Immune modulation by cow's milk components:

Implications for respiratory health

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Thesis

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voor Anouk

List of Abbreviations

$1,25(OH)_2D_3$:	VitD3
Alkaline phosphatase:	ALP
Bovine lactoferrin:	LF
C-C chemokine receptor:	CCR
Chemokine (C-C motif) ligand:	CCL
Cluster of differentiation:	CD
Dendritic cells:	DC
Endotoxin unit:	EU
Epidermal growth factor:	EGF
Fc epsilon receptor I:	FcεRI
Fructooligosaccharides:	FOS
Fucosyllactose:	FL
G protein coupled receptor:	GPR
Galactooligosaccharides:	GOS
Gastrointestinal:	GI
Granulocyte-macrophage colony-stimulating factor:	GM-CSF
Gut-associated lymphoid tissue:	GALT
House dust mite:	HDM
Human milk oligosaccharides:	HMO
Immunoglobulin:	Ig
Innate lymphoid cells:	ILC
Interferon gamma:	IFNγ
Interleukin:	IL
Lipopolysaccharide:	LPS
Mesenteric lymph node:	MLN
monocyte-derived DC:	moDC
Neonatal Fc receptor:	FcRn
Nuclear factor kappa-light-chain-enhancer of activated B cells:	NF-κB
Ovalbumin:	OVA
Pathogen-associated molecular pattern:	PAMP
Peripheral blood mononuclear cells:	PBMCs
Plasmacytoid DC:	pDC
Polyinosinic-polycytidylic acid:	Poly I:C
Programmed death-ligand 1:	PD-L1
Regulatory T cells:	Tregs
Resiquimod:	R848
Respiratory syncytial virus:	RSV
Retinoic acid:	RA
Short chain fatty acids:	SCFA
Sialyllactose:	SL
T helper:	Th
Thymic stromal lymphopoietin:	TSLP
Tight junction:	TJ
Toll-like receptor:	TLR
Transforming growth factor:	TGF
Tumor necrosis factor alpha:	TNF
Very low birth weight:	VLBW

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Chapter 1

General Introduction

Introduction

It has been almost 30 years since Strachan postulated the hygiene hypothesis stating that our Western lifestyle (i.e. a more hygienic environment) may be the underlying cause of the rise in allergy and asthma prevalence in the Western world[1]. To date, an estimated 30% of the world population is suffering from allergies and asthma that are causing dramatic health- and economic effects[2,3]. Additionally, we know that our mucosal immune system and microbiota, shaped in early life, influence disease prevalence later in life. Despite recommendations by the World Health Organization and scientific evidence showing health benefits (e.g. increased cognitive ability, reduced gastrointestinal and respiratory infections and reduced diabetes prevalence) of breastfeeding [4], the majority of children is not exclusively breastfed in the first six months of life. However, epidemiological studies report conflicting results on the association between breastfeeding and allergy prevalence, which may be explained by individual differences in breast milk composition[5,6]. Thus, we are far from understanding the interplay between early life nutrition, microbiota, and immunity and their relation in allergy and asthma prevalence. Hence, there is a pressing need to better understand the impact of early life nutrition on the health of neonate in order to design effective preventive strategies.

Allergies

Allergies are induced by a classical T helper (Th) 2-mediated immune response that is initiated in two phases: the sensitization and effector phase. In the sensitization phase, resident allergen-exposed dendritic cells (DC) mature and migrate in a C-C chemokine receptor type 7 (CCR7)-dependent manner to the draining lymph node and trigger the polarization of naïve T cells into allergen-specific Th2 cells. This allergen-driven programming of DC is enhanced by epithelial-derived chemokines and cytokines (e.g. thymic stromal lymphopoietin (TSLP) and alarmins like interleukin (IL)-33) that are produced upon allergen exposure. These soluble compounds also activate type 2 innate lymphoid cells (ILC2) that enhance the allergic cascade by producing IL-5 and IL-13[7,8]. Allergen-specific Th2 cells play a central role in the induction of allergy by subsequent induction of IL-4 driven IgE class-switching in B cells. Allergen-specific IgE consequently binds to the high affinity Fc epsilon receptor I (FcεRI) on mast cells and basophils. Upon re-exposure, the allergen binds to IgE and

may induce cross-linking of these specific IgE antibodies, which results in the release of soluble mediators (e.g. histamine) that cause characteristic symptoms of IgE-mediated allergy[8]. This Th2-mediated response is thought to have evolved to tackle helminth infections, but is now triggered by harmless antigens (e.g. birch pollen), causing the pathogenesis of allergy. In early life, this pathogenesis may be prevented by exposure to environmental factors (e.g. diet) that trigger the development of a local immunosuppressive microenvironment, which allows tolerogenic responses to be mounted against the particular allergen. An integrated overview of the mucosal immune system is required to understand this complex interplay between diet and local and systemic immunity. We will therefore give a brief introduction of the different players in the gastrointestinal (GI) immune system that play a role in maintaining immune homeostasis.

The larynx-associated and gastrointestinal mucosal immune system

The larynx-associated lymphoid tissue comprises of the palatine- and lingual tonsils[9]. The lymphoid structures in the oropharynx have organized lymphoid structures similar to those found in the intestine (i.e. Peyer's Patches)[9]. Here, structurally intact (i.e. functional) proteins can interact with the immune system before being hydrolyzed in the intestine. These lymphoid structures are covered by epithelial cells similar to M-cells in the gut, which are specialized in antigen uptake. After swallowing, dietary antigens are exposed to enzymatic hydrolysis in the small intestine, where they come into contact with the GI immune system.

The GI tract has a staggering surface area of 400 m^2 that contains 5-20% of all lymphocytes in the body[10]. The GI tract hosts an intimate symbiotic relationship between our microbiota and the mucosal immune system. The microbiota ($3.8*10^{13}$) outnumber the total number of body cells ($3*10^{13}$)[11,12]. The GI immune system is covered by a single layer of epithelial cells, covering a loose layer of connective tissue (called the lamina propria in the gut) that rapidly renews every 3-4 days. Antigens are taken up by epithelial cells, specialized sampling DC[13] or via the neonatal Fcγ receptor (FcRn) if the antigen is coated with IgG. FcRn-mediated signaling induced by breast milk-derived allergen-specific IgG complexes was shown to be essential in the induction of tolerance towards allergens in animal models[14–16].

Epithelium and mucus – the first physical barrier

The epithelial cells of the intestine are covered by a thick layer of secreted mucus that is constantly shed by epithelial goblet cells. Mucus shields proteins expressed on epithelial cells from proteolysis, prevents epithelial cells from dehydration by its high hydrophobicity (i.e. >98% water) and it actively forms the first physical barrier[17]. Unlike the small intestine that consists of a single layer of mucus and where bacteria may come into contact at the villi tops, in the colon the mucus layer consist of two layers that act as a size exclusion filter, preventing direct contact between bacteria and epithelium[17]. Many commensal bacteria are capable of utilizing this outer mucus layer of the colon for their growth. Epithelial cells are covered in this thick layer of mucus. The epithelium is constantly renewed by stem cells that are located at the bottom of the intestinal crypts. In the small intestine, stem cells reside that undergo asymmetrical cell division, that results in one transitional effector cell and one stem cell[18]. These transitional effector cells differentiate into four different epithelial subsets: columnar cells, Paneth cells, goblet cells and endocrine cells that regulate barrier functioning, secrete anti-microbial peptides, produce mucus and hormones, respectively[19]. Columnar cells, also called enterocytes or colonocytes in the small and large intestine, respectively, form the first physical layer of the immune system. These columnar cells have a strong polarized morphology and functionality. Moreover, the apical side of the enterocytes has microvilli, contains specific transporters to import nutrients and expresses Toll-like receptor (TLR) that recognize TLR ligands. This apical TLR signaling was shown to contribute to barrier functioning and immune homeostasis via several mechanisms[20]. Enterocytes are linked to each other by tight junction proteins that regulate paracellular transport and only allow selective transport of small molecules (i.e. proteins of <0.5kDa) while preventing pathogens or pathogen-associated molecular patterns (PAMPs) to pass through the barrier[21].

DC and macrophages

The key innate immune cell that is capable of orchestrating immune responses by activating cells of the adaptive immune system in the periphery are DC. Monocytes and DC differentiate from a common macrophage dendritic cell progenitor cell that gives rise to four distinct human cell types in the blood: two different types of classical DC (CD1c+ DC and CD141+ DC), plasmacytoid DC and monocytes[22,23].

In the intestine, CX3CR1+ DC are capable of continuous sampling of antigens from the lumen, while DC subsets are more specialized to modulate mucosal T cell biology (e.g. CD103+ DC) upon activation[13]. CD103+ DC are derived from pre-DC and may receive their antigens from goblet cells[24]. Interestingly, these DC were shown to migrate to the mesenteric lymph node where they induce FoxP3+ regulatory T cell formation and instruct these cells to migrate back to the lamina propria to suppress local immune responses[25–27]. A local microenvironment comprising dietary components and microbial- or epithelial derived molecules potentially induces the differentiation of pre-DC into CD103+ DC[25]. Not only intestinal CD103+ DC migrate to the lymph node, but also CD103- DC are capable of doing so[28]. Additionally, a population of tissue-resident macrophages (e.g. Langerhans- and microglia cells) are derived from the yolk sack and are already seeded before birth[29]. In contrast to the skin and brains, the GI tract relies on a constant recruitment of monocytes to retain its tissue-resident macrophage population[30]. These macrophages produce IL-10 and TGFβ and are resistant to TLR stimulation[30]. Therefore, it was postulated that the GI tract is in a constant state of primed homeostasis in which the microbiota, diet or microbial derived product trigger a continuous tolerant state of macrophages and DC[31].

Adaptive immunity

The induction of oral tolerance towards dietary antigens is still poorly understood. FoxP3+ regulatory T cells (Tregs) are abundantly present in the intestine but not in secondary lymphoid tissues, implying that they play an active role in the induction and maintenance of tolerance to food antigens. The concept of oral tolerance induction towards food proteins that can suppress subsequent systemic immune responses is already known for over 100 years[32]. However, only recently it was formally shown in mice that normal chow induced short-lived regulatory T cells in the periphery that are mainly detected in the small intestine and in lower numbers in the colon[33]. These regulatory T cells are instructed by CD103+ DC in the mesenteric lymph node to migrate back to the lamina propria where they produce high levels of immunosuppressive cytokines such as IL-10 and TGFβ[32]. Although the exact mechanism of this “bystander tolerance” is poorly understood, it is clear that oral tolerance can subsequently suppress the induction of systemic inflammatory responses[32,34]. As such, antigen-specific induced FoxP3- IL-10 producing Tregs (also

called type 1 Tregs or Tr1) are well-known to inhibit allergic responses (e.g. Tr1 are enhanced in successful immunotherapy)[8]. Similarly, IL-10 producing B cells (Bregs) were elevated after allergen-specific immunotherapy. Breg-derived plasma cells produce high levels of IgG4, which are known to block binding of allergen-specific IgE antibodies to FcεRI, and thereby prevent allergic sensitization[35]. Allergen-specific IgG4 antibodies are of similar affinity than IgE and are produced in at least a 1000-fold excess compared to IgE. This competition is therefore very effective in blocking development of allergic symptoms. Interestingly, Bregs were shown to be induced by type 3 innate lymphoid cells (ILC3) in the palatine tonsils and were less prevalent in allergic individuals[36]. Hence, it is of interest to identify whether dietary compounds (e.g. intact proteins) are capable of restoring the allergy-dependent reduction in ILC3 and Bregs in the pharynx.

Microbiota and microbial-derived metabolites

The microbiota colonizes the newborn's gut upon birth and is further shaped by breast milk in early life – a process essential for the infant to adapt to its new environment[37]. Breastfeeding was shown to affect the microbiota composition in the nasopharynx[38] and gut[39]. In the nasopharynx of six-week old breastfed infants, the lactate producing bacterium *Dolosigranulum* was more abundant compared to bottle-fed infants of the same age. Its prevalence was inversely correlated with wheezing and mild respiratory symptoms[38]. In the gut, the predominant breast milk-derived components that influence microbiota composition are human milk oligosaccharides (HMO). HMO function as an important carbon source for specific microbes in the colon[40]. Breastfed infants show a higher abundance of Bifidobacteria and other bacteria considered as health-promoting bacteria (e.g. *B. longum*, *L. johnsonii* and *L. casei*)[39]. These bacteria produce metabolites such as short chained fatty acids (SCFA). In the last decade, *in vitro*- and animal models showed that SCFA can inhibit local and systemic inflammation and prevent diseases such as allergy and asthma[12,41–46].

Breast milk and early life development

Viviparity has evolved multiple times in vertebrates, implying that it has a strong fitness advantage[47]. Viviparity gives the unique opportunity to nourish the newborn

during pregnancy and in early life, which gives the mother the chance to supply her infant with the best nutrition. Because of this intimate relationship between mother and child, the pre- and postnatal lifestyle of the mother impacts the health of the infant. Moreover, maternal diet and microbial-derived products aid in the development of the mucosal immune system of the unborn child[48]. After birth, the neonate receives a unique mixture of components (e.g. proteins, lactose, fat, lipids, oligosaccharides, vitamins) in the form of breast milk that further enhances the development of the neonate's immune system. We have recently reviewed the effect of this dietary and microbial exposure on fetal and neonatal immune development and will therefore not further introduce it here[49] (**Appendix I**). Thus, breast milk aids in the development of immune homeostasis and tolerance induction. This oral tolerance may suppress subsequent inflammation after exposure to airborne or dietary allergens[32,34]. Moreover, breast milk contains several immunoregulatory cytokines (e.g. TGFβ and IL-10)[50], compounds that promote outgrowth of beneficial bacteria (e.g. human milk oligosaccharides promoting outgrowth of bifidobacteria), antimicrobial proteins that prevent outgrowth of pathogenic bacteria (e.g. lactoferrin (LF), lactoperoxidase) and factors promoting epithelial barrier functioning (e.g. EGF and TGF-β)[34].

Aims of this thesis

Several epidemiologic studies conducted at different sites in the world (New-Zealand, Europe and the U.S.A.) show that the consumption of raw cow's milk in early life is inversely correlated with the development of allergies and asthma. Controlled studies with unprocessed cow's milk in infants are impossible due to safety and ethical reasons and thus causality cannot formally be proven. Therefore, we used animal and *in vitro* models to identify functional allergy and immunomodulating milk components, which subsequently may allow human intervention studies. Therefore, the overall **aim** of this thesis was to investigate the effect of raw cow's milk and its components on maintenance of mucosal immune homeostasis. Since the link between diet and respiratory health is still poorly understood, we summarized potential mechanisms by which cow's milk may improve respiratory health in **chapter 2**.

One of the proteins in cow's milk that may aid in immune homeostasis in early life is LF. Moreover, LF supplementation has been shown to prevent sepsis in low birth

weight infants and prevent viral infections. Although *in vitro* evidence also indicates that LF is an important immunomodulatory protein, its mechanism of action is poorly understood. In **chapter 3** we investigated the effect of LF on human DC differentiation and functioning. It is challenging to investigate the immunomodulatory effect of LF since it binds the highly immunogenic gram-negative constituent LPS. We therefore tested multiple methods to verify that the protein structure was responsible for the immunogenic effect. One of these methods is described in **chapter 4.** In this chapter, we optimized an existing method to remove LPS from protein samples using Triton X-114. Importantly for the immunogenicity of the food protein of interest, we investigated whether the three-dimensional structure of the protein remained intact after applying the optimized Triton X-114 method.

Cow's milk based infant nutrition is currently supplied with lactose-derived (i.e. GOS) or plant-derived oligosaccharides (e.g. FOS). Breast milk contains, in contrast to cow's milk, a wider variety and larger quantity of HMO compared to cow's milk. Hence, it is of interest to identify the functionality of these HMO to improve infant nutrition. One of the few oligosaccharides present in cow's milk that can be isolated and represent a commercial interest is sialyllactose. Upon digestion, a fraction of the milk oligosaccharides may be taken up into the bloodstream and modulate immune cells. Therefore, our second **aim** was to assess the direct effects of milk oligosaccharides on the immune system. Hence, in **chapter 5** we investigated the immunomodulatory effect of 3'SL. Many milk oligosaccharides were shown in literature to exert their immunogenicity via interacting with TLR4. As such, 3'SL was shown to exert immunomodulatory effects in a TLR4 dependent manner in mice. Hence, we investigated the effect of 3'SL on human TLR4 signaling and DC differentiation. Other mucosal factors present in cow's and human milk are TGFβ and the inactive and active form ($1,25(OH)_2D_3$: abbreviated as VitD3) of vitamin D3. These factors are well-known to induce differentiation of monocytes into tolerogenic DC and might thereby modulate activity of the immune system. In **chapter 6** we used these factors as benchmark to assess the effect of the milk trisaccharides 2'FL and 6'SL and the prebiotic galactooligosaccharides (GOS) on DC differentiation. Additionally, we used these TGFβ- and vitD3DC to investigate the effects of 6'SL, 2'FL and GOS on DC maturation.

The first important component of the immune system that comes into contact with milk oligosaccharides are the epithelial cells in the oral cavity. Subsequently, a large

fraction of milk oligosaccharides passes through the GI tract without being absorbed or digested and end up intact in the colon. It is thought that newborns have an immature epithelial layer and that factors in breast milk may aid in epithelial barrier functioning. Additionally, milk oligosaccharides function as an important carbon source that can be utilized by the microbiota, which produce metabolites such as anti-inflammatory SCFA. Although mechanistic evidence in animal models stresses the importance of nutrition on the host, the impact of prebiotic or HMO in early life is poorly understood. Therefore, in **chapter 7** we study the effect of sialyllactose and GOS on epithelial barrier functioning, microbiota composition and SCFA production *in vitro*. Finally, we aimed to extrapolate these *in vitro* findings to an OVA-induced asthma model. In **chapter 8** we used this model to determine whether sialyllactose or raw cow's milk could alleviate OVA-induced asthma via modulating the microbiota (Figure 1). In **chapter 9**, the results of the previous chapters are discussed and integrated with respect to the further mechanistic understanding of the immunomodulatory capacity of raw bovine milk and its components. These insights might aid in rational design of future intervention strategies with raw bovine milk or relevant components derived thereof.

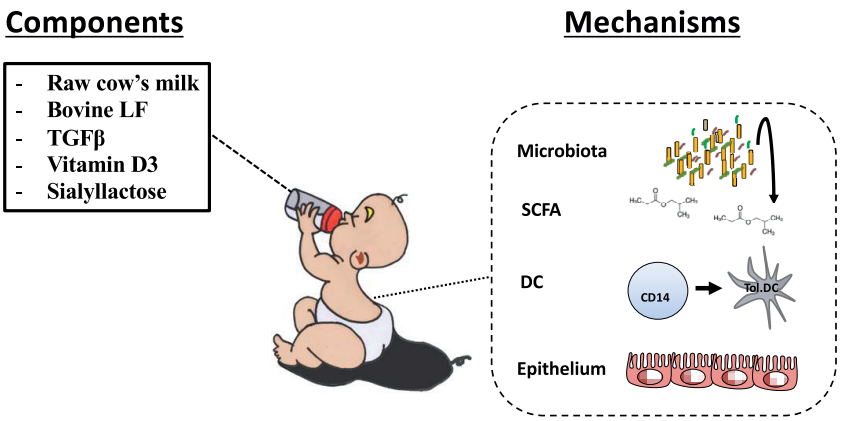

Figure 1. Overview of the investigated (raw cow's milk-derived) components and their effects on different players of the mucosal immune system.

Chapter 2

Cow's milk and immune function in the respiratory tract: potential mechanisms

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Abstract

During the last decades, the world has witnessed a dramatic increase in allergy prevalence. Epidemiological evidence shows that growing up on a farm is a protective factor, which is partly explained by the consumption of raw cow's milk. Indeed, recent studies show inverse associations between raw cow's milk consumption in early life and the prevalence of asthma, hay, fever and rhinitis. A similar association of raw cow's milk consumption with respiratory tract infections is recently found. In line with these findings, controlled studies in infants with milk components such as lactoferrin (LF), milk fat globule membrane, and colostrum IgG have shown to reduce respiratory infections. However, for ethical reasons, it is not possible to conduct controlled studies with raw cow's milk in infants, so formal proof is lacking to date. Because viral respiratory tract infections and aeroallergen exposure in children may be causally linked to the development of asthma, it is of interest to investigate whether cow's milk components can modulate human immune function in the respiratory tract and *via* which mechanisms. Inhaled allergens and viruses trigger local immune responses in the upper airways in both nasal and oral lymphoid tissue. The components present in raw cow's milk are able to promote a local microenvironment in which mucosal immune responses are modified and the epithelial barrier is enforced. In addition, such responses may also be triggered in the gut after exposure to allergens and viruses in the nasal cavity that become available in the GI tract after swallowing. However, these immune cells that come into contact with cow's milk components in the gut must recirculate into the blood and home to the (upper and lower) respiratory tract to regulate immune responses locally. Expression of the tissue homing-associated markers α4β7 and CCR9 or CCR10 on lymphocytes can be influenced by vitamin A and vitamin D3, respectively. Since both vitamins are present in milk, we speculate that raw milk may influence homing of lymphocytes to the upper respiratory tract. This review focuses on potential mechanisms *via* which cow's milk or its components can influence immune function in the intestine and the upper respiratory tract. Unraveling these complex mechanisms may contribute to the development of novel dietary approaches in allergy and asthma prevention.

