869 suppressed 2'FL/GF and CpG induced epithelial-derived galectin-9 and -4 secretion, which subsequently prevented the rise in IL-10 and reduction in IL-13 secretion observed in the HT-29/PBMC co-culture model upon exposure to 2'FL/GF and CpG.

Conclusions: Exposure to 2'FL/GF and CpG or 2'FL/GF promoted Th1-type regulatory effects in HT-29/PBMC or FHs 74 Int/PBMC co-culture respectively, while Th2-type IL-13 was reduced in association with increased galectin-9 release. Galectin-9 and -4 were present in exosomes from HT-29 and the inhibition of exosome biogenesis and secretion inhibited epithelial-derived galectin secretion. The inhibition of exosome biogenesis also affected immunomodulatory effects in IEC/PBMC co-culture suggesting a key role of galectin expressing IEC-derived exosomes in the mucosal immune regulation induced by NDO.

INTRODUCTION

Non-digestible oligosaccharides (NDO) in human milk provide the infant with a unique source of energy and support the growth and development of key organs and systems as well as contributing to the establishment of the microbiome. Specific NDO derived from milk or plant sources such as a 9:1 mixture of short-chain galacto- and long-chain fructo-oligosaccharides (GF) have been manufactured to mimic the amount and structure diversity of NDO in human milk. This NDO mixture was shown to promote the growth of beneficial commensal bacteria as well as supporting the immune system [1]. Direct effects of these NDO were investigated in an *in vitro* co-culture model built to study the crosstalk between intestinal epithelial cells (IEC) and activated peripheral blood mononuclear cells (PBMC). In association with CpG oligodeoxynucleotides or bacterial DNA from *Bifidobacterium breve* M-16V, the GF mixture was found to enhance regulatory type Th1 cytokines, while lowering Th2 type response, driving away from the allergic phenotype [2, 3]. Galectin-9 was identified as the epithelial-derived factor responsible for shaping the phenotype of the immune response [2]. Furthermore, in a murine model for cow's milk or hen's egg allergy, a reduction of allergic symptoms was observed upon combination of GF with *Bifidobacterium breve* M-16V. These effects were associated with increased galectin-9 levels in serum and intestine [4–6]. Additionally, in children affected with atopic dermatitis fed hydrolyzed milk formula supplemented with GF and *Bifidobacterium breve* M-16V, serum galectin-9 levels increased while their symptoms reduced [5, 7]. One year later, these children had a lower risk of developing asthma like symptoms and less asthma medication was prescribed compared to control group [8]. In an *in vitro* IEC/PBMC co-culture model similar immunomodulatory effects were observed upon exposure to 2'-fucosyllactose (2'FL), one of the most abundant NDO in human milk, and GF when combined with CpG, mimicking a bacterial trigger [9]. Both, 2'FL and GF in combination with CpG, promoted Th1-type regulatory immune effects. Epithelial-derived galectin-3, -4 and in particular galectin-9 were thought to be involved in promoting immunomodulation [9].

Galectins are carbohydrate-binding proteins with diverse intracellular and extracellular physiological functions like supporting epithelial homeostasis and immune functions [10]. Galectins are synthetized in the cytosol and lack a signal sequence thus, are thought to be released by non-classical secretion mechanisms [11]. One of the non-conventional pathways by which galectins are thought to be secreted is via extracellular vesicles [11]. Extracellular vesicles (EV) contain biomolecules (proteins, lipids and nucleic acids) which are relevant for intercellular communication that can modulate immune responses locally as well as systemically [12–16]. Exosomes are small extracellular vesicles (30-200 nm) which are originated from multivesicular bodies and

released into the extracellular compartment by fusion with the plasma membrane [12–16]. There, they participate in cell-to-cell communication as well as contributing to transport, storage and release of proteins [12–16] that mediate intercellular communication but also contribute to maintain cellular homeostasis [17, 18]. Many cell types including enterocytes and immune cells are known to secrete exosomes with diverse physiological functions [12, 15]. In particular in the gut, EV secreted from IEC can interact with the immune cells present in the lamina propria and thereby control intestinal homeostasis [13]. Previously, exosomes derived from T84 IEC were shown to interact with dendritic cells which in turn led to T-cell activation [19]. Besides T84, also HT-29 IEC were shown to produce CD63-expressing exosomes [20]. Furthermore, the secretion of exosome-containing galectins has been previously described [21]. Nevertheless, the exact mechanism regarding how galectins are released by IEC remains poorly understood.

Ceramide is an abundant component of the exosomal membrane which is produced upon hydrolysis of sphingomyelin and its accumulation is required for the formation of exosomes [22]. Downregulation of the ceramide production by a neutral inhibitor of sphingomyelinase (nSMase) resulted in the inhibition of the production of exosomes [23]. In this regard, the neutral sphingomyelinase inhibitor GW4869 has been used to potentially block the mechanisms required for the biogenesis and secretion of exosomes [24, 25].

IEC were shown to secrete epithelial-derived galectins upon exposure to NDO and CpG in an *in vitro* co-culture model used to study the crosstalk between HT-29 and immune cells [2, 3, 9, 26]. Due to the carcinogenic background of the HT-29 cell line, we aimed to investigate if other non-carcinogenic epithelial cell lines could promote immunomodulatory effects upon exposure to NDO in the presence or absence of CpG. Hereby, we aimed to study whether NDO alone or in combination with CpG could promote immunomodulatory effects in a human fetal intestinal cell line (FHs 74 Int), similar to the effects shown for HT-29 before [2, 9]. In addition, we studied if the inhibition of exosome biogenesis by GW4869 impaired the epithelial-derived galectin secretion that was promoted upon exposure of IEC to NDO and CpG. Moreover, we studied if the inhibition of galectin secretion interfered with the immunomodulatory effects observed in IEC/PBMC upon exposure to NDO and CpG, to contribute to the understanding of the roles of galectins and exosomes in immunomodulatory responses in the intestinal mucosa.

MATERIAL AND METHODS

Intestinal epithelial cell culture

Human FHs 74 Int (ATCC, CCL-241™) and human HT-29 (ATCC, HTB-38™) cell lines were used as models for IEC. FHs 74 Int were grown in T25 flasks (Greiner Bio-One) using Hybri-care medium (ATCC, 46-X) supplemented with 30 ng/mL epidermal growth factor (EGF), 10% fetal-calf serum (FCS), penicillin (100 U/mL) and streptomycin (100 µg/mL). HT-29 were grown in T75 flasks (Greiner Bio-One) using McCoy 5A medium (Gibco) supplemented with 10% FCS, penicillin (100 U/mL) and streptomycin (100 µg/mL). Medium was refreshed every 2-3 days and cultures were maintained at 37 °C and 5% CO_2. One week before the experiments, IEC were seeded in 12-well transwell inserts (Costar Corning) by diluting 4 times for FHs 74 Int and 8-10 times for HT-29 based on surface area. Confluent IEC monolayers were used to perform co-culture experiments.

Peripheral blood mononuclear cell isolation

Human PBMC were isolated from buffy coats (Sanquin, Amsterdam, The Netherlands). Buffy coats from healthy donors were diluted (1:1) in PBS supplemented with 2% FCS. PBMC fraction was isolated by density gradient centrifugation (1,000 x g, 13 minutes) using Leucosep tubes (Greiner Bio-one). After washing, remaining red blood cells were lysed (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). Purified PBMC were resuspended in RPMI 1640 supplemented with 2.5% FCS, penicillin (100 U/mL) and streptomycin (100 µg/mL) and used for IEC/PBMC co-culture experiments.

IEC/PBMC co-culture

FHs 74 Int and HT-29 were grown in transwell inserts and exposed apically to a 1:1 mixture of 2'FL and GF in 0.25-1% solution (2.5-10 mg/mL, *w/v* %) in the presence of CpG (M362, 0.1 or 0.5 µM, Invivogen). In the basolateral compartment 2 x 10^6 cell/mL PBMC were added, activated with αCD3/αCD28 (0.15 µg/mL and 0.2 µg/mL respectively from Sanquin and BD), and incubated at 37 °C and 5% CO_2 for 24 h. Alternatively, before exposure to 2'FL/GF and CpG, IEC were exposed to 10 µM GW4869 (CAS Number 6823-69-4, Sigma-Aldrich) for 1 h. After 24 h of incubation, the basolateral supernatant was collected and stored at -20 °C for cytokine and galectin analysis. Then, IEC were separated from the PBMC by transferring the inserts to a new plate, washed with PBS and incubated in fresh medium in the presence or absence 10 µM GW4869 for additional 24 h to study the basolateral IEC-derived galectin release. Additionally, the cell culture supernatant was used to isolate HT-29 derived exosomes.

Exosome isolation

Exosomes were isolated from conditioned supernatant using ExoQuick-TC™ ULTRA Isolation Kit (System Biosciences LLC). Exosomes were also isolated from plain RPMI (supplemented with 2.5% FCS and penicillin/streptomycin) as a control. Shortly, conditioned supernatant was collected and after cell debris removal by spinning down (3,000 x g, 15 minutes) ExoQuick-TC was added and incubated overnight at 4 °C. Upon centrifugation (3,000 x g, 10 minutes, 4 °C) the exosome pellet was collected and purified following manufacturer's instructions and stored at -80 °C for further analysis.

Enzyme-linked immunosorbent assay (ELISA)

The cytokine and galectin secretion was studied in the basolateral supernatant of IEC/PBMC co-cultures. Commercially available kits were used to measure IFNγ, IL-13, TNFα (all from Thermo Fisher scientific), IL-10 (U-Cytech), and galectin-3 (R&D systems) following manufacturer's protocol. Human galectin-4 and -9 were measured using antibody pairs (all from R&D systems) as described before [9, 26, 27].

Additionally, a CD63 detection ELISA kit (System Biosciences) was used to measure the exosomes isolated from conditioned supernatant following manufacturer's protocol.

Western blot

Isolated exosome samples were mixed with Laemmli sample buffer (Bio-rad) containing 50 mM dithiothreitol (DTT) and incubated at 95 °C for 5 minutes to reduce and denaturate proteins before loading into a 4-20% SDS-PAGE gel (Mini-PROTEAN® Bio-rad) for separation by electrophoresis. Separated proteins were then transferred into a polyvinylidene difluoride membrane (Transblot Turbo, Biorad) after which the membrane was blocked with 5% milk protein in phosphate-buffered saline containing 0.05% Tween-20. The membranes were then incubated with galectin-4 (1:4000) and galectin-9 (1:100) antibodies (both from R&D). After overnight incubation, the membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000, Dako) for 2 h. After washing, ECL reagent (Cytiva) was used to visualize the proteins. Image J software (Wayne Rasband, National Institutes of Health) was used to analyze the data.

Statistical analysis

All statistical analysis were done using GraphPad Prism software. Data were analyzed using one-way ANOVA followed by Bonferroni's multiple comparison post hoc test. When data did not fit normal distribution, transformation was applied prior to ANOVA analysis. In FHs 74 Int co-cultures the conditions with different concentrations of CpG were analyzed separately as represented by the dotted line. However, within the analysis of CpG-exposed conditions a comparison between medium control and CpG alone was included. Probability values of $p < 0.05$ were considered significant.

RESULTS

FHs 74 Int/PBMC co-culture results

To study if the FHs 74 Int IEC had the ability to crosstalk with immune cells in a IEC/PBMC co-culture model, FHs 74 Int were seeded in transwell inserts and co-cultured with non-stimulated or αCD3/CD28-activated PBMC for 24 h alone or in combination with CpG (0.5 μM). Additionally, after FHs 74 Int co-culture with PBMC, FHs 74 Int were washed and incubated in fresh medium for additional 24 h after which epithelial-derived galectin-9 was measured.

Upon exposure to CpG there was no effect on the levels of galectin-9, IFNγ, IL-10 and IEC-derived galectin-9 secretion of non-stimulated PBMC (**Figure 1**). Co-culture of FHs 74 Int with αCD3/CD28-activated PBMC tended to increase galectin-9 secretion ($p = 0.06$) but did not affect the levels of IFNγ, IL-10 and IEC-derived galectin-9 secretion as compared to non-stimulated PBMC (**Figure 1**). However, exposure of FHs 74 Int to CpG and αCD3/CD28-activated PBMC significantly increased galectin-9, IL-10 and IEC-derived galectin-9 secretion as compared to non-stimulated or αCD3/CD28-activated PBMC (**Figure 1**).

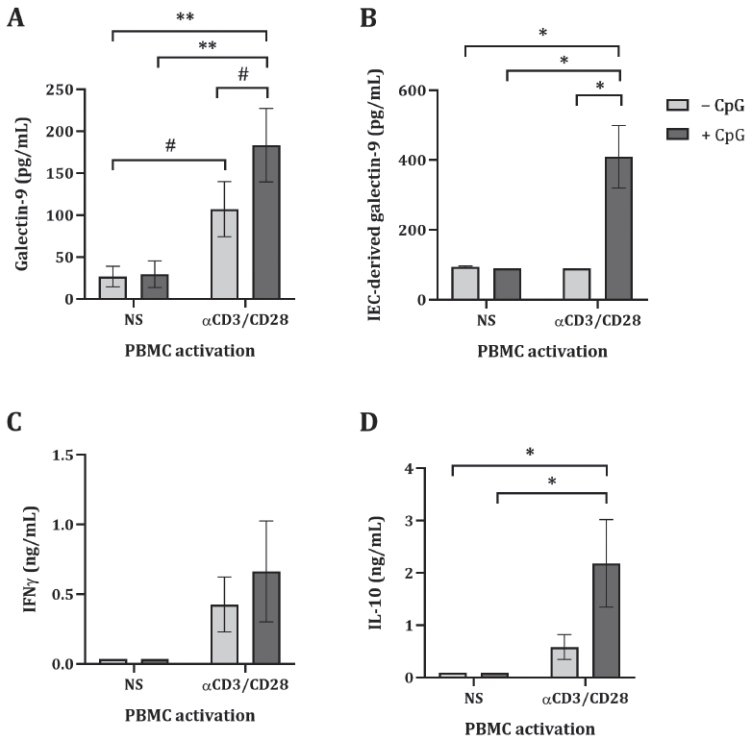

Figure 1. Cytokine and galectin-9 secretion in FHs 74 Int/PBMC co-culture model. FHs 74 Int IEC were stimulated with CpG (0.5 μM) and basolaterally exposed to non-activated (ns) or αCD3/CD28-activated PBMC for 24 h. After incubation, galectin-9 (**A**), IFNγ (**C**) and IL-10 (**D**) were measured. Additionally, after IEC/PBMC co-culture, FHs 74 Int were washed and incubated in fresh medium for additional 24h after which IEC-derived galectin-9 (**B**) was measured. Data represent mean ± SEM of $n = 3$ independent PBMC donors (# $p < 0.1$, * $p < 0.05$, ** $p < 0.01$).

Since only upon stimulation of PBMC with αCD3/CD28 upregulated cytokine secretion is observed, which can be modulated by stimulation with CpG, the following studies were done using only αCD3/CD28-activated PBMC in co-culture with IEC.

NDO modulate cytokine secretion from FHs 74 Int

By means of the FHs 74 Int and PBMC co-culture model, the ability of NDO in combination with CpG to induce immunomodulatory effects was studied. Therefore, FHs 74 Int were exposed to 2'FL, GF and a 1:1 mixture of 2'FL and GF (0.5% *w/v*) in combination with 0.1 or 0.5 μM CpG for 24 h. Additionally, after FHs 74 Int co-culture with PBMC, FHs 74 Int were washed and incubated in fresh medium for additional 24 h after which epithelial-derived galectin-9 was measured.

There was no effect on galectin-9, IFNγ, IL-10, IL-13 or TNFα (**Figure 2A & C-F**) secretion of FHs 74 Int exposed to 2'FL but IEC-derived galectin-9 secretion tended to increase ($p = 0.07$) (**Figure 2B**). Exposure to GF alone significantly increased galectin-9, IFNγ, IL-10 and tended to increase IEC-derived galectin-9 ($p = 0.07$), while significantly decreasing IL-13 levels (**Figure 2A-E**). Significantly increased galectin-9 and IL-10, and decreased IL-13 concentrations were observed upon exposure of FHs 74 Int to a 1:1 mixture of 2'FL and GF (**Figure 2A, D-E**).

Exposure to 0.1 or 0.5 μM CpG did not affect galectin-9, IEC-derived galectin-9, IFNγ, IL-10, IL- 13 or TNFα, except for significantly increased TNFα and a tendency towards increased galectin-9 concentrations ($p = 0.08$) upon exposure to 0.5 μM CpG as compared to medium control (**Figure 2**). Combined exposure to both concentrations of CpG and 2'FL significantly decreased IL-13 and TNFα concentrations (**Figure 2E-F**). Combined exposure to 2'FL and 0.5 μM CpG significantly increased galectin-9 and tended to increase IL-10 ($p = 0.06$) concentrations (**Figure 2A, D**). IEC-derived galectin-9 tended to increase only upon exposure to 2'FL and 0.1 μM CpG ($p = 0.09$) (**Figure 2B**). IEC-derived galectin-3 tended to increase upon exposure to 2'FL and 0.1 μM CpG and significantly increased with GF and 0.1 μM CpG as compared to 0.1 μM CpG alone (**Figure S1**). IEC-derived galectin-4 was under detection limit.

Exposure to GF and 0.1 μM CpG significantly increased IFNγ and IL-10 and tended to increase galectin-9 ($p = 0.06$) and decrease IL-13 ($p = 0.09$) concentrations (**Figure 2A, E**). Meanwhile, when GF was combined with 0.5 μM CpG significantly increased IL-10 and decreased IL-13 and TNFα concentrations were observed (**Figure 2D-F**).

There was no effect on IFNγ and IL-10 concentrations upon exposure to 2'FL/GF and CpG (in both concentrations) (**Figure 2C-D**). However, significantly increased galectin-9 and decreased IL-13 and TNFα concentrations were observed upon exposure to 2'FL/GF and CpG (in both concentrations) (**Figure 2A, E-F**). Only when 2'FL/GF was combined with 0.5 μM CpG significantly increased IEC-derived galectin-9 concentrations were observed (**Figure 2B**).

Taken together, exposure of FHs 74 Int and PBMC to NDO alone significantly modulated cytokine and galectin-9 release. Combined exposure to NDO and CpG further modulated the cytokine secretion from FHs 74 Int and PBMC co-cultures. Although exposure to 2'FL/GF alone and in combination with CpG significantly increased galectin-9 as well as decreasing Th2-type IL-13 and pro-inflammatory TNFα, epithelial derived galectin-9 secretion was only increased upon combined exposure to 2'FL/GF in combination with CpG. Thereby, following studies were done focusing on the 1:1 mixture of 2'FL/GF and CpG.

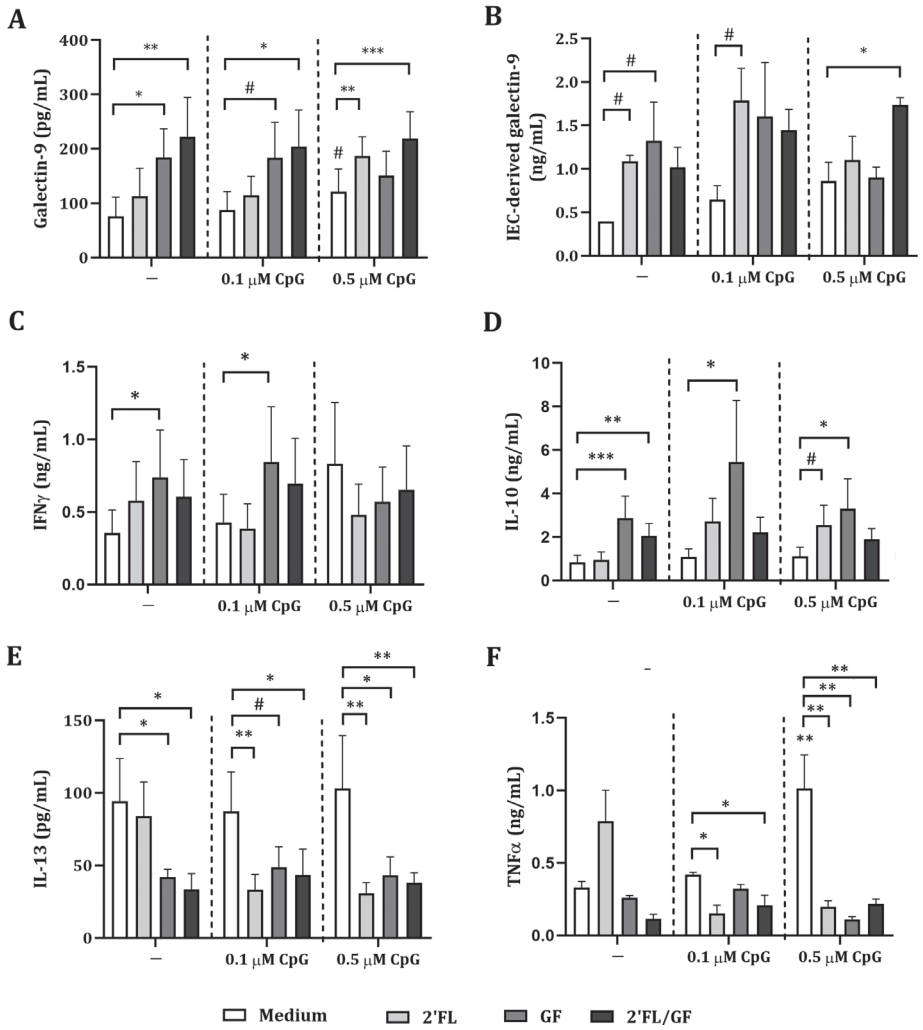

Figure 2. Cytokine and galectin-9 secretion in FHs 74 Int/PBMC co-culture model.
FHs 74 Int IEC were stimulated with 2'FL, GF or 1:1 mixture of 2'FL and GF (0.5 % *w/v*) in combination with CpG (0.1 or 0.5 μM) and basolaterally exposed to αCD3/CD28-activated PBMC for 24 h. After incubation, galectin-9 (**A**), IFNγ (**C**), IL-10 (**D**), IL-13 (**E**) and TNFα (**F**) were measured. Additionally, after IEC/PBMC co-culture, FHs 74 Int were washed and incubated in fresh medium for additional 24h after which IEC-derived galectin-9 (**B**) was measured. Data represent mean ± SEM of $n = 3$ independent PBMC donors. The conditions without CpG and with 0.1 or 0.5 μM CpG were analyzed separately as represented by the dotted line. Within this analysis, a comparison between the medium control and CpG alone was included (# $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Increased IEC-derived galectin-9 secretion upon exposure to 2'FL/GF and CpG

To further study the involvement of IEC-derived galectins in the modulation of cytokine secretion in the IEC/PBMC co-culture model, following studies were done using HT-29 IEC since these were shown to secrete not only IEC-derived galectin-9 but also galectin-3 and -4 upon stimulation with NDO and CpG [9]. Therefore, IEC (HT-29 cell line) were apically exposed 2'FL/GF mixture in combination with 0.5 μM CpG and basolaterally to αCD3/CD28-activated PBMC for 24 h. After IEC/PBMC co-culture, IEC were separated from PBMC, washed and incubated in fresh medium for additional 24 h to study the IEC-derived galectin secretion.

First a dose-response experiment was done using 0.25-1% (*w/v*) 2'FL/GF to determine the optimal concentration at which the most relevant immunomodulatory effects are observed. Results showed that in association with CpG exposure to 0.5% 2'FL/GF, was enough to promote regulatory IL-10 and IEC-derived galectin-9 as well as decreased concentrations of IL-13 and a tendency towards increased IFNγ (**Figure S2**). Thus, following studies were done using 0.5% 2'FL/GF.

Exposure to 2'FL/GF alone did not affect galectin-9, epithelial-derived galectin-9, IFNγ, IL-10, IL-13 and TNFα concentrations (**Figure 3**). Exposure to CpG alone significantly increased IL-10 and decreased IL-13 concentrations as compared to medium control (**Figure 3D-E**). Combined exposure to 2'FL/GF and CpG, significantly increased IEC-derived galectin-9, IFNγ and IL-10, and decreased IL-13 concentrations, without affecting TNFα, as compared to medium control, 2'FL/GF alone and/or CpG alone (**Figure 3B-F**).

Altogether, Th1-type IFNγ and regulatory type IL-10 and IEC-derived galectin-9 were significantly increased, and Th2-type IL-13 was decreased suggesting Th1-type regulatory immunomodulatory activity of 2'FL/GF and CpG in the IEC/PBMC model.

Besides IEC-derived galectin-9 secretion, the secretion of galectin-3 and -4, were studied upon exposure of HT-29 and PBMC to 2'FL/GF and CpG. There was no effect on IEC-derived galectin-3 concentrations (**Figure S3**). Exposure to 2'FL/GF or CpG alone did not affect the concentrations of IEC-derived galectin-4. However, upon combined exposure to 2'FL/GF and CpG, significantly increased IEC-derived galectin-4 were observed (**Figure S3**).