Introduction

In the Western world, the prevalence of chronic inflammatory diseases, including allergies, has increased dramatically in the last few decades, while the number of serious infectious diseases has declined rapidly[51]. An inverse correlation, indicating a "protective effect" of infectious diseases against chronic inflammatory diseases (e.g. allergy and asthma), was postulated in 1989 by Strachan, who formulated the hygiene hypothesis[1]. The hygiene hypothesis suggests that the exposure to viruses and bacteria is essential to induce a T-helper (Th)1 response, which balances the immune system and protects against Th2-mediated diseases. With the discovery of additional T cell subsets such as Th17 cells and regulatory T cells (Tregs), this paradigm had to be revised. For example, it was demonstrated that suppressive dendritic cells (DCs) induced by helminths restored the disturbed Th1/Th2 balance by induction of Tregs[52]. The immune education of DCs was suggested to be an important step toward understanding the complex relation between infectious diseases and allergies[53]. Th2 responses are now known to be enhanced by the production of type 2 cytokines (e.g. TSLP, IL-25, IL-33) secreted by group 2 innate lymphoid cells and epithelial cells[7,54]. Thus, different cell types are responsible for Th2-mediated diseases such as allergies. Allergy is initiated as an aberrant immune response towards a harmless antigen (allergen). *Via* activation of Th2 cells, the allergen triggers the production of allergen-specific IgE by B cells that binds to high-affinity FcεR1 on effector cells like mast cells and basophils. Effector cells release soluble factors (e.g. histamine) upon secondary exposure to the allergen that cause immediate type I allergic symptoms. The term "atopic march" refers to the sequence of IgE responses and clinical symptoms initiated in early life[55].

In the first year of life, the mucosal immune system is shaped by microbial colonization and dietary components, which contributes to health later in life[49]. Viral infections during this critical period also impact health later in life. For example, respiratory syncytial virus (RSV) infection in early life was shown to increase the risk of wheezing up to 11 years of age[56] and allergic sensitization and development of asthma into adulthood[57–59]. The exact mechanism by which allergy and viral infection in the upper airways results in the development of asthma is not yet elucidated. However, Holt and Sly[60] proposed a mechanism in which viral infection can trigger excessive type I interferon production that can result in upregulation of FcεR1 expression on airway resident DCs. FcεR1-mediated signaling in DCs has been suggested to contribute to allergic airway inflammation depending on the environmental stimuli[61]. In mice,

cross-linking of virus-specific IgE on these airway DCs results in the production of Th2 cytokines and the chemoattractant CCL28, recruiting effector Th2 cells to the airways[62,63]. However, recent evidence shows that not all asthma patients have this typical Th2 profile in early life[64]. Nevertheless, atopy and viral infections in early life are risk factors for asthma development. Therefore, preventive strategies for asthma, such as dietary interventions, should be targeted at early life to suppress allergen- or viral-induced airway inflammation.

In Europe, rapid evolutionary changes are found in the lactase persistence gene suggesting health benefits of cow's milk consumption to humans (Box 1). The existing epidemiological evidence shows that consumption of cow's milk in early life is associated with a lower prevalence of allergies, respiratory tract infections, and asthma. This suggests that milk components (e.g. proteins, sialylated oligosaccharides, and vitamins) may contribute to the protection against the development of allergies[65] and respiratory viral infections[66]. Since raw cow's milk may contain pathogenic bacteria, intervention studies in infants are impossible due to safety risks. Nevertheless, a recent mouse study showed a causal relation between raw milk consumption and the protection against house dust mite (HDM)-induced asthma, which was not seen in mice receiving heated milk[67]. The mechanisms underlying this protective effect of raw cow's milk remains speculative. Therefore, in this review we discuss potential mechanisms by which dietary components, using cow's milk as an example, can protect against airway inflammation.

Box 1.

The consumption of cow's milk in Europe and the Middle East already dates back to the Neolithic cultural period. Milk fatty acids were traced by carbon isotope analysis on Middle Eastern pottery, showing that cow's milk was already processed since 6500 BC[68]. This introduction of processed ruminant milk might explain why it was adopted so quickly, despite lactose intolerance. Lactase persistence (i.e., the capacity to digest lactose into adulthood) seems to have arisen around 5500 BC in the European population due to a specific mutation in the gene encoding the lactase enzyme[69]. Its rapid expansion in the ancestral population suggests high selective pressure[70]. This makes it appealing to speculate that this mutation confers health benefits to the host by consuming cow's milk.

Homology Between Cow's Milk and Breast Milk

By comparing the immunomodulatory components in breast milk with those in cow's milk, conserved mechanisms could be identified, which contribute to

immune homeostasis in early life. Overall bovine and human milk contain similar components. However, the concentration or presence of several specific components (e.g. β-lactoglobulin specific for cow's milk) may differ. For a complete overview comparing breast milk and cow's milk, we refer to a review by van Neerven *et al.* (2012) who compared the composition of breast milk to cow's milk[65]. We briefly describe several immunomodulatory components in cow's milk that are used in this review to illustrate potential mechanisms by which cow's milk may affect respiratory health.

Systematic reviews conclude that TGFβ consumption in early life protects against allergies in humans and animal models[71,72]. Strikingly, the active forms of TGFβ1 and TGFβ2 are identical between cow and human[73]. Although articles report different concentrations of TGFβ in breast and cow's milk, there is a consensus that TGFβ2 is several fold more abundant compared to TGFβ1[71,74]. Remarkably, the concentrations of TGFβ1 and TGFβ2 are approximately fivefold more abundant in cow's milk compared to breast milk[65]. The concentration of TGFβ1 in cow's milk decline significantly after processing and are non-detectable in processed milk[75].

Bovine lactoferrin (LF) has 77% homology to human lactoferrin on mRNA level and 69% on protein level[76]. Nevertheless, LF is taken up by the human lactoferrin receptor and exerts similar bioactivities as human lactoferrin on human colon epithelial cells such as induction of proliferation, differentiation, and TGFβ expression[77]. Similarly, bovine IL-10 is 76.8% homologous and affects human cells[50]. Cow's milk shows lower IgA and higher IgG levels compared to human milk[65].
While the quantities of proteins in human milk are quite similar to cow's milk, the oligosaccharide composition is completely different. In contrast to cow's milk, human milk is unique among mammals in its high and diverse levels of complex oligosaccharides[40]. Cow's milk contains only small amounts and a non-diverse profile of oligosaccharides, which is dominated by sialylated oligosaccharides. Therefore, this review will only address the effect of sialylated oligosaccharides present in cow's milk. In colostrum, the concentrations of sialylated oligosaccharides range between 0.23–1.5 and 1–3.3g/L in cows and humans, respectively[78]. The concentrations of sialylated oligosaccharides in mature bovine milk are approximately 10-fold lower compared to colostrum[78]. Most sialylated oligosaccharides in human- and cow's milk are monomeric [e.g. 3'-sialyllactose (3'SL) and 6'-sialyllactose (6'SL)] and are present in very low concentrations in infant formulas[79].

Vitamin A and D, which are not specific for cow's milk, are essential for the development of the mucosal immune system. Vitamin A can be obtained from different dietary sources and can be converted to its active metabolite retinoic acid (RA) by epithelial cells and DCs in the gut[80]. Breast milk contains, depending on the vitamin D status of the mother, low levels of vitamin D3, and additional vitamin D3 supplementation is recommended for infants[81]. Cow's milk contains similar concentrations of vitamin A, vitamin D3, and $1,25(OH)_2D_3$(active form of vitamin D3) compared to breast milk[65,82].

Epidemiological Evidence for the Immune Modulatory Role of Cow's Milk on Respiratory Health

It is now well established that children growing up on a farm less often develop allergies and asthma[83]. Of the different environmental factors investigated in these epidemiological studies, contact with farm animals, endotoxin levels in house dust, and the consumption of farm milk (i.e., cow's milk with an unknown heating status) showed the strongest association with the protection of childhood asthma and allergy[83,84]. The consumption of farm milk was associated with higher Treg numbers in blood, which were negatively associated with asthma and serum IgE levels[85]. Moreover, increased demethylation of the FOXP3 gene and increased FoxP3+ T cell numbers were detected in PBMC cultures of children who were exposed to farm milk, suggesting that farm milk consumption induces an immunoregulatory phenotype.

Raw cow's milk consumption in the first year of life showed an inverse correlation with the prevalence of atopy and doctors-diagnosed asthma in farmers and non-farmers[86]. This study showed that raw cow's milk consumption in the first year of life is inversely associated with atopic sensitization and asthma independently of the farming environment. Children who consumed raw cow's milk produced higher IFNγ levels upon whole blood stimulation[87]. Since IFNγ production is associated with a Th1 profile, this finding—even though counterbalancing Th2 responses—is in contrast to studies showing a regulatory phenotype induced by farm milk consumption in the first year of life[85]. Nevertheless, both studies show that either raw cow's milk or farm milk is associated with lower total serum IgE levels and allergic diseases[85,87]. Other epidemiological studies have specifically addressed the question whether heating of farm milk influences its effect on allergic diseases.

Loss *et al*. (2011), showed that the protective effect of cow's milk on asthma and hay fever incidence was only noted in children who consumed raw milk and not in children who consumed high heat-treated shop milk (>85°C)[88]. Indeed, the thermosensitive whey proteins BSA, α-lactalbumin, and β-lactoglobulin were associated with the protective effects. Similar trends were found for LF and total IgG. No associations were found between microbiological communities or cell counts in the milk, showing that the protective effect was not primarily caused by bacteria in the raw cow's milk[88]. A follow-up study investigated the association between raw, boiled, or commercially available cow's milk consumption and the occurrence of common infections in infants (2–12 months of age). In comparison to ultra-heat-treated milk, raw milk consumption in the first year of life was inversely associated with the occurrence of rhinitis, otitis, and respiratory tract infections at 12 months of age. In addition, soluble CRP levels were lower in the infants that received raw cow's milk. Interestingly, respiratory tract infections and fever were also reduced in infants receiving boiled cow's milk[66]. It was suggested that the milk fat globule membrane contributes to this negative association between boiled milk consumption and respiratory tract infections[66]. Indeed, non-heat-sensitive cow's milk components may also contribute to the induction of a regulatory phenotype[85]. Nevertheless, these studies show that the thermosensitive fraction of the milk (i.e., proteins, most likely whey fraction) is an important driver of the protection against not only allergies and asthma but also viral infections, fever, and inflammatory conditions in the upper airways.

These epidemiological findings cannot be confirmed in controlled intervention studies in infants due to safety risks. However, controlled trails with infants fed experimental infant formulas rich in immune-related bovine milk components have shown effects on respiratory tract infections. Infants fed with a bovine milk fat globule membrane preparation rich in IgG and LF showed a reduced prevalence of acute otitis media and showed lower pneumococcal-specific IgG levels in serum[89]. Similarly, infants of 4–6 months of age receiving infant formula supplemented with LF showed fewer respiratory illness[90,91]. A reduction in respiratory tract infections was also observed in an intervention study with children of 1–6 years of age receiving bovine colostrum that is extremely rich in IgG[92]. These findings indicate that bovine milk components may prevent respiratory tract infections in early life.

Passage Through the Gastrointestinal Tract

After swallowing milk components, allergens, or pathogens, they pass through the GI tract and are exposed to different pH levels and proteases, varying from pancreatic, gastric, or peptidases on the enterocytic brush border. In adults, there is little evidence that intact dietary proteins can reach the circulation in homeostasis[93]. In early life, however, the digestion of proteins is lower compared to adults, which has several causes. First, the gastric acid production in infants only reaches the levels of adults after 6 months of age. Infants therefore have a higher pH in the stomach compared to adults. Lower gastric acid levels impair the activity of pepsins. Second, concentrations of other proteases (e.g. chymotrypsin and enterokinase) are significantly lower in the small intestine of neonates (10–60% of that of adults) compared to adults[94]. Thus, in infants, a significant fraction of milk proteins reaches the small intestine intact and may interact with intestinal immune cells (e.g. epithelial cells and sampling DC). For instance, 10% of the orally fed bovine IgG (bIgG) can be found in stool of infants, compared to <0.1% in adults[95]. In addition, the infants gut is in a "leaky state"[96], which may promote sensitization to allergens and bacteria- or virus-induced inflammation. On the other hand, it is a window in which (milk-derived) components have an opportunity to induce tolerance.

Some milk proteins are less sensitive to the low pH and proteases and pass the GI tract intact or can even be activated by an acidic environment or protease activity. For instance, TGFβ, which is present in milk in its latent form first needs to be activated before exerting any effector function. The activation of this exogenous latent TGFβ can be triggered by multiple factors such as macrophages membrane-bound receptor TSP-1, αvβ-3/5/6 and αvβ8 integrins, ROS, low pH during passage of the stomach, and proteases[97,98]. Thus, TGFβ can be activated by binding integrins in the upper airways or by activation in the stomach and small intestine. Significant amounts of the abundant milk protein LF reach the small intestine intact and retain their functional activity in both adults and infants[76].

In contrast to proteins, milk oligosaccharides escape enzymatic hydrolysis in the small intestine and low pH of the stomach and are fermented in the colon[99]. By escaping degradation in the small intestine, they function as a carbon source for the microbiota in the colon and can be converted into metabolites such as short-chain fatty acids (SCFAs). In breastfed infants, the genus *Bifidobacterium* is commonly present, which

comprises mainly comprises mainly of *Bifidobacterium bifidum, Bifidobacterium* longum subsp. *infantis*, and *Bifidobacterium breve*. Of the three, *B. longum infantis* has the right machinery to ferment sialylated oligosaccharides directly[100,101] and is unique in its ability to import and degrade low-molecular-weight oligosaccharides[102]. Indeed, several *B. longum* strains were capable of converting 3'SL and 6'SL, which are abundantly present in bovine milk, into SCFA *in vitro*[103]. Nevertheless, it is unknown whether the concentrations of sialyllactose present in cow's milk alters the microbiota *in vivo*. *B. longum* is abundantly present in breast-fed neonates and is thought to confer various health benefits (e.g. enhanced barrier functioning and anti-inflammatory effects) to the host[104]. Although most of these sialylated oligosaccharides are fermented by these *Bifidobacteria*, a small fraction of oligosaccharides reaches the circulation intact[105,106]. Therefore, sialylated oligosaccharides might impact immunity directly. Interestingly, it has been shown that the microbial community in the upper respiratory tract can be differentially modulated by breast milk compared to formula-fed children[38]. Interestingly, breastfed infants showed a higher prevalence of *Dolosigranulum* that was negatively associated with respiratory tract infections[38]. Children with asthma show a lower nasal microbiota composition and higher abundance of *Moraxella*[107]. *Moraxella* was not associated with asthma in children who were exposed to a farming environment, which was in contrast to children who were not exposed to a farming environment. This indicates that the farming environment might protect the children from the detrimental effects of *Moraxella*. To date, it is unknown whether raw cow's milk alters the nasopharyngeal microbiota composition and if this influences susceptibility toward upper respiratory tract infections or allergies.

Binding of Bovine IgG to Respiratory Pathogens

One mechanism by which food components could modulate immunity in the (upper) respiratory tract is by preventing contact between pathogens or allergens and the host immune system. In early life, maternal antibodies are essential for passive protection of the infant against viral infections. Interestingly, maternal RSV-specific antibodies in amniotic fluid were recently shown to protect mouse pups from RSV infection for at least 1 week after birth[108]. Human antibodies are found against conserved parts of the pre-fusion F protein of human RSV and metapneumovirus (PMV) that cross-neutralize bovine RSV[109]. This cross-reactivity could also work *vice versa* if bovine

IgG could recognize conserved patterns on human RSV. Cross-reactive antibodies to other human pathogens have also been demonstrated in bovine milk and colostrum. Indeed, as reviewed by van Neerven, feeding colostrum of cows vaccinated against specific human pathogens protected children from subsequent infections[110]. Interestingly, bovine IgG was shown to bind human RSV and to induce phagocytosis *via* FcγRII receptors on macrophages, neutrophils, and monocytes[111]. The binding of bovine IgG to RSV also directly neutralizes RSV, as shown by protection of Hep2 cells from infection with RSV *in vitro*[111]. Bovine IgG isolated from cow's milk does not only bind to human viruses but was also found to bind to inhaled allergens (e.g. HDM)[112]. In addition, bovine IgG inhibits translocation of Pam3CSK4 over the epithelial barrier, thereby suppressing the production of pro-inflammatory cytokines *in vitro*[113]. Thus, bovine IgG can neutralize RSV infection *in vitro* and might also play a role in preventing sensitization by binding allergens and supporting barrier functioning by preventing binding of TLR ligands to the epithelium.

Next to bovine IgG, milk oligosaccharides have also been shown to prevent binding of viruses to host cells[40]. Viruses use lectin-like structures to adhere and infect host cells. It was hypothesized that breast-fed infants developed less otitis caused by viral infections (e.g. RSV and influenza) due to the decoy receptor activity of milk oligosaccharides[40]. However, as stated in the review by ten Bruggencate *et al.* (2014). it is to date uncertain which sialylated oligosaccharides can serve as a decoy receptor for human respiratory infecting viruses[78]. Thus, IgG and sialylated oligosaccharides present in cow's milk might shield allergens or virus pathogens from inducing infection and inflammation (Figure 1).

The First Host Barrier: The Epithelium

When the allergen or virus is not neutralized, it will encounter the epithelial cell layer of mucosal tissues. This epithelial cell layer is the first line of defense in mucosal tissues. Epithelial cells are covered by a thick layer of mucus that keeps harmful compounds from entering the body. Epithelial cells act as the first physical barrier and the first responders of the innate immune system. The epithelial cells protect against inflammation and sensitization by preventing bacteria and virus entry and leakage of allergens into the mucosal tissue. The integrity of the epithelial barrier in the upper airways[114] and intestine[20] is regulated by tight junctions (TJs).

Homeostasis is maintained by hyporesponsiveness of epithelial cells toward bacterial constituents, because inflammation disrupts barrier functioning[115]. Therefore, the barrier functioning of the upper airways is under constant threat of environmental factors, including viral infections (e.g. RSV) and allergens[116]. Breaching of the barrier can results in tissue modifications in the upper airways as seen in patients suffering from allergic rhinitis or sinusitis[117].

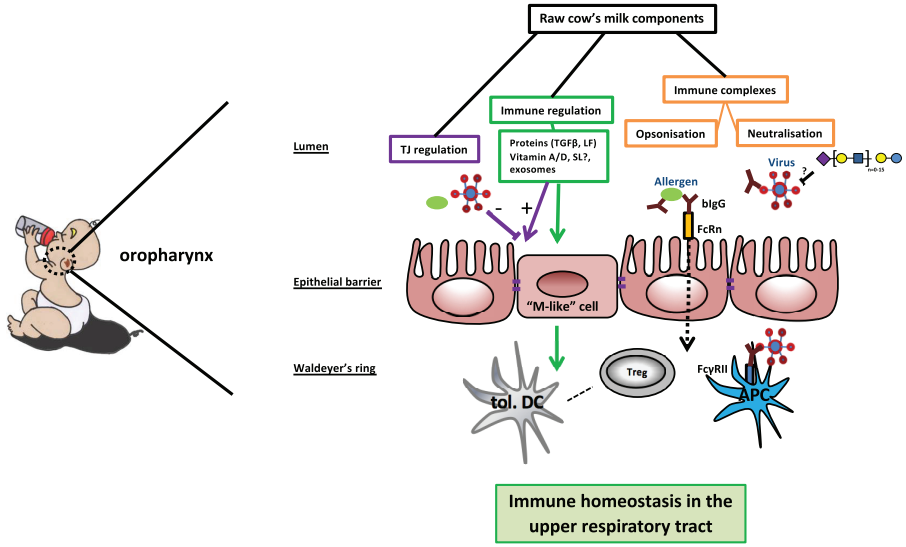

Figure 1. Potential mechanisms of cow's milk-induced immune homeostasis in the upper respiratory tract. In the oropharynx, raw cow's milk components can contribute to immune homeostasis via different mechanisms. First, bovine IgG can bind and possibly even neutralize bacteria, viruses, or allergens. Immune complexes are transported over the epithelial barrier by neonatal Fc receptor (FcRn) or transported via M-like cells to reach the mucosal tissue in the Waldeyer's ring. The immune complexes can bind to FcγRII on antigen-presenting cells (APCs), leading to phagocytosis and clearance of the pathogens—as well as antigen presentation to (regulatory) T cells. Second, sialylated oligosaccharides may function as decoy receptors for viruses in the lumen of the oropharynx, preventing viral adhesion. Further, the expression of tight junction proteins can be enhanced by several milk components, thus strengthening the mucosal barrier against breaching by allergens and pathogens. Finally, several milk components contribute to immune regulation by inducing the differentiation into tolerogenic dendritic cells (tol. DC) and immunoregulatory T cells (Tregs). In this way, raw cow's milk can promote a local microenvironment that contributes to immune homeostasis in the upper airways.

Tight Junctions

The integrity of the epithelial barrier is maintained by structural elements including adherens junctions, desmosomes, and TJs. TJs consists of a "ziplock-like" structure of multiple protein strands that are connected to the cytoskeleton, allowing selective transport across the barrier[118]. Cytosolic scavenger proteins (e.g. ZO-1) link the actin cytoskeleton to paracellular located proteins: the claudins and occludins. The

claudin family consists of transmembrane proteins, which *via* the interaction of claudin strands, are connected to each other by extracellular loops[119]. Claudins can be subdivided into pore-forming (e.g. claudin-2) and sealing claudins (e.g. claudin-4) that increase and decrease permeability, respectively[119]. Notably, pro-inflammatory cytokines (e.g. IL-6 and TNF) increase the expression of the pore-forming claudin-2 and thereby reduce the epithelial barrier function[120]. The function and distribution of occludins is highly influenced by its phosphorylation status that is regulated by protein kinase C[121]. TJ proteins are key in maintaining epithelial barrier function and are shown to regulate proliferation on gene expression level[122]. For further reading about TJ proteins, we would like to refer to other excellent reviews specifically about occludins[121], claudins[119], or the function and morphology of TJs in general[21,118].

Many allergens have protease activity that could breach barrier functioning[123]. For instance, the protease activity of one of the major HDM allergens, Der p 1, was shown to disrupt the cleavage site in the extracellular loops of claudins and occludins[124]. In contrast, RSV disrupts barrier functioning by remodeling the actin cytoskeleton and interfering with cytosolic scavenger proteins[125]. Disruption of barrier functioning in the upper airways results in increased exposure of allergens and viral particles to the underlying immune system (Figure 1), which could result in chronic inflammatory diseases such as asthma and allergies[126]. In early life, the epithelial barrier is more permeable compared to adults. Closure of the barrier occurs only after a few weeks in humans, while in mice, this is a more gradual process that develops during weaning[96,127].

Effect of Milk Components on Barrier Functioning

Breast milk contains many growth factors that facilitate gut maturation. Neonates receiving infant formulas were shown to have a higher gut permeability compared to breast-fed neonates. This stresses the importance of identifying functional milk components that promote barrier functioning[96]. Currently, there is no *in vivo* evidence on effects of cow's milk on epithelial barrier functioning. Nevertheless, at least two recent studies investigated the effect of cow's milk on epithelial cells *in vitro*. To study barrier functioning of dietary components *in vitro*, most studies use colon carcinoma cell lines (Caco-2 or HT-29). It is to date impossible to study the effect of dietary components on barrier functioning in the upper airways since no human oropharyngeal epithelial cell lines are available that form TJs. Caco-2 cells express enzymes that are expressed in the fetal intestine and are biochemically and

morphologically similar to ileal enterocytes[128]. These *in vitro* models are thus one of the few limited models available to study the effect of dietary components on TJ regulation. The anti-inflammatory properties of milk components on the epithelium are reviewed by Chatterton *et al.* (2013)[73]. In this review, the role of dairy components on epithelial barrier function in terms of epithelial proliferation, differentiation, and TJ regulation is addressed.

The first study that looked at the effect of cow's milk *in vitro* showed that cow's milk induces the expression of the pore-forming TJ protein claudin-2 in Caco-2 cells[129]. However, no differences were observed in permeability, which was proposed to be counteracted by the milk-induced increase of endogenous TGFβ expression. A second study stimulated HT-29 cells with raw milk versus pasteurized cow's milk preparations. The authors showed with microarray analysis that raw milk induced the expression of genes related to immunity compared to the pasteurized cow's milk or medium control[130]. This study showed that the thermosensitive milk fraction (i.e., proteins) induced the expression of immune-related pathways and thereby indirectly barrier functioning.

One of the proteins in cow's milk that is important for epithelial barrier functioning is TGFβ. Apart from these exogenous sources of TGFβ, TGFβ is endogenously produced. In the gut, TGFβ is most prominently expressed in epithelial cells compared to its expression in the underlying lamina propria[131]. TGFβ1 is capable of promoting barrier functioning by regulating TJ expression and proliferation. TGFβ1 induces the expression of claudin-4 and protein kinase C expression *in vitro*, both strengthening the barrier[132,133]. On the other hand, TGFβ inhibits the proliferation of epithelial cells[131,134]. Interestingly, the production of endogenous TGFβ1 by epithelial cells is regulated through a positive feedback loop by other milk proteins like lactoferrin that triggers an intracellular cascade that results in the production of TGFβ1. Bovine and human lactoferrin were shown to have similar effects on barrier functioning. Moreover, in low concentrations, human lactoferrin induces differentiation of epithelial cells, whereas it stimulates proliferation in higher concentrations[76].

In high concentrations, sialylated milk oligosaccharides affect the cell cycle and induce differentiation of intestinal epithelial cells[135]. In the colon, these oligosaccharides are fermented by the microbiota. These microbes produce SCFAs that also impact barrier functioning. As reviewed by Tan *et al.* (2017), SCFAs reduce paracellular permeability

and induces the expression of TJ genes and MUC2 expression, thus strengthening the epithelial barrier[12] that may subsequently protect the host against infections[136].

Another milk ingredient shown to have immunomodulatory effects is vitamin D. More specifically, the inactive and circulating form of vitamin D3 ($25(OH)_2D_3$) is converted to the active form ($1,25(OH)_2D_3$) by the enzyme 1α-hydroxylase, which is highly expressed in the kidney and lowly expressed in epithelial cells[137]. Epithelial cells transport the inactive form of vitamin D3 over the membrane, which can be subsequently systemically metabolized[138]. The conversion locally by epithelial cells of dietary inactive vitamin D3 into the active form can create a microenvironment containing active vitamin D3. Stimulation of Caco-2 cells with $1,25(OH)_2D_3$ was shown to result in the induction of E-cadherin, which indirectly promotes the transcription of ZO-1 and induces differentiation[139]. In support of this, blocking vitamin D receptor transcription resulted in a decreased transepithelial electrical resistance and expression of ZO-1 and E-cadherin and claudin 1, 2, and 5 but not occludin[140]. Thus, it is evident that vitamin D3 contributes to epithelial barrier function by regulating TJ protein expression. Less is known about the effect of vitamin A on barrier functioning. RA was shown to enhance differentiation of epithelial cells, as indicated by the increase in alkaline phosphatase (ALP) expression. In contrast, RA also decreased the expression of claudin-2, resulting in a decrease in permeability of the Caco-2 model[141]. Thus, several components present in cow's milk promote epithelial barrier functioning (Figure 1).

Do Milk Components Promote Immune Homeostasis?

The nasal mucus is cleared to the back of the throat every 10–15 minutes by the movement of cilia. Thus, it is likely that allergens and viruses are trapped in this thick layer of mucus and are subsequently swallowed. The oropharynx (throat) is the place where milk components, bacteria, viruses, and allergens may interact before they are digested. Lymphoid tissues in the upper airways are the lingual tonsils, tubal tonsils, palatine tonsils, and adenoids, together forming the Waldeyer's ring[9]. Uptake of antigens by the tonsils occurs *via* M-like cells in specialized induction sites, which are composed of follicles containing both myeloid and lymphoid cells[142]. Similarly, in the GI tract, antigens can be taken up by columnar epithelial cells (transcellular), M cells, neonatal Fc receptor-mediated uptake[143], or direct uptake by specific sampling subsets of DCs[13].

The mucosal immune system is capable of distinguishing between harmful and harmless compounds resulting in inflammation or tolerance, respectively. Food components are important non-self-antigens to which an immune response constantly needs to be suppressed. This type of tolerance induction is known as oral tolerance. Food does not only trigger local tolerance but also systemic tolerance, and thus food makes the systemic and mucosal immune systems relatively unresponsive to these food antigens. Breast milk contains many components that dampen immune responses. It is suggested that this regulatory milieu induced to breast milk components favors tolerance inductions towards other harmless antigens such as allergens[34]. This suppression of immune responses is antigen specific and long lasting.

The consumption of farm milk is associated with higher regulatory FoxP3+ T cell numbers, which were negatively associated with doctors-diagnosed asthma and IgE levels[85]. We here address several potential cow's milk components that might promote these regulatory responses. Literature supports that raw cow's milk contains a multitude of components, including proteins and vitamins, that promote the development of human "tolerogenic" or regulatory monocyte-derived DCs (moDCs) *in vitro*.

Cow's milk and colostrum contain several immunoregulatory cytokines such as TGFβ and IL-10. Interestingly, a population of tolerogenic IL-10 producing DCs (IL-10 DCs) with similar characteristics to *in vitro* monocyte-derived DCs, differentiated in the presence of IL-10, were identified in human blood[144,145]. Not only human IL-10 but also bovine IL-10, which has 70% homology to human IL-10, was shown to induce a dose-dependent reduction of CD80/CD86 expression and IL-12 and TNF production [50]. DCs with low CD86/CD80 expression in the presence of TGFβ or IL-10 are known to polarize naive T cells into FoxP3+ T cells[146]. IL-10 DCs also express PD-L1 which is critical for the induction of T cell anergy. Similarly, moDC differentiated in the presence of LF showed inhibited cytokine responses and surface marker expression upon stimulation with TLR ligands[147].

TGFβ is an unique pleiotropic cytokine that is produced by leukocytes and epithelial cells[148]. The dual role of TGFβ was shown in a recent review, which showed that TGFβ-induced SMAD proteins are key in balancing immunity[149]. DCs from the lamina propria are essential for inducing FoxP3 expression in naive T cells, which requires an exogenous source of TGFβ[150]. Interestingly, pups of mice exposed to

airborne allergens developed oral tolerance towards the allergen that was dependent on milk-derived TGFβ[151]. In addition, TGFβ and IL-10 inhibit type I interferon production by pDCs[152]. Immunosuppressive cytokines such as IL-10 and TGFβ in milk are important in maintaining immune homeostasis and the suppression of type I interferon production. These immunosuppressive cytokines in cow's milk could be essential for inducing a regulatory milieu, which subsequently may result in tolerance towards allergens.

Antigen-specific IgG in breast milk was shown to protect against OVA-induced asthma in a mouse model by inducing regulatory responses. Moreover, pups of mothers that were exposed to antigen aerosols during lactation resulted in a regulatory immune response that protected them from developing asthma[14]. The proposed mechanism involves binding of IgG to neonatal Fc receptor (FcRn), which resulted in the expansion of antigen-specific Tregs. Bovine IgG shows some affinity for human FcRn[153] and is specific for human allergens[112], and it is therefore possible that the uptake of bovine IgG–allergen complexes induces FoxP3+ T cells (Figure 1). These functional properties of milk proteins are lost upon heating. Another heat-sensitive fraction of bovine milk that has been suggested to induce immune regulation is exosomal microRNA[154].

The role of milk oligosaccharides in the induction of oral tolerance remains inconclusive. 6'SL was shown to alleviate OVA-induced food allergic symptoms by promoting IL-10-producing T cells[155]. In contrast, pups fed milk that contained 3'SL had more severe induced colitis compared to pups fed milk devoid of 3'SL. *Ex vivo* cultures of mesenteric lymph node (MLN) DC showed direct TLR4 activation by 3'SL[156]. However, 3'SL did not induce TLR4-mediated activation of human immune cells *ex vivo*[157]. In addition, sialylated milk oligosaccharides were shown to alter the microbiota composition and growth in infants[158]. These changes in microbiota composition in turn impact the production of SCFAs that were shown to be essential, together with vitamin A, in oral tolerance induction[159]. In summary, the direct immunomodulatory effect of sialylated oligosaccharides remains inconclusive. Rather than having direct effect on the immune system, sialylated oligosaccharides may promote immune homeostasis indirectly by promoting the outgrowth of SCFA-producing bacteria.

In the gut, a subset of migratory DC expressing the integrin CD103 are known to convert vitamin A into RA. In mouse models, RA induces the differentiation of naive

T cells into Tregs *in vivo*[25]. These findings were confirmed in vitro in humans by differentiating moDC in the presence of RA. These RA DCs expressed CD103 and were capable of polarizing naive T cells into Tregs[160] or FoxP3– IL-10-producing T cells[27]. Similarly, under steady-state conditions, lung macrophages produce RA and TGFβ toward harmless airborne antigens and induce antigen-specific Tregs[161]. Thus, dietary vitamin A triggers endogenous RA production that is essential to induce Tregs in the gut and the lung. However, it is unknown whether dietary vitamin A contributes in the upper airways to induce antigen-specific Tregs. The active form of vitamin D3, $1,25(OH)_2D_3$ (VitD3), halts the differentiation of monocytes into moDC *in vitro* and does not affect pDCs[162]. These VitD3 DCs are less sensitive to TLR ligands and develop a semi-mature phenotype upon stimulation. These authors show that this semi-mature phenotype is instrumental for priming naive T cells to become Tregs and to induce T cell anergy[163]. In summary, cow's milk contains a variety of components that are known to promote immune homeostasis and induce regulatory responses by human immune cells *in vitro* (Figure 1). Therefore, we hypothesize that these immune regulatory effects aid in tolerance induction toward allergens or suppress immune responses in the upper airways that could aid in the protection against asthma exacerbation.

Systemic Responses: the Gut–Lung Axis

Several recent studies have shown that immune responses triggered in the GI tract can influence immunity in the respiratory tract. Evidence for the existence of this so-called gut–lung axis is increasing, although the exact mechanisms involved are not yet completely understood[163–166]. The importance of TLR signaling by commensal microbiota in relation to airway immunity was demonstrated in multiple studies[167–169]. Mice treated with antibiotics before influenza infection showed a higher viral load in the lungs, reduced CD4+ T cells responses and reduced influenza specific antibody titers compared to control mice. Intrarectal administration of TLR agonists could restore immune responses to influenza infection in this model. To clear the influenza infection, commensal bacteria or TLR agonists were needed to induce inflammasome-dependent cytokine release (IL-1β and IL-18). These cytokines allowed lung DCs to migrate to the mediastinal lymph node where they activate specific T cells[167]. Another study in mice showed that oral administration of a bacterial extract (OM-85) reduced the viral load in the respiratory tract after influenza infection. The bacterial

extract also boosted specific polyclonal antibodies against *Klebsiella pneumoniae* and *Streptococcus pneumoniae*, which protected the mice against these airway pathogens[168]. Furthermore, germ-free mice showed increased susceptibility to pulmonary infection with *K. pneumonia*e, which could be restored by i.p. injection of LPS[169]. These studies indicate that there is cross-talk between the commensal microbiota and immunity in the respiratory tract *via* TLR signaling (Figure 2).

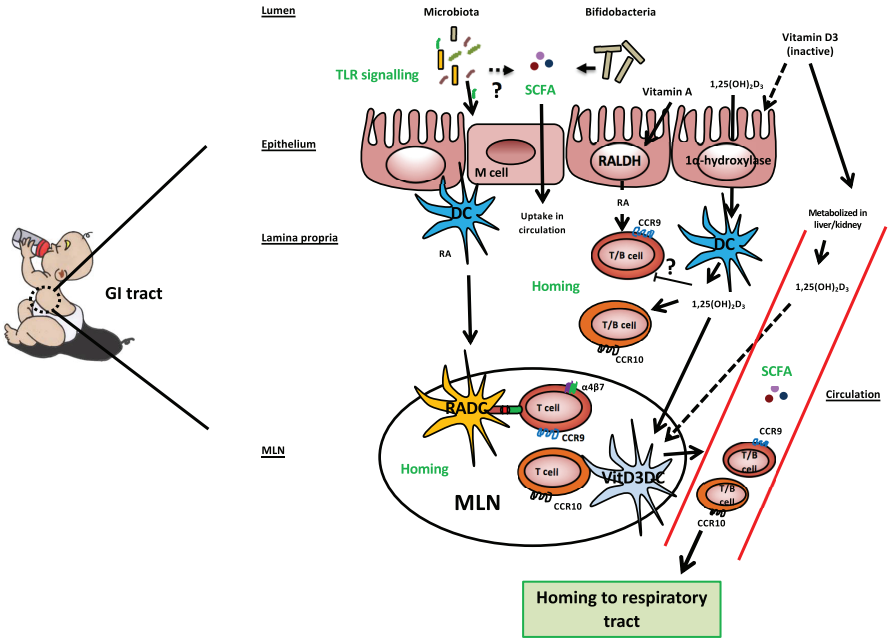

Figure 2. Mechanisms involved in the gut-lung axis linked to milk components. TLR signaling by microbiota in the gut results in improved airway immunity although the exact mechanism in this gut–lung axis is unknown. Microbiota, specifically bifidobacteria, may ferment sialylated oligosaccharides present in cow's milk into SCFAs, which are taken up into the circulation. Cow's milk contains vitamin A and the inactive- and the active form ($1,25(OH)_2D_3$) of vitamin D3. Vitamin A and vitamin D3 can be taken up by epithelial and dendritic cells (DCs) and converted by the enzymes RALDH and 1α-hydroxylase into retinoic acid (RA) and $1,25(OH)_2D_3$ (VitD3), respectively. However, the majority of vitamin D3 is taken up into the circulation and converted into its active form in the kidneys. In the lamina propria, RA may induce expression of gut homing markers CCR9 and α4β7 on T cells and CCR9 on B cells directly or prime DC to induce the expression of these markers in the mesenteric lymph node (MLN). VitD3 may induce the expression of homing marker CCR10 on T and B cells directly or indirectly via VitD3-primed DC (VitD3 DC). In addition, VitD3 downregulates the expression of CCR9 on lymphocytes in a direct way. Lymphocytes expressing CCR9 have a homing capacity toward the small intestine, while lymphocytes expressing CCR10 have a homing potential toward the colon and respiratory tract, providing a potential mechanism of the gut–lung axis.

Another link between airway immunity and the gut microbiota is the release of SCFAs. SCFAs are metabolites produced by bacteria in the gut from dietary non-digestible fibers. One type of non-digestible fibers is sialylated oligosaccharides present in cow's milk. As mentioned earlier, milk oligosaccharides are fermented in the colon into SCFAs[99]. B. longum infantis has been shown to ferment sialylated oligosaccharides directly[100,101]. After release into the colon, SCFAs such as acetate and to a lesser extent propionate are taken up into the circulation in mice [44] and humans[45]. SCFAs bind to metabolite-sensing G protein-coupled receptors, and signaling influences gene expression via induction of histone deacetylases[45]. Both acetate and propionate bind via GPR41, which is expressed on various tissues and cells including enteroendocrine cells and PBMCs[170]. Several studies indicate that SCFAs play an important role in the gut–lung axis by regulating immune activation in the lung[44,45] (Figure 2). A high-fiber diet was prevented against allergic airway disease in mice[44,45]. This protective effect was shown to be mediated by acetate produced by the microbiota. Direct oral administration of acetate resulted in higher Treg numbers in the lung and protection against HDM-induced allergic airways disease [45]. In another study, oral administered propionate did not affect Treg numbers in the lung, but resulted in hematopoiesis in the bone marrow of DCs that were found in the lungs. These DCs had a more immature phenotype (lower levels of MHCII and CD40) and therefore a reduced capacity of activating Th2 cells[44]. These studies demonstrate that microbial metabolites produced in the intestines can have an effect on immune function in the airways. In addition, other microbial components such as TLR ligands may be taken up in the circulation and impact immunity in the respiratory tract. TLR stimulation in the gut could activate DCs leading to the activation of lymphocytes in the mediastinal lymph node. Upon activation, these lymphocytes can migrate to the lung and potentially to the gut. Besides, microbiota can have an indirect effect via SCFA production, as SCFAs in the circulation can affect DCs and Tregs in the respiratory tract. Currently, direct effects of SCFAs on the induction of homing markers on DCs or lymphocytes are not known.

After activation, lymphocytes can migrate (i.e., home) to tissues depending on their homing marker (e.g. selectins, integrins, and chemokine receptors) expression. These receptors can bind to tissue-specific ligands (e.g. addressins and chemokines) expressed by the endothelium. In humans, mucosal vaccination was used as a model to show that the site of induction of a mucosal immune response resulted in IgA production in restricted mucosal tissues. Holmgren and Czerkinsky showed that specific IgA

antibodies are produced in the upper respiratory tract and gut in cholera toxin B (CTB) vaccinated individuals. In contrast, intranasal vaccination with CTB resulted in specific IgA production in both upper and lower respiratory tract and genital tract, but not in the gut. Furthermore, rectally vaccinated individuals only produced specific IgA locally in the rectum[171]. The fact that orally administered antigens result in effector cells being present both in the gut and the upper respiratory tract indicates that homing markers might overlap. Well-studied homing marker interactions in humans are among others, CCR9 binding to locally produced CCL25 in the small intestine[172,173] and CCR10 binding to CCL28 produced in the airways and colon[174]. For B cells, there is no clear homing marker that differentiates between upper and lower respiratory tract homing as CCR10 expressed on B cells binds CCL28 produced locally in lower and upper respiratory tracts. In contrast, T cells express CCR10 to bind CCL28 produced in the lower respiratory tract and salivary glands[63,175], while T cells express CCR3 to bind CCL28 in the nasal mucosal[176]. Another homing marker that could be important in migration between gut and lung is CCR6 as its ligand CCL20, which are expressed in both tissues[177].