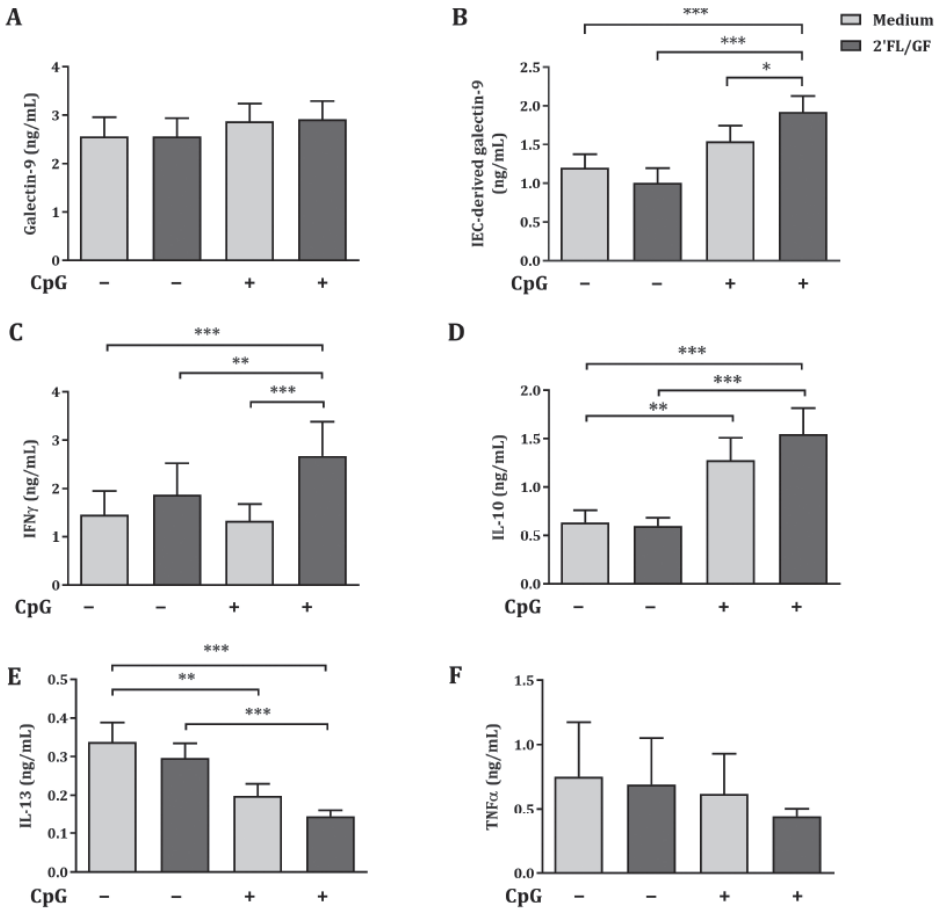

Figure 3. Cytokine and galectin-9 secretion in IEC/PBMC co-culture.
HT-29 IEC were stimulated with a 1:1 mixture of 2'FL and GF (0.5% *w/v*) in combination with CpG (0.5 μM) and basolaterally exposed to αCD3/CD28-activated PBMC for 24 h. After incubation, galectin-9 (**A**), IFNγ (**C**), IL-10 (**D**), IL-13 (**E**) and TNFα (**F**) were measured. Additionally, after IEC/PBMC co-culture, HT-29 IEC were washed and incubated in fresh medium for additional 24 h after which IEC-derived galectin-9 (**B**) was measured. Data are represented as mean ± SEM of $n = 6$ (E), $n = 7$ (A, B, E) or $n = 8$ (A, C, D) independent PBMC donors (# $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

In order to study the connection between epithelial-derived galectin secretion and the cytokine secretion in the IEC/PBMC co-culture correlations analysis were done. The correlation coefficient r and the probability values (p) are summarized in the **Table 1** below. Th1-type IFNγ secretion was positively correlated with epithelial derived galectin-3, and -4. Epithelial-derived galectin-4 and -9, but not galectin-3 correlated positively with IL-10 secretion. Meanwhile, only galectin-9 correlated negatively with

IL-13 secretion. Although in this experiment, no correlation was observed between IFNγ secretion and IEC-derived galectin-9, in the correlations of the dose-response experiments, a positive correlation was observed (**Figure S2**).

Table 1. Correlations between cytokines in the IEC/PBMC co-culture and epithelial-derived galectins.

	IFNγ	IL-10	IL-13
IEC-derived galectin-3	+ r = 0.06 $p = 0.001$ (**)	n.s. r = 0.17 $p = 0.4$	n.s. r = − 0.33 $p = 0.11$
IEC-derived galectin-4	+ r = 0.45 $p = 0.01$ (*)	+ r = 0.43 $p = 0.015$ (*)	n.s. r = 0.41 $p = 0.045$
IEC-derived galectin-9	n.s. r = 0.21 $p = 0.3$	+ r = 0.67 $p < 0.001$ (***)	− r = − 0.7 $p = 0.0002$ (***)

(−) negative correlation ; (n.s.) non-significant correlation; (+) positive correlation

Epithelial-derived galectin-9 concentrations were significantly increased upon exposure to 0.5% 2'FL/GF and CpG after IEC/PBMC co-culture. Exposure of HT-29 to CpG and 0.5% 2'FL/GF significantly increased Th1-type IFNγ and regulatory IL-10 and epithelial-derived galectin-9, while decreasing Th2-type IL-13. The cytokine secretion in IEC/PBMC co-culture significantly correlated with epithelial-derived galectin-9 secretion, which suggests that epithelial-derived galectin-9 is involved in the immunomodulatory effects observed upon exposure to 2'FL/GF and CpG in the IEC/PBMC co-culture model.

Galectins are released by HT-29 through exosomes in IEC/PBMC co-culture

Exosomes are implicated in cell-to-cell communication and galectins have relevant roles in modulating several immune processes. In order to study if HT-29 secrete galectins via exosomes, after exposure to NDO and CpG, IEC-derived supernatant was collected and exosomes were isolated. To confirm the presence of exosomes in the supernatant, CD63 expression was measured by ELISA. As a control, exosomes were isolated from RPMI medium supplemented with FCS, which gave the lowest CD63 signal (represented as dotted line) (**Figure 4A**). In IEC supernatant from IEC/PBMC co-cultures conditioned with medium and CpG, increased CD63 secretion was observed. This was further increased by the addition of 2'FL/GF (**Figure 4A**).

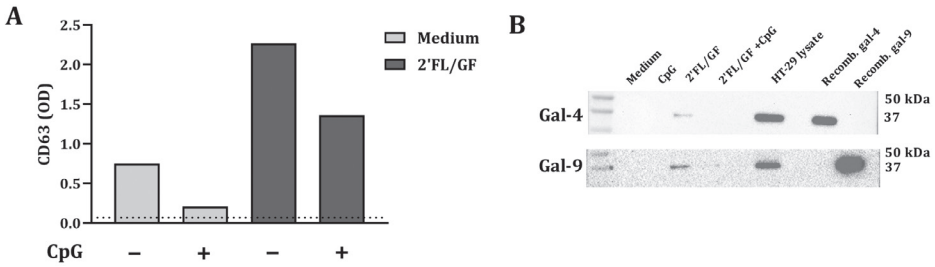

Figure 4. Galectin-4 and -9 are present in CD63-expressing exosomes.
IEC were apically exposed to 0.5% (*w/v*) 2'FL/GF alone or in combination with 0.5 µM CpG and basolaterally to αCD3/CD28-activated PBMC. After 24 h incubation, IEC were separated from PBMC, washed and incubated in fresh medium for additional 24 h after which the basolateral supernatant was collected and exosomes isolation was performed. The presence of exosomes was confirmed by measuring CD63 marker (**A**) by means of ELISA. The dotted line represent CD63 present in culture medium (non-exposed to IEC). Additionally, the presence of galectin-4 and -9 within CD63-expressing exosomes was studied by western blot (**B**) of $n = 1$.

Additionally, the presence of galectin-4 and -9 in the exosomes was studied by western blot. Galectin-4 and -9 expression was found in 2'FL/GF stimulated exosome samples (**Figure 4B**). However, galectin-4 and -9 were not measured in medium samples or in samples exposed to CpG alone or in combination with 2'FL/GF (**Figure 4B**).

Exposure to GW4869 suppressed IEC-derived galectin-4 and -9 secretion

To study how IEC secrete galectins we apically exposed IEC (HT-29 cell line) to 10 µM GW4869, the neutral sphingomyelinase inhibitor which functions as an inhibitor of the exosome biogenesis, and after 1h incubation we exposed IEC to CpG alone or in combination with 0.5% 2'FL/GF and co-cultured with activated-PBMC. After 24 h co-culture, IEC were separated, washed and incubated again with 10 µM GW4869 for 24 h after which IEC supernatant was collected for epithelial-derived galectin measurement.

IEC-derived galectin-9 was significantly increased upon exposure to CpG alone or in combination with 0.5% 2'FL/GF as compared to medium control and/or CpG alone (**Figure 5A**). Exposure to GW4869 suppressed IEC-derived galectin-4 and -9 secretion as observed upon exposure to CpG alone or 2'FL/GF and CpG (**Figure 5A-B**). There was no effect on IEC-derived galectin-3 and -4 upon exposure to CpG alone and only IEC-derived galectin-4 was significantly increased upon exposure to 0.5% 2'FL/GF and CpG as compared to medium control or CpG alone (**Figure 5B-C**).

The secretion of IEC-derived galectin-4 and -9 was suppressed upon exposure to GW4869 inhibitor.

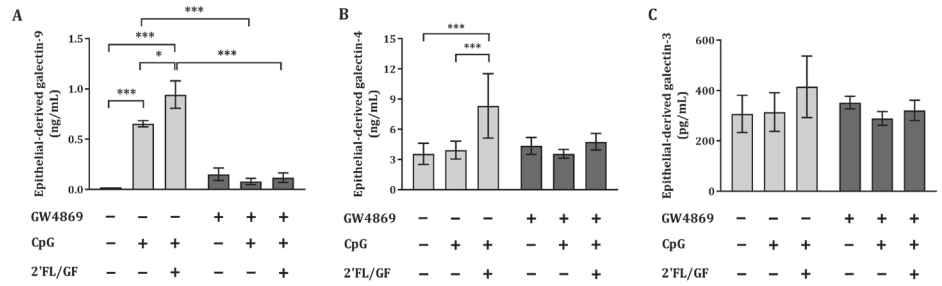

Figure 5. Exposure to GW4869 suppressed epithelial-derived galectin-4 and -9 secretion.
IEC were apically exposed to 10 µM GW4869 for 1 h after which 0.5% (*w/v*) 2'FL/GF mixture and CpG were added. In the basolateral compartment αCD3/CD28-activated PBMC were added and incubated. After 24 h incubation, IEC were separated from the PBMC fraction, washed and incubated in fresh medium or in the presence of 10 µM GW4869 for additional 24h after which the basolateral supernatant was collected and epithelial-derived galectin-9 (**A**), -4 (**B**) and -3 (**C**) were measured. Data are represented as mean ± SEM of $n = 6$ (**A, B**) or $n = 4$ (**C**) independent PBMC donors (# $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Exposure to GW4869 suppresses IL-10 and IL-13 secretion in IEC/PBMC co-culture

To study the effects of the inhibitor of exosome biogenesis GW4869 in the cytokine secretion in HT-29/PBMC co-culture, we studied the secretion of IFNγ, IL-13 and IL-10 concentrations upon exposure to CpG and 2'FL/GF and CpG in the presence and absence of 10 µM GW4869.

There was no effect on IFNγ concentrations upon exposure to CpG alone or in combination with 2'FL/GF (**Figure 6A**). However, significantly increased IL-10 and decreased IL-13 concentrations were found upon exposure to CpG alone or in combination with 2'FL/GF as compared to medium control (**Figure 6B-C**). When IEC were exposed to GW4869 in the presence of 2'FL/GF and CpG, and co-cultured with activated-PBMC, the reduction in IL-13 secretion as well as the increase in IL-10 concentrations was suppressed (**Figure 6B-C**).

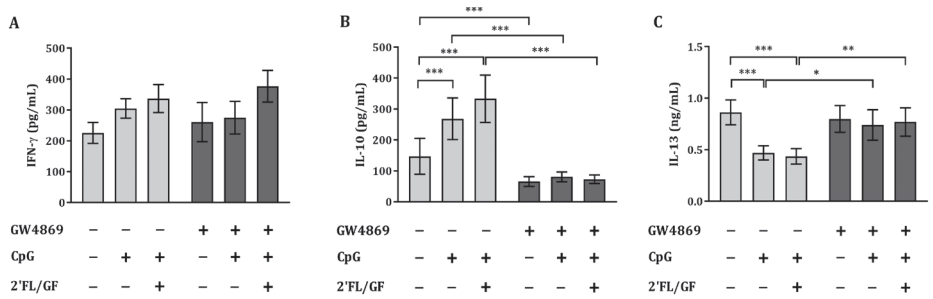

Figure 6. Exposure to GW4869 suppressed IL-10 increased and the decrease in IL-13 secretion.
IEC were apically exposed to 10 µM GW4869 for 1 h after which 0.5% (*w/v*) 2'FL/GF mixture and 0.5 µM CpG were added. In the basolateral compartment αCD3/CD28-activated PBMC were added and incubated. After 24 h incubation, the basolateral supernatant was collected and IFNγ (**A**), IL-10 (**B**) and IL-13 (**C**) were measured. Data are represented as mean ± SEM of $n = 6$ (B,C) or $n = 5$ (A) independent PBMC donors (# $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

The reduction in Th2-type IL-13 and increase in regulatory type IL-10 promoted upon exposure to 2'FL/GF and CpG was suppressed upon exposure of IEC to GW4869 inhibitor.

DISCUSSION

IEC-derived galectin-9 was identified as a key factor contributing to immune regulation in an *in vitro* co-culture model combining IEC and immune cells [2,3,9,28] as well as *in vivo* in a murine food allergy prevention model [5], which was secreted upon exposure to NDO and a bacterial trigger such as TLR9 agonist. In these *in vitro* studies, the HT-29 cell line was used as a model of intestinal epithelial cells.

Primary FHs 74 Int or HT-29 in IEC/PBMC co-culture

Our aim was to study whether a non-carcinogenic intestinal epithelial cell line would be able to modulate immune effects of NDO and CpG, similar to what was previously shown for the HT-29 cell line [2, 9]. Therefore, the human fetal intestinal cell line FHs 74 Int was used in co-culture with human PBMC. In line with previous studies, only upon activation of PBMC, CpG-induced upregulation of, regulatory IL-10 and galectin-9 were found [29]. The addition of NDO enhanced the regulatory responses while lowering Th2-type IL-13 secretion [2, 3, 9, 26]. Previous studies also showed upregulated Th1- type IFNγ upon exposure to NDO and CpG. However here, we used a lower concentration of CpG, thereby IFNγ secretion did not reach significance.

Unlike in the HT-29 cell line, in the FHs 74 Int/PBMC co-culture, already in the absence of CpG, GF or 2'FL/GF alone were able to modulate the secretion of cytokines like Th1-type IFNγ, Th2-type IL-13 as well as regulatory IL-10 and galectin-9. Even though the FHs74 and HT-29 cells have a similar response to CpG when co-cultured with activated PBMC, in absence of CpG the NDO did enhance galectin-9 release by FHs74 cells but not by HT-29 cells. This suggests that primary epithelial cells such as the fetal FHs 74 Int are more responsive to NDO exposure than the carcinogenic HT-29 cell line derived from an adult. By inducing the release of galectin-9 in the FHs 74 Int/PBMC co-culture, the NDO may have been able to modulate the inflammatory response of the activated-PBMC. We hypothesize that the FHs 74 Int may have responded differentially to the inflammatory milieu compared to the HT-29, which may have resulted in the increased sensitivity to the effects of NDO. Other studies indicated that the FHs 74 Int cell line was more susceptible to inflammation than other adult cell lines [30, 31].

In addition, when NDO were combined with CpG and exposed to the FHs 74 Int/PBMC co-culture, upregulated regulatory-type Th1 and downregulated Th2-type cytokines were observed, similar to what was previously observed in HT-29 or T84 and PBMC co-cultures [2, 9]. However, even though the HT-29 cells were found to release also galectin-4 and galectin-3 beyond galectin-9 [2, 9, 26], the primary FHs 74 Int epithelial cells only secreted galectin-9 and -3, but not galectin-4. Also others found anti-inflammatory effects in primary fetal epithelial cells using NDO. Downregulated IL-8 secretion was observed in TNFα-activated FHs 74 Int exposed to NDO [30]. Furthermore, in FHs 74 Int exposed to human milk, growth-related effects of immature epithelial cells [32] and regulation of inflammatory responses [33] were observed. This supports the ability of bioactive components in human milk such as human milk oligosaccharides (HMOS) to play a role in IEC maturation and regulation of IEC function. These studies support the potential use of FHs 74 Int primary IEC *in vitro* to study the modulation of immune responses upon exposure to NDO. Other non-carcinogenic human intestinal epithelial cell lines are also available such as HIEC-6, H4 or NCM-460. However, the FHs 74 Int is a primary human intestinal cell line isolated from the fetal small intestine, it has been validated as a model of intestinal epithelium in newborns [33] which is of interest to study the regulation of immune responses in early life.

Galectins and immune-regulation

The immunomodulatory properties of a mixture of 2'FL and GF in combination with CpG observed in the present study are in line with previous studies [2, 3, 9]. Exposure of HT-29 to 2'FL/GF resulted in upregulated Th1-type IFNγ and regulatory IL-10 and galectin-9, while downregulating Th2- type IL-13 and pro-inflammatory TNFα. Additionally, an upregulation of IEC-derived galectin-9 and -4 was observed. The

increased secretion of epithelial-derived galectins was described before upon exposure to 2'FL or GF in association with CpG [9]. Also in the current study, IEC-derived galectin-9, -4 and -3 secretion was positively correlated with IFNγ, while galectin-9 and -4 were positively correlated with IL-10 and negatively with IL-13 secretion in the HT-29/PBMC co-culture. These correlations were particularly pronounced for IL-10 and IL-13 and IEC-derived galectin-9, which emphasizes the involvement of epithelial-derived galectins in supporting immunomodulation. Similar to the results with the HT-29 cells, also FHs 74 Int primary IEC show the same immunomodulatory effects already in the absence of CpG exposure, while only modulating galectin-9 secretion, therefore we hypothesize that galectin-9 has a major role in mucosal immune modulation by NDO. In a murine food allergy model, NDO were found to enhance galectin-9 expression in IEC, while increasing the serum levels in association with modulation of the mucosal immune response and allergy protection [5].

Galectin secretion via exosomes

Little is known about the cellular mechanisms involved in the secretion of galectins and how these can be influenced by NDO. Since galectins lack a N-terminal signaling sequence to direct them through the endoplasmic reticulum for secretion, un-conventional transport pathways are thought to be the routes used for galectin secretion. One of the routes by which galectins are secreted into the extracellular milieu are extracellular vesicles [11, 21]. Although it's still unknown how galectins are recruited into extracellular vesicles, various studies have demonstrated the localization of galectin-3, - 4 and -9 inside multivesicular bodies [11, 21].

Here we show that CD63-expressing exosomes were released by the IEC into the basolateral compartment after IEC/PBMC co-culture, and these exosomes were found to contain galectin-9 and -4 as measured by western blot. Depending on the experimental condition, the amount of exosomes isolated differed which might have hampered the detection of galectin-9 and -4 for all conditions. As was shown previously using the HT-29/PBMC model [26], NDO facilitate CpG induced galectin-9 release by IEC and this may have taken place during the first 24 h, thus during the co-culture with PBMC. The CpG and 2'FL/GF condition may therefore have already caused the exosome release which might explain a more limited signal observed after the additional 24 h of epithelial cell culture following the co-culture with PBMC. However, since galectins were measured in the IEC supernatant by means of ELISA, also in the 2'FL/GF and CpG condition, we hypothesize that in all conditions the IEC-derived galectins were secreted via exosomes, similar to what was observed for the 2'FL/GF condition. To study if exosomes were involved in the mechanism by which NDO and CpG promoted the secretion of galectins, IEC were exposed to GW4869, a specific neutral sphingomyelinase

(nSMase) inhibitor known to block the exosome biogenesis and thereby their release [25]. GW4869 suppressed epithelial-derived galectin-9 release and partially galectin-4, indicating that the inhibition of exosome biogenesis by GW4869 might have hampered the recruitment of galectin-9 and -4 into the multivesicular bodies as well as their release into the basolateral compartment. However, galectin-3 secretion was not affected by 2'FL/GF and CpG which suggests that only the secretion of the newly formed galectins was inhibited by GW4869, but not the constitutive secretion of galectin-3 from IEC. Although most of the galectin-9 and -4 secretion was blocked by exposure to exosome biogenesis inhibitor GW4869, it can't be excluded that the residual galectin-9 and -4 secretion is derived from other secretion mechanisms such us through direct transport across the membrane [11].

Involvement of galectins on immunomodulation

The secretion of epithelial-derived galectin-9 and -4 were positively correlated with IL-10 and negatively with IL-13 secretion in the IEC/PBMC co-culture. Blocking the galectin-9 and -4 release induced by 2'FL/GF and CpG through the inhibition of exosomes secretion from IEC was found to prevent the increase of IL-10 secretion, while blocking the reduction in IL-13 secretion. These results, imply an essential role for epithelial-derived galectin-9 and -4 in the regulation of the immunomodulatory effects observed in the IEC/PBMC co-culture upon exposure to NDO and CpG. These results are in accordance with a previous study showing the essential immunomodulatory role for galectin-9 in this co-culture model using either lactose or Tim3-Fc fusion protein to block galectin-9 [2].

In conclusion, exposure to NDO promoted Th1-type regulatory effects in IEC, while driving away from the Th2 phenotype, in association with CpG in the HT-29/PBMC co-culture. In the primary FHs 74 Int/PBMC co-culture, NDO induced similar immunomodulatory effects already in the absence of CpG in association with enhanced galectin-9 secretion. Galectin-9 and -4 were present in epithelial-derived exosomes and inhibition of exosome biogenesis by IEC inhibited galectin release, blocking the immunomodulatory effects of 2'FL/GF and CpG. This demonstrates an essential role for galectin-9 from IEC-derived exosomes in NDO induced mucosal immune regulation.