Thus, the homing potential of immune cells is affected by the site of induction and is dependent on local production of tissue-specific stromal factors. Recent evidence suggests that it can also be modified by dietary components. Of all dietary components, the effect on homing is best studied for RA and VitD3 (Figure 2). Dietary vitamin A as a source of RA is essential for efficient homing of T cells to the GALT[178,179]. In mice, RA production by mucosal CD103+ DCs[179,180] or stromal cells in the MLN[181] is essential for efficient differentiation of naive T cells into FoxP3+ Tregs that express the gut homing markers α4β7 and CCR9 in the MLN. Interestingly, human RA-primed CD103+ DCs were also shown to induce differentiation of naive T cells into IL-10-producing T cells expressing gut homing markers *in vitro*[27]. RA is also a factor that regulates B cell proliferation, differentiation, and class switching [182]. Moreover, RA and TGFβ1 induce IgA class switching[183]. Similarly to the effects observed on T cells, RA derived from GALT-DCs alone was shown to induce gut homing markers on B cells[184]. Interestingly, vitamin D3 blocks the upregulation of RA-induced gut homing marker expression on T cells[185,186] although this was not observed by Baeke *et al.* (2011)[187]. The majority of dietary vitamin D3 is taken up along the GI tract and converted into its active form $1,25(OH)_2D_3$ in the kidney and becomes systemically available[138]. In addition, the inactive form of vitamin D3 is shown to be converted into its active metabolites by DCs and epithelial cells[137,185]. Dietary supplementation

of vitamin D3 to HIV-infected patients was shown to induce CCR10 expression on Tregs[188]. This finding is in line with *in vitro* studies showing VitD3 induces CCR10 expression on human B and T cells[185,187,189]. Interestingly, RA also induces CCR10 expression in human B cells and acts even synergistically with VitD3[189]. The balance of vitamin A and vitamin D3 may thereby regulate homing of lymphocytes to gut or respiratory tract, respectively (Figure 2). However, it should be noted that the concentrations of vitamin A and vitamin D3 are relatively low in cow's and breast milk. In addition, the active metabolites of these vitamins can be endogenously produced (e.g. by stromal cells) in the secondary lymphoid tissues. In summary, we are only beginning to unravel the complex interplay between gut and lung. Hence, we can only speculate about the mechanisms by which cow's milk through sialylated oligosaccharides and vitamin A and D could affect microbiota composition or homing of lymphocytes, respectively.

Concluding Remarks

The existing epidemiological evidence suggests that the consumption of raw cow's milk contributes to protection against allergies and asthma and respiratory tract infections. In this review, we discussed potential mechanisms by which cow's milk and its components may exert these immunological effects. Bovine IgG can bind to bacterial and viral pathogens, enhance phagocytosis, and may neutralize pathogens. Other milk components like TGFβ promote epithelial barrier functioning by upregulation of TJ genes and might favor the differentiation of Tregs that can reduce inflammation locally. Finally, recent evidence shows an interplay between gut and lung. We speculate about the effect of milk components on trafficking of lymphocytes from the intestine to the upper airways through modulation of homing receptors and microbiota. Further unraveling the impact of milk components on local responses in the respiratory tract, microbiota and immune trafficking are necessary to fully understand their effects on allergy, infection, and asthma.

Chapter 3

Bovine Lactoferrin Modulates Dendritic Cell Differentiation and Function

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Abstract

Lactoferrin is an abundant glycoprotein in bovine milk that has immunomodulatory effects on human cells. Bovine lactoferrin (LF) binds lipopolysaccharides (LPS) with high affinity and is postulated to act via TLR4-dependent and -independent mechanisms. It has been shown that LF modulates differentiation of human monocytes into tolerogenic dendritic cells. However, in a previous study, we showed that LPS also mediates differentiation into tolerogenic dendritic cells (DC). Since LF binds LPS with high affinity, it remains to be investigated whether LF or LPS is mediating these effects. We, therefore, further investigated the LPS-independent effect of LF on differentiation of human monocytes into dendritic cells (DC). Human monocytes were isolated by magnetic cell sorting from freshly isolated PBMCs and cultured for six days in the presence of IL-4 and GM-CSF with or without LF or proteinase K treated LF to generate DC. These immature DC were stimulated for 48 h with LPS or Poly I:C + R848. Cell surface marker expression and cytokine production were measured by flow cytometry. DC differentiated in the presence of LF produced higher IL-6 and IL-8 levels during differentiation and showed a lower expression of CD1a and HLA-DR. These LFDCs showed to be hyporesponsive towards TLR ligands as shown by their semi-mature phenotype and reduced cytokine production. The effect of LF was abrogated by proteinase K treatment, showing that the functional effects of LF were not mediated by LPS contamination. Thus, LF alters DC differentiation and dampens responsiveness towards TLR ligands. This study indicates that LF can play a role in immune homeostasis in the human GI tract.

Introduction

Bovine lactoferrin (LF) is an abundant glycoprotein in cow's milk that is 69% identical to human lactoferrin at the protein level[76]. LF is an extensively researched protein that has been shown to exert antimicrobial and antiviral activity[190]. The involved anti-pathogenic mechanisms, which are mostly investigated in vitro, range from depriving iron, antimicrobial activity by bioactive peptides and decoy receptor activity. These mechanisms may underlie the protective effect against sepsis by LF supplementation in very low birth weight (VLBW) infants[191–195]. LF was shown to protect against sepsis in VLBW infants in either breast fed or formula fed, showing the need for additional supplementation[191]. Subsequent analysis of this study showed that LF inhibits the progression of invasive fungal infections[192], indicating immunomodulatory effects of LF. In line with these findings, LF was shown to induce the prevalence of regulatory T cells in VLBW infants[194]. Although these studies indicate an immunoregulatory role of LF in humans, little mechanistic evidence is available to date.

In infants, LF ends up in the intestine intact and is only partly hydrolyzed into bioactive peptides with antibacterial activity[196]. Moreover, intact human lactoferrin was found in stool and urine of VLBW infants and resisted trypsin and chemotrypsin treatment *in vitro*[197]. LF was shown to largely resists gastric hydrolysis in vivo in adults[198]. However, LF is completely degraded in the small intestine of adults[199]. Thus LF may, in contrast to adults, retain its bioactivity throughout the gastrointestinal tract and may even become systemically available in infants[197,200,201]. This could be explained by lower concentrations of proteases in the small intestine in infants compared to adults[94]. Additionally, breast milk contains protease inhibitors, which may limit degradation of milk proteins in the GI tract of infants[202].

LF has specific domains that bind iron with high affinity, a high isoelectric point (pI around 9) and an overall nett positive charge with high cationic peptide regions, which is crucial for its bactericidal activity[203]. Due to these biochemical properties, LF may bind multiple receptors (e.g. intelectin-1 and DC-SIGN) with low affinity[76,204,205]. LF was shown to bind the human lactoferrin receptor (i.e., intelectin-1) on Caco-2 cells and, dependent on the concentration, induce proliferation or differentiation of these epithelial cells[77].

Additionally, LF is known for its binding activity to lipopolysaccharides (LPS). Binding of LF to LPS may result in neutralization of LPS, which is hypothesized to play an important role in the immune regulatory role of LF[206]. In contrast, LF was shown to activate monocytes during DC differentiation, which resulted in diminished TLR activation[147]. Similarly, human LF was shown to induce the differentiation into anergic macrophages that were hyporesponsive towards TLR ligands[207]. However, LF binds LPS with a high binding affinity[208]. Since LPS induces differentiation of monocytes into tolerogenic DC as well[157], it is of interest to investigate the true immunomodulatory potential of LPS-free LF. A previous study showed that LPS-free LF induces the expression of pro-inflammatory cytokines on porcine derived macrophages in a TLR4-independent manner[209]. It is however unknown whether this TLR4-independent signaling of LF affects the functionality of human monocytes and dendritic cells. We therefore investigated whether LF, independently of bound LPS, is capable of inducing differentiation of human monocytes into tolerogenic dendritic cells.

Materials and Methods

Isolation of LF

The whey fraction from bovine colostrum was collected after spinning the milk at 100.000 *g* for 45 min (Ultracentrifuge Avanti J301, Beckman Coulter, Brea, CA, USA). This casein and fat-free fraction was stored at −20 °C until further use. These whey proteins were thawed and diluted with washing buffer containing 0.01 M KH_2PO_4 and 0.1 M NaCl, pH 6.5. Samples were centrifuged at 23.500 *g* for 20 min and the supernatant was carefully collected through a filter paper to remove casein traces. Ion exchange chromatography (Akta Purifier, Pharmacia, Stockholm, Sweden) was used to isolate LF from the whey proteins. The Hiprep SP FF 16/10 column was preconditioned by rinsing the column with 100 mL washing buffer (3 mL/min; 12 mS/cm) and elution buffer (3 mL/min; 85 mS/cm). Whey proteins were loaded on the preconditioned column with a flow rate of 3 mL/min (HiLoad P50 pump, Pharmacia). After the complete volume of whey protein has run over the column, it was connected to the AKTA. Unbound matrix proteins were washed from the column with washing buffer (3 mL/min). LF was eluted from the column using an increasing gradient of 0.1–1 M NaCl. The preparation was desalted and concentrated on a 10 kDa Ultracel PLGC membrane using an Ultrafiltration Cell model 8200 (Amicon/Millipore).

Isolation and Culturing of Monocyte-Derived DC

Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation from buffy coats obtained from healthy anonymous donors (Sanquin blood bank, Nijmegen, The Netherlands) as described previously[157]. A written informed consent was provided before blood collection. In short, 1:1 diluted blood in phosphate buffered saline (PBS) (Mg^{2+} and Ca^{2+} free, Lonza, BE17-516F, Basel, Switzerland) was loaded on Ficoll-Paque (Amersham Bioscience, Uppsala, Sweden) and centrifuged for 20 min at 500 *g* without brake. The PBMC layer was collected and washed three times with PBS. Cells were spun down and the pellet was resuspended with anti-human CD14 magnetic beads (BD Biosciences, 557769, Franklin Lakes, NJ, USA). CD14+ cells were isolated on a separation magnet (BD IMagnet, BD Biosciences) according to the manufacturer's instructions. 100.000 monocytes per well were cultured in 96 cells wells flat bottom plates in RPMI 1640 (GIBCO, MA, USA, 22409-015) and 10% FCS (GIBCO, 10270-106), normocin (100 µg/mL, Invivogen, anti-nr-1, San Diego, CA, USA), penicillin and streptomycin (100 U/mL, GIBCO, 14150-122). Monocytes were differentiated into dendritic cells by culturing them for six days in the presence of 20 ng/mL IL-4 (Peprotech; 200-04, Rocky Hill, CT, USA) and GM-CSF (Peprotech; 300-03, Rocky Hill, CT, USA) with or without 10 µg/mL, 250 µg/mL LF (FrieslandCampina) or 10 nM VitD3 (Sigma-Aldrich, D1530, St. Louis, MO, USA). After six days, immature DC were matured with 1 µg/mL LPS (*Escherichia coli*, Sigma, L2880, St. Louis, MO, USA) or 3 µg/mL R848 (Invivogen: tlrl-r848-5) and 20 µg/mL Poly I:C (Sigma, P1530) for 48h.

Isolation and Staining of moDC

After 6 days (immature-) or 8 days (mature-) moDC were incubated on ice (while shaking) for 30 min in ice cold FACS buffer (PBS (Lonza, BE17,516F) containing 0.5% BSA fraction V (Roche, 10735086002, Basel, Switzerland), 2.0 mM EDTA (Merck, 108418, Kenalworth, NJ, USA) and 0.05 NaN_3) to facilitate the detachment of DC from the surface. Surface marker expression was analyzed by using fluorochrome-conjugated antibodies directed against CD14 (FITC; BD Biosciences, 555397), CD86 (V450; BD Biosciences, 560357), CD83 (FITC; BD Biosciences, 556910), HLA-DR (APCef780; eBiosciences, 47-9956-42, MA, USA), CD80 (PE-Cy5; BD Biosciences, 559370), PD-L1 (PE-Cy7; BD Biosciences, 558017), CD1a (PerCP/Cy5-5; BioLegend, 300130, San Diego, CA, USA). 10 µg/mL of human Fc Block (BD Bioscience, 554220) was added to the antibody mixture to block non-specific binding. Compensation beads (eBiosciences, 01-2222-41) stained with

single antibodies were run for every experiment. Cells were washed with 200 µL FACS buffer and stained by incubating the antibody mixture for 30 min in the dark at 4 °C. Before measuring DRAQ7 (Abcam; ab109202, Cambridge, UK) was added and incubated for 10 min in the dark to stain nonviable cells. Cells were resuspended in 100 µL FACS buffer and acquired on a BD FACS Canto II (BD Biosciences) and analyzed using the FlowJo software V10.

Quantification of Cytokine Levels in Supernatants

Levels of IL-8, IL-6, IL-10, TNF and IL-12p70 were measured in the supernatants of moDC cultures using cytometric bead array technique (BD Biosciences). Individual flex-sets for IL-8 (558277), IL-6 (558276), TNF (560112), IL-10 (558274) or IL-12p70 (558283) were run according to the manufacturer's instructions.

Proteinase K Treatment and SDS-Page

LF was treated with 100 µg/mL proteinase K for 1 h at 46 °C followed by 10 min on 95 °C to inactivate the enzyme activity. 1 µg of LF and proteinase K treated LF were loaded on a SDS-page gel (Mini-PROTEAN TGX Precast SDS-page gel, Biorad, Berkeley, CA, USA) and run on 120 V for 1 h. The gel was stained with GelCode (Thermo Scientific, 24590, MA, USA) according to the manufacturer's instructions.

LPS Detection

LF and Triton X-114 treated LF was tested for LPS contamination by a recombinant factor C LAL assay that was performed according to the manufacturers recommendations (EndoZyme recombinant factor C assay, Hyglos; 609050, Bernried am Starnberger See, Germany).

Triton X-114 Treatment

LPS was removed from LF by an optimized Triton X-114 method (Amresco, cat. # M114, OH, USA) [210]. In short, 2% *v*/*v* Triton X-114 was added to the sample and the mixture was stirred for 30 min at 4 °C and thereafter transferred to a 41 °C water bath for 10 min. The micelles were spun down by centrifugation for 10 min at 20.000 *g* at 25 °C. The upper layer was collected and treated with 10 mg/mL Bio-beads SM-2 (cat. # 152-8920) under constant stirring at 4 °C to remove Triton X-114 traces.

TLR4 Reporter Assay

HEK-293 cells expressing human TLR4, CD14 and MD-2 and harboring a pNIFTY construct (Invivogen, Toulouse, France) were grown on selective medium containing DMEM and Glutamax (Fisher Emergo, Landsmeer, The Netherlands) supplemented with 10% FCS, 100 µg/mL penicillin/streptomycin (Sigma, St. Louis, MO, USA), Zeozin (50 µg/mL), Normocin (100 µg/mL) and HygroGold (45 µg/mL) (Invitrogen, Carlsbad, CA, USA) in an atmosphere of 5% CO_2 at 37 °C. HEK-293 cells were seeded at 3×10^5 cells/mL and cultured overnight before stimulation the next day. NF-κB activation was measured after 24-h stimulation by adding Bright-Glo™ (Promega, Fitchburg, MA, USA) substrate to cells. The plate was shaken and luminescence was measured using a spectramax M5 (Molecular Devices, Sunnyvale, CA, USA).

Statistics

Data was assessed for normality using a D'Agistino and Pearson omnibus test. A repeated measures ANOVA with Tukey's multiple comparison test or Friedman test with Dunn's multiple comparison post-hoc test was performed for normal and non-normal distributed data, respectively. Data is represented as mean ± standard error of the mean (SEM). Graphpad Prism V. 5.0 was used for all statistical analyses.

Results

Monocytes were differentiated into monocyte-derived DC (moDC) in the presence or absence of LF. Upon differentiation into moDC, monocytes lost CD14 expression and gained CD1a expression. Although LFDC were negative for CD14, a lower percentage of cells gained CD1a expression (Figure 1A). LFDC showed a higher expression of CD86 and PD-L1 (Figures 1B-C) and a significantly lower expression of HLA-DR when differentiated in the presence of 250 µg/mL LF (Figure 1D). These phenotypical changes induced by LF during differentiation were accompanied by a dose-dependent increase in IL-8 production (Figure 1E) and increase in IL-6 production when cultured in the presence of 250 µg/mL LF (Figure 1F). Interestingly, in contrast to the profound effects of LF during the differentiation of monocytes into moDC, LF did not induce phenotypic changes on moDC that were already differentiated (Figure S1). Moreover, moDC stimulated with LF or LF + Poly I:C and R848 for two days did not show phenotypic changes compared to moDC or moDC + Poly I:C and R848, respectively.

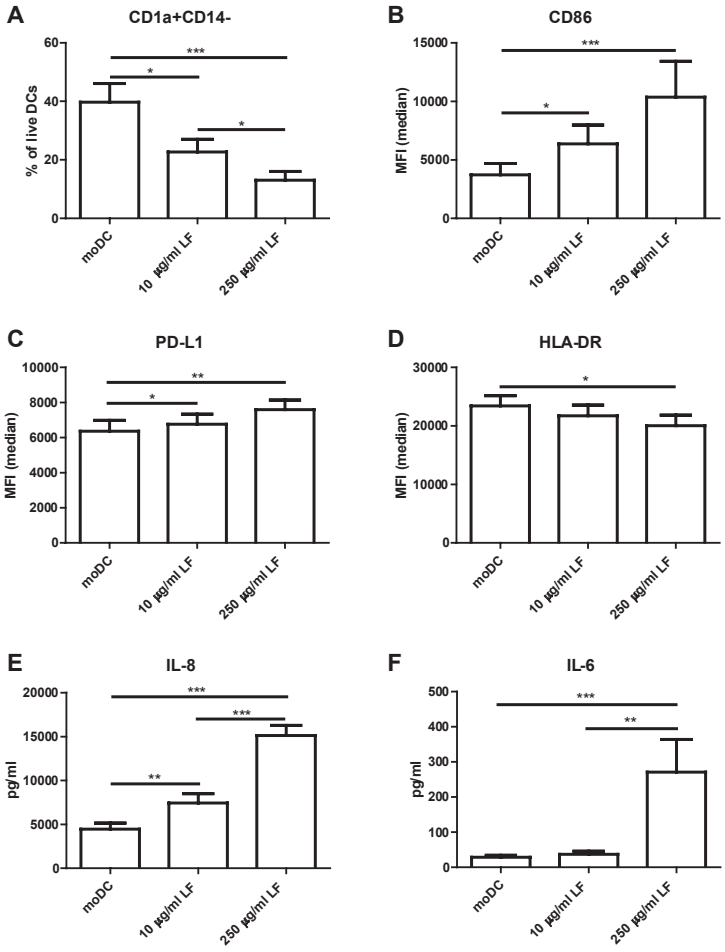

Figure 1. LF modulates DC differentiation. Monocytes were differentiated into moDC by culturing them for six days in the presence of IL-4 and GM-CSF with or without LF (10 or 250 µg/mL LF). (**A**) The percentage CD1a+CD14– DC and the median fluorescent intensity (MFI) of (**B**) CD86, (**C**) PD-L1 and (**D**) HLA-DR is shown. The production of (**E**) IL-8 and (**F**) IL-6 was measured in the supernatant by CBA. The mean ± SEM of four independent experiments with 12 different donors is shown. Significance is indicated by *** = p <0.001, ** = p <0.01 and * = p <0.05.

Next, we investigated the responsiveness of LFDC by stimulating the cells with LPS. LFDC showed to be hyporesponsive towards LPS as observed by the reduced induction of the maturation marker CD83 (Figure 2A) and costimulatory molecules CD86 (Figure 2B), PD-L1 (Figure 2C) and CD80 (Figure S2A) upon LPS stimulation. HLA-DR expression was lower on mature LFDC compared to mature moDC (Figure 2D). In line with their phenotype, LFDC also produced lower cytokine levels,

showing a lower production of IL-10 (Figure 2E), IL-6 (Figure S2B), TNF (Figure S2C) and abrogated levels of IL-12p70 (Figure 2F). Since DC were differentiated and subsequently stimulated in the presence of LF, we wanted to exclude the possibly that the hyporesponsiveness towards LPS was caused by neutralization of LPS by LF. We showed that the surface marker expression was unaffected by replacing ¾ of the medium and that the cells were also hyporesponsive towards R848 and Poly:IC stimulation, indicating that the effect was not mediated by LPS neutralization (Figure S3).

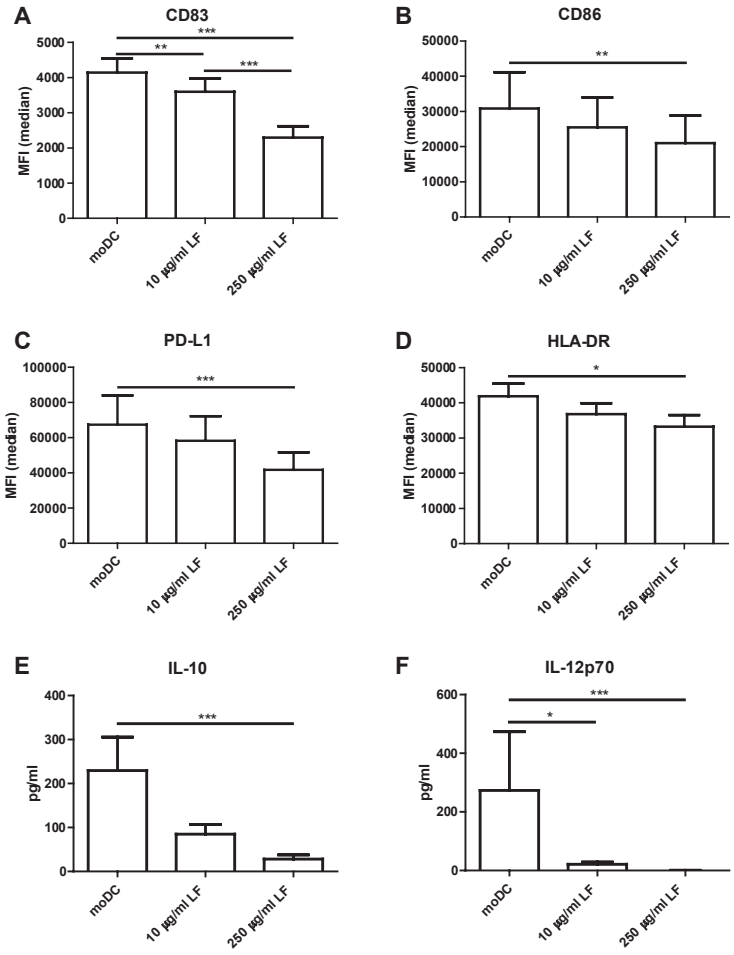

Figure 2. LFDC are hyporesponsive for LPS stimulation. Immature DC that were cultured in the presence or absence of LF were stimulated with 1 µg/mL LPS for 48 h. The median fluorescent intensity (MFI) of (**A**) CD83, (**B**) CD86, (**C**) PD-L1 and (**D**) HLA-DR is shown. The production of (**E**) IL-10 and (**F**) IL-12p70 was measured in the supernatant by CBA. The mean ± SEM of four independent experiments with 12 different donors is shown. Significance is indicated by *** = p <0.001, ** = p <0.01 and * = p <0.05.

LF is reported to bind LPS and has been postulated to induce immunomodulation via TLR4-dependent mechanisms. However, previously we showed that low concentrations of LPS can also LPS induce these phenotypic changes. We therefore measured the concentration of LPS in LF by an endozyme LAL assay. LF used in this study showed concentrations of 2.6 EU LPS/mg LF (Figure S4A). We therefore applied an optimized Triton X-114 method[210] to reduce LPS levels. This method reduced the endotoxin levels to 0.58 EU LPS/mg LF (Figure S4A). Despite this five-fold decrease in LPS levels, the immunomodulatory activity of LF remained the same (Figure S4). Nevertheless, we have previously shown that low concentrations of LPS induce endotoxin tolerance[157]. We therefore wanted to be absolutely certain that the functional effects of LF were not mediated by LPS. Therefore, we treated LF with proteinase K to degrade the protein and release any potentially bound endotoxins. We confirmed that LF was completely degraded after proteinase K treatment as observed on SDS-page gel (Figure S5A), Additionally, we showed by using a TLR4 reporter assay that the NF-κB inducing capacity of LPS was unaltered by proteinase K treatment (Figure S5B), indicating that the LPS released from bound to LF remains functional. Interestingly, the dose-dependent increase of CD86 (Figure 3A) and PD-L1 (Figure S6B) and decreased CD1a (Figure 3B) expression induced by LF was reduced to moDC levels after proteinase K treatment. Similarly, the LF induced production of IL-6 (Figure 3C) and IL-8 (Figure S6A) during DC differentiation was abrogated after degradation of the protein. Thus, the LF-induced phenotype on immature DC was completely abolished by proteinase K treatment of the protein, showing that the effect is mediated by LF and not by LPS. In line with these findings, the responsiveness towards LPS of LFDC was completely restored to that of moDC upon proteinase K treatment as shown by the expression of CD86 (Figure 3D), CD83 (Figure 3E), CD80 and PD-L1 (Figure S7A, B) and the production of cytokines such as IL-6 (Figure 3F), IL-12p70, TNF and IL-10 (Figure S7D–F).

Discussion

In this paper, we show that LF modulates human DC differentiation and function. Additionally, we show that its immunomodulatory capacity is not mediated by LPS. With this study, we confirm and expand on the study of Puddu *et al.* (2011), that showed that LF is capable of inducing differentiation into tolerogenic DC[147]. Since trace amounts of LPS can already induce a tolerogenic phenotype[157], it is necessary to

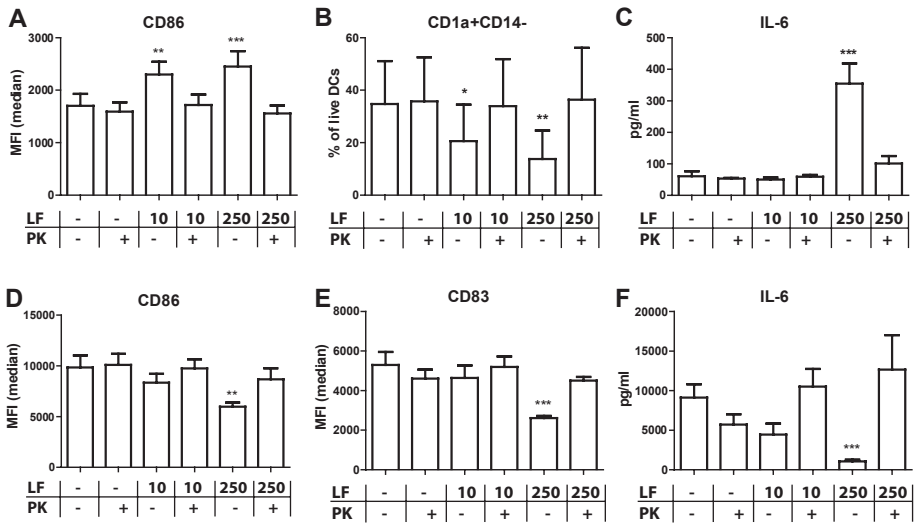

Figure 3. Proteinase K treatment of LF restores responsiveness towards LPS. (**A**–**C**) monocytes were cultured in the presence of IL-4 and GM-CSF with or without LF or proteinase K treated LF. (**D**,**E**) These immature DC were subsequently stimulated with 1 μg/mL LPS for 48 h. The median fluorescent intensity (MFI) of (**A**,**D**) CD86 and (**E**) CD83 and (**B**) the percentage CD1a+CD14– DC is shown. (**C**,**F**) The production IL-6 was measured in the supernatant by CBA. The mean ± SEM of 3 different donors is shown. Significance is indicated by *** = p <0.001, ** = p <0.01 and * = p <0.05.

assess that LF and not LPS is responsible for the effect. Here, we show that LF alters the differentiation of monocytes into DC, resulting in a phenotypical distinct DC type which is hyporesponsive towards several TLR ligands.

It has been proposed that the GI tract is in a constant state of low-grade inflammation or "primed homeostasis" due to the constant exposure to commensal bacteria and microbial products in which monocytes are recruited toward the GI tract[31,211]. In line with this thought, a local micro-environment comprised of dietary and microbial factors may steer monocyte differentiation. We therefore investigated the effect of LF on monocyte differentiation into DC. It is well established that monocytes lose CD14 expression and gain CD1a expression during differentiation into moDC. In the presence of LF, DC lose CD14 expression and gain less CD1a expression compared to conventional differentiated moDC. CD1a is an important functional marker for immature DC. Moreover, CD1a+ DC produce higher IL-12p70 levels and lower IL-10 levels upon stimulation and show a lower internalization capacity compared to CD1a-DC[212,213]. Potential explanations for the reduced CD1a expression on LFDC can be induction of PPARγ activity[213], differentiation into more macrophage-like

cells due to IL-6-mediated autocrine M-CSF production[214], or inhibition of GM-CSF signaling[215]. Interestingly, an embryonic fibroblast cell line transfected with the human lactoferrin gene showed inhibited GM-CSF levels upon stimulation[216]. LF is, in contrast to moDC, internalized into the nucleus of human monocytes[147]. This finding makes it appealing to speculate about direct effect of internalized LF on the GM-CSF promotor in monocytes. Additionally, LF induces the production of IL-6 and IL-8 within 24 h of differentiation (data not shown). Although IL-6 has been shown to be an important cytokine capable of modulating DC differentiation[205] and boosting autocrine G-CSF production[214], Puddu *et al.* (2011) showed that the effects of LF are not mediated by IL-6. In line with these findings we show that IL-6 production was not elevated if monocytes are differentiated in the presence of 10 μg/mL LF and yet this concentration is sufficient to induce DC that are hyporesponsive towards TLR ligands.

LFDC produced much lower cytokine levels and upregulated costimulatory molecules markers to a lesser extent compared to moDC upon contact with TLR ligands. Moreover, they showed a semi-mature ($CD83^{int}CD86^{int}$) phenotype, which is postulated to induce polarization of naive T cells in regulatory T cells[162]. Thus, LF induces the differentiation of monocytes into more tolerogenic DC. In contrast to the effect of LF on monocytes, we show that LF does not induce phenotypical changes on human moDC nor modulate Poly I:C and R848-induced inflammation. These findings are in line with previous research, showing internalization of LF into the nucleus of human monocytes and not in moDC[147]. Their findings propose that the uptake of LF is mediated by a receptor expressed on human monocytes that is not expressed on moDC, which could explain the distinct effect of LF on both cell types. LF binds to intelectin-1 (i.e., intestinal lactoferrin receptor), which is expressed on epithelial cells[77]. To date, no evidence suggests expression of intelectin-1 on myeloid cells. LF also binds DC-SIGN which is expressed on monocytes and moDC[205]. However, the observed effects are also not likely mediated via DC-SIGN since its expression increases upon differentiation into DC[217]. Additionally, it has been suggested that LF binds with low affinity to several other receptors (e.g. RAGE and MNR). Interestingly, human LF was also shown to bind to soluble CD14[218]. Since CD14 expression is lost upon differentiation of monocyte into moDC, it is tempting to speculate that our phenotypic changes are induced via CD14. However, LF in concentration used in this study did not activate NF-κB on CD14-MD-2:TLR4 expressing HEK cells (data not shown). Recently, CD14 was shown to be essential

for endocytosis of the TLR4 complex[219], showing that its function is more than just facilitating LPS to bind TLR4. We therefore hypothesize that LF:LPS complexes can be internalized on monocytes in an unknown CD14-mediated manner.

This study shows, in line with the literature, that bovine LF, as well as human LF, induces differentiation of monocytes into hyporesponsive DC[147] and macrophages[207], respectively. However, due to the high binding affinity of LF to LPS, it is essential to investigate whether LPS or LF is responsible for this tolerogenic phenotype. Importantly, we showed that our sample did contain traces of LPS as measured by a LAL assay. In this assay, a recombinant factor C protein is used to detect LPS, which binds the lipid A part of the molecule[220]. LF was also shown to bind the lipid A part of the molecule[208], which could shield LPS from binding TLR4 and apparently not factor C. According to the LPS contamination measured in our sample (i.e., 2.6 EU/mg), the concentrations of LF used in our study of 0.25 mg/mL and 0.01 mg/mL, thus, contain 0.65 EU and 0.026 EU, respectively. We have shown that LPS contamination of >0.5 EU induces the differentiation into tolerogenic DC[157]. The concentrations of LPS measured in LF in this study could, thus, theoretically explain the tolerogenic DC phenotype. Nevertheless, we demonstrated that the effects of LF are lost upon proteinase K treatment and that Triton X-114-treated LF shows the same immunomodulatory capacity compared to non-treated LF. We thereby exclude the possibility that effects of LF are mediated by endotoxin tolerance. This finding is in line with earlier research showing that the induced expression of pro-inflammatory genes in porcine macrophages is mediated in a TLR4-independent manner[209].

This study adds on to the current understanding of the role of LF in immune regulation. Additionally, LF is well-known for its anti-pathogenic activity. Hence, LF supplementation to infants and immunocompromised individuals has been studied to investigate its efficacy against inflammatory conditions. Moreover, several clinical studies in children have been conducted with LF, all showing no adverse effects of LF supplementation[221]. LF supplementation to breast milk or infant formulas has been shown to reduce the incidence of sepsis VLWB infants[191–195]. These VLWB infants often suffer from excessive gut inflammation and have an impaired. Moreover, LF supplementation to children epithelial barrier functioning, which may result in necrotizing enterocolitis[222]. Additionally, several studies show that LF fortification to early life nutrition may alleviate symptoms of viral infections[90,91,223,224] <5 years of age and to infants in the first year of life showed a reduction of the incidence of rotaviral

gastroenteritis[223] and lower respiratory tract infections[91]. Similarly, LF supplementation to infant nutrition resulted in a lower incidence of symptoms of respiratory illness (e.g. running nose, coughing, wheezing) compared to infants receiving non-fortified formula[90]. LF supplementation has also been investigated for its additive effect in HIV therapy in children by measuring several immune parameters and viral titers. Interestingly, phagocytic activity, CD14/TLR2 expression and IL-12p70/IL-10 ratio in CD14+ was increased in children receiving LF supplementation[224]. Thus, apart from functioning as a direct anti-pathogenic protein, LF may inhibit infections by its immunomodulatory capacity. Since, LF is poorly digested in the GI tract of infants and a fraction of the protein is taken up intact and reaches the circulation[197,200,201], it may impact the functionality of monocytes and DC in vivo. Larger cohorts should validate the protective effect of LF supplementation against viral infections and sepsis and investigate its immunomodulatory potential *in vivo*.

Conclusions

Taken together, our results show that LF inhibits DC differentiation which hampers their responsiveness towards TLR ligands. Additionally, we showed that these effects are diminished after degrading the protein, formally showing that the LF-induced differentiation of monocytes into hyporesponsive DC is not mediated by endotoxin tolerance. This study indicates that LF may promote immune homeostasis in the gastrointestinal tract.

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The authors would like to thank Wim Mengerink and Evelien Kramer for isolation of LF.

Supplementary Files

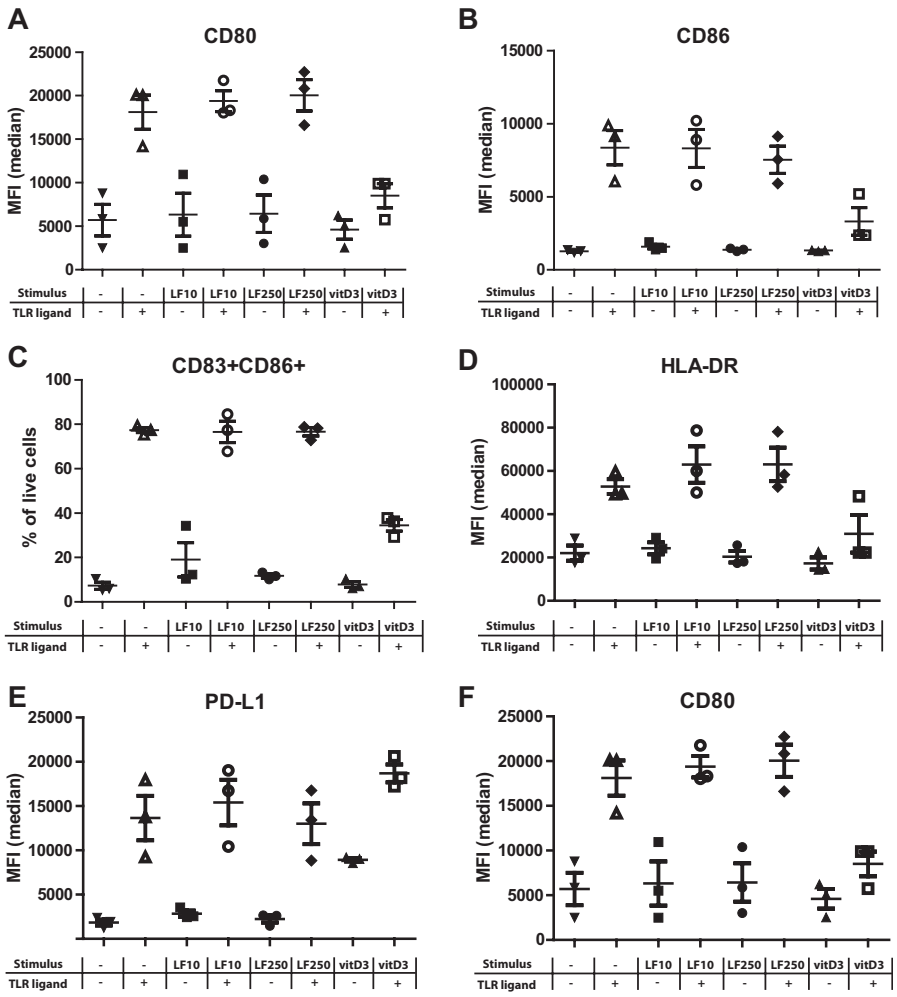

Figure S1. LF does not modulate immature DC activation. Monocytes were differentiated into moDC by culturing them for six days in the presence of IL-4 and GM-CSF. These immature DC were stimulated with 20 µg/mL Poly I:C and 3 µg/mL R848 in the presence or absence of LF. The median fluorescent intensity (MFI) of (**A**) CD8, (**B**) CD86, (**D**) HLA-DR, (**E**) PD-L1, (**F**) CD80 and (**C**) the percentage of CD83+CD86+ DC is shown. The expression of three individual donors with mean and standard deviation is shown in a scatter plot.

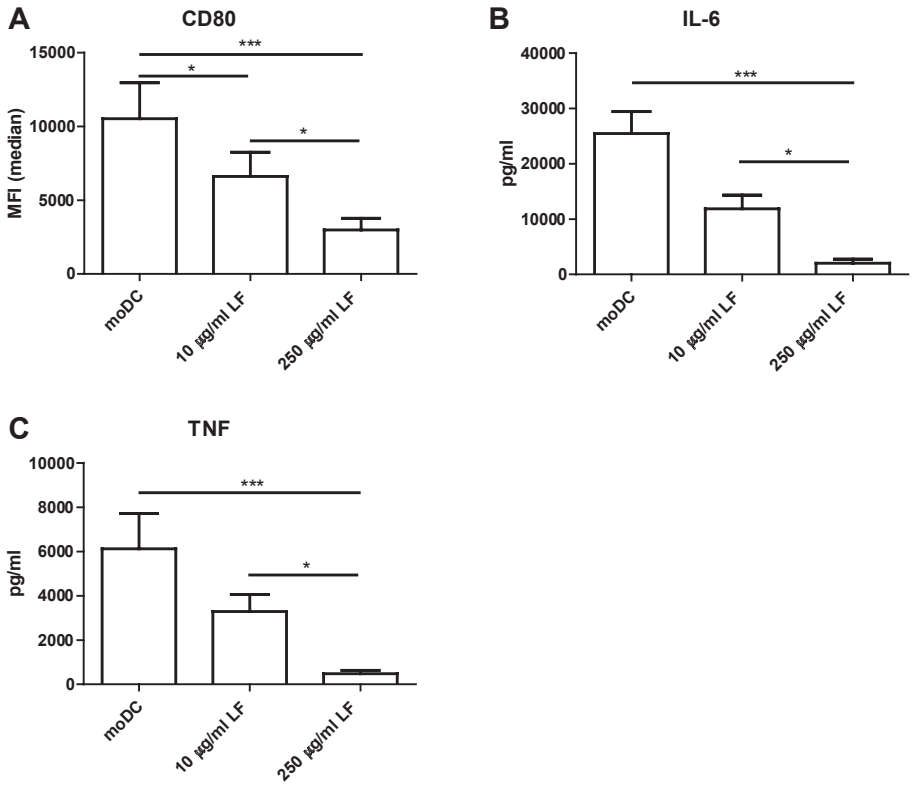

Figure S2. LFDC are hyporesponsive for LPS stimulation. Immature DC that were cultured in the presence or absence of LF were stimulated with 1 µg/mL LPS for 48 h. The median fluorescent intensity (MFI) of (**A**) CD80 is shown. The production of (**B**) IL-6 and (**C**) TNF was measured in the supernatant by CBA. The mean ± SEM of four independent experiments with 12 different donors is shown. Significance is indicated by *** = p <0.001, ** = p <0.01 and * = p <0.05.

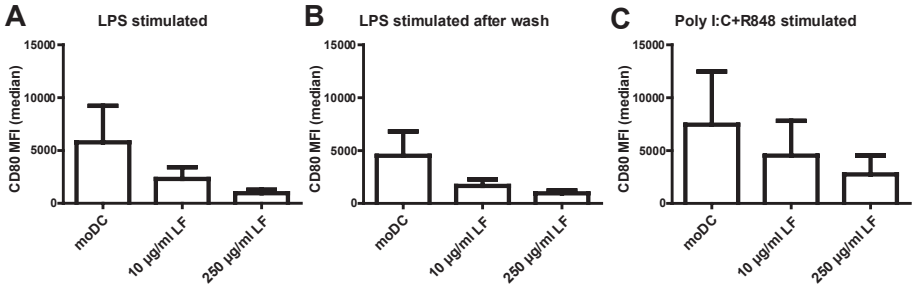

Figure S3. Hyporesponsiveness of LFDC is not mediated by decoy activity. Immature DC that were cultured in the presence or absence of LF were stimulated with 1 µg/mL LPS (**A**) without or (**B**) with replacing ¾ of the medium or (**C**) 20 µg/mL Poly I:C and 3 µg/mL R848 for 48 h. The median fluorescent intensity (MFI) of CD80 is shown. The mean ± SEM 3 different donors is shown.