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SUPPLEMENTARY FIGURES

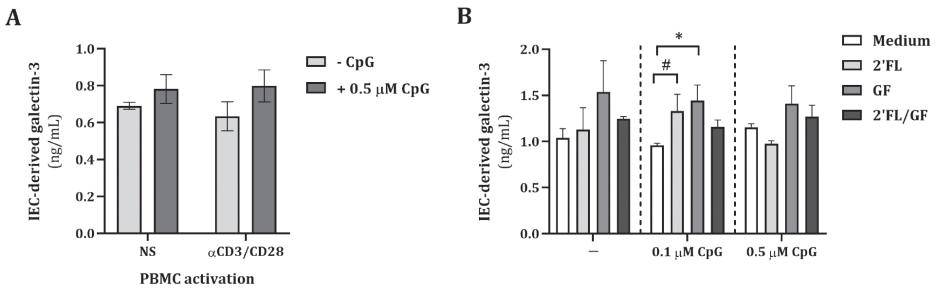
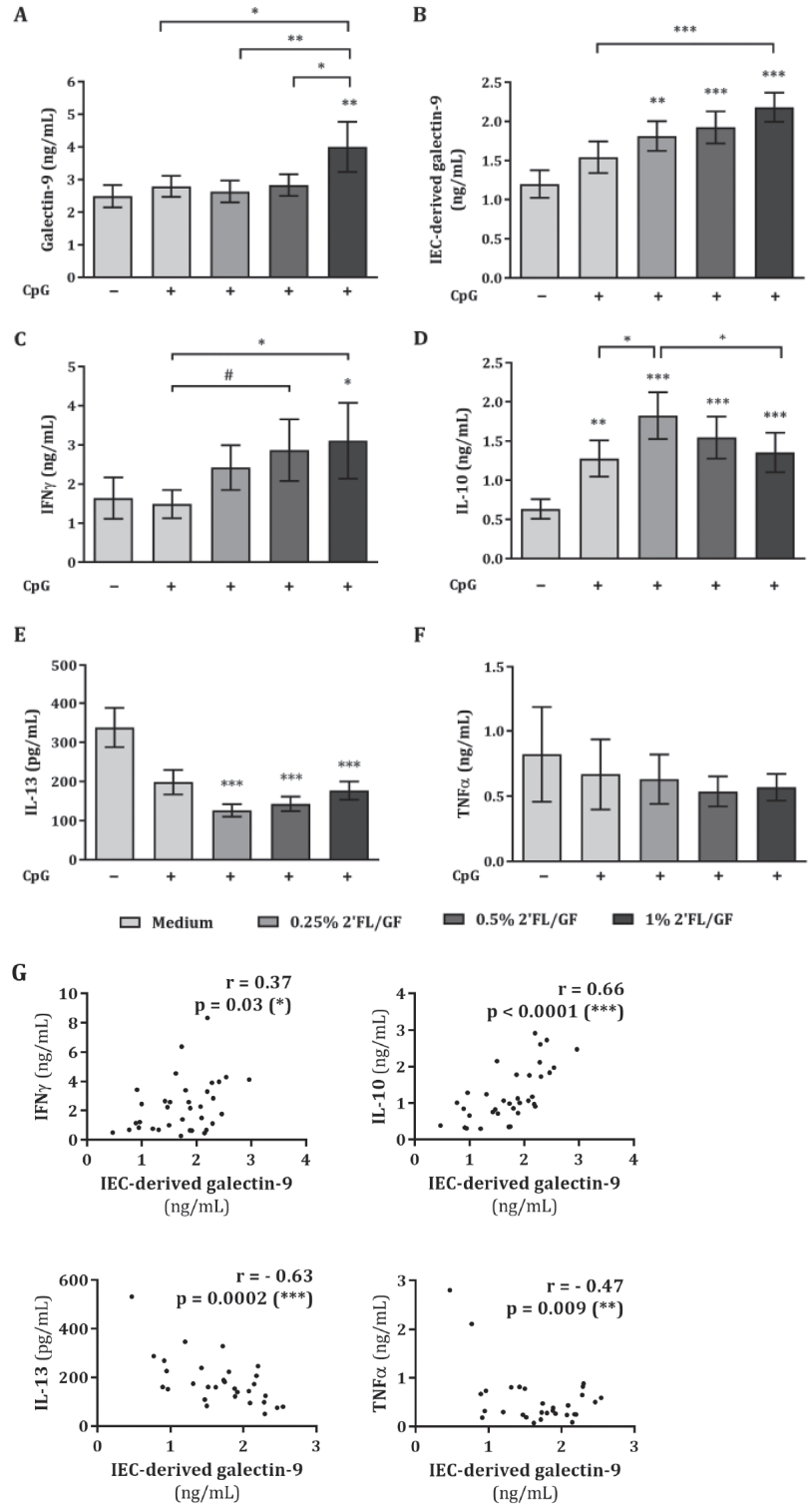


Figure S1. Galectin-3 secretion in FHs 74 Int/PBMC co-culture model.
FHs 74 Int IEC were stimulated with 0.5 µM CpG and basolaterally exposed to non-activated (NS) or αCD3/CD28-activated PBMC for 24 h. Alternatively, FHs 74 Int were stimulated with 0.1 or 0.5 µM CpG in the presence of 0.5% (*w/v*) 2'FL, GF or a 1:1 mixture of 2'FL and GF, and basolaterally to αCD3/CD28-activated PBMC for 24 h. After IEC/PBMC co-culture, FHs 74 Int were washed and incubated in fresh medium for additional 24 h after which IEC-derived galectin-3 was measured. Data represent mean ± SEM of $n = 3$ independent PBMC donors (# $p < 0.1$, * $p < 0.05$).

> Figure S2. Dose-response effects and correlations in IEC/PBMC co-culture.
HT-29 IEC were stimulated with 2'FL, GF or 1:1 mixture of 2'FL and GF (0.25-1% *w/v*) in combination with CpG (0.5 µM) and basolaterally exposed to αCD3/CD28-activated PBMC for 24 h. After incubation, galectin-9 (**A**), IFNγ (**C**), IL-10 (**D**), IL-13 (**E**) and TNFα (**F**) were measured. Additionally, after IEC/PBMC co-culture, HT-29 IEC were washed and incubated in fresh medium for additional 24 h after which IEC-derived galectin-9 (**B**) was measured. The correlations between IEC-derived galectin-9 and the cytokines in IEC/PBMC co-culture are also shown (**G**). Data are represented as mean ± SEM of $n = 7$ (A, B, C, D, E, F) or $n = 8$ (D) independent PBMC donors (# $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).



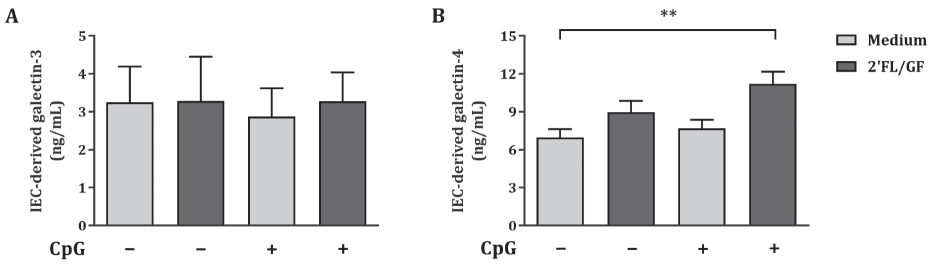


Figure S3. Epithelial-derived galectin-3 and -4 secretion in IEC/PBMC co-culture.
After IEC/PBMC co-culture, IEC were washed and incubated in fresh medium for additional 24 h (total 48 h; 24 h in IEC/PBMC co-culture and additional 24 h of IEC culture in fresh medium), after which basolateral supernatant was collected and epithelial-derived galectin-3 (**A**) and -4 (**B**) were measured. Data are represented as mean ± SEM of $n = 6$ (A) or $n = 8$ (B) independent PBMC donors (# $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

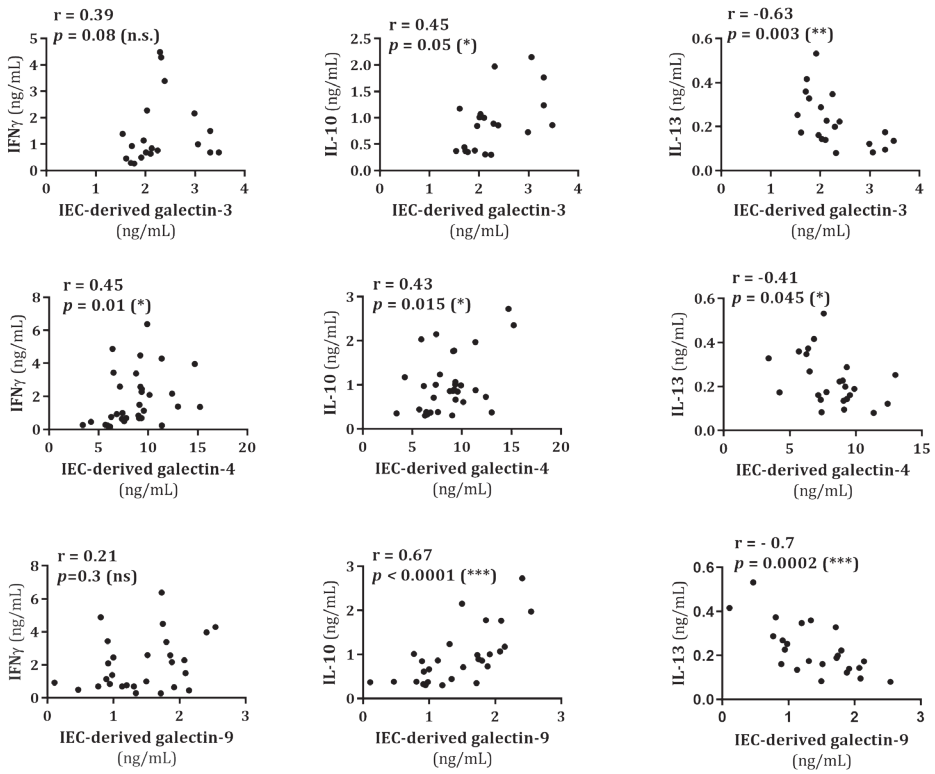
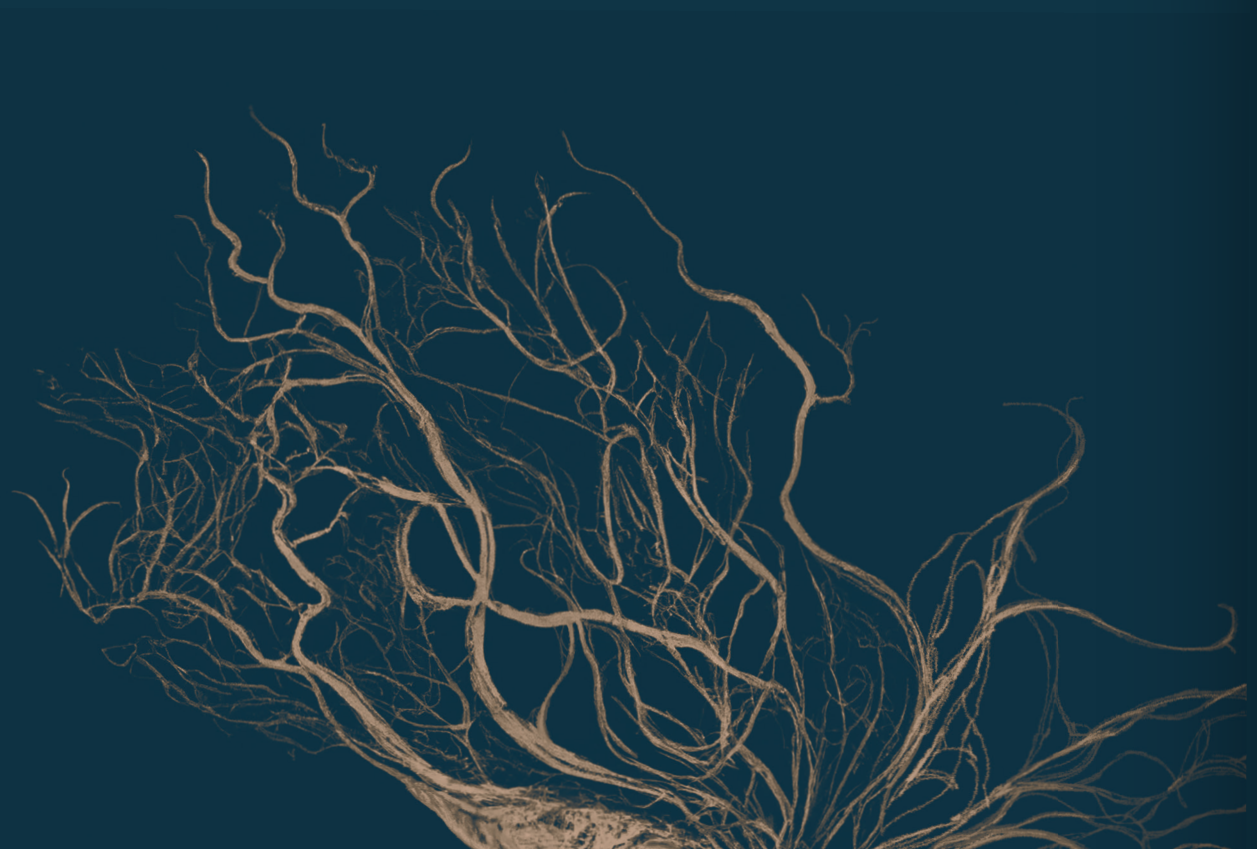


Figure S4. Correlation plots epithelial-derived galectins and cytokines in IEC/PBMC co-culture.



CHAPTER | 7

A fermented milk matrix containing postbiotics supports Th1- and Th17-type immunity in vitro and modulates the influenza-specific vaccination response in vivo in association with altered serum galectin ratios

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ABSTRACT

During a specific milk fermentation process with *Bifidobacterium breve* C50 and *Streptococcus thermophilus* 065 (Lactofidus™), postbiotics with possible immunomodulatory properties are produced. We investigated the effects of this fermentation product (FP) *in vitro* using a model that allows crosstalk between intestinal epithelial (IEC) and immune cells. IEC were exposed to FP and αCD3/CD28-activated peripheral blood mononuclear cells after which the mediator secretion was measured. Additionally, using a murine influenza vaccination model, immune development was assessed. Mice were fed an AIN93G diet containing FP or lactose as control. Vaccine-specific immunity was measured as delayed-type hypersensitivity (DTH) and correlated to intestinal and systemic immunomodulation levels. *In vitro*, exposure to FP enhanced IFNγ, TNFα and IL-17A concentrations. Moreover, IEC-derived galectin-3/galectin-9 and galectin-4/galectin-9 ratios were increased. *In vivo*, dietary intervention with FP increased vaccine-specific DTH responses as compared to the lactose-receiving group. Although no effects on humoral immunity and vaccine-specific T-cell responses were detected, an enhanced systemic serum galectin-3/galectin-9 and galectin-4/galectin-9 ratio correlated with a shift in RORγ (Th17) mRNA expression over regulatory TGFβ1 in the ileum. This was also positively correlated with the increased DTH response. These results indicate that FP can enhance epithelial galectin-3 and -4 over galectin-9 release, and boost adaptive immunity by promoting Th1- and Th17-type cytokines under inflammatory conditions *in vitro*. Similar variations in galectin and immune balance were observed in the vaccination model, where FP improved the influenza-specific DTH response.

Keywords: postbiotics; vaccination; influenza; galectins; fermentation; adaptive immunity

INTRODUCTION

The development of the mucosal immune system constitutes a crucial stage in early life and its development represents a decisive period for the establishment of a balanced mucosal immune and systemic immune function [1, 2]. Diet plays a pivotal role by providing all the necessary nutrients for growth and development of a healthy gut and supporting the establishment of a balanced microbiome and a proper maturation of the immune system. Breastfeeding is considered the gold standard for infant nutrition, and as such, the World Health Organization recommends exclusive breastfeeding during at least the first six months of life, which can be extended up to two years or beyond next to complementary food introduction [3].

Current research is focused on developing alternative nutritional interventions for those infants that are unable to receive enough breastmilk. Although human milk is always the preferred option for infant nutrition, fermented milk-based infant formulas are being developed [4] and studied for their ability to modulate the immune function [5]. Fermented milk-based formulas are obtained by fermentation of a milk matrix with lactic acid-producing bacteria, followed by heat-inactivation of viable bacteria. This fermentation process results in the formation of bioactive components known as postbiotics. Postbiotics are defined as bioactive compounds produced by food-grade microorganisms in a fermentation process (including microbes, cell constituents and metabolites) that in adequate amounts promote health and/or well-being of the host [4, 6–8]. Postbiotics refer to soluble factors such as enzymes, proteins, polysaccharides, short-chain fatty acids and peptidoglycans, known to promote diverse local as well as systemic effects, among which immunomodulation and anti-inflammation stand out [7].

Specific fermented infant formulas containing postbiotics are commercially available and their possible beneficial effects have been systematically reviewed [9]. The most extensively studied postbiotics are derived from *Lactobacillus*, *Streptococcus* and *Bifidobacterium* strains due to their use as probiotics [6] as well as in regard to the beneficial effects shown in cell-mediated immunity and inflammation [10]. One of the most studied fermented formula is obtained through a unique fermentation process of a milk matrix (Lactofidus™) using two bacterial strains namely *Bifidobacterium breve* C50 and *Streptococcus thermophilus* 065, known to generate bioactive components such as 3'galactosyllactose; a non-digestible oligosaccharide naturally occurring in human milk [11]. Several clinical trials have described improved gut and immune parameters upon dietary intervention with infant formula fermented by *B. breve* C50 and *S. thermophilus* 065, summarized by Salminen et al. [4]. Those clinical trials showed that healthy infants receiving infant formula supplemented with fermentation products

from *B. breve* and *S. thermophilus*, had lower severity of acute diarrhea episodes [12], enhanced thymus size [13], lowered fecal calprotectin and increased secretory IgA [14]. Furthermore, systemic effects such as an increased anti-poliovirus IgA response was also seen in infants fed an infant formula supplemented with fermentation products [15]. Additionally, in infants at high risk of atopy who were fed a fermented infant milk formula, fewer cases of positive skin-prick test to cow's milk were observed [16]. *In vitro* fermentation products (FP) derived from a fermentation of a milk matrix with *B. breve* C50 and *S. thermophilus* 065 were shown to promote immunomodulatory effects in dendritic cells by increasing IL-10 release [17] as well as stimulating a Th1 immune response in mice [18].

In an *in vivo* influenza vaccination model, specific non-digestible oligosaccharides effectively improved vaccine-specific immune responses by promoting Th1-type immunity [19–23]. Furthermore, *in vitro* and *in vivo* combined exposure to non-digestible oligosaccharides and specific bacteria or bacterial fragments (bacterial CpG DNA) has previously been shown to drive regulatory-type Th1 responses, among others, via instruction of epithelial-derived galectin release [24–27].

Regarding the immunoregulatory capacities of milk-based fermented formula shown *in vitro* and *in vivo*, we hypothesize that FP could be able to improve the response to an influenza vaccine in a murine vaccination model by exerting an effect in the gut. Hence, the aim of this study was to investigate the possible immunomodulatory effects of a fermented milk matrix containing possible bioactive fermentation products (FP) produced following the Lactofidus™ fermentation process, and to study its effect in the influenza vaccination model. An established *in vitro* co-culture model combining human intestinal epithelial as well as immune cells [24, 25, 28] was used to study the effect of FP on the epithelial cell and immune cells crosstalk. Additionally, a murine influenza vaccination model was used to study the effects of a dietary intervention with FP on vaccination responses. FP was found to modulate the galectin-3 and galectin-4 over galectin-9 balance systemically, which correlated with an increased influenza-specific delayed-type hypersensitivity (DTH) response.

MATERIALS AND METHODS

In vitro IEC/PBMC co-culture model

Intestinal epithelial cell culture

Human intestinal epithelial cells (IEC), HT-29 cell line (ATCC, HTB-38), were cultured in 75 cm^2 cell culture flasks (Greiner Bio-One) using Mc Coy 5A medium

(Gibco, Invitrogen) supplemented with 10% heat-inactivated fetal-calf serum (FCS), penicillin (100 U/mL) and streptomycin (100 µg/mL) (Sigma-Aldrich). HT-29 cells were kept in an incubator at 37 °C and 5% CO_2. Cells were passaged once a week and medium was refreshed every 2-3 days.

Peripheral blood mononuclear cell isolation

Buffy coats from healthy donor (Sanquin, Amsterdam, The Netherlands) were used to isolate human peripheral blood mononuclear cells (PBMC) by density gradient centrifugation (1,000 x g, 13 min). After centrifugation, the pellet was washed with PBS supplemented with 2% FCS. The remaining erythrocytes were lysed using 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. The isolated PBMC fraction was resuspended in RPMI 1640 supplemented with 2.5% FCS, penicillin (100 U/mL) and streptomycin (100 µg/mL).

IEC/PBMC co-culture model

One week prior to the experiment, HT-29 cells were diluted five to eight times based on cell surface area and seeded in transwell inserts (12-well, 0.4 µm polyester membrane, Costar Corning Incorporated). When HT-29 reached confluency, they were apically exposed to 0.25–0.5% FP (*w/v*). In the basolateral compartment, 2 x 10^6 cells/mL of αCD3/CD28-activated PBMC (clone CLB-T3/2 and clone CLB-CD28 respectively, both 1:10,000, Sanquin, The Netherlands) were added. After 24 h of incubation (37 °C, 5% CO_2), the basolateral supernatant was collected and stored at –20 °C for cytokine measurements.

Enzyme-linked immunosorbent assay (ELISA)

The cytokine secretions were analyzed in the basolateral supernatant from IEC/PBMC co-cultures. Commercially available kits were used to determine IFNγ, TNFα, IL-17A, IL-13, TGFβ1 (all from Thermo Fisher scientific), IL-10 (U-Cytech) and galectin-3 (R&D systems) according to the manufacturer's protocol. Human galectin-4 and -9 were measured using antibody pairs. In short, high-binding Costar 9018 plates were incubated overnight with 0.75 µg/mL human galectin-4 or -9 affinity purified polyclonal antibody. Non-specific binding was blocked with 1% bovine serum albumin (BSA) in PBS for one h, after which samples were added and incubated for 2 h at room temperature. After washing, biotinylated galectin-4 or -9 affinity purified polyclonal antibody (0.75 µg/mL) was incubated for 1 h. Then, plates were washed and streptavidin-HRP was incubated for 1 h. After washing, tetramethylbenzidine was used as a substrate to develop the reaction (TMB, Thermo Fisher scientific), which was stopped with 1 M H_2SO_4. Optical density was measured at 450 nm.

In vivo influenza vaccination model

Animals

Six-week-old C57Bl/6JOlaHsd female mice were purchased from Envigo and housed under conventional conditions with a light/dark cycle of 12 h/12 h (lights on from 7.00 am–7.00 pm) at controlled relative humidity (relative humidity of 50–55%) and temperature (21 ± 2 °C) with access to food and water *ad libitum*, in the animal facility of Utrecht University. Upon arrival, mice were randomly grouped as three mice per cage in filter-topped makrolon cages (22 cm x 16 cm x 14 cm, floor area 350 cm^2, Tecnilab BMI) with wood-chip bedding (Tecnilab BMI); tissues and a plastic shelter were available as cage enrichment at the animal facility. The animals received standard diets for one week until the start of the experiments. The C57Bl/6JOlaHsd female mice were previously used to establish this influenza vaccination model and further studies are also available [19–23,29].

This study was conducted in accordance with institutional guidelines for the care and use of laboratory animals established by the Animal Ethics Committee of Utrecht University, and all animal procedures were approved under the Ethical license of the national competent authority, securing full compliance the European Directive for the use of animals for scientific purposes.

Vaccination protocol and dietary intervention

One week after acclimatization, mice were fed AIN93G diet or the AIN93G diet containing FP or lactose (SNIFF Spezialdiäten GmbH) until the end of the experiment by a researcher blinded to the experimental treatments. AIN93G diets were mixed with 0.5% or 2.5% (*w/w*) FP. As a control for the amount of lactose present in the fermented milk matrix, the lactose diets were mixed with 0.25% and 1.25% (*w/w*), respectively. The percentages of FP and lactose were exchanged against an equal amount (*w/w*) of total carbohydrates present in the control diet.

On days 0 and 21, the mice received a subcutaneous vaccination under isoflurane anesthesia using Influvac season 2015/2016 (Abbot Biologicals B.V.). The mice (n = 9 per experimental group) received a total volume of 100 µL containing 90 µg/mL hemagglutinin from three strains of the influenza virus. A negative control group, referred as sham (n = 3), received injections with 100 µL PBS. Delayed-type hypersensitivity (DTH) reactions were induced 9 days after booster vaccination by intradermal injection of 20 µL Influvac into the ear pinnae of the right ear. As basal line, 20 µL PBS was injected in the left ear pinnae. Ear thickness was measured in duplicate before intradermal challenge and 24 h thereafter using a digital micrometer

(Mitutoyo). The antigen-specific delayed-type hypersensitivity (DTH) responses were calculated using the following formula: DTH = Right ear thickness (@24 h–@0 h)–Left ear thickness (@24 h–@0 h). After measuring the ear thickness, mice were anesthetized and sacrificed. Then, the ears were punctured and the weight of the ear puncture was measured.

Vaccine-specific immunoglobulins and galectins in serum

At the end of the experiment, mice were anesthetized and blood was collected by retro-orbital bleeding of the eye. Blood samples were centrifuged (14,000 rpm, 10 min) and serum was stored at −80 °C until analysis of vaccine-specific antibodies by means of ELISA. Vaccine-specific antibody titers were measured as described previously [19]. Briefly, 96-well high-binding plates (Costar Corning Incorporated) were coated with 1:100 diluted Influvac in PBS. As blocking reagent, 2% BSA in PBS was used. Serial dilutions of pooled serum containing vaccine-specific antibodies was done and used for standard curve calculations. Biotinylated anti-IgG1 and anti-IgG2a antibodies (Becton Dickinson) were diluted 1:100 and incubated for 1 h, after which streptavidin-HRP (Sanquin, Amsterdam, The Netherlands) was added. Optical density was measured at 490 nm with a microplate reader (Bio-Rad). Mouse serum galectin-4 and -9 (both from R&D systems) were measured by means of ELISA according to the protocol described in Section 2.1.4. for human galectin-4 and -9. Purified mouse anti-goat antibodies (0.2 mg/mL for galectin-4 and -9), recombinant mouse cytokines and biotinylated goat anti-mouse antibodies (0.2 mg/mL for galectin-4 and -9) were purchased from R&D systems. Non-specific binding was blocked using PBS supplemented with 5% goat serum (Dako). Mouse galectin-3 concentrations were measured according to manufacturer's protocol (R&D systems).

Cell isolation from tissues

Lymphocytes were isolated from the spleens and mesenteric lymph-nodes (MLN) of mice sacrificed after the ear thickness measure, 24 h after challenge. Single-cell splenocyte suspensions were obtained by crushing the spleen through a 70 µm nylon cell strainer using a syringe. The splenocyte cell suspensions were incubated with a lysis buffer (8.3 g NH_4Cl, 1 g $KHCO_3$ and 37.2 mg EDTA dissolved in 1 L demi water, filter-sterilized) for 4 min on ice to remove the red blood cells. Cell suspensions were resuspended in RPMI 1640 medium (Lonza) supplemented with 10% heat-inactivated FCS, 100 U/mL penicillin, 100 µg/mL streptomycin and 20 µM β-mercapto-ethanol (Thermo Fisher scientific).

Flow cytometry of immune cells

Spleen and MLN single cell suspensions (0.5-1 x 10^6 cells/well) were incubated with anti-mouse CD16/CD32 (Mouse BD Fc Block, BD Biosciences, San Jose) in PBS supplemented with 1% BSA and 5% FCS for 15 min on ice to block non-specific binding sites. Subsequently, cells were incubated for 30 min with the following antibodies: CD4-PerCP Cy5.5, CCR6-PE (both from Biolegend) CD8a-PECy7, CD69-PE, CD25-Alexa Fluor 488, CD3-PerCP Cy5.5, CD27-PE, CD19-APC and B220-FITC (all from Thermo Fisher scientific). For intracellular staining, cells were first fixated and permeabilized with Foxp3 Staining buffer set (Thermo Fisher scientific) according to manufacturer's protocol, followed by incubation with Foxp3-PECy7 (Thermo Fisher scientific), RORγT-Alexa Fluor 647 (BD) or Tbet-eFluor 660 (Biolegend). Dead cells were excluded using Fixable Viability Dye eFluor® 780 (Thermo Fisher scientific). Stained cells were measured by FACS Canto II (BD Biosciences) and analyzed using Flowlogic software version 7 (Inivai Technologies).

Generation of bone marrow-derived dendritic cells (BMDC)

Naïve mice (donor mice) were sacrificed on day 24 and bone marrow cells were isolated from the femur as previously described [23,30,31]. Bone marrow cells were resuspended in RPMI 1640 supplemented with 10% FCS, penicillin (100 U/mL) and streptomycin (100 µg/mL). Bone marrow cell suspensions (0.5 x 10^6 cell/mL) were cultured in the presence of 20 ng/mL recombinant mouse granulocyte macrophage colony-stimulating factor (GM-CSF) (Prospec) in a petri dishes (Corning). On day 3, medium was refreshed, and on day 6, bone marrow derived-dendritic cells (BMDC) were harvested. Immature BMDC were then loaded with the Influvac vaccine (0.9 µg/mL) and incubated for 24 h (37 °C, 5% CO_2).

Ex vivo re-stimulation assay

Spleens were aseptically removed and single cell suspensions were obtained as described in Section 2.2.4. Splenocytes (5 x 10^6) were co-cultured with BMDC (5 x 10^5) either or not loaded with Influvac in U-bottom 96-well plates at 37 °C, 5% CO_2. After 5 days of incubation, supernatants were collected and stored at −20 °C for cytokine analysis. IFNγ, IL-13 (both from R&D) were measured by means of ELISA according to the protocol described in Section 2.1.4. for galectin-4 and -9. Purified rat anti-mouse antibodies (1 µg/mL for IFNγ and 2 µg/mL for IL-13), recombinant mouse cytokines and biotinylated rat anti-mouse antibodies (1 µg/mL for IFNγ and 400 ng/mL for IL-13) were purchased from BD Biosciences. TNFα (Biolegend), IL-10 and IL-17A concentrations (both from Thermo Fisher scientific) were measured by ELISA according to manufacturer's protocol.

qPCR analysis of gene expression

Ileum and colon samples from mice sacrificed after DTH measurement were collected in RNA later (Invitrogen) and stored in −80 °C until mRNA isolation. Tissues were homogenized and RNA was isolated using a NucleoSpin® RNA Plus kit (Macherey-Nagel) in combination with DNAse (Qiagen) to remove contaminating DNA. Complementary DNA (cDNA) was synthesized using an iScript™ cDNA synthesis kit (Bio-Rad) according to the manufacturer's protocol. Quantitative analysis was performed on a CFX96 real-time PCR detection system with the use of IQ™ SYBR® Green Supermix (both from Bio-Rad). Commercially available primers for TGFβ1, TGFβ3, RORγ, Foxp3, TNFα, IL-10, Tbx21, galectin-3, -4 and -9, were obtained and GAPDH and PPIP5K1 (all from Qiagen) were used as reference genes. Relative mRNA expression was calculated as 100 x $2^{Ct\ reference - Ct\ gene\ of\ interest}$ [32]. Additionally, a custom-designed primer (**Table 1**) was used for, TNFα (Biolegio), previously validated [23].

Table 1. Sequence of custom-made primer and corresponding accession number.

Gene ID	Accesion Number	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')
TNFα	NM_013693.3	AAC GGC ATG GAT CTC AAA GA	TTT CTC CTG GTA TGA GAT AGC AAA TC

Statistical analysis

All statistical analyses were done using GraphPad Prism software. Data were transformed if they did not fit normal distribution prior to ANOVA analysis. IEC/PBMC co-culture datasets were analyzed using one-way ANOVA followed by Bonferroni's post-hoc test. The sample size of the *in vivo* vaccination study was calculated based on the DTH results from previous studies. The *in vivo* datasets from the vaccination model were analyzed using a one-way ANOVA followed by Bonferroni's test with selected pairs. Probability values of $p < 0.05$ were considered significant.

RESULTS

Exposure of IEC to FP enhances Th1- and Th17-type cytokines in the IEC/PBMC co-culture

A model to study the crosstalk between IEC and innate as well as adaptive immune cells was used to investigate the immunomodulatory effects of FP. Therefore, IEC were apically exposed to FP and basolaterally to αCD3/CD28-activated PBMC for 24 h, after which cytokines were analyzed. Exposure of IEC to activated PBMC and 0.25% or 0.5% FP resulted in significantly increased Th1-type IFNγ and TNFα

concentrations as compared to medium (**Figure 1A-B**). Th17-type IL-17A concentrations were significantly upregulated upon exposure to 0.5% FP, as compared to medium (**Figure 1C**). Meanwhile, Th2-type IL-13 and regulatory-type IL-10 and galectin-9 concentrations were not affected upon exposure to FP in the IEC/PBMC model (**Figure 1D–F**). The secretion of Th1- and Th17-type cytokines was promoted in the IEC/PBMC model upon exposure to FP.

IEC-derived galectin-3, -4 and-9 after IEC/PBMC co-culture

In order to analyze the epithelial cell responsiveness, after the IEC/PBMC co-culture, the IEC were washed and kept in incubation with fresh medium for additional 24 h, after which IEC-derived mediator release was measured. Due to their involvement in the regulation of many immune processes, IEC-derived galectin concentrations were studied in the basolateral compartment. Exposure to 0.5% FP resulted in significantly increased IEC-derived galectin-3, -4 and -9 (**Figure 2A–C**) as compared to control or 0.25% FP. IEC-derived galectin-9 was significantly increased upon exposure to 0.25% FP (**Figure 2C**). Meanwhile, a tendency towards increased epithelial-derived galectin-3 and -4 ($p = 0.08$ and $p = 0.06$ respectively, **Figure 2A-B**) was observed upon exposure to 0.25% FP, compared to medium. Another regulatory mediator known to be produced by epithelial cells is TGFβ1, but FP did not enhance TGFβ1 concentrations above medium background levels (data not shown).

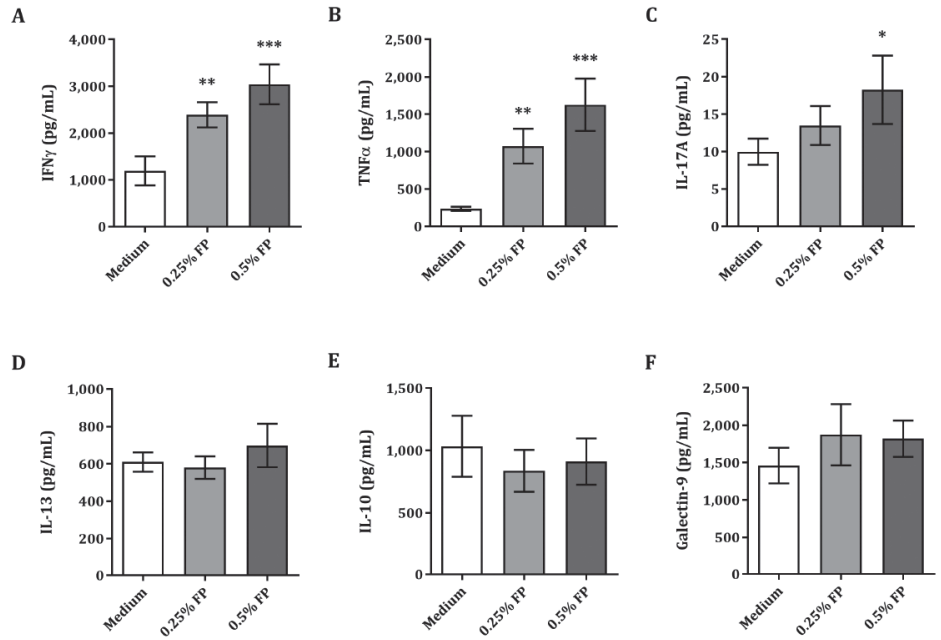


Figure 1. Cytokine release in IEC/PBMC co-culture.
IEC were apically exposed to 0.25% or 0.5% FP and basolaterally to αCD3/CD28-activated PBMC. IFNγ (**A**), TNFα (**B**), IL-17A (**C**) IL-13 (**D**), IL-10 (**E**) and galectin-9 (**F**) concentrations were measured in the basolateral supernatant after 24 h co-culture. Data are represented as mean ± SEM of $n = 5–6$ independent PBMC donors. Significant differences are shown as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

As the rise in galectin-3 and -4 release upon 0.5% FP exposure appeared greater than the rise in galectin-9 release, the ratios between IEC-derived galectins was calculated to illustrate the balance between these inflammatory and regulatory galectins. No significant effect was found in the ratio of galectin-4 over galectin-3 (**Figure 2D**), whereas the ratio of galectin-3 over galectin-9 (**Figure 2E**) and galectin-4 over galectin-9 (**Figure 2F**) showed a significant increase in the 0.5% FP conditions. Hence, exposure of IEC to FP in the IEC/PBMC model resulted in significantly increased IEC-derived galectin-3, -4 and -9, while the ratio of galectin-4 over galectin-9 as well as the ratio of galectin-3 over galectin-9 significantly increased upon exposure to 0.5% FP.

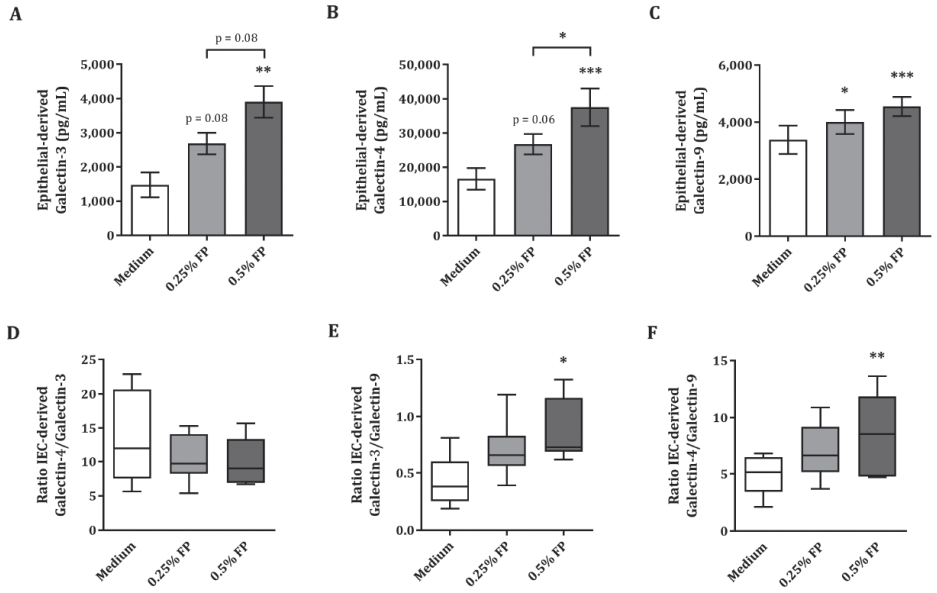
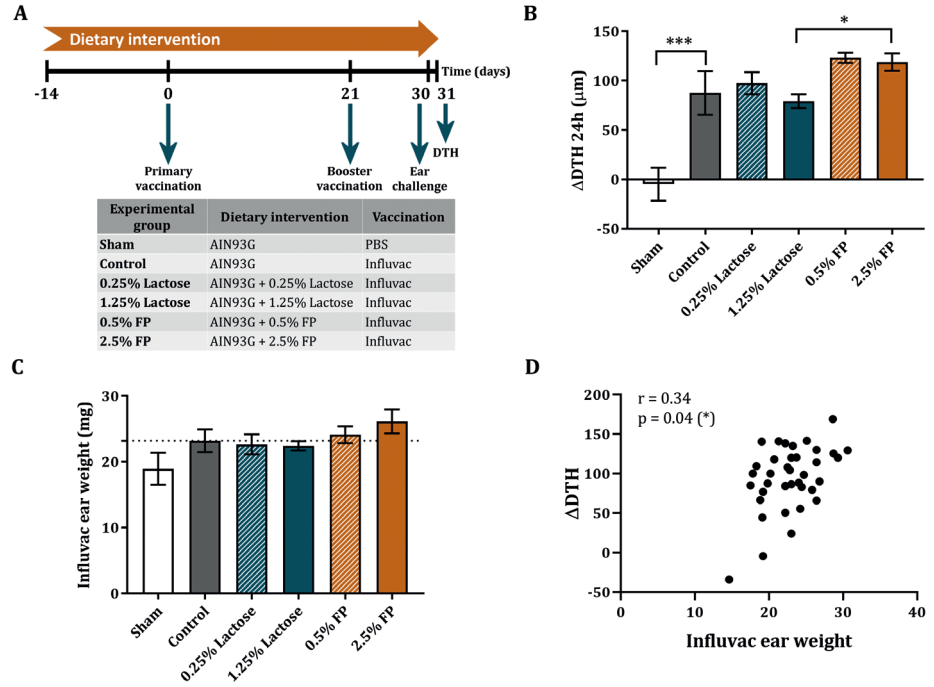


Figure 2. Epithelial-derived galectin release after IEC/PBMC co-culture.
IEC were apically exposed to 0.25% or 0.5% FP and basolaterally to αCD3/CD28-activated PBMC for 24 h. After IEC/PBMC co-culture, IEC were washed and incubated with fresh medium for additional 24 h to measure epithelial-derived galectin-3 (**A**), -4 (**B**) and -9 (**C**) concentrations. Additionally, the ratios between epithelial-derived galectins are shown. The galectin-4 and -3 ratio (**D**), the galectin-3 and -9 ratio (**E**) and the galectin-4 and -9 ratio (**F**). Data are represented as mean ± SEM of $n = 5–6$ independent PBMC donors. Significant differences are shown as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

In light of the immunomodulatory profile shown by FP in the IEC/PBMC model, further studies were done to determine the capacity of FP on the improvement of influenza vaccination responses *in vivo*.

Dietary intervention with FP improves the vaccine-specific DTH response

On day 30, the C57BL/6OlaHsd mice received a subcutaneous injection with the vaccine in the ear pinnae, after which, on day 31, the ear swelling was measured as DTH to determine the T-cell-dependent cellular response to vaccination. A significant increase in the influenza-specific DTH response was seen in all vaccinated mice as compared to the non-vaccinated sham mice (**Figure 3B**). The DTH response did not differ between the vaccinated mice receiving lactose diet as compared to vaccinated mice receiving control diet (**Figure 3B**). Although 0.5% FP did increase the DTH reaction compared to its appropriate lactose control, it did not reach the level of significance. However, increasing the dose to 2.5% FP significantly enhanced the DTH response compared to its appropriate lactose control group (**Figure 3B**).



Experimental group	Dietary intervention	Vaccination
Sham	AIN93G	PBS
Control	AIN93G	Influvac
0.25% Lactose	AIN93G + 0.25% Lactose	Influvac
1.25% Lactose	AIN93G + 1.25% Lactose	Influvac
0.5% FP	AIN93G + 0.5% FP	Influvac
2.5% FP	AIN93G + 2.5% FP	Influvac

Figure 3. The effect of a dietary intervention with FP on the vaccine-specific DTH response and ear weight in an influenza vaccination model.
The study design is shown (**A**). The increase in DTH response after ear challenge (**B**) and the Influvac ear weight (**C**) 24 h after challenge are shown. The Spearman correlation between the ΔDTH response and the ear weight is shown (**D**). Data are represented as mean ± SEM of sham ($n = 3$) and vaccinated mice ($n = 9$). Significant differences are show as * $p < 0.05$, *** $p < 0.001$.

Although no significant effect was observed in the weight of the Influvac injected ears, a similar pattern compared to the ΔDTH response was observed (**Figure 3C**). In addition, the differences detected in the weight of the Influvac injected ears significantly correlated with the ΔDTH response ($r = 0.34$, $p = 0.04$) (**Figure 3D**). Due to the significantly increased vaccine-specific DTH response observed in the mice receiving 2.5% FP diet, further analyses were done in this group and its respective lactose control group.

Influvac-specific IgG1 and IgG2a in serum and *ex vivo* cytokine secretion

In order to measure the humoral responsiveness to the vaccine and the impact of the dietary intervention with FP, the serum of the mice was collected and vaccine-specific IgG1 and IgG2a were measured. There was an increase in IgG1 and IgG2a levels in vaccinated mice compared to non-vaccinated sham mice (**Figure 4A, B**). However,

vaccine-specific IgG1 and IgG2a levels were not affected by the dietary interventions with FP or lactose (**Figure 4A, B**).

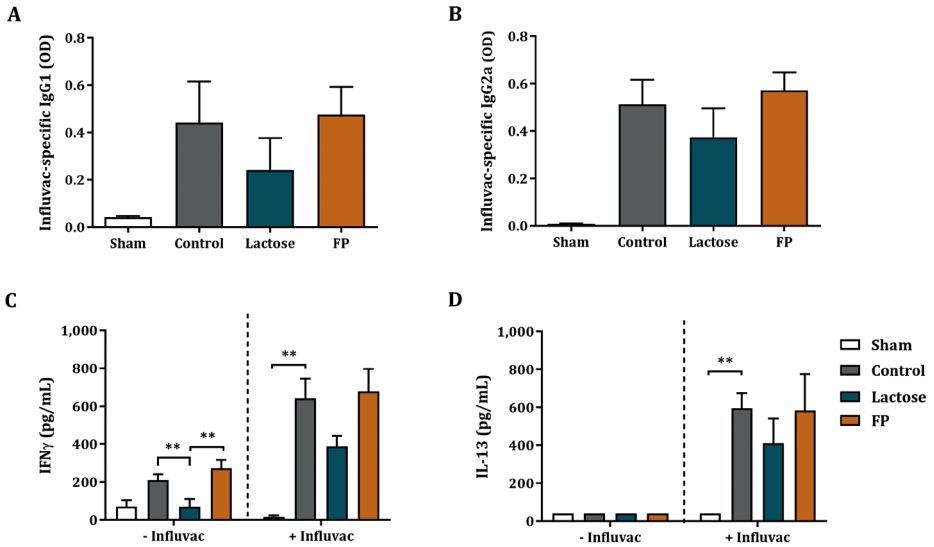


Figure 4. Serum immunoglobulins and cytokine production after *ex vivo* re-stimulation with influenza-loaded BMDC.
Influvac-specific IgG1 (**A**) and IgG2a (**B**) concentrations were measured in the serum. Additionally, BMDC were obtained from donor mice and loaded with or without 0.9 μg/mL Influvac for 24 h. On day 31, BMDC were co-cultured with fresh spleen cell suspensions from sham as well as vaccinated mice for 5 days, after which the supernatants of the co-culture were collected and the cytokine secretion was analyzed. IFNγ (**C**) and IL-13 (**D**) concentrations are shown. Data are represented as mean ± SEM of sham (n = 3) and vaccinated mice (n = 9). Significant differences are show as ** $p < 0.01$.

In order to investigate the effect of FP in the systemic vaccination response both T and B-cell subsets of the spleen and MLN were analyzed by flow cytometry. Regulatory T-cells were identified as $CD4^+CD25^+Foxp3^+$, RORγ positive cells were identified as $CD4^+CCR6^+ROR\gamma^+$ and, lastly, activated Th1 type cells were identified as $CD4^+CD69^+Tbet^+$. Activated B-cells were identified as $CD3^-CD19^+B220^+CD27^+$ and activated $CD8^+$ T-cells as $CD8^+CD69^+$.

In the spleen, the frequency of regulatory T-cells was significantly increased, and the frequency of activated Th1-type cells was decreased in vaccinated mice as compared to non-vaccinated mice; however, this was not affected by the dietary intervention (**Figure S1**). Dietary intervention with FP did also not have an effect in the T- and B-cell frequencies of the spleen, even though in the lactose control group the frequency

of activated B- and $CD8^+$ T-cells was increased as compared to the control diet (**Figure S1**). In the MLN, no significant differences were found in either T- or B-cell populations of vaccinated mice as compared to non-vaccinated mice. Dietary intervention with FP also did not affect the T and B-cell frequencies in the MLN (**Figure S1**). The frequency of Th1 positive T-cells in the MLN was significantly increased in the mice receiving lactose as compared to control and FP (**Figure S1**).

Using an *ex vivo* re-stimulation model, vaccine-specific T-cell responses were investigated. Splenocyte cell suspensions were co-cultured with BMDC either loaded or not with Influvac for 5 days. After co-culture, the cytokine concentrations were analyzed. Co-culture of splenocytes with non-loaded BMDC resulted in a relatively small non-specific background increase of IFNγ concentrations, while IFNγ was significantly increased when using Influvac loaded BMDC (**Figure 4C**). Similarly, increased IL-13 concentrations were found in co-cultures of splenocytes from vaccinated mice as compare to non-vaccinated mice in co-culture with Influvac-loaded BMDC (**Figure 4D**). Dietary intervention with lactose or FP did not further increase IFNγ and IL-13 concentrations (**Figure 4C-D**). TNFα, IL-17A and IL-10 concentrations were under detection limit.

Dietary intervention with FP did not have affect the Influvac-specific IgG1 and IgG2a levels or the frequency of B-cell populations in spleen of MLN. Influenza-specific *ex vivo* re-stimulation induced IFNγ secretion was not altered in the FP group.

Shift in Th17/T-regulatory mRNA expression in ileum

The effect of dietary intervention with FP was also assessed locally in the intestine. Therefore, ileal as well as colonic sections were subjected to qPCR analysis, which obtained detectable levels of RORγ, TGFβ1 and TGFβ3. Meanwhile, Foxp3, TNFα, IL-10 and Tbx21 were below detection limits. Although no effects were observed in the relative mRNA abundance of RORγ (**Figure 5A**), the TGFβ1 relative mRNA abundance was decreased in the ileum of vaccinated mice as compared to non-vaccinated mice (**Figure 5B**). Even though in the FP diet group an increasing pattern of RORγ mRNA expression and a decreasing pattern of TGFβ1 expression was shown, this did not reach statistical significance (**Figure 5A-B**). The TGFβ3 mRNA expression did tend to decrease in the ileum of the mice receiving FP diet as compared to control (**Figure 5C**).

Additionally, to represent the Th17 immune versus regulatory balance in the ileum, the ratio of Th17 marker RORγ and regulatory marker TGFβ was calculated (RORγ/TGFβ1 and RORγ/TGFβ3). The RORγ/TGFβ1 ratio was significantly increased in vaccinated mice as compared to non-vaccinated mice (**Figure 5D**). No significant effect

was found in RORγ/TGFβ1 ratio between control and FP groups (**Figure 5D**), although the FP showed a shift towards RORγ over TGFβ1 compared to the control and lactose groups. In addition, the correlation between RORγ/TGFβ1 ratio and ΔDTH response was studied, which showed a significant positive correlation (r = 0.59, $p = 0.004$, **Figure 5E**). No effect was found in the RORγ/TGFβ3 ratio (**Figure 5F**) and no correlation was found between the RORγ/TGFβ3 ratio and ΔDTH (r = 0.28, $p = 0.24$, **Figure 5G**).