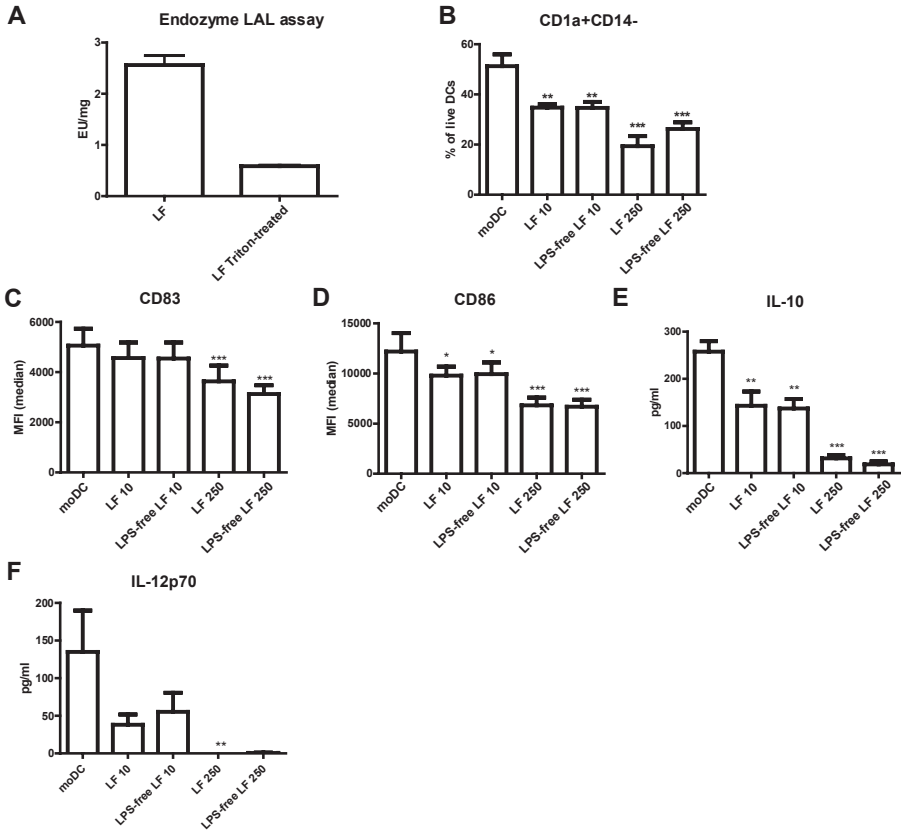

Figure S4. DC modulatory activity of LF is not reduced by Triton X-114 treatment. (**A**) An Endozyme LAL assay was used to detect LPS in LF before or after applying an optimized Triton X-114 method. Immature DC were cultured in the presence or absence of Triton X-114 treated or non-treated LF. (**B**) The percentage CD1a+ CD14– DC is shown on immature DC. These immature DC were stimulated with 1 µg/mL LPS for 48 h. Median fluorescent intensity (MFI) of (**C**) CD83 and (**D**) CD86 is shown. The production of (**E**) IL-10 and (**F**) IL-12p70 was measured in the supernatant by CBA. The mean ± SEM of 3 different donors is shown. Significance is indicated by *** = p <0.001, ** = p <0.01 and * = p <0.05.

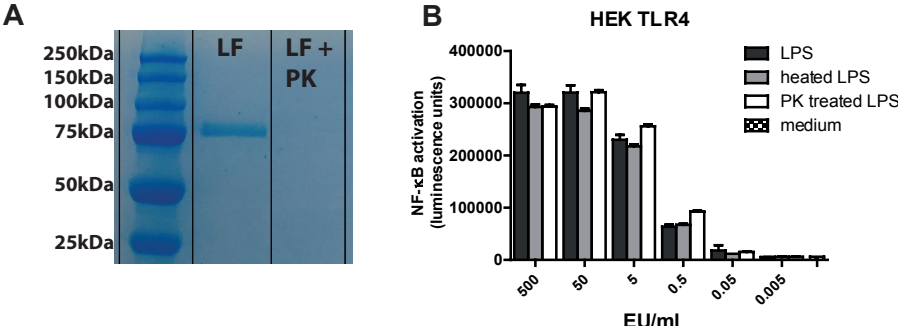

Figure S5. Proteinase K treatment does not affect NF-κB activation via TLR4 by LPS. (**A**) 1 µg/mL LF was loaded before and after proteinase K treatment on SDS-PAGE gel. (**B**) LPS, proteinase K treated LPS and heated LPS was tested for its NF-κB activation in a TLR4 reporter assay.

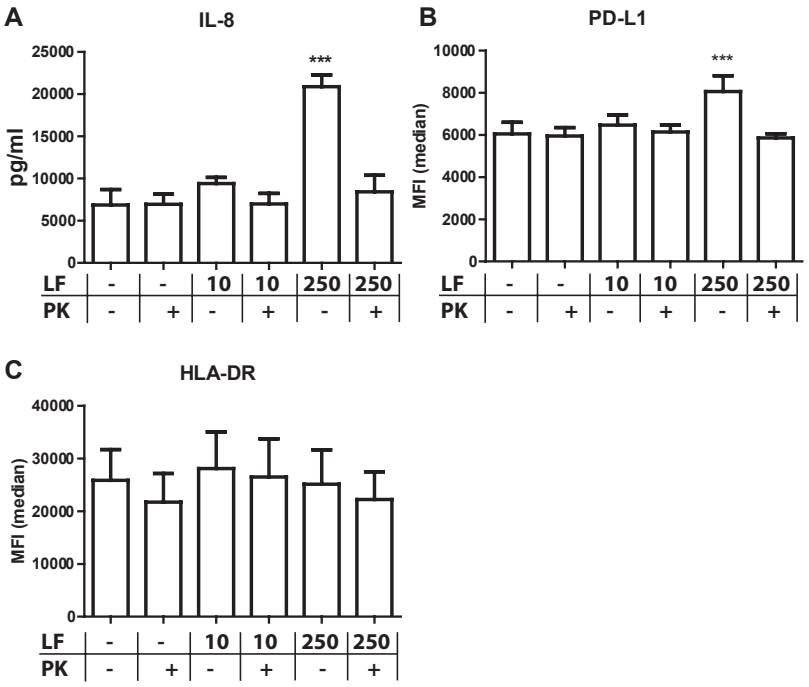

Figure S6. Proteinase K treatment of LF abrogates its effect on DC differentiation. Monocytes were cultured in the presence of IL-4 and GM-CSF with or without LF or proteinase K treated LF. (**A**) The production of IL-8 was measured in the supernatant by CBA. The median fluorescent intensity (MFI) of (**B**) PD-L1 and (**C**) HLA-DR is shown. The mean ± SEM of 3 different donors is shown.

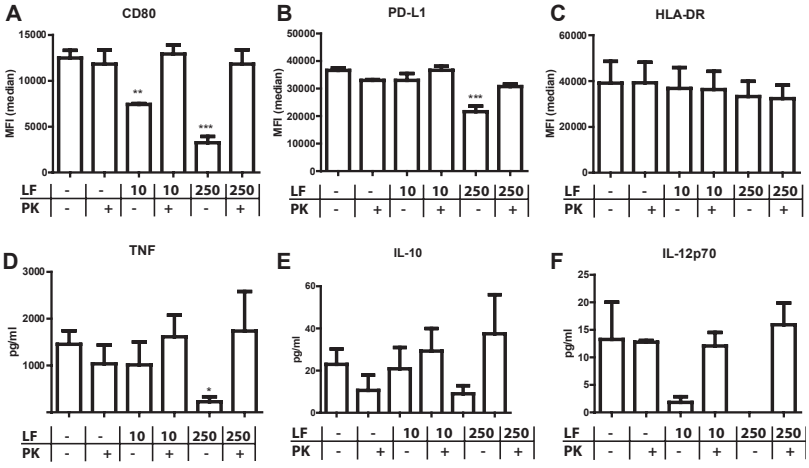

Figure S7. Proteinase K treatment of LF restores responsiveness towards LPS. Monocytes were cultured in the presence of IL-4 and GM-CSF with or without LF or proteinase K treated LF for six days and subsequently stimulated with 1 µg/mL LPS for 48 h. The median fluorescent intensity (MFI) of (**A**) CD80, (**B**) PD-L1 and (**C**) HLA-DR is shown. The production of (**D**) TNF, (**E**) IL-10 and (**F**) IL-12p70 was measured in the supernatant by CBA. The mean ± SEM of 3 different donors is shown. Significance is indicated by *** = p <0.001, ** = p <0.01 and * = p <0.05.

Chapter 4

Optimized Triton X-114 Assisted Lipopolysaccharide (LPS) Removal Method Reveals the Immunomodulatory Effect of Food Proteins

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Abstract

Investigations into the immunological response of proteins is often masked by lipopolysaccharide (LPS) contamination. We report an optimized Triton X-114 (TX-114) based LPS extraction method for β-lactoglobulin (BLG) and soy protein extract suitable for cell-based immunological assays. Optimization of an existing TX-114 based phase LPS extraction method resulted in >99% reduction of LPS levels. However, remaining TX-114 was found to interfere with LPS and protein concentration assays and decreased viability of THP-1 macrophages and HEK-Blue 293 cells. Upon screening a range of TX-114 extraction procedures, TX-114-binding beads were found to most effectively lower TX-114 levels without affecting protein structural properties. LPS-purified proteins showed reduced capacity to activate TLR4 compared to non-treated proteins. LPS-purified BLG did not induce secretion of pro-inflammatory cytokines from THP-1 macrophages, as non-treated protein did, showing that LPS contamination masks the immunomodulatory effect of BLG. Both HEK293 cells expressing TLR4 and differentiated THP-1 macrophages were shown as a relevant model to screen the protein preparations for biological effects of LPS contamination. The reported TX-114 assisted LPS-removal from protein preparations followed by bead based removal of TX-114 allows evaluation of natively folded protein preparations for their immunological potential in cell-based studies.

Introduction

Increasing food demand creates a need to search for more sustainable food systems. The number of studies focused on the functionality, processing, and industrial application of alternative proteins is rapidly increasing[225]. Nutritional and immunological aspects of these novel proteins are not well-known and introducing them to the human diet requires insight into these aspects[225,226]. *In vitro* cell culture models are a useful strategy to study the nutritional value and immunological potential of novel proteins[226,227]. LPS (lipopolysaccharide) is a major endotoxin found in food protein preparations and other food extracts such as polysaccharides[210]. LPS is a pathogen associated molecular pattern found in the outer membrane of most gram-negative bacteria and is capable of initiating a strong innate immune response upon bacterial infection in humans[228–230]. Soluble LPS particles form a complex with lipopolysaccharide-binding protein (LBP), which is transferred to CD14 and subsequently interacts with Toll-like receptor (TLR)4 and MD-2 to activate the NF-κB pathway. This activation results in the secretion of pro-inflammatory cytokines, like IL-1β, IL-6 and TNF[228,229]. Investigation of the effect of food-derived proteins on the immune response is therefore masked by high levels of immunomodulatory LPS. Removal of LPS from protein preparations reveals the unbiased immunomodulatory effects that proteins may induce. A vast array of LPS extraction procedures have been published over the years and these methods often include several chromatographic steps, such as ion exchange, hydrophobic interaction chromatography, and gel filtration[231–233]. These methods are laborious, and do not fully eliminate biological activity of residing LPS[234]. Polymyxin B (PMB) is an antibiotic that has been used as alternative strategy to eliminate LPS activity and even though PMB was shown to prevent TLR4 signaling its presence does not prevent IL-1β secretion in monocyte cultures[235]. Other compounds which have been experimentally shown to eliminate the immunogenic potential of LPS include paeonol[236] and gedunin[235] although their mode of action potentially interferes with the immunogenic properties of proteins under investigation. Proanthocyanins isolated from different food sources[237] and lactoferrin[238] have also been identified as potent binding partners of LPS. However, the activity of LPS removal agents that act by interaction with LPS is influenced by the chemical properties of the protein, often require pH adjustment and do not guarantee complete dissociation and removal of protein-bound LPS[231–233]. A technique that triggers the dissociation of LPS from the protein irrespective of protein chemical properties is a two-phase detergent-based (e.g. Triton X-114, TX-114) extraction.

Recently, this method was described as an efficient way of endotoxin removal from protein preparations of animal origin[239], recombinant proteins and antibodies[240–242], plasmids[243] and viral proteins[244]. TX-114 is an aqueous surfactant that assists LPS into forming micelles, which subsequently aggregate into a surfactant enriched phase at a temperature of 22˚C. After phase separation, the lipophilic LPS-rich fraction can be separated from the hydrophilic protein phase by means of centrifugation[233,239]. Although this method was shown to retain the biological activity and the structure of proteins[245,246], TX-114 was also reported to be toxic at low concentrations to cells in culture[239] urging elimination of this extraction agent from protein preparations to allow unbiased investigation of the immunogenic potential of proteins.

In this study we demonstrate that LPS and TX-114 can be effectively removed from protein preparations to levels that do not interfere with cell viability, LPS and protein concentration assays, and *in vitro* immunological read-outs and do not impact on protein structure. We further show that LPS- and TX-114 purified β-lactoglobulin does not induce secretion of pro-inflammatory cytokines from THP-1 macrophages.

Materials and Methods

Proteins

Bovine β-lactoglobulin (BLG) (Sigma, cat. # L0130) was dissolved in sterile phosphate buffered saline (PBS, Life Technologies, cat. # 18912-014) or MQ H_2O. Soy protein extract (SPE) was obtained by extracting the proteins from soy flour (Sigma Aldrich, cat. # S9633) according to the procedure described by L'Hocine *et al.*(2006)[247]. Protein concentration was determined by absorbance at 280 nm using a NanoDrop ND1000 spectrophotometer.

Triton X-114-assisted LPS-extraction method

A previously published TX-114 based LPS extraction protocols[240,248] was modified to optimize for LPS removal and removal of remaining TX-114. TX-114 (Amresco, cat. # M114) was added to the protein solution (BLG at 10 mg/ml and SPE at 14 mg/ml in PBS) to a final TX-114 concentration of 2% v/v. The solution was incubated at 4˚C for 30 min with constant stirring. Subsequently, the sample was transferred to a water bath set at 37˚C and incubated for 10 min followed by centrifugation at 20,000 g for 20 min at 37˚C. The upper part containing the protein was separated from the

TX-114 layer by means of pipetting and the LPS concentration was determined. To investigate whether repeated TX-114-assisted extraction increased LPS removal efficiency, the extraction procedure was repeated one, two and three times. Bio-Beads SM-2 (Bio-Rad, cat. # 152-8920)[249] with high affinity for Triton were added to the collected supernatant and incubated overnight at 4°C with constant stirring. The ratio of beads to protein solution was calculated based on the assumption that 1 g of beads adsorbs 0.07g of Triton. Using sedimentation, the Bio-Beads were removed from the samples. A similarly TX-114 treated protein free buffer control was included to determine TX-114 remaining concentrations using absorption at 280 nm.

TX-114 removal

Several approaches were evaluated for their ability to eliminate TX-114 from protein preparations. First, 8 ml protein solution was subjected to dialysis for 24 h against distilled water using a Slide-A-Lyzer 10kDa cassette (Life Technologies, cat. # 66380). Second, a volume of 1 ml of 0.1% TX-114 in buffer and 1 ml of 1.0 mg/ml of BLG were applied to a HiTrap Desalting column (GE Healthcare Life Sciences, cat. # 29-0486-84). Third, samples were centrifuged at 21,000 g and 25˚C for 10 and 20 min and at 37˚C for 10 and 20 min. Fourth, samples were centrifuged using 0.22 µm (cat. # 8160) and 0.45 µm (cat. # 8162) spin X filter columns (Costar) at 10,000 g at 37˚C for 4 min. Last, high-affinity Triton binding Bio-Beads SM-2 (Bio-Rad, cat. # 152-8920) were tested for their efficacy to remove Triton and used according to the manufacturer manual.

Measurement of LPS concentration

The LPS concentration was measured using the commercially available Endozyme Recombinant Factor C assay (Hyglos, cat. # 609050) according to the protocol of the manufacturer. The measurements were conducted in triplicate using a Tecan Infinite 200Pro plate reader. The positive control (MQ) was spiked with a concentration of 0.45 EU/l of LPS, and results for all tests were considered valid when the value of recovered LPS concentration was between 50 and 200% of this value. LPS units were converted from EU (Endotoxin Unit) to concentrations in pg/ml by assuming that 1 EU corresponds to 100 pg of standard endotoxin EC-5[250]. It should be kept in mind that LPS concentration strongly depends on the source of the endotoxin, since endotoxin activity is highly variable, depending on the bacterial strain.

SDS-PAGE

Non-reducing SDS-PAGE was used to evaluate the influence of the LPS removal procedure on protein content and quality. Samples were loaded onto a 12.5% polyacrylamide gel followed by staining using GelCode Blue Stain Reagent (Life Technologies, cat. # 24592).

Far-UV circular dichroism

Protein samples (BLG, SPE) at 0.1 mg/ml in PBS were placed in a quartz cuvette with an optical path of 0.1 cm. Far-UV circular dichroism (CD) spectra were recorded in a Jasco J-1500 spectropolarimeter at 25˚C. The wavelength range was set from 260 to 190 nm with 0.5 nm resolution, 4.0 sec response time, and 1.0 nm band width. Data were collected as averages of eight scans at a scanning speed of 50 nm/min. Spectra were corrected by subtracting the buffer baseline. Measurements were performed as independent duplicates.

Intrinsic tryptophan fluorescence

Emission fluorescence spectra of protein samples (BLG, SPE) at 0.01 mg/ml in PBS were recorded at 25˚C using a Varian Cary Eclipse spectrophotometer. The excitation wavelength was set at 295 nm (5 nm bandwidth) and the emission intensity was recorded from 310 to 500 nm (5 nm band width) at a scan rate of 120 nm/min and a 0.5 nm data interval. Spectra were corrected for buffer and represent an average of two scans. Measurements were performed as independent duplicates.

Cell culture

HEK-Blue-hTLR4 and HEK-Blue-hTLR2 cells (InvivoGen hkb-htlr4 and hkb-htlr2) were obtained by co-transfection of the human TLR4/TLR2, MD-2 and CD14 co-receptor genes and an inducible secreted embryonic ALP (SEAP) reporter gene into HEK293 cells. Stimulation of TLR4 or TLR2 with LPS activates NF-κB and AP-1 (activator protein 1), which induce the production of SEAP. SEAP activity was determined with the commercially available Quanti-Blue assay (InvivoGen, cat. # rep-qb1). The cells were grown in DMEM, 4.5 g/l glucose, 10% (v/v) fetal bovine serum, 50 U/ml penicillin, 50 mg/ml streptomycin, 100 mg/ml Normocin, 2 mM L-glutamine at 37°C in a humidified atmosphere at 5% CO_2. For the cytotoxicity and activation of TLR4 and TLR2 receptors assays cells were plated at a density of $1.25x10^3$ cells per well in a 96-well plate and incubated for 24 h. Next day the medium was replaced by 150 µl of medium containing SPE or BLG. The human monocytic

leukemia cell line THP-1 (American Type Culture Collection, Rockville, Md.) was grown as described previously[251]. Macrophage differentiation was induced by treating THP-1 monocytes (10^6 cells/ml) for 48h with 100 ng/ml phorbol 12-myristate 13-acetate (PMA, cat. # P1585) in 96-well cell culture plates containing 100 µl of cell suspension or 24-wells cell culture plates containing 500 µl of cell suspension in each well. It has been demonstrated that this differentiation method of THP-1 cells results in the expression of macrophage specific surface markers CD11b and CD36 and also phagocytic activity[252,253].

Cytotoxicity assay

The cytotoxic effect of TX-114 was determined using a cell viability test (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega, cat. # G3580). HEK-Blue-hTLR4 and HEK-Blue-hTLR2 cells were incubated with 2, 1, 0.5, 0.25, and 0.13 mg/ml of BLG or 2.8, 1.4, 0.7, 0.35, and 0.175 mg/ml of SPE. Phosphate buffered saline (PBS), PBS spiked with 0.005% of TX-114 (PBS/0.005% triton) and 0.1% of TX-114 were used as controls. After 24h incubation of both cell lines a volume of 20 µL of CellTiter 96 AQueous One Solution Reagent was added to each well followed by 1 h of incubation at 37°C. Absorbance was measured at 485 nm with FilterMax F5 Multi-Mode microplate readers and the percentage of viable cells was calculated in relation to cells cultured in pure medium. All experiments were performed in triplicate.

Activation of TLR4 and TLR2 receptors

HEK-Blue-hTLR4 and HEK-Blue-hTLR2 cells were incubated with 2, 1, 0.5, 0.25, and 0.13 mg/ml of BLG or 2.8, 1.4, 0.7, 0.35, and 0.175 mg/ml of SPE. The LPS standard curve, MQ water and MQ water spiked with 0.005% of Triton X-114 were used as controls. After 24h incubation in the presence of the stimuli the supernatant was collected and SEAP activity was measured using the Quanti-Blue assay according to the protocol of the manufacturer. The absorbance was measured at 620 nm after 1 h of the incubation at 37°C. All samples were measured in triplicate. TLR4/TLR2 receptor stimulation was expressed relative to the level of SEAP activity of cells cultured in pure medium.