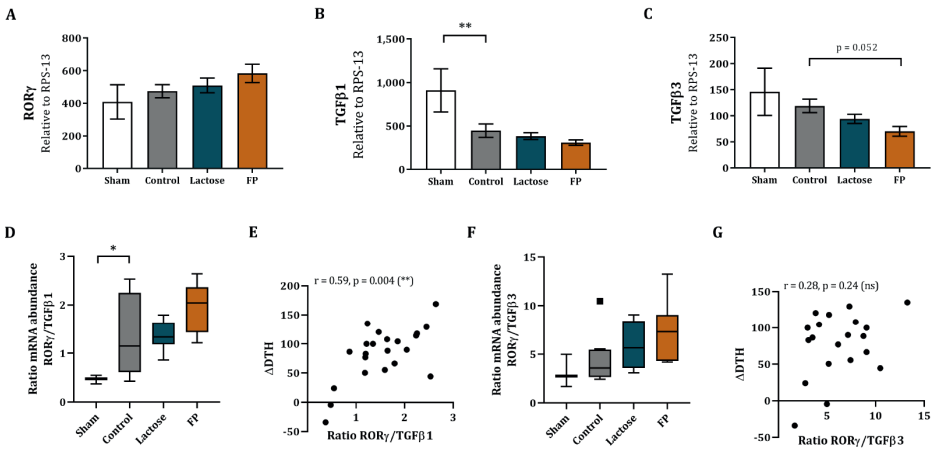


Figure 5. Relative mRNA expression in ileum and correlation with ΔDTH.
Relative mRNA expression of RORγ (**A**), TGFβ1 (**B**) and TGFβ3 (**C**) were measured in the ileum using RT-qPCR. RORγ/TGFβ ratios were calculated to represent Th17/regulatory balance in ileum. The ratio of RORγ over TGFβ1 mRNA abundance (**D**) and RORγ over TGFβ3 (**F**) are shown. Additionally, Spearman correlations of ΔDTH and RORγ over TGFβ1 ratio (**E**) as well as correlations of ΔDTH and RORγ over TGFβ3 ratio (**G**) are shown. Data are represented as mean ± SEM of sham ($n = 3$) and vaccinated mice ($n = 9$). Significant differences are show as * $p < 0.05$, ** $p < 0.01$.

In the colon, the relative mRNA abundance of RORγ in vaccinated mice was decreased as compared to non-vaccinated mice (**Figure S2**). No effects were found in the relative mRNA abundance of galectin-3, galectin-4, galectin-9, TGFβ1 or TGFβ3 in vaccinated mice as compared to non-vaccinated mice (**Figure S2**). TGFβ3 mRNA expression was significantly increased by FP and lactose, as compared to control. The TGFβ1, RORγ and galectin-3, -4, and -9 mRNA abundance was not affected upon dietary intervention with FP (**Figure S2**).

Galectin-3, -4 and -9 mRNA expression in ileum and concentrations in serum

Besides the immune markers, the impact of the dietary intervention by FP was studied locally on the gene expression of galectins in the ileum and colon. No effect of the vaccination or the FP diet was found in the relative mRNA abundance of galectin-3, -4 and -9 (**Figure S3**).

Galectin concentrations were also measured in the serum of the mice. No significant differences were observed in galectin-3, -4 and -9 concentrations in the serum of vaccinated mice as opposed to non-vaccinated mice (**Figure 6A–C**). Galectin-3 and -4 concentrations were not affected upon dietary intervention with FP or lactose as compared to control group. However, for the group fed the FP diet, an increasing pattern was observed (**Figure 6A-B**), while galectin-9 concentrations in serum were reduced as compared to both the control and lactose group (**Figure 6C**). As a result, the galectin-4/galectin-9 as well as the galectin-3/galectin-9 ratio were significantly increased (**Figure 6D-E**, respectively) in the FP group as compared to the control and lactose group. The galectin-4/galectin-3 ratio was not affected (**Figure S4**).

In order to connect the effects observed, such as the increase in DTH as well as of RORγ/TGFβ1 ratio in the intestine, with the serum galectin concentrations, the galectin-4/galectin-9 and galectin-3/galectin-9 ratio were correlated to RORγ/TGFβ1 ratio in the ileum as well as to ΔDTH. The serum galectin-4 over galectin-9 ratio showed a positive correlation to ΔDTH (r = 0.56, $p = 0.0098$, **Figure 6F**) and to the ratio of RORγ/TGFβ1 mRNA expression in the ileum (r = 0.38, $p = 0.045$, **Figure 6G**). Furthermore, the serum galectin-3/galectin-9 ratio was found to be significantly correlated to ΔDTH (r = 0.44, $p = 0.049$, **Figure 6H**) as well as to the ratio of RORγ/TGFβ1 mRNA expression in ileum (r = 0.41, $p = 0.03$, **Figure 6I**). Meanwhile, RORγ/TGFβ3 ratio did not show a significant correlation (**Figure S4**) with serum galectin-4/galectin-9 ratio (r = 0.25, $p = 0.2$) or galectin-3/galectin-9 ratio (r = 0.3, $p = 0.12$).

Dietary intervention with FP significantly reduced galectin-9 concentrations in the serum, which contributed to significantly increased galectin-4 over galectin-9 as well as galectin-3 over galectin-9 ratios. This shift in serum galectin-4/galectin-9 and galectin-3/galectin-9 correlated with an increase in DTH as well as to an increase in the ratio of RORγ/TGFβ1 mRNA expression in the ileum.

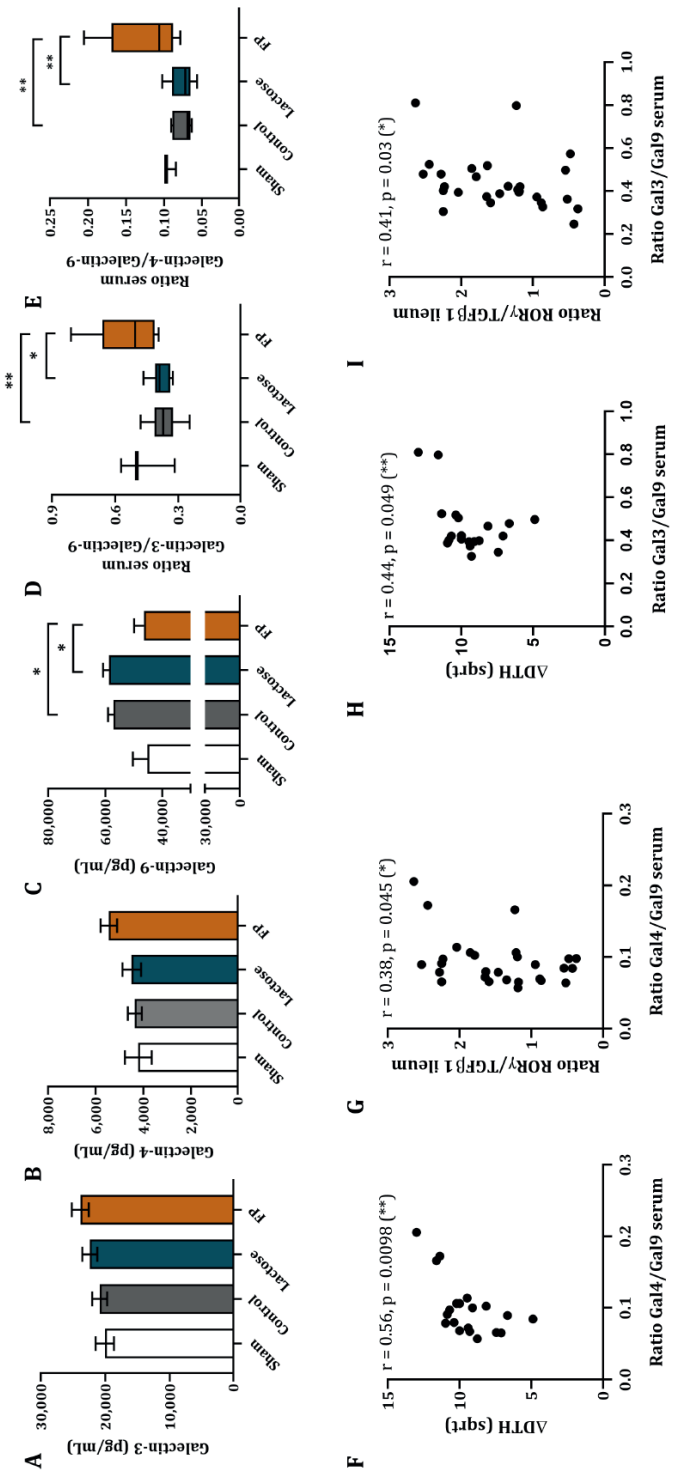


Figure 6. Galectin-3, -4 and -9 concentrations in serum.
Serum galectin-3 (**A**), galectin-4 (**B**) and galectin-9 (**C**) concentrations were measured. The galectin-4/galectin-9 ratio (**D**) and galectin-3/galectin-9 ratio (**E**) are shown. Serum galectin ratios were correlated to ΔDTH and RORγ/TGFβ1 ratio in the ileum. Spearman correlation of galectin-4/galectin-9 ratio and ΔDTH is shown (**F**). Galectin-4/galectin-9 ratio was also correlated with RORγ/TGFβ1 ratio (**I**) were calculated using a Pearson and Spearman correlation, respectively. galectin-3/galectin-9 ratio and ΔDTH (**H**) as well as to the RORγ/TGFβ1 ratio (**G**). Additionally, correlations of Data are represented as mean ± SEM of sham ($n = 3$) and vaccinated mice ($n = 9$). Significant differences are show as * $p < 0.05$, ** $p < 0.01$.

DISCUSSION

Postbiotics are known to improve immune as well as gut parameters in healthy and pre-term infants [4]. An *in vitro* co-culture model developed to study the crosstalk of IEC and immune cells was used to investigate the immunomodulatory capacity of FP derived from the fermentation of a milk matrix with *Bifidobacterium breve* C50 and *Streptococcus thermophilus* 065 (Lactofidus™). Studies using the same model showed the immunomodulatory capacity of non-digestible oligosaccharides in association with CpG ODN, a TLR9 agonist, under inflammatory conditions [21, 22, 28]. In the current study, apical exposure of IEC to FP, and basolaterally to αCD3/CD28-activated PBMC, resulted in significantly increased Th1-type IFNγ and TNFα, as well as Th17-type IL-17A concentrations. Moreover, Th2-type IL-13, regulatory-type IL-10 and galectin-9 were not affected, which indicates that FP can boost the adaptive immunity by promoting Th1- and Th17-type cytokine release in this model. Unlike the studies with specific non-digestible oligosaccharides, namely a 9:1 mixture of short-chain galacto- and long-chain fructo-oligosaccharides (GF) [24, 28] or 2'-fucosyllactose (2'FL) [22] and TLR9 agonist CpG ODN, FP did not boost IL-10 nor lowered IL-13, which evidences selective FP immunomodulatory properties. However, as opposed to non-digestible oligosaccharides in association with CpG ODN, FP promoted a strong Th1- and Th17-type response with less regulatory component already in the absence of CpG ODN, again emphasizing the relevance of the particular properties of FP.

IEC-derived galectin-9 has been identified as a key factor contributing to immunomodulation by previous studies using the IEC/PBMC model [24, 25, 28]. For the purpose of this study, we used the HT-29 cell line as a model for IEC. HT-29 have previously been shown to differentially respond to diverse microbial or immune triggers mimicking responses in human intestinal biopsies [33, 34]. The HT-29 also were shown to have a similar immunomodulatory effect compared to the differentiated T84 epithelial cell model when co-cultured in transwells with PBMC. Similar to HT-29, also the T84 cell line expressed and secreted galectin-9 upon apical exposure to TLR9 agonist CpG ODN, resulting in increased IFNγ secretion by the underlying PBMC [35]. The involvement of IEC-derived and/or systemic galectin-9 as an immunomodulatory factor has also been substantiated in dietary intervention studies done in food allergy prevention models as well as in human infants [26, 27]. The confirmation of findings in the HT-29/PBMC co-culture model in pre-clinical settings further validates the choice of HT-29 as a model for intestinal epithelial cells to study the crosstalk between IEC and immune cells.

Besides galectin-9, the association of IEC-derived galectin-3 and -4 in promoting immunomodulatory effects in the IEC/PBMC model was already reported upon exposure to non-digestible oligosaccharides and CpG ODN [22]. Galectins are carbohydrate-binding proteins that function to modulate innate and adaptive immune responses. Secreted by epithelial as well an immune cells, galectins are key players in inflammatory and regulatory immune processes [36]. Galectin-3 as well as -4 were shown to have anti-inflammatory as well as pro-inflammatory activities in diverse immune processes [37, 38]. Besides its role in the stabilization of lipid rafts, apical protein trafficking and cell adhesion [38], galectin-4 was shown to exacerbate intestinal inflammation by stimulating $CD4^+$ T-cells to produce IL-6 in a murine colitis model [39]. Contrarily, galectin-4 has also been described as an anti-inflammatory agent by selectively modulating T-cell responses in an experimental colitis model [40]. Similarly, galectin-3 also showed anti-inflammatory properties by contribution to ameliorate mucosal inflammation in a murine colitis model [41]. In this regard, galectin-9 has been shown to regulate inflammatory responses and collaborates with TGFβ to instruct regulatory T-cell development [42, 43]. In the current study, exposure to 0.5% FP resulted in significantly increased IEC-derived galectin-3, -4 as well as -9, which indicates that not only galectin-9, but also galectin-3 and -4 might be involved in the immunomodulatory effects promoted by FP. However, upon exposure to FP, the ratio of IEC-derived galectin-3 or galectin-4 over galectin-9 were significantly increased, suggesting a more immunostimulatory over regulatory profile induced by FP.

Due to the ability of FP in boosting the adaptive immunity by promoting Th17- and Th1-type cytokines in the IEC/PBMC model, the capacity of a diet containing FP in improving a vaccination immune response was studied. Thereby, an established *in vivo* influenza vaccination model was used [19] in which mice received a dietary intervention with FP or lactose as a control. Dietary intervention with non-digestible oligosaccharides can modulate the vaccine-specific DTH response, a Th1-related parameter [20–23, 29]. In line with these studies, here, we show that the vaccine-specific DTH was increased, although not significantly, upon dietary intervention with 0.5% FP compared to the lactose control diet. However, increasing the dose of the dietary intervention to 2.5% FP resulted in a significant increase in the DTH response. The increase in vaccine-specific DTH *in vivo* and the increase in Th1- and Th17-type cytokines as well as the increased IEC-derived galectin-3 or galectin-4 over galectin-9 ratio seen in the IEC/PBMC model *in vitro* highlight the ability of FP to support Th1- and Th17-type immunity possibly in association with the modulation of galectin expression.

Despite the increase in DTH observed in mice receiving the FP-containing diet, the vaccine-specific IgG1 and IgG2a levels were not affected by the dietary intervention

with FP. Previous studies described similar effects in mice receiving a GF supplemented diet [19]. Contrarily, significantly increased IgG1 and IgG2a levels were found in mice receiving a 2'FL supplemented diet [23]. This suggests that FP supplementation might affect the T-cell rather than B-cell immune responses, emphasizing the selective mechanisms derived from different dietary interventions.

Changes in the phenotype of T- and B-cells were studied in the spleen and MLNs of the mice and Influvac re-stimulation was performed *ex vivo*. The increase in DTH did not translate into an increase in the percentage of Th1 or Th17 type T-cells in the spleen or MLN, measured as $CD69^+Tbet^+$ or $CCR6^+ROR\gamma^+$, nor enhanced Influvac-specific IFNγ release, suggesting that such an effect is obtained through distinct mechanisms.

Regulatory T-cells as well as Th17 cells are found in intestinal mucosal immune responses and are known to protect the host from exaggerated effector T-cell responses. Th17 cells were described to have a critical role in host defense and vaccine-induced memory immune responses, by promoting the recruitment of Th1-type cells through the upregulation of chemokines, among other processes [44, 45]. TGFβ is a key mediator involved in regulating the differentiation of naïve T-cells into regulatory as well as Th17- type. Thus, the Th17/Treg balance is key in maintaining gut immune homeostasis [46–48]. In order to study the effects of the dietary intervention with FP in the Th17/Treg balance in the intestine, RORγ and TGFβ1 mRNA expression of the ileum and colon were studied. Dietary intervention with FP was found to enhance the balance of RORγ over TGFβ1, which was calculated as a reflection of the Th17/Treg balance. This ratio was enhanced in the intestine upon systemic vaccination and correlated positively with the DTH response. This indicates that the modulation of the intestinal immune system by means of a dietary intervention with FP might be able to affect the systemic vaccine-specific immune response.

In addition to TGFβ1, mRNA expression of TGFβ3 was measured. Dietary intervention with 2'FL significantly increased mRNA expression of TGFβ3 in a murine influenza vaccination model [23]. In the current study, as opposed to 2'FL, relative mRNA abundance of TGFβ3 tended to decrease upon dietary intervention with FP as compared to control, showing a similar trend as seen for TGFβ1 mRNA expression. However, as opposed to the RORγ/TGFβ1 ratio, the RORγ/TGFβ3 ratio and ΔDTH were not correlated. Therefore, in addition to these immune markers, the role for galectins in the immunomodulatory effect of the FP diet were further studied.

Circulating galectins are being considered as relevant biomarkers for supporting the diagnosis of several chronic disorders [49]; even in response to viral infections

such as influenza, plasma galectin-9 levels were found to be a relevant biomarker for disease prognosis [50]. In order to determine the relevance of circulating galectins in our model and link them to the epithelial-derived galectin concentrations seen *in vitro*, serum concentrations of galectin-3, -4 and -9 were studied. Dietary intervention with FP showed decreased galectin-9 concentrations, while no effects were found in galectin-3 and -4 concentrations. Indeed, galectin-3, -4 and -9 could also be measured in the intestine and serum of the mice in the vaccination model. Even though different responses were observed regarding the modulation of galectin levels in the murine vaccination model as compared to the *in vitro* IEC/PBMC co-culture, these might derive from the specific conditions mimicked in the models. While the IEC/PBMC co-culture represents a generic inflammation *in vitro*, the vaccination model focuses on antigen-specific immune responses *in vivo*, where more complex immune processes are studied. In spite of the distinct individual galectin concentrations observed in the serum of the mice, the galectin-4/galectin-9 ratio as well as the galectin-3/galectin-9 ratio were significantly increased upon exposure to FP in the *in vivo* model, similar to the IEC/PBMC co-culture model. This points towards a similar role of these types of galectins in orchestrating the immune activation and highlights the translational value of the *in vitro* co-culture model, which includes both IEC as well as immune cells, when studying effects on immune activation. Thus, validating the relevance of the results observed in less complex *in vitro* models as compared to *in vivo* models. Moreover, the use of *in vitro* models could contribute to evaluating the effects of bioactive components in order to select the most promising intervention and condition to be confirmed in animal studies, thereby contributing to the reduced use of animals.

Furthermore, the serum galectin-4/galectin-9 ratio as well as the galectin-3/galectin-9 ratio appeared to be correlated to the increased vaccine-specific DTH. This supports the idea that the circulating galectin balance was affected by the FP diet, which as a consequence might have an effect in the vaccine-specific immune response as measured by means of the DTH response. Little is known about the role of galectins in vaccination. However, galectins have shown dual-regulatory capacities in the promotion or inhibition of viral infections depending on the surrounding conditions and localization [51, 52]. This study reveals a potential involvement of systemic galectins in the improvement of vaccine immune responses. Further research is needed to study the contribution of specific subtypes of galectins in this regard.

Altogether, a Th17- and Th1-type immunomodulatory capacity of FP was shown in the IEC/PBMC model associated with increased epithelial-derived galectin-4 and galectin-3 over galectin-9. Although there was no significant difference in serum galectin-3 and -4 upon dietary intervention with FP, similar to the *in vitro* IEC/PBMC

model, increased galectin-4 or galectin-3 over galectin-9 ratios were observed in the murine influenza vaccination model. This was associated with improved vaccine immune response determined as increased DTH response. More research is needed in order to unravel possible mechanisms implicated and deciphering the bioactive components responsible for the effects observed.

Institutional Review Board Statement: The experimental procedures in this study were approved by the Ethical Committee for Animal Research of the Utrecht University and Central Commission for Animal use (approval numbers: DEC2015.II.243.038 and AVD108002016460) and complied with the principles of good laboratory animal care following the European Directive on the protection of animals used for scientific purposes.

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SUPPLEMENTARY FIGURES

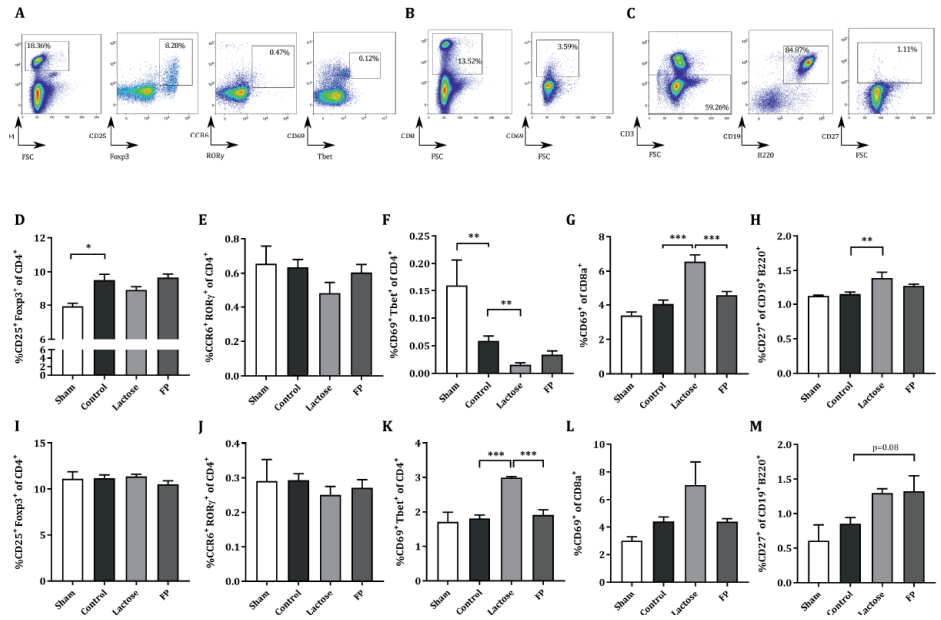


Figure S1. Flow cytometry analysis of T- and B-cell populations in spleen and MLN.
On day 31, the spleens and MLNs were collected and cell suspensions were obtained. Representative $CD4^+$ T-cell (**A**), $CD8^+$ T-cell (**B**) and B-cell (**C**) plots are shown. Percentages of regulatory T-cells (**D**), Th17 (**E**), Th1 (**F**), activated $CD8^+$ T-cells (**G**) were determined in the $CD4^+$ or $CD8^+$ lymphocyte populations in the spleen. The percentage of $CD19^+B220^+$ B-cells (**H**) in the spleen is also shown. Respectively, T- and B-cell populations for MLN are shown (**I-M**). Data are represented as mean ± SEM of sham (n = 3) and vaccinated mice (n = 9). Significant differences are show as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

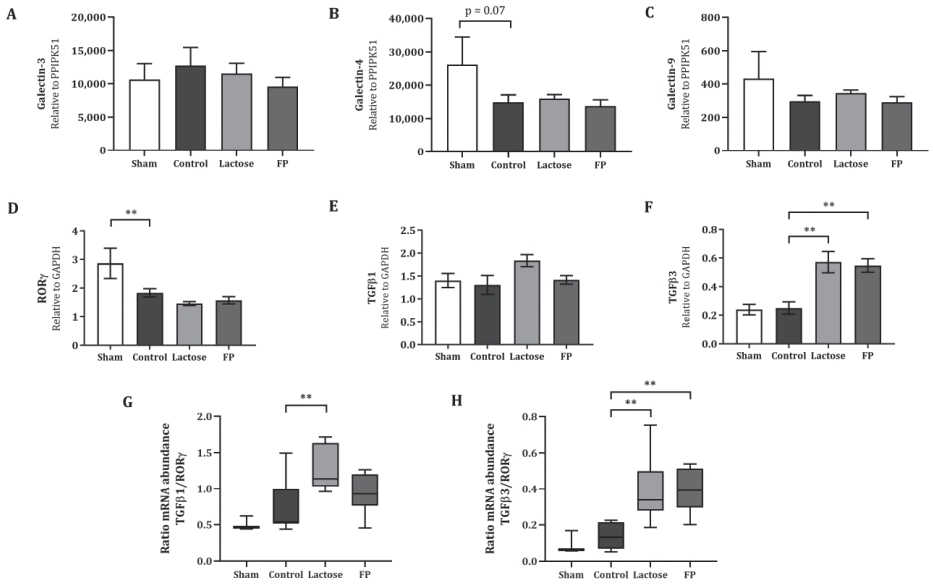


Figure S2. Relative mRNA expression in the colon.
Relative mRNA expression of galectin-3 (**A**), -4 (**B**), -9 (**C**), RORγ (**D**), TGFβ1 (**E**) and TGFβ3 (**F**) were measured in the colon using RT-qPCR. TGFβ1/RORγ ratio (**G**) and TGFβ3/RORγ ratio (**H**) were calculated to represent regulatory/Th17 balance in the colon. Data are represented as mean ± SEM of sham ($n = 3$) and vaccinated mice ($n = 9$). Significant differences are show as ** $p < 0.01$.