Cytokine secretion by THP1 derived macrophages

Differentiated, adherent THP-1 macrophages were washed once with sterile PBS and once with complete RPMI 1640 medium. Cells were co-cultured for 24 h with 100 µl

of non-purified and LPS-purified BLG at protein concentrations of 100, 25 and 6.25 μg/ml. After 24h of incubation the supernatant was collected and human cytokine concentrations (IL-6, IL-8, IL-1β and TNF) were determined using the cytometric bead array (CBA) kit (Human Inflammatory Cytokine Kit, BD Bioscience, cat. # 551811) according to the manufacturer's instructions. The samples were analyzed by flow cytometry (BD FACS Canto II, BD Bioscience). The results were normalized to cytokine levels of unstimulated macrophages cultured in the medium.

Gene expression analysis using Q-PCR

THP-1 macrophages were incubated with titrated amounts of LPS (10^{-15} to 10^{-5} g/ml *E. coli* 0111:B4; Sigma) for 6 h. Cells were washed once with PBS and lysed using 200 μl TRIzol per well. RNA isolation, cDNA synthesis and RT-qPCR were performed as reported previously [30]. Investigated genes and used primers were IL-1β (FWD: GTGGCAATGAGGATGACTTGTTC; REV: TAGTGGTGGTCGGAGATTCGTA), IL-8 (FWD: CTGATTTCTGCAGCTCTGTG; REV: GGGTGGAAAGGTTTGGAGTATG), IL-10 (FWD: GTGATGCCCCAAGCTGAGA; REV: CACGGCCTTGCTCTTGTTTT), IL-12p40 (FWD: CTCTGGCAAAACCCTGACC; REV: GCTTAGAACCTCGCCTCCTT), TNF (FWD: TTCTGCCTGCTGCACTTTG; REV: GGGTTCGAGAAGATGATCTG) and NF-κB (FWD: TGAGTCCTGCTCCTTCCA; REV: GCTTCGGTGTAGCCCATT). Relative fold-changes to non-stimulated THP-1 macrophages were calculated following normalization to GAPDH (FWD: TGCACCACCAACTGCTTAGC; REV: GGCATGGACTGTGGTCATGAG) and using the $2^{-\Delta\Delta Ct}$ method.

Statistics

Results were expressed as mean ± SD of three to five independent measurements. Statistical analysis was carried out by GraphPad Prism 4 software. One-way ANOVA test with Tukey post-hoc ($p<0.05$, shown as one asterisk) was used to evaluate the significance if not specified differently in Figure legend.

Results

Triton X-114 needs to be removed from protein preparations before in vitro applications

Even though TX-114 presents an effective LPS-extraction agent for LPS-contaminated protein preparations, residual detergent remaining in the treated protein solution has been reported to be toxic to cells in culture[239] and hamper both determination of protein by absorbing at 280 nm and LPS concentration by interfering with most commercially available LPS detection assays. These observations motivated the need to lower the remaining TX-114 concentration after the purification procedure. TX-114 concentrations were conveniently determined under conditions where no protein is present using the characteristic to absorb light at 280 nm in a linear concentration-dependent manner (Figures 1A-B). This allowed estimation of the concentration of TX-114 in PBS spiked with 0.45 EU/l of LPS, after applying the TX-114 treatment. After one cycle of TX-114 purification the concentration of TX-114 remaining in LPS spiked PBS was estimated to be 0.12% (v/v) and this concentration was increasing with an increasing number of purification cycles (Figure 1C). A TX-114 concentration of 0.12% (v/v) was found to interfere with the EndoZyme assay giving rise to false negative results (Figure 1D). At the same time, a concentration of TX-114 in the medium equal to or higher than 0.006 % (v/v) significantly decreased the viability of THP-1 derived macrophages (Figure 1E). To evaluate the ability of a range of methods to effectively extract TX-114, PBS was spiked with 2% (v/v) TX-114 and subjected to centrifugation, dialysis, Hi-Trap desalting and spin X column purification or treatment with Triton-binding Bio-Beads (Figure 2). The use of Bio-Beads presented the most effective extraction of TX-114 by reducing TX-114 levels down to 0.005% (v/v). This concentration was significantly lower comparing to other conventional methods (Figure 2) and sufficiently low to permit reliable quantification of LPS with the EndoZyme assay (Figure 1D), UV-based determination of the protein concentration and was not cytotoxic to HEK 293 (Figure S1) or THP-1 derived macrophages (Figure 1E).

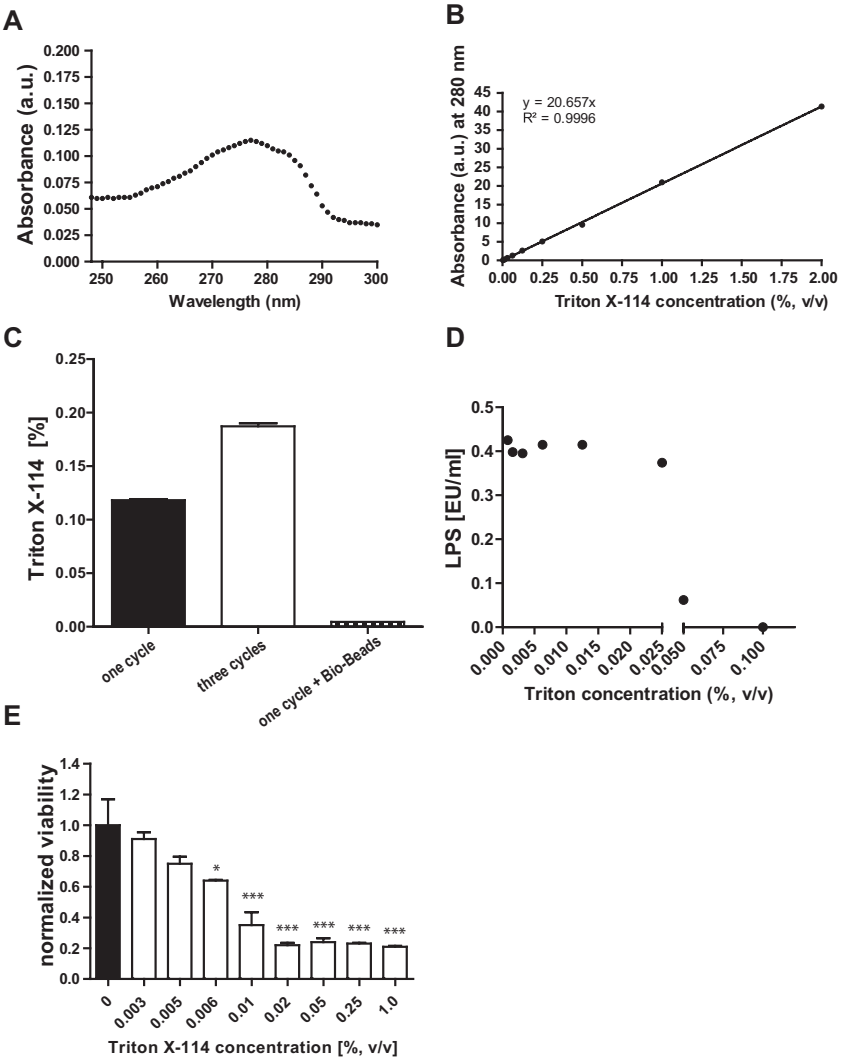

Figure 1. Optimization of TX-114 removal method from beta-lactoglobulin (BLG) and soy protein extract (SPE). (**A**) Triton concentrations can be quantified in protein-free solutions using spectroscopic absorbance at 280 nm**.** Absorbance spectrum of 0.005% (v/v) TX-114 solution in PBS was determined by a NanoDrop ND1000 spectrophotometer. (**B**) TX-114 in PBS dose-dependent absorption at 280 nm. Results are corrected for PBS background and represent an average of three independent measurements. (**C**) Concentration of TX-114 in PBS spiked with LPS (0.45 EU/l) after applying the TX-114 treatment described in Materials and Methods measured after one TX-114 cycle, three TX-114 cycles or after one TX-114 cycle followed with Bio-Beads treatment. (**D**) TX-114 reduces LPS detection with EndoZyme recombinant factor C assay in a dose-dependent manner. LPS concentration was measured in PBS spiked with 0.45 EU/l of LPS and decreasing concentration of TX-114. (**E**) Concentration of TX-114 in the medium equal or higher than 0.006 % (v/v) decreases the viability of THP-1 derived macrophages. Viability of THP-1 macrophages cultured for 24 h in the presence of TX-114 in the medium expressed as relative to cells grown in TX-114 free medium (n=1).

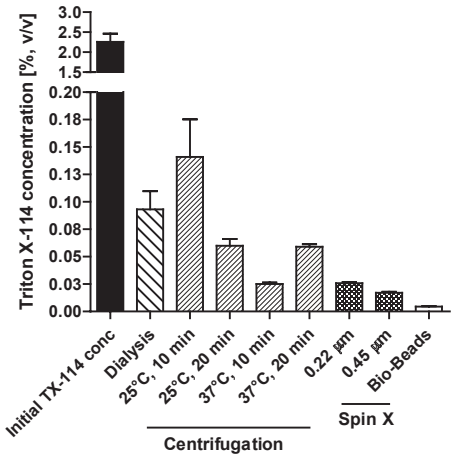

Figure 2. Extraction of remaining TX-114 from protein extract with high-affinity Bio-Beads results in most effective lowering of detergent concentration to non-toxic levels. Comparison of different TX-114 extraction methods. Starting from an initial TX-114 concentration of - 2% (v/v), the concentration of this detergent is effectively lowered by the application of dialysis, various centrifugation conditions, spin-X column and Bio-Bead assisted purification. Application of Bio-Beads results in most efficient TX-114 extraction down to 0.005% (v/v).

TX-114 assisted LPS extraction does not affect protein content and structure

TX-114-assisted LPS removal does not result in loss of proteins as protein concentrations of BLG and SPE, determined by OD280, were similar before and after application of the purification procedure (Figure 3A). Using SDS-PAGE, SPE showed a range of bands corresponding to β-conglycinin and glycinin similar to a previously reported migration profile [247]. The TX-114-assisted LPS extraction procedure did not affect this band distribution and similar results were obtained for BLG (Figure 3B) suggesting that quaternary structural arrangements were not affected by the treatment. Further, the LPS removal procedure did not affect the secondary and tertiary structural organizations estimated from Far-UV CD and intrinsic tryptophan fluorescence (Figures 3C-F). The CD and tryptophan fluorescence spectra largely overlap when comparing non-treated with LPS- and TX-114 extracted BLG and SPE and the wavelength of maximum tryptophan fluorescence intensity, indicative of a folded protein structure, did not differ significantly for BLG. For SPE, intrinsic tryptophan and CD peak positions for both LPS-extracted and non-treated samples were not significantly different although a small degree of variation appeared in signal intensity for both read-outs.

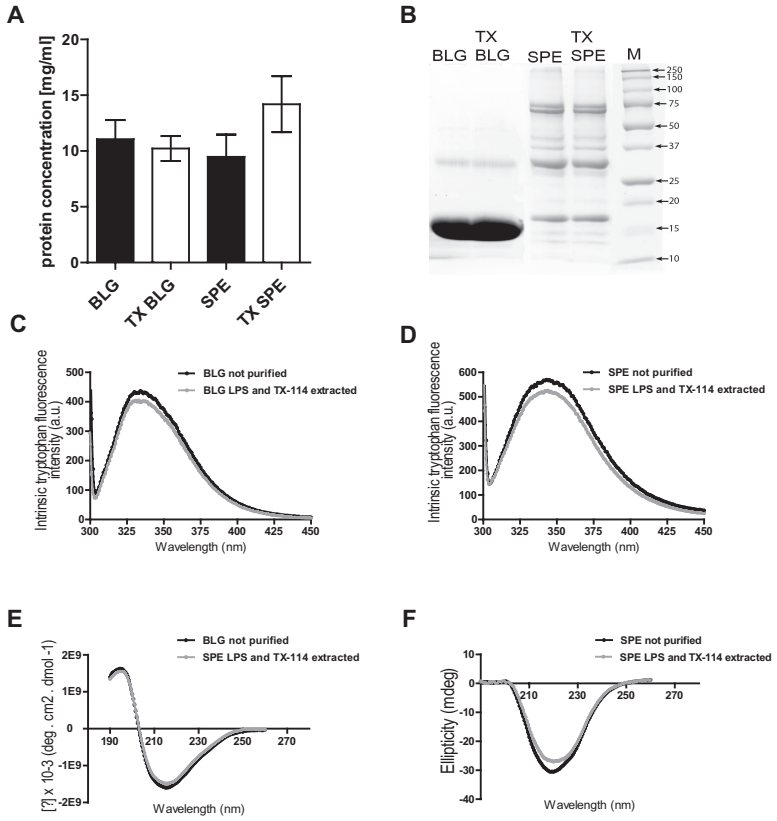

Figure 3. Protein yield and structure are retained upon application of the TX-114 assisted LPS extraction procedure. (**A**) BLG and SPE were dissolved in PBS at an initial concentration of - 10 mg/ml and protein concentrations were determined again after application of the LPS extraction procedure. (**B**) Non-reducing SDS-PAGE of not purified BLG, SPE and LPS- and Triton-extracted (TX BLG, TX SPE), M - molecular weight marker. (**C,D**) Intrinsic tryptophan fluorescence and far-UV CD spectra (**E,F**) of BLG and SPE. BLG concentrations used for far-UV CD were determined spectroscopically using absorbance at 280 nm and resulting CD spectra were normalized for molar ellipticity. SPE CD spectra were distorted at wavelength values below 205 nm as a result of high (>800) voltage.

LPS extraction by TX-114 significantly reduces LPS contamination of β-lactoglobulin and soy protein

The initial concentration of LPS in BLG and SPE was estimated to be 1216 EU/ml and 6 EU/ml, respectively. TX-114-assisted LPS extraction reduced the LPS concentration in BLG to 0.87 EU/ml and in SPE to 0.05 EU/ml demonstrating in purification efficiencies of 99.9% and 99.2%, respectively (Figure 4A). Repeated (one, two or three times) LPS-extraction did not lower LPS levels further (Figure 4B) but caused an increase of the residual TX-114 concentration in the protein sample (Figure 1E).

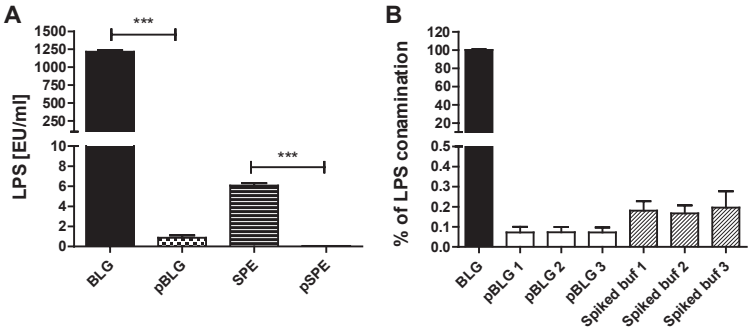

Figure 4. TX-114 assisted LPS extraction reduce LPS levels in BLG and SPE extracts to the levels below 1 EU/ml. (**A**) LPS concentration in protein preparations before (BLG, SPE) and after purification (pBLG, pSPE) with TX-114 method. (**B**) Repeated TX-114-assisted LPS extraction does not affect LPS extraction efficiency. BLG or PBS spiked with 0.45 EU/l of LPS were treated once, twice or three times with a 2% (v/v) TX-114 solution and subjected to incubation at 4˚C for 30 min followed by a 10 min incubation at 37˚C and centrifugation.

Stimulation of TLR4 and TLR2 receptors by β-lactoglobulin and soy protein

Figure 5 shows the levels of TLR4 and TLR2 receptor mediated signaling in HEK 293 cells incubated with non-treated and TX-114 treated BLG and SPE. Incubation of HEK-Blue 293 cells with non-treated BLG resulted in high TLR4 activation (on average 10-fold higher than medium control) reaching the plateau for all tested concentrations (Figure 5A). The level of TLR4 stimulation was significantly lower (up to 88%), but followed a concentration-dependent pattern, upon incubation of cells with LPS-purified BLG. Incubation of non-treated SPE with HEK-Blue 293 cells resulted in low activation of TLR4 (2-fold higher than medium control). This was observed only for the highest concentration of protein at 2.8 mg/ml, (corresponding with 1.2 EU/ml of LPS) and was reduced but not eliminated by treatment with TX-114 (Figure 5C). These data suggest that SPE itself, in the absence of LPS, may also induce TLR4 activation. A dose-dependent activation of TLR2, at lower level compared to TLR4, was observed upon incubation of HEK-Blue 293 cells with non-treated BLG which was significantly reduced upon extraction of LPS from the protein (Figure 5C). An activation of TLR2 by LPS-purified BLG at the concentration of 2 mg/ml (0.17 EU/ml of LPS contamination) may be explained by an immunomodulatory effect of BLG itself as LPS at a concentration of 1 EU/ml did not induce TLR2 activation. However, contamination of the BLG preparation with other TLR2 ligands could not be excluded. Collectively, these data show that the described LPS extraction procedure sufficiently lowers LPS concentrations to reveal the immunomodulatory effects of proteins.

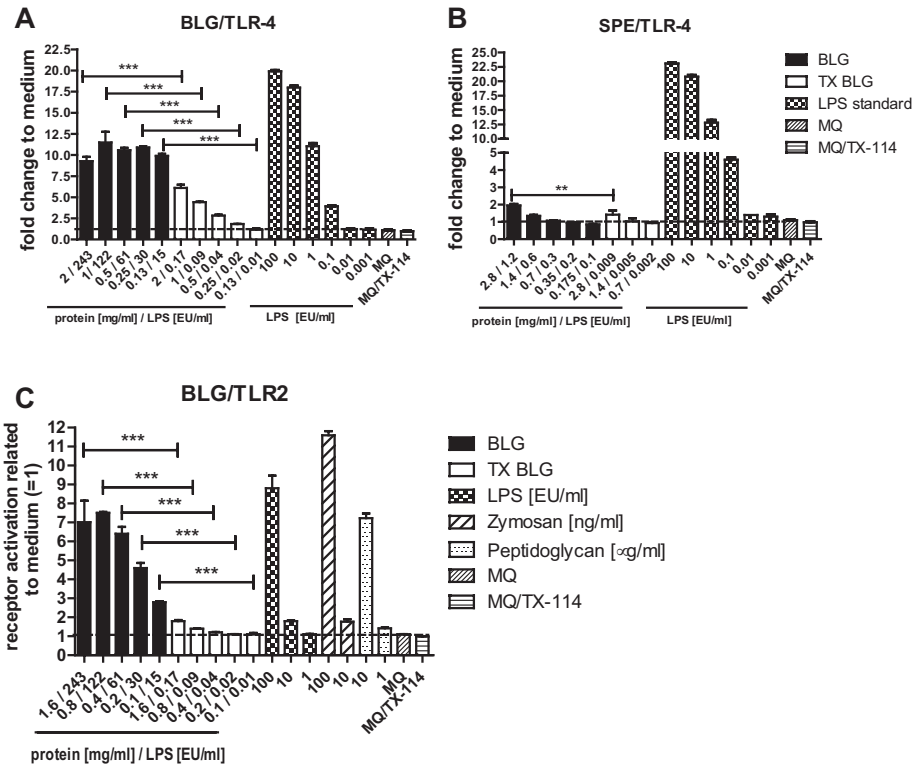

Figure 5. Reduced TLR4 (A,B) and TLR2 (C) activation upon incubation of HEK-Blue 293 with LPS purified protein preparations. HEK-Blue 293 cells were incubated 24 h with non-treated BLG (BLG, black bars **A, C**), BLG purified with TX-114 method (TX BLG, white bars **A, C**), non-treated SPE (SPE, black bars B), SPE purified with TX-114 method (TX SPE, white bars B). The samples were tested in a range of dilutions and the corresponding concentration of protein (mg/ml) and LPS (EU/ml) in each dilution is presented on the x axis. The results are expressed as the relative to unstimulated cells (medium control =1). Statistically significant differences between corresponding dilutions of LPS-purified and non-treated preparations are shown ($p<0.05$). LPS standard curve, MQ water and MQ water spiked with 0.005% of TX-114 (MQ/TX-114) were used as controls.

THP-1 macrophages as a model to study immunomodulatory potential of BLG

To establish the potential of THP-1 derived macrophages to serve as a model to study the immunogenic potential of proteins we first analyzed the sensitivity threshold of THP-1 macrophages to *E. Coli* derived LPS by analyzing gene expression of inflammatory cytokines and measurement of released IL-8 levels in the medium (Figures 6A- B). LPS concentrations of 10 pg/ml and higher induced a pro-inflammatory gene response in THP-1 macrophages by significantly increasing TNF expression. Gene expression levels of IL-1β and IL-8 were increased albeit not

significantly (Figure 6A). These observations at the mRNA level directly translated into increased levels of IL-8 upon incubation of THP-1 cells with LPS at a concentration of ≤50 pg/ml (Figure 6B). Next, we incubated THP-1 macrophages with non-treated and LPS-purified BLG to investigate its immunomodulatory potential in the absence of interfering LPS. Non-treated BLG at a concentration of 25 μg/ml (304 pg/ml of LPS) significantly increased the level of IL-8 while higher concentration of BLG of 100 μg/ml increased significantly the secretion of IL-1β and IL-6 (Figures 6C-E). Incubation of cells with LPS-purified BLG diminished the secretion of all three cytokines (IL-1β, IL-8 and IL-6) when compared to non-treated BLG, which was shown to be contaminated with LPS. These observations indicate that LPS can be eliminated from protein preparation in such a manner that the isolated natively folded protein-induced immunogenic effects on immune cells in culture can be reliably measured in the absence of LPS.