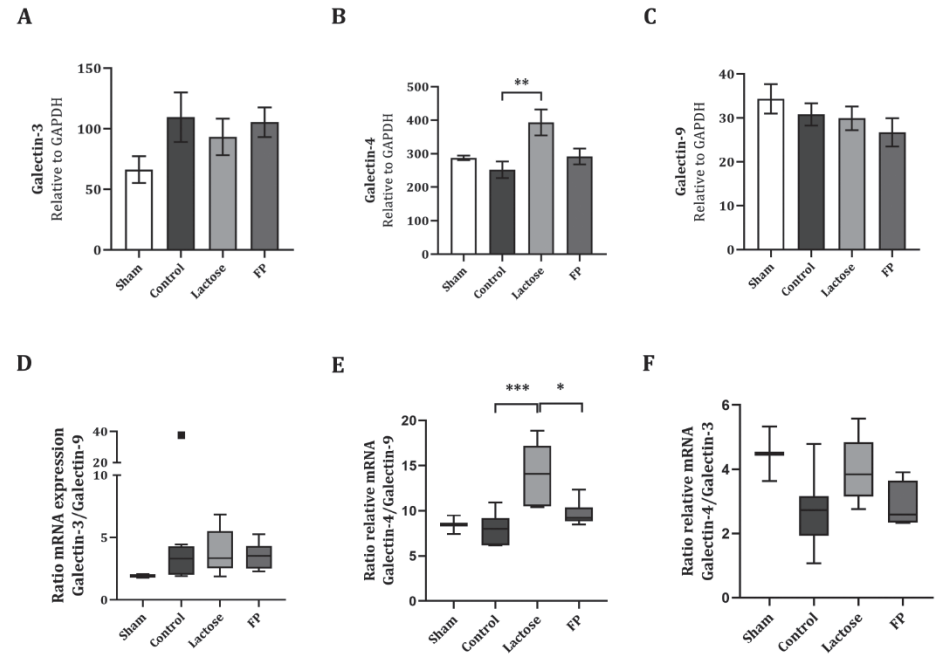


Figure S3. Ratio mRNA expression and serum galectins.
Relative mRNA expression of galectin-3 (**A**), -4 (**B**), -9 (**C**), RORγ (**D**), TGFβ1 (**E**) and TGFβ3 (**F**) were measured in the ileum using RT-qPCR. TGFβ/RORγ ratios were calculated to represent regulatory/Th17 balance in the colon. Data are represented as mean ± SEM of sham ($n = 3$) and vaccinated mice ($n = 9$). Significant differences are show as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

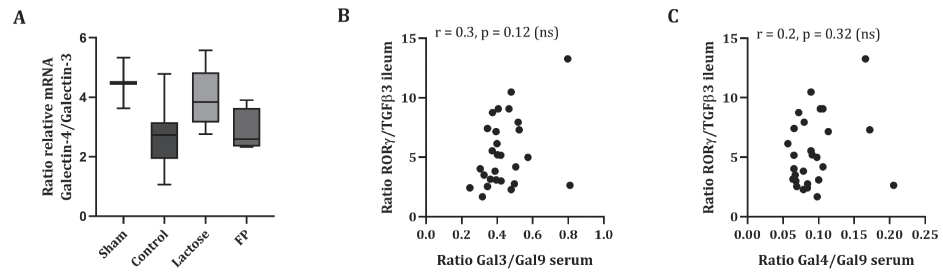
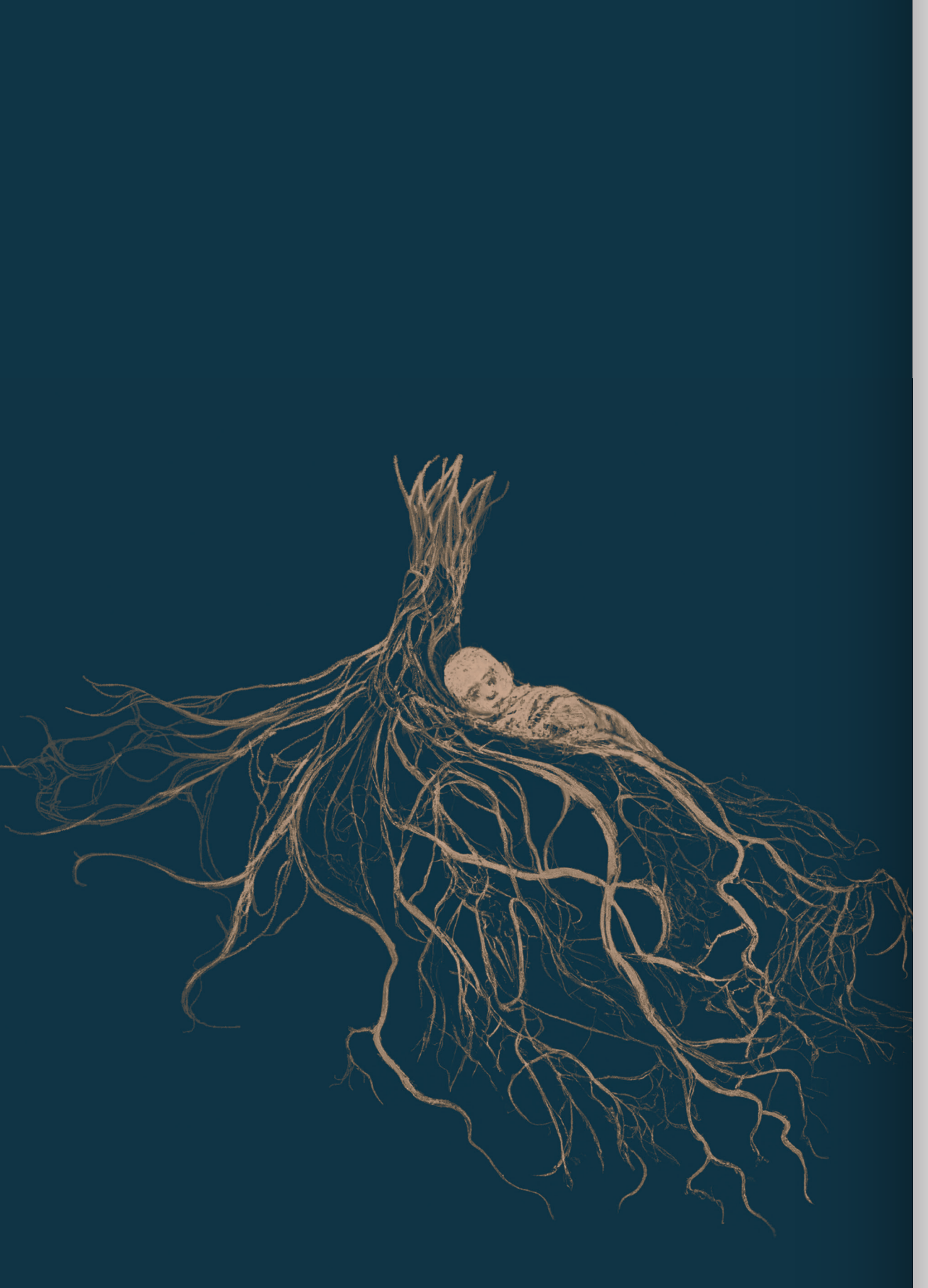


Figure S4. Ratio galectin-4/galectin-3 mRNA in ileum and correlations of RORγ/TGFβ3 and serum galectins.
The ratio of galectin-4/galectin-3 mRNA expression (**A**) is shown. Additionally, RORγ/TGFβ3 ratio in ileum were correlated to serum galectin3/galectin-9 ratio (**B**) and to serum galectin-4/galectin-9 ratio (**C**) using Spearman correlation. Data are represented as mean ± SEM of sham ($n = 3$) and vaccinated mice ($n = 9$).



CHAPTER | 8

General discussion

Newborns are vulnerable at birth due to the immaturity of their immune system as well as their gastro-intestinal tract (GIT) [1–3]. The prevalence of infections is high in newborns and the vaccination response is limited [4, 5]. Due to the maturation and developmental changes occurring, early life is considered a relevant period which provides a window of opportunity [2]. It is of great interest to study the key elements that support the growth and development of the newborn and to establish relevant dietary strategies.

Although the WHO recommends exclusive breastfeeding of newborns until 6 months of age [6], according to recent data in the European region only around 25% of the newborns are exclusively breastfed [7]. This suggests that it is relevant not only to promote breastfeeding practices, but also to study key components in breast milk to provide alternatives when breastfeeding or provision of human milk is not possible. In addition, human milk may contain novel components supporting the growth, the microbiome, and also the immune function and health of newborns of which the impact and mechanism of action is currently poorly understood.

This thesis aims to understand the contribution of pre- and post-biotics in the development of intestinal mucosa as well as systemic immunity with the aim to support the development of balanced immune responses in early life. For this purpose, novel *in vitro* models to study the interactions between intestinal epithelial cells (IEC) and innate immune cells were used.

Beneficial effects of human milk and HMOS

Breastfeeding is the preferred nutrition for the newborn since it provides all the necessary nutrients to support the GIT, immune maturation and growth, establishment of the microbiome as well as providing protection against infections [8,9]. Despite being a source of macronutrients such as proteins, carbohydrates, lipids and water, many bioactive components are also found in human milk. These include, human milk oligosaccharides (HMOS), immunoglobulins, polyunsaturated fatty acids, growth factors, lactoferrin, antioxidants and cytokines such as IL-10 or TGFβ that contribute to the protective effects and attenuate inflammation [9, 10]. Besides, human milk contributes to the colonization of Bifidobacteria and Lactobacillus which selectively ferment the HMOS available in human milk [11]. These species are present as commensal bacteria in the GIT of breastfed infants and are known to confer health benefits [8]. The neonatal microbiota is established shortly after birth however, its diversity and density increase and develop up until 3 years of age [2]. Nutritional factors and antibiotic treatment

early in life are known to directly impact the development of the gut microbiota and subsequently the development of the immune system [2, 12, 13].

Bioactive components in breast milk such as HMOS are known to have specific functions in the IEC as well as immune cells that promote the maturation and development of the GIT and immune system. The most relevant effects have been described and are summarized in **Chapter 2**. Besides the protective effects of HMOS reviewed in **Chapter 2**, recent studies have also described the ability of HMOS in promoting gut function and intestinal homeostasis by supporting the function of goblet cells [14], protecting against the development of necrotizing enterocolitis [15] and lowering the need to receive antibiotic treatment in early life [16].

Although the concentration and types of HMOS present in human milk differ with the lactational stage [17] and may depend on factors such as maternal nutrition [18], genetic predisposition [19] or geographic localization [20], the diversity of HMOS structures is unique to humans and not observed in other mammals [21, 22]. Most research focused on the most abundant HMOS such as 2'-fucosyllactose (2'FL) [23, 24], however other less abundant structures like 3'-galactosyllactose (3'GL) [25] also showed immunomodulatory capacities in immature IEC [26–28]. In clinical trials, infant formula containing 2'FL was shown to be safe and well tolerated [29], showed similar growth as breastfed infants [30], similar concentration of inflammatory cytokines in serum as breastfed infants [31] and reported less respiratory infections [32]. In fact, most infant formulas derive from cow's milk which are known to have a very limited amount of non-digestible oligosaccharides (NDO) which also contain less diverse NDO structures including low concentrations of fucosylated NDO, as opposed to human milk [21, 22]. In order to support the growth and development of infants receiving formula, and based on the amount and structure diversity of HMOS in human milk, supplementation of infant formula with NDO mixtures such as short-chain galacto- and long-chain fructo-oligosaccharides (GF) is being used. The addition of specific structures naturally occurring in human milk such as 2'FL or 3'GL is of interest. However, more research is needed regarding specific effects of these structures. Thus, studying the immunomodulatory capacities of these HMOS and their contribution to mucosal homeostasis is of relevance, since supplementing infant formulas with specific oligosaccharides or mixtures of NDO may improve the composition of available infant formulas.

Various mechanisms have been described by which the biological functions and beneficial effects of human milk in the GIT and immune system may occur. On one hand, HMOS are known to block pathogens entering the host cell by mimicking cell entry

receptors or by blocking viral replication inside the cell. In addition, HMOS such as 2'FL are known to bind enteric pathogens like Rotavirus and Norovirus directly [33–36], which might also prevent viral entry to host cell and thus, support protection against infections. In addition, HMOS can promote the intestinal epithelial barrier directly by supporting the maturation of the IEC [26, 37, 38] as well as indirectly by means of the short chain fatty acids produced upon fermentation of HMOS by beneficial commensal bacteria, which are anti-inflammatory and known to support barrier integrity [39–45].

In addition, HMOS might promote the development of the immune system by targeting factors that are key in the modulation of immune responses and in pathogen recognition and also by interacting with immune cells underneath the epithelial barrier or present in circulation upon absorption to systemic circulation [46]. These HMOS also exert direct effects on structural cells and cells of the innate and adaptive immune system. Previous research showed that 2'FL reduced inflammation by attenuating TLR4 signaling [47], while 3'GL modulated TLR3 signaling [26]. Besides, 2'FL and 3'GL showed anti-inflammatory [48] and immunomodulatory effects in dendritic cells (DC) [49], monocytes [50] and T-cells [51]. Furthermore, HMOS were reported to bind galectins [52] which are key regulatory mediators in inflammatory and immune processes that act as glycan binding receptors [53] but can also be secreted as soluble mediators and have an effect in immune responses.

Investigating the specific effects of the bioactive components in breast milk, will help to better understand the beneficial effects of breast milk as well as providing opportunities to develop improved alternatives for the newborns that can't be breastfed. Since breastfeeding is known to protect against infections in infancy, interventions using similar immunomodulatory components as those present in human milk are of interest for infants who are not breast-fed.

Supportive role of 2'FL in mucosal immune defense against viral triggers

Due to the vulnerability to infections of newborns, more studies regarding potential interventions to help in the fight against pathogens are needed. Studying what and how bioactive components in human milk provide protection against infections will help to improve available infant formulas in order to support the protection of infants who are not breastfed.

To study if specific HMOS like 2'FL could have beneficial immunomodulatory effects against a viral trigger, we developed a model to mimic an enteric viral infection and studied the regulatory role of IEC in maintaining mucosal immune homeostasis against a viral trigger (**Chapter 3**). We observed that upon stimulation of the intestinal epithelial cell line HT-29 with a synthetic analog of double-stranded RNA (dsRNA); poly I:C (pIC), the secretion of cytokines and chemokines like IL-8, CCL20, GROα and CXCL10 was promoted, which are relevant for viral defense and mucosal immunity [54–56]. The upregulation of IL-8 and GROα was also observed during rotavirus (RV) infection of HT-29 cells [57] suggesting that with this model we were able to mimic to some extent an enteric viral infection.

Interestingly, we observed that the mode of delivery of pIC affected the secretion of cytokines relevant to promote viral defense. Only upon complexation of pIC with the cationic lipid-based reagent Lyovec™ (LV-pIC), upregulated CCL5, IFNβ and IFNλ1 were observed, as opposed to naked pIC stimulation. In HT-29 cells infected with RV, increased IFNβ secretion was observed. This increase was thought to be mediated by cytosolic RIG-I and MDA-5 activation [58–60]. The internalization of pIC into the cytosol obtained through complexation of pIC might have resulted in a stronger activation of cytosolic RIG-I and MDA-5, which subsequently led to increased IFNβ secretion. Stimulation with naked pIC might not have achieved the threshold activation level for RIG-I and MDA-5 signaling, thus limiting the anti-viral response initiated by the cells, suggesting that LV-pIC mimicked more closely a viral enteric infection.

Upon a viral infection, IEC provide a physical barrier to prevent the passage of viral antigens to the lamina propria, as well as secreting mediators to attract immune cells to fight against pathogens. In addition, DC underneath the IEC can take up viral antigens, present them to naïve T-cells in the Peyer's patches or mesenteric lymph nodes (MLN) and promote differentiation of T-cells. By doing this, they contribute collectively to the development of adaptive anti-viral immune responses. In **Chapter 3**, we investigated the role of IEC in instructing DC to drive T-cell polarization, and in regulating intestinal homeostasis and local viral defense. We observed that in IEC and monocyte derived DC (moDC) co-cultures exposed to LV-pIC, less pronounced CCL20, CXCL10 and CCL5 secretion was observed as compared to moDC-exposed to LV-pIC. These results suggest lower activation and reduced attraction of immune cells to the site of infection, and as a result, less inflammation. In addition, LV-pIC exposed moDC secreted increased amounts of IL-12p70 which also led to increased Th1-type IFNγ secretion in the DC/T-cell assay, indicating a strong pro-inflammatory effect, as opposed to LV-pIC exposed IEC/moDC cultures, supporting the regulatory role of IEC in generating local immune defense and maintaining intestinal homeostasis.

In **Chapter 3**, we also studied the potential role of 2'FL in modulating the immune response against a viral trigger. Exposure of HT-29 to LV-pIC and 2'FL promoted the secretion of CCL20 and CXCL10. Both of these epithelial mediators are known to recruit innate and adaptive immune cells to the site of infection and thereby, support resolution of the viral threat [54]. Thus, 2'FL might support the epithelial function in local immune defense against a viral trigger. Previously, 2'FL was shown to reduce the severity and incidence of RV-induced diarrhea by promoting intestinal maturation and supporting immune cell populations in a neonatal rat and piglet models [61–64] as well as promoting anti-inflammatory effects *in vitro* [47]. Further studies are needed to determine whether the effects observed for 2'FL might lead to protection against infections and if so, studying the mechanisms by which these effects are mediated is of relevance. Preliminary data obtained in this model regarding the potential protective effects of HMOS should be further studied in more complex models using inactivated viruses and/or viral proteins as well as *in vivo* using viral infection models. In addition, this model could be used to study other components with potential protective effects against viral triggers.

Although no differences were observed upon exposure to 2'FL in the cytokine and chemokine secretion in IEC/moDC and moDC cultures, significant differences were observed in the secretion of galectins. Increased galectin-4 and -9 secretion was observed in IEC/moDC cultures stimulated with 2'FL and LV-pIC, suggesting a role of galectins in supporting mucosal immune defense against viral triggers. Galectins act as regulators of various immune processes as well as epithelial homeostasis [65, 66]. In particular, galectin-9 could support many steps in the viral immune defense by acting as a damage associated molecular pattern (DAMP) [67], promoting maturation of DC [68], inducing regulatory T-cell differentiation [69], suppressing $CD4^+$ Th and $CD8^+$ Tc [70], reducing tissue damage by controlling apoptosis [70–72], or promoting migration of immune cells [73]. Besides, galectin-9 is key in modulating the crosstalk of macrophages and $CD4^+$ T-cells for the control of bacterial infection [74]. Galectins were shown to promote innate immune responses by acting as alarmins upon recognition of damage-associated signals but have also shown to be involved in the resolution of inflammation [65]. IEC actively respond to environmental stimuli by secreting epithelial-mediators like galectins that act as regulators of innate and adaptive immunity and thereby they may be able to support viral defense, while maintaining mucosal homeostasis.

Various mechanisms are in place to control and maintain intestinal homeostasis for which IEC act as key regulators. Studying the roles of epithelial mediators is relevant to better understand the regulatory role of IEC and potentially develop strategies to support those mechanisms. Expanding our knowledge regarding the ability of IEC to control

and maintain intestinal homeostasis will result in the development of more efficient recommendations to prevent and treat immune related disorders.

NDO as immunomodulatory agents

A mixture of NDO derived from plant and milk sources that resembles the amount and structure diversity of HMOS in human milk has been developed and is being used to supplement infant formula. Studied for its immunomodulatory and prebiotic effects [11] as well as immune effects observed in clinical trials [75–77], this specific mixture containing (9:1) short-chain galacto- and long-chain fructo-oligosaccharides (GF) was shown to promote Th1-type regulatory immune effects in IEC/PBMC co-culture model in association with CpG ODN, which are unmethylated dinucleotides mimicking a bacterial trigger [78].

The crosstalk between IEC and the underlying immune cells is key to maintain mucosal homeostasis upon exposure to harmless antigens that reach the intestinal lumen or to induce effector responses to fight infectious agents like viruses and pathogenic bacteria [79–81]. To study these interactions an *in vitro* co-culture model combining IEC and innate and adaptive immune cells was used. In this model an inflammatory environment is mimicked by stimulating peripheral blood mononuclear cells (PBMC) with αCD3/αCD28, representing the immune cells from the lamina propria. In **Chapter 5**, we demonstrated that activation of the PBMC was needed to promote the secretion of cytokines sensitive to modulation by NDO.

Since the biological activity might be dependent on the size of NDO, studying the specific effects of NDO with different degree of polymerization (DP) is of relevance. The DP of NDO represents the amount of chains of monosaccharides forming a specific molecule. As such, short-chain galacto-oligosaccharides (GOS) are composed of multiple structures with DP chains between 2-8, while long-chain fructo-oligosaccharides (lcFOS) contain over 23 monosaccharide chains [11]. Being GOS the main component in the GF mixture and having identified the structures with varying DP sizes present in GOS before [3], in this thesis, we studied the immunomodulatory effects of specific DP fractions. In **Chapter 4**, we demonstrated that in association with CpG, the DP3-5 fraction of GOS was the most active, out of the studied DP fractions, in promoting Th1-type regulatory effects in the IEC/PBMC co-culture model, similar to the effects shown for GF before [78]. These results suggest that DP3-5 sized GOS might be key to support Th1- and regulatory-type immunomodulatory effects. Thus, it might be relevant to study NDO with similar size in this co-culture model to gain insights about the direct immunomodulatory effects of these components.

Most studies have investigated if specific bacterial strains are capable of fermenting NDO with specific DP sizes. However, few studies have studied the direct effects of varying DP-sized NDO in IEC or immune cells. Some studies have reported changes in gene expression upon absorption of NDO by enterocytes [82, 83]. In this regard, Ito et al. observed that only in rats receiving DP4-sized fructo-oligosaccharides enhanced production of IFNγ and IL-10 by $CD4^+$ T-cells was promoted [84]. However, this was not seen in rats receiving longer-sized fructo-oligosaccharides, suggesting that shorter NDO might be absorbed by enterocytes and thereby be able to promote immunomodulation as opposed to longer DP-sized NDO. Similar to the rat study, also a 9:1 mixture of short chain and long chain FOS as well as lactulose enhanced IFNγ and IL-10 secretion on top of the effect of CpG in a similar co-culture model used to study GOS DP [85, 86]. All these studies suggest that NDO like GOS/lcFOS, GOS or FOS/lcFOS may generate direct immunomodulatory effects mainly via interaction of their short-chain NDO with epithelial and/or immune cells.

Additionally, in **Chapter 4** and **5** respectively, we demonstrate that in association with CpG, β-3'GL and 2'FL, HMOS structures naturally present in human milk, with short DP3-sized structures, supported similar Th1- and regulatory-type immunomodulatory effects, as shown by increased IFNγ and IL-10 secretion in the same IEC/PBMC co-culture model. In addition, a reduction in Th2-type IL-13 secretion was observed. Furthermore, in **Chapter 6** we showed that even a 1:1 mixture of 2'FL and GF also supported Th1- and regulatory type-immunomodulation, when associated with CpG. Due to the Th1 and regulatory polarizing capacity of β-3'GL, 2'FL and 2'FL/GF, these NDO might support mucosal immunity and thereby, could be used in strategies designed to prevent Th2-skewed immune related diseases such as allergies, and to support Th1 immune polarization required in early life for immune maturation or for proper immune defense [1, 2, 8, 87, 88]. In addition, it may help to support immune responses in elderly since aging is associated with a decline in Th1 immunity [89–91]. Clinical studies showed that dietary supplementation with GOS or FOS was safe and well tolerated by healthy adults [92], and supplementation resulted in increased Bifidobacteria counts which for reasons still unknown, are decreased in elderly [93, 94]. In addition, increased IL-10 and decreased IL-6 and TNFα production was observed in PBMC from healthy elderly receiving GOS [95] indicating possible immunomodulatory and anti-inflammatory effects which could be beneficial. More research is needed to determine whether supplementation with NDO in elderly might be of benefit in supporting immune responses. Nevertheless, the mechanisms contributing to immune development and immune senescence may differ and thereby, different strategies might be needed for different stages in life.

The involvement of galectin-9 in promoting the Th1- and regulatory-type immunomodulatory effects of GF in association with CpG was previously reported in the IEC/PBMC co-culture model [78] and showed a protective effect of a dietary intervention using GF and *Bifidobacterium breve* M16V which increased galectin-9 levels and protected against food allergy *in vivo* [96]. In **Chapter 4, 5** and **6**, we observed that not only galectin-9 but also galectin-4 and/or -3 were upregulated upon CpG and NDO exposure in the IEC/PBMC co-culture model. In addition, the upregulation of epithelial-derived galectins correlated with the Th1- and regulatory-type immune effects suggesting the involvement of galectins in the promotion of such effects. Furthermore, and to confirm this, in **Chapter 6** we used an inhibitor of neutral sphingomyelinase to block the release of galectins by exosomes from HT-29 cells. There, we identified the presence of galectin-9 and -4 in epithelial-derived exosomes, and observed that blocking the biogenesis and secretion of exosomes from IEC resulted in downregulated galectin-9 and -4 secretion, which indicates their secretion through exosomes from HT-29 cells. Furthermore, blocking the exosome secretion, and thereby galectin-9 and -4 secretion resulted in the loss the immunomodulatory properties of CpG and 2'FL/GF (no increase in IL-10 and decrease in IL-13 secretion were observed). These results confirmed the involvement of these galectins in the immunomodulatory effects promoted by NDO and CpG in the IEC/PBMC co-culture model. These findings are in line with previous studies were lactose or Tim3-Fc fusion protein were used to block galectin-9 in the co-culture model [78, 85]. Due to the ability of exosomes to mediate intercellular communication locally as well as systemically, and regarding the observation of galectin-containing exosomes, studying the implications of galectins in promoting local as well as systemic immune responses is of interest.

Most of the studies shown in this thesis are done using the HT-29 cell line as a model for IEC. Although these models might be useful to obtain pre-clinical data, they might not fully resemble the characteristics of a healthy human enterocyte due to the carcinogenic phenotype of this cell line. In spite of the fact that these models have shown their translational relevance, the results described in this thesis should be confirmed using other more complex models such as *in vivo* pre-clinical or clinical studies.

For the purpose of this thesis, we did however investigate whether similar immunomodulatory effects were observed in a non-carcinogenic epithelial cell line. In **Chapter 6** we demonstrated the capacity of a human primary fetal intestinal cell line, namely FHs 74 Int, in modulating the cytokine secretion upon exposure to 2'FL, GF or 2'FL/GF mixture and CpG in the IEC/PBMC co-culture model. Using these cells, we observed that some immunomodulation was already obtained upon exposure to GF or 2'FL/GF alone (in the absence of CpG). This effect differed from the studies with HT-29

since there, CpG exposure was required to facilitate the immunomodulatory effects of the NDO. Nevertheless, epithelial-derived galectin-9 secretion was also increased in FHs 74 Int/PBMC co-cultures upon combined exposure to NDO and CpG, which emphasizes the relevance of these epithelial triggers in supporting the immunomodulatory effects observed upon inflammatory conditions, independent of the genetic background of the epithelial cells used. Recent research emphasized the involvement of galectins in physiological functions with unique roles in cell-to-cell communication, inflammation, immune responses or cell signaling [65, 97], which highlights the relevance of our observations. Although little is known about the mechanism by which NDO can promote such immunomodulatory effects, the involvement of galectins in processes like cell signaling, inflammation and development of immune responses points to a key role of galectins in the mechanism by which NDO promote Th1- and regulatory-type immune effects.

2'FL supporting mucosal immune development

Dendritic cells residing in the GIT are particularly relevant to maintain mucosal homeostasis by generating protective immune responses upon sampling of antigens reaching the gut lumen. When antigen recognition occurs, DC migrate to the MLN and instruct naïve T-cell differentiation [79, 80]. In addition, to promote mucosal homeostasis, IEC signal to the DC via cell-cell contact or by secreting epithelial-derived factors that can condition DC towards a tolerogenic phenotype, which subsequently promotes the differentiation of $CD4^+$ T-cells into regulatory cell phenotype in the Peyer's patches or draining lymph nodes [79–81].

To study this, we used an IEC/moDC *in vitro* co-culture model followed by a DC/T-cell assay, mimicking the sequential steps required to induce an adaptive immune response. In **Chapter 5** we observed that IEC exposed to 2'FL and CpG in IEC/PBMC model (after washing of IEC and co-cultured with immature moDC) were capable to condition DC to express less CD80 maturation marker and to promote increased IFNγ and IL-10 secretion in an allogeneic DC/T-cell assay. These effects were not observed for IEC exposed to GF and CpG suggesting structure-specific effects in this co-culture model even though in the IEC/PBMC model both GF and 2'FL generate a similar immunomodulatory outcome. These data indicate a possible role of 2'FL in promoting mucosal immune maturation by conditioning DC to instruct a Th1- and regulatory-type immune response. This may skew away from the Th2 prone immune status after birth [8, 88] and help to build proper mucosal immune functions, while maintaining homeostasis [1, 2, 8]. Previously, moDC conditioned with pooled HMOS (in the absence of IEC), increased IL-10 secretion and generated regulatory T-cells from DC/T-cell co-cultures [49]. However, no effect was observed on IFNγ or on the percentage of Th1 cells [49]. These studies indicate the ability of HMOS to promote immunomodulation and thus support immune development. Further research is needed to better understand the complex mechanisms involved in such immune effects.

Studying the IEC/DC crosstalk upon exposure to specific NDO might help to understand the interactions occurring between IEC and immune cells and to study how to promote regulatory type immune development contributing to maintain mucosal homeostasis.

Postbiotics-A strategy to support vaccine-specific immune responses

The immune response to vaccines is immature at birth and depends on several factors including feeding habits, microbiome as well as pre-existing immunity among others [98]. Therefore, it might be possible to support vaccination immune responses by specific dietary interventions. Previously, a dietary intervention with 2'FL was shown to support influenza-specific vaccination immune responses in a murine influenza vaccination model by inducing T- and B-cell immunity [99].

In **Chapter 7** we studied the ability of a fermentation product (FP) containing postbiotics in supporting an influenza-specific vaccination response. Postbiotics containing bioactive components are obtained upon fermentation of food products using food-grade microorganisms such as short chain fatty acids, proteins, enzymes and organic acids, but also microbial components like lipoteichoic acids, peptidoglycans and cell surface proteins [100, 101]. The specific fermentation process of a milk matrix using *Bifidobacterium breve* C50 and *Streptococcus thermophilus* was shown to generate 3'-GL, a structure naturally occurring in human milk [102]. Supplementation of infant formula with postbiotics is being studied as a strategy to supplement infant milk formula with the purpose to support the growth and health of infants due to their immunological effects [103, 104]. Although the mechanisms implicated in the beneficial effects promoted by specific postbiotics in the host are not yet known, several studies have described functional properties such as anti-inflammatory, anti-oxidant and immunomodulatory effects [100, 101].

In **Chapter 7** we observed that exposure to a specific FP promoted the secretion of IFNγ, TNFα and IL-17A cytokines in the IEC/PBMC co-culture model, which indicates the ability of FP to support strong Th1- and Th17-type immune responses. In addition, IEC-derived galectin-3, -4 and -9 were increased by FP, already in the absence of CpG.

As shown in previous studies, bacterial DNA which might be available in the FP, can have similar effects as CpG in the IEC/PBMC co-culture model [78] and thus, might have played a role in supporting the immunomodulatory effects of the FP. Besides, in clinical trials, specific FP were found to improve GIT as well as immune parameters [104]. When galectin ratios were calculated in the *in vitro* IEC/PBMC studies, this translated into increased galectin-3 over -9 and galectin-4 over -9 ratios, indicating immunostimulatory rather than regulatory immune effects, which corresponded with the observed increase in Th1, Th17 and pro-inflammatory mediators. It might be beneficial to promote Th1-type immunity as these are low or compromised in life stages such as in early life. Even though the implication of galectins in diverse steps of viral infections has been described before [105, 106], little is known about the implications of galectins upon vaccination.

To study whether a dietary intervention with FP could be beneficial to support immune responses to vaccines *in vivo*, we used a murine influenza vaccination model and observed improved influenza-specific immune responses as measured by DTH, an indicator of a Th1 driven vaccination response. Vaccine-specific immunoglobulins were not affected in vaccinated mice receiving FP diet, as compared to control, suggesting a T-cell rather than B-cell mediated mechanism. However, no phenotypic changes were observed in T-cells from spleen nor MLN. Interestingly, circulating galectin ratios were found increased, similar to the findings in the IEC/PBMC model, and correlated positively to vaccine-specific DTH response. These results suggest that circulating galectins might be involved in supporting improved vaccine-specific immunity. In line with the observations in **Chapter 6** regarding the secretion of galectins via exosomes by IEC, also here epithelial-derived galectins produced in the GIT after ingestion of a diet containing FP, might have resulted in the secretion of galectin-containing exosomes by IEC. These can then reach the systemic circulation and might have supported the systemic vaccine-specific effects measured as an increased DTH response in the ear resulting in an overall improved vaccination response. These results highlight the relevance of studying the involvement of epithelial-mediators, and in particular galectin-9, in supporting local as well as systemic effects. Furthermore, it emphasizes the key role of exosomes in regulating intercellular communication.

Currently data concerning the contribution of galectins in the success of vaccination responses is largely lacking and further research is needed to unravel the mechanisms by which galectins may influence the vaccination response. Also the effects of specific FP on vaccination responses in infants are yet largely unknown. One clinical study did show promising results as increased anti-poliovirus IgA titers were observed in infants fed formula containing FP [107]. Studying the specific effects of bioactive components

in FP will help to better understand the key components and mechanisms by which postbiotics promote beneficial effects in the host, but more studies using FP in dietary interventions to support vaccination responses are certainly needed to gain more insights in this regard.

Conclusions and future directions

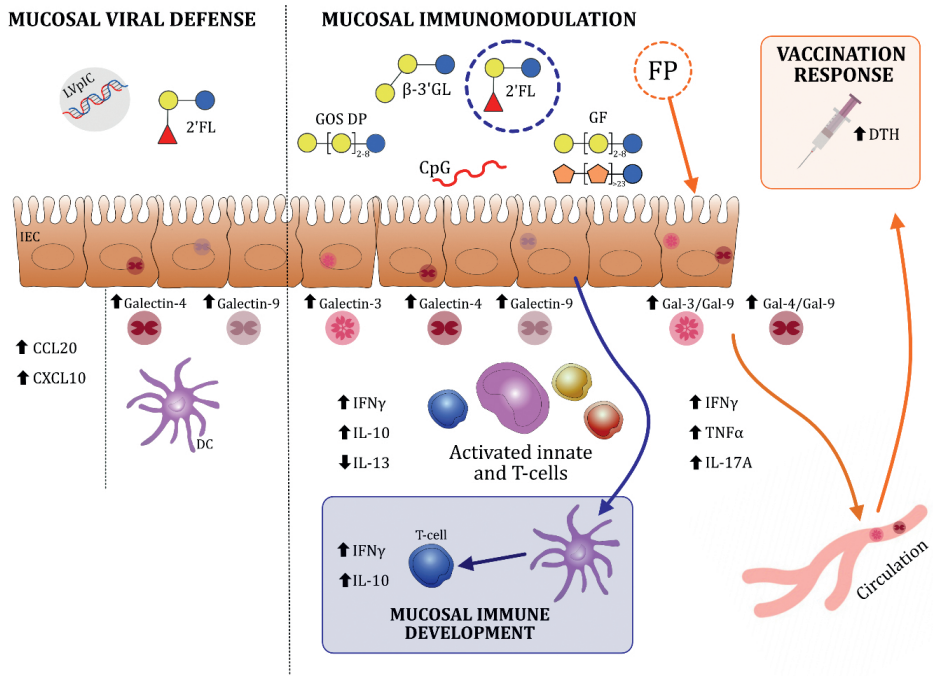
The results in this thesis demonstrate the relevance of IEC in regulating innate and adaptive immune responses during inflammation or upon a viral trigger by secreting galectins as key mediators to recruit immune cells and to maintain intestinal homeostasis.

Furthermore, we describe the ability of specific HMOS structures such as β-3'GL and 2'FL in modulating the cytokine secretion towards Th1- and regulatory type in an inflammatory setting, similar to the more generally used prebiotics GOS, GOS/lcFOS or FOS/lcFOS. This supports the idea that also the HMOS may be directly involved in the maturation and development of the immature immune system of the newborn, beyond their possible effects via the microbiome. In addition, we confirmed the involvement of epithelial-derived galectins in the modulation of the Th1- and regulatory type cytokine secretion by NDO and CpG, and we proved that these galectins are derived from exosomes secreted by IEC.

Besides, we demonstrated the ability of specific FP containing postbiotics in supporting an influenza-specific vaccination response, which further expands the interest in studying dietary interventions with postbiotics for their immunomodulatory and anti-inflammatory properties in a clinical setting, since supplementation of infant formula with FP was shown to be safe, well-tolerated and supported adequate growth in healthy term infants [102].

In conclusion, this thesis gains insight regarding the ability of NDO to support Th1- and regulatory type immunomodulation as shown *in vitro* in a model using IEC and PBMC. In addition, novel *in vitro* models were developed combining epithelial cells and immune cells, which can be used to study mucosal viral defense or mucosal immune development. The immunomodulatory effects of the dietary components were associated with IEC-derived galectins. In addition, FP were shown to support immunomodulation in the IEC/PBMC model. Furthermore, the immunomodulatory effects of FP were then confirmed *in vivo* using a pre-clinical model designed to study immune responses to vaccination, which highlights the translational relevance of *in vitro* co-culture models. All these effects are represented and summarized in **Summarizing figure**. Overall, the

ability of NDO and FP to promote immunomodulation indicates the capacity of these components to boost mucosal immune maturation in early life.



Summarizing figure. An overview of the effects promoted by NDO and postbiotics. Exposure of IEC to LV-pIC and 2'FL promoted the secretion of CCL20 and CXCL10 from IEC. Meanwhile, in IEC/moDC co-cultures exposed to LV-pIC and 2'FL, increased IEC-derived galectin-4 and -9 were found. These might contribute to mucosal viral defense. GOS DP, β-3'GL, 2'FL or 2'FL/GF in combination with CpG significantly increased Th1-type IFNγ and IL-10, while decreasing Th2-type IL-13 secretion from activated-PBMC (represented as activated innate and T-cells). These modulation on cytokine secretion was associated with increased IEC-derived galectin-3, -4 and/or -9 secretion. Galectin-4 and -9 were found to be secreted via exosomes to the basolateral milieu. In addition, 2'FL was shown to condition DC to instruct Th1 and regulatory-type immune responses indicating the ability of 2'FL to support mucosal immune development. In IEC/PBMC, FP increased the secretion of Th1-type IFNγ and TNFα, and Th17-type IL-17A. Also increased IEC-derived galectin-3 over -9 and galectin-4 over -9 ratios were observed. Furthermore, in an influenza murine vaccination model, a dietary intervention with FP increased DTH (a Th1 related parameter) with increased serum galectin-3 over -9 and galectin-4 over -9 ratios.

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ANNEX

NEDERLANDSE SAMENVATTING

Het maag-darmkanaal (MDK) en het immuunsysteem van een pasgeborene zijn bij de geboorte nog niet volgroeid en moeten zich ontwikkelen en aanpassen aan de omgeving buiten de baarmoeder. Een goede ontwikkeling van het MDK en het immuunsysteem in het vroege leven is cruciaal om de pasgeborene te beschermen tegen infecties of allergieën, die bij zuigelingen veel voorkomen.

De uitrijping van het MDK en het immuunsysteem vindt voornamelijk plaats in de eerste 3 levensjaren, een periode waarin een goede ontwikkeling van de pasgeborene centraal staat. Het is bekend dat voeding de ontwikkeling van het MDK en het immuunsysteem rechtstreeks beïnvloedt. Moedermelk wordt beschouwd als de optimale voeding voor pasgeborenen, omdat bekend is dat het bescherming biedt tegen infecties en maag-darmaandoeningen en daarnaast de kans op obesitas, diabetes of inflammatoire darmziekten op latere leeftijd vermindert. De WHO beveelt exclusieve borstvoeding gedurende ten minste 6 levensmaanden aan. Volgens recente Europese studies krijgt echter slechts 25% van de pasgeborenen de eerste 6 maanden uitsluitend borstvoeding. Het is daarom relevant om het geven van borstvoeding te bevorderen. Bovendien zal onderzoek naar de specifieke effecten van de bioactieve bestanddelen in moedermelk bijdragen aan een beter begrip van de gunstige werking van moedermelk en mogelijkheden bieden om betere alternatieven te ontwikkelen voor pasgeborenen die geen borstvoeding kunnen krijgen. Dit proefschrift beoogt specifieke effecten te bestuderen van humane melk oligosachariden (HMOS), niet-verteerbare oligosachariden (NVO) en postbiotica. Het onderzoek wordt gedaan in darmepitheelcellen (DEC) en cellen van het aangeboren en verworven immuunsysteem, om te begrijpen hoe bioactieve componenten de uitrijping en ontwikkeling van het MDK en het immuunsysteem kunnen bevorderen en daardoor een gezonde ontwikkeling in het vroege leven kunnen ondersteunen.

Er is geen twijfel over dat moedermelk de meest optimale voeding is voor de pasgeborene. De mechanismen waardoor moedermelk bescherming biedt en de ontwikkeling van het immuunsysteem en het MDK van de pasgeborene ondersteunt, blijven echter slecht begrepen. De DEC in het MDK zijn betrokken bij de opname van voedingsstoffen, maar vormen ook een barrière tegen binnendringende ziekteverwekkers of schadelijke antigenen. De wisselwerking tussen DEC en immuuncellen onder de DEC-barrière, mede door de uitscheiding van epitheliale mediatoren, is van essentieel belang voor de bevordering van adequate immuunreacties en de handhaving van de intestinale homeostase. Galectines zijn een voorbeeld van mediatoren die door DEC worden uitgescheiden om immuuncellen te reguleren en te instrueren.

Om de communicatie tussen DEC en immuuncellen te bestuderen worden *in vitro* co-culture modellen gebruikt waarin beide type cellen worden gecombineerd. De meeste van deze modellen gebruiken de humane HT-29 cellijn als model voor DEC. Hoewel deze cellijn een carcinogene achtergrond heeft, is gebleken dat de HT-29 cellijn een goed model is om interacties tussen DEC en immuuncellen te bestuderen.

Moedermelk bevat veel bioactieve componenten zoals HMOS, die in hoge mate aanwezig zijn. In **Hoofdstuk 2** beschrijven wij de diversiteit aan HMOS-structuren in moedermelk en de vele functies van deze HMOS, bijvoorbeeld via effecten op DEC en immuuncellen, om bescherming te bieden in het vroege leven.

DEC vormen een fysieke barrière om de gastheer te beschermen tegen pathogenen en dragen daarnaast bij aan de modulatie van de immuunrespons tegen pathogenen in het lumen van het MDK. Epitheliale mediatoren zijn betrokken bij de verdediging tegen o.a. virus infecties en zorgen voor het aantrekken en activeren van immuuncellen om zo een goede immuunrespons tegen virale pathogenen te bevorderen. In **Hoofdstuk 3** hebben wij een model opgezet om deze verdedigingsmechanismen van de gastheer tegen een specifieke virale trigger, namelijk poly I:C, te bestuderen. Daar zagen wij dat DEC geactiveerd werden door deze stimulus, maar over-activatie van onderliggende immuuncellen trad niet op, wat de homeostase van de darm kan ondersteunen. Bovendien hebben wij aangetoond dat blootstelling van DEC aan 2'-fucosyllactose (2'FL), een HMOS die in overvloed aanwezig is in moedermelk, de lokale virale afweer kan ondersteunen door de secretie van galectine te moduleren, zo kan het mogelijk bescherming bieden tegen virale infectie in het vroege leven.

De communicatie tussen DEC en aangeboren en verworven immuuncellen in de lamina propria is essentieel voor het behoud van immuunhomeostase. Van NVO, in combinatie met synthetisch CpG DNA (dat een bacteriële trigger nabootst), is bekend dat het de secretie van epitheliale galectines beinvloedt in een model waarin DEC en perifeer bloed mononucleaire cellen (PBMC) worden gecombineerd. In **Hoofdstuk 5** bestudeerden wij de immunomodulerende effecten van 2'FL. Door gebruik te maken van een DEC/PBMC co-cultuur model zagen wij dat 2'FL in combinatie met CpG een Th1-type regulerende immuunrespons ondersteunde met een vermindering van de Th2-type cytokine IL-13. Bovendien bevorderde de blootstelling aan 2'FL en CpG de uitscheiding van epitheliaal galectine-3, -4 en -9. Interessant is dat deze galectines positief correleren met Th1- en/of regulatoire cytokines, hetgeen suggereert dat epitheliale galectines betrokken zijn bij de immunomodulerende effecten van 2'FL en CpG. Bovendien zagen wij dat DEC geconditioneerd met 2'FL en CpG monocyt afgeleide dendritische cellen konden instrueren om de ontwikkeling van naïeve T-cellen aan te sturen.

Deze resultaten wijzen op het vermogen van HMOS zoals 2'FL om de ontwikkeling van het immuunsysteem direct te ondersteunen.

Een mengsel van NVO, bestaande uit (9:1) korte-keten galacto-oligosachariden en lange-keten fructo-oligosachariden (GF) afkomstig uit lactose en plantaardige bronnen, is ontwikkeld om de hoeveelheid en de structuurdiversiteit van HMOS in moedermelk na te bootsen en wordt gebruikt als toevoeging aan kunstmatige zuigelingenvoeding. De belangrijkste component van dit specifieke NVO-mengsel zijn korte-keten galacto-oligosachariden (GOS) die meerdere structuren bevatten met een polymerisatiegraad (PG) tussen 2 en 8. In **Hoofdstuk 4** hebben wij met behulp van een DEC/PBMC co-cultuurmodel vastgesteld dat in combinatie met CpG, GOS PG3-5 de meest actieve PG-fractie is in het ondersteunen van een Th1-type regulerende immuunrespons, vergelijkbaar met de immunomodulerende effecten die eerder werden aangetoond voor GF en CpG. Bovendien bestudeerden wij de immunomodulerende effecten van β-3'-galactosyllactose (β-3'GL), een specifieke structuur die van nature voorkomt in moedermelk. Hier zagen wij dat blootstelling aan β-3'GL of PG3 in aanwezigheid van CpG een regulatoire Th1-respons bevorderde met een verhoogde uitscheiding van galectine-9 door het epitheel. Deze resultaten wijzen op het vermogen van GOS PG3-5, en specifieker die van β-3'GL, om mucosale immunomodulatie te ondersteunen.

In **Hoofdstuk 6** onderzochten wij de bijdrage van epitheliale galectines aan de immunomodulerende effecten bij blootstelling aan NVO en CpG. De uitscheiding van galectines via exosomen is al eerder beschreven, maar het precieze mechanisme blijft onduidelijk. Hier hebben wij gebruik gemaakt van een exosoom-biogeneseremmer GW4869 om de epitheliale galectine uitscheiding, die wordt bevorderd door blootstelling aan een mengsel van 2'FL en GF (2'FL/GF) in combinatie met CpG, te blokkeren. De galectines bleken aanwezig in exosomen afkomstig uit DEC. Bovendien resulteerde de remming van exosoom-biogenese in het tegengaan van de door 2'FL/GF en CpG geïnduceerde uitscheiding van epitheel afkomstig galectine-9. Dit leidde vervolgens tot het tegengaan van zowel de door 2'FL/GF en CpG veroorzaakte toename in regulerend IL-10 als de afname in Th2-type IL-13. Deze resultaten suggereren dat epitheliale galectines betrokken zijn bij de immunomodulerende effecten door 2'FL/GF en CpG in dit co-culture model.

Gezien de carcinogene achtergrond van HT-29 cellen, wilden wij onderzoeken of soortgelijke immunomodulerende effecten ook worden waargenomen bij blootstelling van NVO en CpG aan een primaire humane darmepitheelcellijn. Zoals aangetoond in **Hoofdstuk 6**, bleek ook bij gebruik van FHs 74 Int, een foetale darmcellijn, dat NVO in combinatie met CpG immunomodulerend werkt. Dit wijst op het vermogen van deze

structuren om mucosale immuunreacties te beïnvloeden. Interessant is dat NVO ook al in afwezigheid van CpG immunomodulerende effecten liet zien, hetgeen mogelijk samenhangt met het nog onvolgroeide fenotype van de foetale darmcellijn.

Naast NVO worden ook andere strategieën bestudeerd om kunstmatige zuigelingenvoeding aan te vullen. Vanwege de aanwezigheid van bioactieve bestanddelen en hun voordelen in het MDK worden formules op basis van gefermenteerde melk bestudeerd. Tijdens de fermentatie van een melkmatrix met micro-organismen van levensmiddelenkwaliteit, worden bioactieve bestanddelen geproduceerd die naast microbiële metabolieten ook niet-levensvatbare microbiële bestanddelen bevatten, gezamelijk postbiotica genoemd. In **Hoofdstuk 7** onderzochten wij de immunomodulerende effecten van een specifiek fermentatieproduct (FP) dat postbiotica bevat afkomstig van *Bifidobacterium breve* C50 en *Streptococcus thermophilus*. Blootstelling aan FP kon de secretie van Th1- en Th17-type cytokines bevorderen, hetgeen wijst op het vermogen van FP om de immuunactivatie in het DEC/PBMC co-cultuurmodel te ondersteunen. Bovendien bleek een dieetinterventie met een specifiek FP in een muis model voor griepvaccinatie, de galectine concentraties in het bloed te beïnvloeden en de influenza specifieke Th1 type respons te verbeteren. Dit resultaat laat zien dat een specifiek FP dat postbiotica bevat de vaccinatierespons en het immuunsysteem kan ondersteunen.

Ten slotte worden in **Hoofdstuk 8** de bevindingen en de algemene conclusies van dit proefschrift besproken die inzichten kunnen verschaffen om strategieën te ontwikkelen ter ondersteuning van de ontwikkeling van mucosale immuniteit in het vroege leven. Bovendien onderschrijven de verschillende hoofdstukken van het proefschrift de translationele waarde van de ontwikkelde *in vitro* modellen waarin structurele cellen en immuuncellen, die van belang zijn voor de ontwikkeling van het MDK en de immuunrespons vroeg in het leven, worden gecombineerd.

RESUMEN EN ESPAÑOL

El tracto gastro-intestinal (TGI) y el sistema inmunitario del recién nacido son inmaduros al nacer y necesitan desarrollarse para adaptarse al entorno extrauterino. El desarrollo adecuado del TGI y el sistema inmune en los primeros años de vida, es crucial para promover la maduración del sistema inmunitario, y para apoyar la protección contra las infecciones o las alergias tan frecuentes en los bebés.

Los 3 primeros años de vida representan una ventana de oportunidad para establecer un correcto desarrollo del recién nacido ya que la maduración del TGI y del sistema inmune ocurre principalmente durante ese periodo de vida. Se sabe que la nutrición tiene una influencia directa en la maduración del TGI y del sistema inmunitario. La leche materna es considerada como nutrición óptima para los recién nacidos, ya que protege contra el desarrollo de las infecciones y los trastornos gastrointestinales, además de reducir la incidencia en el desarrollo de la obesidad, la diabetes o la enfermedad inflamatoria intestinal. La OMS recomienda la lactancia materna exclusiva al menos durante los primeros 6 meses de vida. Sin embargo, según estudios europeos recientes, sólo el 25% de los recién nacidos son alimentados exclusivamente con leche materna durante los primeros 6 meses de vida. Por lo tanto, es necesario promover la lactancia materna. Además, investigar sobre los efectos específicos de los componentes bioactivos presentes en la leche materna, ayudará a comprender mejor sus efectos beneficiosos, así como a proporcionar oportunidades para desarrollar mejores alternativas para los recién nacidos que no pueden recibir lactancia materna. Esta tesis tiene como objetivo estudiar los efectos específicos de los oligosacáridos de la leche materna (OLM), los oligosacáridos no digeribles (OND) y los postbióticos en las células epiteliales intestinales (CEI) y las células inmunes innatas y adaptativas, con el fin de entender cómo estos componentes bioactivos pueden promover la maduración y el desarrollo del TGI y el sistema inmune, y por lo tanto, apoyar el desarrollo de los recién nacidos en los primeros años de vida.

No hay duda de que la leche materna es una opción inmejorable para la nutrición del recién nacido. Sin embargo, los mecanismos por los cuales la leche materna proporciona protección y apoya la maduración del sistema inmune y el desarrollo del TGI, siguen sin conocerse. Las CEI en el TGI están involucradas en la absorción de nutrientes pero también proporcionan una barrera contra patógenos invasores y antígenos dañinos. Las interacciones entre las CEI y las células inmunitarias subyacentes a la barrera intestinal, mediante la secreción de mediadores intestinales, tienen gran importancia para promover respuestas inmunitarias apropiadas y para mantener la homeostasis intestinal. Un tipo de estos mediadores son las galectinas, que son secretadas por las CEI para regular

e instruir a las células inmunitarias. Para estudiar estas interacciones, comúnmente se utilizan modelos *in vitro* combinando CEI y células inmunitarias. La mayoría de estos modelos, utilizan la línea celular HT-29 como modelo de CEI. A pesar de su procedencia carcinogénica, la línea celular HT-29 ha demostrado ser una herramienta útil para estudiar las interacciones entre las CEI y las células inmunitarias.

La leche materna contiene gran cantidad de compuestos bioactivos como los OLM, los cuales son muy abundantes. En el **Capítulo 2**, se describe la diversidad de estructuras de OLM presentes en la leche materna y las múltiples funciones que estos tienen en las CEI y en las células inmunitarias, con el fin de proporcionar protección en los primeros años de vida.

Las CEI proporcionan una barrera física para proteger al hospedador de patógenos, y también contribuyen a la modulación de las respuestas inmunitarias en contra de patógenos que llegan al lumen intestinal. Mediante la atracción y la activación de las células inmunitarias y mediante la secreción de mediadores intestinales implicados en la defensa viral, las CEI son capaces de promover respuestas inmunológicas en contra de patógenos virales. En el **Capítulo 3** se establece un modelo para estudiar la respuesta inmunitaria del hospedador en contra de un activador viral específico denominado poly I:C. Se observa que las CEI pueden regular la homeostasis intestinal mediante la modulación de la secreción de mediadores epiteliales. Además, se observa que la exposición de las CEI a la 2'-fucosillactosa (2'FL), un OLM abundante en la leche materna, podría modular la defensa viral local mediante la modulación de la secreción de galectinas y por lo tanto, podría ser capaz de proporcionar protección en los primeros años de vida.

Las interacciones de las CEI con las células inmunes innatas y adaptativas que ocurren en la lámina propia, son cruciales para mantener la homeostasis intestinal. Los OND en combinación con el ADN CpG (imitando un activador bacteriano), modulan la secreción de galectinas derivadas del epitelio, en un modelo que combina CEI y células mononucleares de sangre periférica (CMSP). En el **Capítulo 5** se estudian los efectos inmunomoduladores de la 2'FL. Utilizando un modelo de co-cultivo CEI/CMSP se observa que en combinación con CpG, la 2'FL apoyó una respuesta inmunitaria reguladora de tipo Th1, con una reducción de la citoquina IL-13 de tipo Th2. Además, la exposición a 2'FL y CpG promueve la secreción de galectinas-3, -4 y -9 derivadas del epitelio. Curiosamente, encontramos que éstas galectinas se correlacionan con las citoquinas de tipo Th1 y reguladoras, lo cual sugiere que las galectinas derivadas del epitelio están involucradas en los efectos inmunomoduladores promovidos por 2'FL y CpG. Asimismo, observamos que las CEI condicionadas con 2'FL y CpG pueden instruir

a las células dendríticas derivadas de monocitos (CDmo) para impulsar el desarrollo de células T naive. Estos resultados apuntan a la capacidad de los OLM, como el 2'FL, para apoyar el desarrollo inmunitario.

Una mezcla de OND compuesta por (9:1) galacto-oligosacáridos de cadena corta y fructo-oligosacáridos de cadena larga (GF), derivados de lactosa y fuentes vegetales, ha sido diseñada para imitar la cantidad y la diversidad de estructuras de OND presentes en la leche materna, la cual está siendo utilizada para suplementar fórmulas artificiales infantiles. Los componentes mayoritarios de esta mezcla específica de OND son galacto-oligosacáridos de cadena corta (GOS) que contienen múltiples estructuras con grado de polimerización (GP) entre 2 y 8. En el **Capítulo 4**, mediante un modelo de co-cultivo CEI/CMSP, se identifica que en combinación con CpG, la fracción GP3-5 de GOS es la más activa en cuanto a la promoción de una respuesta inmunitaria reguladora de tipo Th1, parecida a la respuesta observada anteriormente ante la exposición a GF y CpG. Además, se estudian los efectos inmunomoduladores de la β-3'-galactosillactosa (β-3'GL), una estructura específica incluida en GOS y presente de manera natural en la leche materna. Se observa, que la exposición a β-3'GL (con tamaño GP3) y CpG, promueve una respuesta reguladora de tipo Th1 con un aumento de la secreción de galectina-9 derivada del epitelio. Estos resultados indican la habilidad de GOS GP3-5 y más específicamente de β-3'GL, en el apoyo a la inmunomodulación de las mucosas.

En el **Capítulo 6** se estudia la contribución de las galectinas derivadas del epitelio a los efectos inmunomoduladores observados tras la exposición a OND y CpG. La secreción de galectinas mediante exosomas ha sido descrita anteriormente. Sin embargo, el mecanismo exacto sigue sin conocerse. Mediante el uso de un inhibidor de la biogénesis de exosomas, denominado GW4869, se bloquea la secreción de galectinas derivadas del epitelio promovida por la exposición a una mezcla de 2'FL y GF (2'FL/GF) en combinación con CpG. Se observa la presencia de galectinas en exosomas derivados de CEI. Además, la inhibición de la biogénesis de los exosomas, suprime la secreción de galectina-9 derivada del epitelio inducida por 2'FL/GF y CpG. Posteriormente, esto conduce a la supresión del aumento de la IL-10 reguladora, así como a la supresión en la reducción de la IL-13 tipo Th2 inducida por 2'FL/GF y CpG. Estos resultados sugieren la participación de las galectinas derivadas del epitelio en la promoción de los efectos inmunomoduladores.

Considerando la procedencia carcinogénica de la línea celular HT-29, se estudia si el uso de una línea celular primaria derivaría en efectos inmunomoduladores similares a los observados tras la exposición a OND y CpG en HT-29. Como se muestra en el **Capítulo 6**, se observa que al utilizar FHs 74 Int, una línea celular intestinal fetal, los

NDO y CpG también promueven efectos inmunomoduladores, lo que indica la capacidad de estas estructuras para apoyar las respuesta inmune de las mucosas. Curiosamente, ya en la ausencia de CpG, los OND son capaces de promover efectos inmunomoduladores, posiblemente debido al fenotipo inmaduro de la línea celular intestinal fetal.

Además de los OND, se están estudiando otras estrategias para suplementar las fórmulas artificiales infantiles. Se está considerando el uso de fórmulas artificiales a base de leche fermentada debido a la presencia de compuestos bioactivos así como por los beneficios en el TGI. Durante la fermentación de una matriz láctea utilizando microorganismos de grado alimentario, se producen componentes bioactivos que además de los metabolitos microbianos, también incluyen componentes microbianos no viables, denominados postbióticos. En el **Capítulo 7**, se investigan los efectos inmunomoduladores de un producto de fermentación (PF) específico que contiene postbióticos derivados de Bifidobacterium breve M16V y Streptococcus thermophilus. En un modelo de co-cultivo CEI/CMSP se observa que la exposición a PF promueve la secreción de citoquinas de tipo Th1 y Th17, lo cual indica la capacidad de PF para apoyar la secreción de citoquinas pro-inflammatorias. Además, una intervención dietética con un PF específico demuestra una mejora en la respuesta específica a la influenza de hipersensibilidad de tipo retardada (HTR), un párametro tipo Th1 indicador de la capacidad de respuesta a la vacuna. Estos resultados destacan la capacidad de PF específicos que contienen postbióticos para favorecer la respuesta inmunitaria de la vacunación.

Por último, en el **Capítulo 8** se discuten los hallazgos y las conclusiones generales de ésta tesis, los cuales pueden proporcionar ideas para desarrollar estrategias con el fin de apoyar la maduración inmune de las mucosas en los primeros años de vida. Asimismo, esta tesis reafirma el valor traslativo desde los modelos *in vitro* a los modelos preclínicos *in vivo*, mediante el estudio de elementos fundamentales para el desarrollo del TGI y de la respuesta inmunitaria en los primeros años de vida.

LABURPENA EUSKARAZ

Jaioberriaren traktu gastrointestinala (TGI) eta sistema immunologikoa heldu gabe daude eta garatu egin behar dira utero kanpoko ingurunera egokitzeko. Bizitzako lehen urteetan, TGI-aren eta sistema immunologikoaren garapen egokia funtsezkoa da sistema immunologikoaren heltzea sustatzeko eta haurrek oso ohiko dituzten infekzio edo alergien aurkako babesa emateko.

TGI-aren eta sistema immunologikoaren heltzea nagusiki bizitzako lehen 3 urteetan gertatzen da, eta horrek jaioberriaren garapen zuzena ezartzeko aukera bat suposatzen du. Jakina da elikadurak eragin zuzena duela TGI-aren eta immunitate-sistemaren heltzean. Jaioberrientzako amaren esnea elikadura ezin hobea da, obesitatearen, diabetesaren edo hesteetako gaixotasun inflamatorioaren intzidentzia murrizteaz gain, infekzioen eta nahaste gastrointestinalen garapenetik babesten duelako. Osasunaren Mundu Erakundeak (OME) edoskitze esklusiboa gomendatzen du gutxienez bizitzako lehen 6 hilabeteetan. Hala ere, Europako azken ikerketen arabera, jaioberrien %25-ak bakarrik jasotzen du edoskitze esklusiboa bizitzako lehen 6 hilabeteetan. Horregatik edoskitze naturala sustatzea beharrezkoa da. Gainera, amaren esnean dauden osagai bioaktiboen efektu espezifikoei buruzko ikerketek haien ondorio onuragarriak hobeto ulertzen lagunduko dute, baita edoskitze naturala jaso ezin duten jaioberrientzako aukera hobeak garatzen ere. Tesi honek amaren esneko oligosakarido-ek (AEO), oligosakarido ez-digerigarri-ek (OED) eta postbiotikoek hesteetako zelula epiteliarretan (HZE) eta immunitate naturaleko eta sortzetiko immunitateko zeluletan duten efektu espezifikoak aztertzea du helburu, osagai bioaktiboek TGI-aren eta sistema immunologiko-aren heltzea nola susta dezaketen ulertzeko eta beraz, bizitzako lehen urteetako garapenean nola laguntzen duten jakiteko.

Zalantzarik gabe, amaren esnea da jaioberriarentzat elikadurarik egokiena. Hala ere, jaioberria babesteko eta bere sistema immunologikoaren heltzea eta TGI-aren garapena ezartzeko erabiltzen diren mekanismoak ezezagunak dira. TGI-eko HZE-ek nutrienteen xurgapenean parte hartzeaz gain, patogeno inbaditzaileen edo antigeno kaltegarrien aurkako hesi bat ere eskaintzen dute. HZE eta heste-hesiaren azpian dauden immunitate zelulen arteko elkarrekintzak, heste-bitartekarien sekrezioaren bitartez, erantzun immune egokiak sustatzeko garrantzi handia dute, baita hesteetako homeostasia mantentzeko ere. Bitartekari horietako mota bat HZE-ek sekretatzen dituzten galektinak dira eta beraien helburua zelula immunologikoak erregulatu eta bideratzea da. Interakzio horiek aztertzeko, HZE-ek eta zelula immunologikoak konbinatzen dituzten *in vitro* ereduak erabiltzen dira. Eredu horietako gehiengoak HT-29 zelula-lerroa erabiltzen dute HZE-en eredu gisa. HT-29 zelula-lerroak jatorri kartzinogenoa izan arren, HZE-en

eta zelula immunologikoen arteko elkarrekintzak aztertzeko tresna erabilgarria dela frogatu da.

Amaren esneak konposatu bioaktibo ugari ditu, hala nola oso ugariak diren AEO-ak. Amaren esnean dauden AEO-en egituren aniztasuna eta hauek bizitzako lehenengo urteetan babesa emateko HZE-etan eta zelula immunologikoetan dituzten funtzio anitzak deskribatzen dira **2.-en atalean**.

HZE-ek hesi fisiko bat eskaintzen dute ostalaria patogenoetatik babesteko eta hestelumenera iristen diren patogenoen aurkako erantzun immunologikoen modulazioan laguntzeko. Zelula immunologikoak erakarri eta aktibatuz, eta defentsa birikoan parte hartzen duten hesteetako bitartekariak sekretatuz, HZE-ak patogeno birikoen aurkako erantzun immuneak sustatzeko gai dira. Poly I:C izeneko aktibatzaile biral zehatz baten aurka ostalariaren erantzun immunea aztertzeko eredu bat aurkezten da **3.-en atalean**. Bertan, bitartekari epitelialen sekrezioa modulatuz HZE-ek hesteetako homeostasia erregula dezaketela ikusi zen. Gainera, amaren esnean ugaria den 2'-fucosillaktosa (2'FL) AEO-aren esposizioak galektinen sekrezioa modulatuz tokiko defentsa birikoa erregulatu dezake eta beraz, bizitzako lehen urteetan babesa emateko gai izan daiteke.

Hesteetako homeostasia mantentzeko HZE-en eta immunitate naturaleko eta sortzetiko immunitateko zelulen arteko elkarrekintzak funtsezkoak dira. HZE-ak eta odol periferikoko zelula mononuklearrak (OPZM) konbinatzen dituen eredu batean, OED-ak CpG DNA-rekin konbinatzean (bakteria-aktibatzaile bat imitatuz), epiteliotik eratorritako galektinen sekrezioa modulatzen dutela ikusten da. **5.-en atalean** 2'FL-aren efektu immunomodulatzaileak aztertzen dira. HZE/OPZM elkar-kultibo eredu bat erabiliz CpG-arekin konbinatzean, 2'FL-k Th1 motako erantzun erregulatzailea eta Th2 motako IL-13 zitokinaren murrizketa sustatzen dela ikusten da. Horrez gain, 2'FL eta CpG-ren esposizioak epiteliotik eratorritako 3, 4 eta 9 galektinen sekrezioa sustatzen du. Hain zuzen ere, galektina hauek Th1 eta erregulatzaile motako zitokinekin korrelazionatuta daudela ikusten da, epiteliotik eratorritako galektinak 2'FL eta CpG-k sustatutako efektu immunodulatzaileetan parte hartzen dutela adieraziz. Era berean, 2'FL eta CpG-rekin baldintzatutako HZE-ek monozitoetatik eratorritako zelula dendritikoei T zelulen garapena bultzatzeko aginduak bidali ditzaketela ikusten da. Ondorioz, 2'FL bezalako AEO-ek bizitzako lehen urteetan garapen immunologikoa sustatzeko gaitasuna dutela adierazten da.

Amaren esnean aurkitzen diren AEO-en egitura kopurua eta aniztasuna imitatzeko OED-ez osatutako nahasketa bat diseinatu da, laktosatik eta landare iturrietatik eratorritako kate laburreko galakto-oligosakaridoz eta kate luzeko frukto-oligosakaridoz

(GF) osatutakoa, haurrentzako formuletan osagarri bezala erabiltzen dena. OED-en nahasketa espezifiko honen osagai nagusiak kate laburreko galakto-oligosakaridoak (GOS) dira, 2 eta 9 arteko polimerizazio maila (PM) dituzten egitura anitzez osatuta daudenak. **4.-en atalean** HZE/OPZM elkar-kultibo eredu bat erabiliz, 3-5PM GOS frakzioak CpG-rekin konbinatuta, Th1 motako erantzun immune erregulatzailea sustatzen duen frakziorik aktiboena dela identifikatzen da, aurrez GF eta CpG-rekin ikusitako erantzun antzerakoa sustatzen duelarik. Horrez gain, GOS-en eta amaren esnean aurkitzen den egitura espezifiko baten, β-3'-galaktosillaktosaren (β-3'GL) efektu immunomodulatzaileak aztertzen dira. β-3'GL (3-ko PM duena) eta CpG-ren esposizioak Th1 motako erantzun erregulatzailea sustatzen duela ikusten da, epiteliotik eratorritako galektina-9 sekrezioa handiagotuz. Emaitza hauek 3-5PM GOS-ek eta zehazkiago β-3'GL-ek mukosaren immunomodulazioari eusteko gaitasuna dutela adierazten dute.

OED eta CpG-ren esposizioaren ondoren ikusitako efektu immunomodulatzaileetan epiteliotik eratorritako galektinen ekarpena aztertzen da **6.-en atalean**. Exosomen bidezko galektinen sekrezioa lehenago deskribatu da. Hala ere, mekanismo zehatza oraindik ezezaguna da. GW4869 izeneko, exosomen biogenesiaren inhibitzaile bat erabiltzen da, CpG-rekin konbinatutako 2'FL eta GF-en nahasketa (2'FL/GF) baten eraginez sustatutako epiteliotik eratorritako galektinen sekrezioa blokeatzeko. Exosometan HZE-etatik eratorritako galektinen presentzia ikusten da. Horrez gain, exosomen biogenesiaren inhibizioak 2'FL/GF eta CpG-k eragindako epiteliotik eratorritako galektina-9-ren sekrezioa ezabatzen du. Honek, gerora, 2'FL/GF eta CpG-k eragindako IL-10 zitokina erregulatzailearen igoera ezabatzea ekartzen du eta baita, Th2 motako IL-13 zitokinaren murrizketa ere. Emaitza hauek epiteliotik eratorritako galektinek efektu immunomodulatzaileak sustatzeko duten inplikazioa aditzera ematen dute.

HT-29 zelula-lerroaren jatorri kartzinogenoa kontutan hartuta, zelula lerro primario bat erabiltzean OED eta CpG-ren esposizioaren ondoren antzeko efektu immunomodulatzaileak ekarriko ote dituen aztertzea nahi izan dugu. **6.-en atalean** erakusten den bezala, FHs 74 Int-a erabiliz (hesteen zelula-lerro fetala), OED eta CpG-k efektu immunomodulatzaileak sustatzen dituztela ikusten da, egitura horiek mukosaren erantzun immunologikoak eusteko duten gaitasuna dutela adieraziz. Interesgarria da, CpG-rik ezean ere, OED-ek efektu immunomodulaltzaileak sustatzeko gai izan zirela, ziurrenik fetuaren hesteetako zelula-lerroaren fenotipo heldugabearen ondorioz.

OED-ez gain, haurrentzako formuletan osagarri bezala erabiltzeko konposatuak bilatzeko beste estrategia batzuk aztertzen ari dira. Konposatu bioaktiboen presentziak eta TGI-ean ekartzen dituen onurak direla eta, esne hartzituan oinarritutako formula

artifizialak aztertzen ari dira. Elikagai-mailako mikroorgnismoak erabiliaz, esne-matrize baten hartziduran osagai bioaktiboak sortzen dira, mikrobioen metabolitoez gain, postbiotiko izeneko bideragarriak ez diren osagai mikrobiotikoak ere dituztenak. *Bifidobacterium breve* M16V eta *Streptococcus thermophilus*-etik eratorritako postbiotikoak dituen hartzidura-produktu (HP) espezifiko baten eragin immunomodulatzaileak ikertzen ditugu **7.-en atalean**. HZE/OPZM elkar-kultibo eredua erabiliz, HP-ren esposizioak Th1 eta Th17 motako zitokinen sekrezioa sustatu zuela ikusten da, HP-k zitokina inflamatorioen sekrezioa sustatzeko gaitasuna duela adieraziz. Horrez gain, HP espezifiko batekin egindako interbentzio dietetiko baten, gripearen aurkako hipersentikortasun atzeratua (HA) neurtzean (txertoen erantzunaren Th1 motako adierazgarria den parametroa), hobekuntza sustatzeko ahalmena zuela frogatzen da. Emaitza hauek postbiotikoetan dauden HP-ak txertoaren erantzun immunologikoa hobetzeko gaitasuna dutela nabarmentzen dute.

Azkenik, **8.-en atalean** tesi honen aurkikuntza eta ondorio orokorrak eztabaidatzen dira, bizitzako lehen urteetan mukosaren heltze immunologikoan laguntzeko estrategiak garatzeko asmoz. Era berean, tesi honek in vitro ereduetatik eredu in vivo prekilnokoetarako balio translazionala berresten du, bizitzako lehen urteetan TGI-a eta erantzun immunologikoak garatzen dituzten oinarrizko elementuak aztertuz.

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ABOUT THE AUTHOR

Veronica Ayechu Muruzabal was born on June 1st, 1988 in Pamplona, Spain. After graduating from high school (Elkar Hezi Ikastetxea, Oñati, Spain) she moved to Pamplona, where she started her Bachelor studies at Universidad de Navarra where she graduated in Pharmacy and Nutrition.

In 2014 she moved to Utrecht, The Netherlands where she pursued a master degree in Drug Innovation at Utrecht University. Thereafter, she worked as a Junior Researcher at the Pharmacology division of Utrecht University in collaboration with Danone Nutricia Research, after which she joined the Infection and Immunity PhD program under the supervision of Prof. Dr. Johan Garssen from the division of Pharmacology and affiliated to Danone Nutricia Research, Dr. Linette E.M. Willemsen from the division of Pharmacology and Dr. Belinda van't Land from Danone Nutricia Research. Her PhD project focused on understanding the role of human milk oligosaccharides, non-digestible oligosaccharides and postbiotics on mucosal immune development. The results of this work are described in this thesis.

During her PhD thesis, she participated in several international conferences an received a travel grant to attend the IMPARAS 2018 meeting in Napoli. In 2019, she was awarded with a prize for best poster presentation at the EAACI in Lisbon. In 2021, she received a registration prize to attend the WIRM meeting. In addition, on that same year she received an award for best poster presentation from the XII ISIN conference in Barcelona (held online).

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