Discussion

Our work aimed at optimizing an LPS extraction procedure from proteins to enable functional investigation of the immunological properties of these proteins without the interfering effect of LPS or LPS extraction agents.

TX-114 based phase separation was shown to be an efficient, simple and cost-effective strategy to significantly reduce or even eliminate endotoxin contamination from biological preparations[232,239–241,243–246,248,254]. Despite the scientific need for LPS removal to establish the immunological activity of natively folded proteins, only few studies reported on the experimental conditions that affect the efficiency of endotoxin removal from protein extracts using TX-114. Reported protocols vary in incubation conditions and TX-114-removal strategies after LPS-extraction[234,239,240,253], which we have shown to critically influence the application of the purified protein in *in vitro* assays[248]. Lopes *et al.* (2010) showed that separation of LPS into a TX-114 micelle-rich phase is more effective at higher TX-114 concentrations and that extraction efficiency is positively correlated with increasing incubation temperature (33-41°C)[248]. We therefore used a concentration of TX-114 of 2% (v/v) and incubated the TX-114-protein mixture at a temperature of 37°C, resulting in an LPS extraction efficiency of 99.9% for BLG and 99.2% for SPE after a single TX-114 treatment. When using TX-114 at a concentration of 1% (v/v), some studies observed improved LPS-extraction efficiency after repeated treatment of the protein solution with

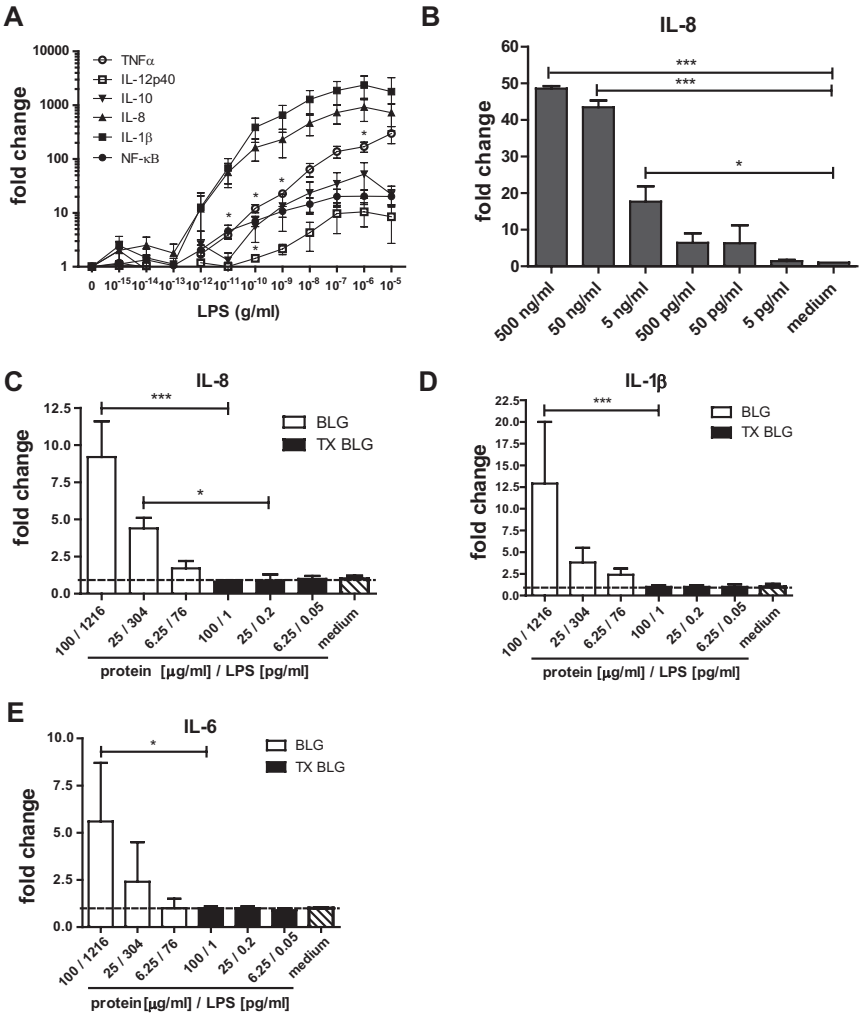

Figure 6. THP-1 derived macrophages as a model to study immunomodulatory potential of non-purified and LPS-purified BLG preparation. (**A**) LPS dose-dependence and differential gene expression of inflammatory cytokines and NF-κB in THP-1 macrophages. The data obtained for 3 repetitions is normalized to GAPDH and relative to unstimulated macrophages cultured in medium. Statistically significant differences relative to non-stimulated THP-1 macrophages were calculated with Student's *t*-tests: * = p<0.05. (**B**) LPS dose-dependent secretion of IL-8 by THP-1 macrophages. THP-1 cells were incubated 24h with increasing concentration of LPS and IL-8 concentration in supernatant was determined with flow cytometry. The results and statistically significant differences are related to unstimulated cells medium control =1). (**C-E**) Dose dependent secretion of pro-inflammatory cytokines from THP-1 macrophages incubated 24h with non-purified BLG (BLG) and BLG purified with TX-114 method (TX BLG). The results are expressed as the relative to unstimulated cells (medium control =1). Statistically significant differences between corresponding dilutions of purified and non-purified BLG preparations are shown (p<0.05).

TX-114[239,240,246]. We observed that the efficiency of LPS extraction did not increase further with additional cycles of 2% (v/v) TX-114 treatment. The observed differences may be explained by interactions between LPS and proteins resulting in the formation of protein–endotoxin complexes, as described before for different types of proteins[206,255]. Such endotoxin-protein complexes are able to activate Toll-like receptor 4 and induce the secretion of pro-inflammatory cytokines as it has been shown previously for lactoferrin[238]. Protein associated LPS is difficult to remove during purification procedures[233] although Reichelt and colleagues showed affinity chromatography with TX-114 as an effective method to remove tightly bound endotoxin from recombinant proteins[241]. Although to our knowledge the interaction between LPS and BLG and soy proteins have not been reported but can also not be excluded. Therefore, the application of optimized TX-114 (2%) for studied proteins provided sufficient detergent and micelle forming ability to dissociate also protein-bound LPS as no secretion of pro-inflammatory cytokines was observed after incubation of macrophages with purified BLG/soy protein sample. Nevertheless, the efficiency at which endotoxin is removed, especially when tightly associated, from a protein may depend on various factors including the type of interactions between the endotoxins and the protein. A number of proteins and peptides with different physicochemical characteristics[244,255–258] were shown to interact with LPS via electrostatic interactions for basic proteins and hydrophobic interactions or formation of dynamically stable calcium bridges played a role for neutral and acidic proteins[232]. Moreover, it has been shown that the concentration of the protein itself effects significantly the amount of disaggregated endotoxin and the formation of protein-LPS complexes. The protein concentration was also shown to has significant effect on protein-LPS binding and endotoxin removal by ultrafiltration membranes (300,000 NMWCO)[259]. Because many variables influence the LPS extraction efficiency, it is challenging to develop one method that is generally suitable for LPS removal from different protein sources and it cannot be concluded that the method described here is indeed suitable to extract LPS from all these different proteins. However, the TX-114 method applied in this study was demonstrated to efficiently extract LPS from two proteins with highly different physio-chemical profiles including BLG, a protein of animal origin belonging to the lipocalin family and SPE, a mixture of storage proteins, mostly globulins, isolated from soy.

The protein structure-function paradigm dictates that any change in protein structure, induced by the LPS extraction procedure may directly translate into an alternative

immunological profile[260]. Although the TX-114 treatment does not influence the biological activity and functionality of proteins[240,242,246] a limited number of studies focus on the structural properties of the proteins after treatment with TX-114[245]. BLG and SPE studied here, did not show differences in secondary, tertiary and quaternary structure upon treatment as determined by SDS-PAGE, far-UV circular dichroism spectroscopy and intrinsic tryptophan fluorescence. Moreover, TX-114 treatment did not result in loss of BLG and SPE based on protein concentration and SDS-PAGE. However, based on literature[261], the significant decrease in protein concentration after TX-114 treatment of hydrophobic proteins can be expected making the procedure less suitable for highly hydrophobic proteins.

A major disadvantage of using TX-114 to extract LPS removal is that residual detergent persists in the protein phase after extraction resulting in toxicity of the preparations to living cells. We observed that 0.1% of TX-114 in the medium cause 35% decrease in the viability of HEK 293 cells while a concentration of 0.006% and higher was already cytotoxic for THP-1 macrophages. TX-114 concentrations of 0.02% (v/v) were previously reported to influence cellular activity of PMA-stimulated neutrophils[239] demonstrating that, even though all cell types investigated responded to TX-114, different cell types may vary in their sensitivity towards this detergent. Virtual elimination of residual TX-114 after LPS extraction therefore seems crucial for applications using cell based read-outs. We further reported that TX-114 can interfere with common protein and LPS concentration determination assays. Previously published procedures often disregard this phenomenon giving rise to conclusions based on TX-114 induced bias in concentration assays[234,244,246,248]. Buetler and colleagues (2011)[234] showed that glycolaldehyde-modified BLG preparations were unable to induce inflammatory signaling in receptor for RAGE-expressing cells after TX-114 assisted extraction of LPS. The authors concluded that cell activation shown for non-treated preparations was connected to a LPS-like lipophilic contamination. Interestingly, data presented by the same authors for glycolaldehyde-modified BLG preparations purified using affinity chromatography, which similarly resulted in efficient reduction of LPS contamination, demonstrated that incubation of cells with these preparations induced expression of TNF. Despite similar virtual elimination of LPS in both preparations, the TX-114-assisted LPS extracted glycolaldehyde-modified BLG did not induce inflammatory signaling while proteins treated by affinity chromatography did. This apparent inconsistency may be explained by the impact of TX-114 remaining after LPS-extraction in the TX-

114 treated BLG. Although centrifugation was applied in this last study to remove TX-114 from BLG preparations we observed that centrifugation reduced TX-114 levels to ±0.025% (v/v), a concentration that unambiguously affects cell viability. Similarly, Jensen and colleagues (2008)[246] showed that TX-114 phase separation can be used to remove LPS from (His)6-tagged proteins and that biological activity of the treated recombinant protein was retained. These authors performed multiple cycles of TX-114 extraction followed by dialysis to remove residual TX-114 levels. However, the TX-114 concentration after dialysis was not determined, while we observed that dialysis does not sufficiently lower TX-114 concentrations to levels that do not interfere with *in vitro* and cell-assays. Above examples show the need to actively remove remaining TX-114 levels from treated protein solutions and to monitor levels of TX-114 after LPS-extraction. Based on our experiments comparing a range of TX-114 removal procedures, the application of Triton-binding Bio-Beads was found to reduce the TX-114 concentration to a non-cytotoxic concentration of 0.005% (v/v) without influencing the protein structure and yield upon treatment as previously reported[242,243,245]. The optimized TX-114 based LPS-extraction procedure described in this manuscript allowed investigation of the immunologic potential of protein preparations without interference of LPS and TX-114. For this, we used HEK293 cells expressing the LPS receptor subunits (TLR4, CD14 and MD-2) and THP-1 macrophages that are naturally sensitive to endotoxins. Similar to Schwarz and colleagues (2014)[262] we observed that incubation of HEK 293 cells with LPS showed activation of TLR4 and -2 at LPS concentrations higher than 1 pg/ml and 100 pg/ml, respectively. LPS at a concentration of 10 pg/ml and higher increased the expression of pro-inflammatory cytokines from THP-1 macrophages. Consistent with these findings, LPS concentrations of 10 and 20 pg/ml were reported to induce secretion of IL-6 and TNF from murine bone marrow-derived dendritic cells[263] and activation of monocytes isolated from human blood, especially CD1c+ dendritic cells[262]. Compatibility of the outcomes suggests that both HEK293 cells expressing the LPS receptor subunits and differentiated THP-1 macrophages may be used as a relevant model for the screening of protein preparations for biological effects of LPS contamination. The biological effects of LPS depend not only on its concentration but also on the source of endotoxin. It has been shown that the primary structure of the lipid A moiety, but also a specific conformation enhance its biological activity by enabling binding to the Toll-like receptors and accessory proteins, LPS binding protein (LBP), CD14 and the (TLR4)–MD-2 complex[229,264,265]. That suggests that comparable LPS amounts present in protein preparations may cause different

biological effects dependently of the source of contamination. Therefore, before an application in immunological *in vitro* studies each protein preparation should be tested individually in the functional screening assay for LPS activity like presented in this paper HEK 293 cell line transfected with TLR-4 receptor. Incubation of TLR4 transfected HEK293 cells with LPS-extracted BLG resulted in stimulation of TLR4 but significantly higher levels were achieved by non-treated BLG demonstrating that contaminating LPS interferes with reliable immunogenic profiling of protein preparations. Similarly, while non-treated BLG induced large-scale upregulation of IL-8, IL-1β and IL-6 in THP-1 macrophages, upon LPS-extraction of BLG this effect was eliminated. The BLG protein preparations, under these conditions and up to a concentration of 100 μg/ml, did not show immunomodulatory properties. This finding is in line with that of Brix and colleagues (2003)[266] who showed that endotoxin is a major immunostimulatory component present in commercial β-lactoglobulin preparations.

Conclusion

Investigation of immunomodulatory properties of food proteins requires an unequivocal conclusion on the source of the observed immunological activity. We optimized an LPS extraction method consisting of a two-step procedure involving TX-114 assisted LPS extraction followed by treatment of the protein preparations using high-affinity triton-binding beads to reduce the detergent residuals to non-toxic levels. The procedure was further validated with functional assays using HEK293 cells expressing LPS receptor subunits and differentiated THP-1 macrophages to demonstrate that the TX-114 based LPS extraction method allows measurement of the immunologic potential of proteins without the interfering effect of LPS or Triton.

Acknowledgements
We thank Robin van den Braak who supported the optimization of the described procedure.

Supplementary files

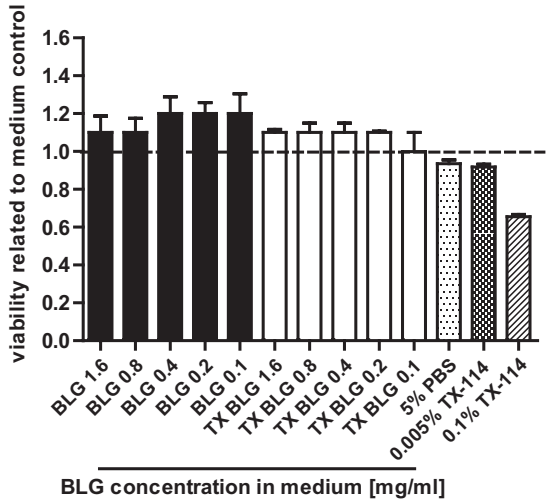

Figure S1. Triton X-114 at a concentration of 0.005% (v/v) does not influence the viability of HEK-Blue 293 cells. HEK-Blue 293 cells were cultured for 24 h in the presence of non-treated BLG (BLG) and BLG purified with TX-114 (TX BLG) and the viability of cells was measured with CellTiter 96 AQueous One Solution Cell Proliferation Assay. Results are expressed as relative to unstimulated cells (medium control =1). PBS, PBS spiked with 0.005% (v/v) TX-114 and 0.1 % (v/v) TX-114 were used as controls.

Chapter 5

Induction of human tolerogenic dendritic cells by 3'-sialyllactose via TLR4 is explained by LPS contamination

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Abstract

The human milk oligosaccharide 3'-sialyllactose (3'SL) has previously been shown to activate murine dendritic cells (DC) in a TLR4-mediated manner *in vivo*. The aim of this study was to investigate whether 3'SL has similar immunomodulatory properties on human DC. Initial experiments indicated that the presence of 3'SL during DC differentiation resulted in phenotypically distinct DC. Moreover, 3'SL induced semi-mature (tolerogenic) DC, that produced IL-10 but abrogated IL-12p70 and TNF levels upon stimulation with several TLR ligands. These effects of 3'SL were completely abolished by blocking TLR4 signaling. To exclude possible LPS contamination in the commercially available 3'SL, a highly sensitive LAL assay was performed. Unexpectedly, LPS was detected in commercial 3'SL from different suppliers. After removal of LPS from the 3'SL, its ability to modify DC differentiation was studied. LPS removal from 3'SL completely abrogated the functional and phenotypical changes. In conclusion, in contrast to what has been reported in murine *ex vivo* cultures, LPS-free 3'SL does not activate NF-κB via TLR4 in humans. Removal of LPS from (oligo)saccharide preparations is therefore an absolute necessity to study their potential immunomodulatory function.

Introduction

The mucosal immune system is largely shaped during the first months of life under the influence of microbial colonization and diet[49]. The resulting proper development of tolerance in early life will prevent the development of allergies, autoimmune diseases and chronic inflammation of the mucosa later in life[127]. Human milk is an unique exogenous source of oligosaccharides and proteins which promote growth and immune function[40,49,158]. Well-studied dietary factors such as vitamin A and D derivatives retinoic acid and $1,25(OH)_2D_3$ (VitD3), respectively, can induce regulatory responses[27,162]. Human milk oligosaccharides (HMOs) are also postulated to regulate mucosal immune function, although its mechanism is poorly understood[40].

HMOs escape enzymatic hydrolysis and are fermented in the colon, facilitating the outgrowth of (beneficial) bacteria. Low levels of HMOs can be absorbed in the small intestine and reach systemic circulation[105,106]. HMOs consist of the disaccharide lactose at the reducing end that can be elongated into either a trissacharide containing a neutral (fucose) or acidic (sialic acid) fraction or is further elongated into more complex oligosaccharides. The binding of the sialic acid N-acetylglucosamine to the backbone via either a α-2,3- or α-2,6 linkage results in 3'SL or 6'SL, respectively. 3'SL and 6'SL are abundant acidic oligosaccharides present in human and bovine milk[78]. Most infant nutrition does not contain such natural HMOs which are replaced by the lactose- or plant-derived oligosaccharides galactooligosaccharide (GOS) and fructooligosaccharide (FOS)[267].

To date it has been appreciated that the architecture of HMOs is essential for their effect on the immune system[267]. Human milk components, including HMOs are postulated to modulate TLR signaling on immune cells and induce regulatory responses[268,269]. Moreover, lactose- or plant derived oligossacharide mixtures were shown to activate different cells of the innate immune system via TLR4[270–272]. The acidic (i.e. sialic acid containing-) HMO fraction – but not the neutral HMO fraction – was shown to induce IFNγ- and IL-10-producing T cells in human mononuclear cell cultures[273,274]. These studies indicate that the acidic HMO fraction is capable of modulating Th2 responses. Oral treatment with the natural milk trisaccharides 6'SL and 2'FL in an OVA induced food allergy model also induced regulatory immune responses and attenuated allergic symptoms[155]. In contrast, Kurakevich and colleagues (2013) demonstrated that mouse pups receiving milk devoid of the acidic 3'SL were

less prone to develop colitis and isolated MLN DC were shown to be activated directly by 3'SL in a TLR4-dependent manner as the cause of inflammation[156]. On the other hand, TLR4 activation was shown to be essential for the proper immune education[275]. Activation of the immune system by such HMOs via TLR4 therefore may be essential for immune development in early life. The aim of the current study was to investigate whether 3'SL exerts similar effects on human immune function.

Materials and Methods

Isolation and culturing of monocyte-derived DC

Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation from buffy coats obtained from healthy anonymous donors (Sanquin blood bank, Nijmegen). First, the blood was diluted 1:1 in phosphate buffered saline (PBS) (Mg^{2+} and Ca^{2+} free, Lonza, BE17-516F). The diluted blood was loaded on Ficoll-Paque (Amersham Bioscience, Uppsala, Sweden) and centrifuged for 5 minutes at 200 g followed by 15 minutes at 500 g both without brake. The PBMC layer was collected and washed three times in 10 ml PBS. After the third wash, the cells were spun down and the pellet was resuspended with anti-human CD14 magnetic beads (BD Biosciences, 557769). CD14+ cells were isolated on a separation magnet (BD IMagnet, BD Biosciences) according to the manufacturer's instructions. This positive selection resulted in a highly pure population (96.5 ± 2.1%; n =5) of monocytes. Monocytes were reconstituted in RPMI 1640 (Gibco, 22409-015) and 10% FCS (Gibco, 10270-106), normocin (100 µg/ml, Invivogen, anti-nr-1), penicillin and streptomycin (100U/ml, Gibco, 14150-122) and cultured in flat bottom 96 wells plates (100.000 cells/well). Monocytes were cultured for six days in the presence of 20 ng/ml IL-4 (Peptrotech; 200-04) and GM-CSF (Peprotech; 300-03) with or without 1 µg/ml – 1 mg/ml Triton X-114 treated- or commercially available 3'SL (Carbosynth; OS04397 or SantaCruz; SC-216626) or ultrapure LPS (0.0001-100 EU/ml) from *E. coli* OIII:B4 (Invivogen; tlrl-3pelps). TLR4 signaling was blocked by adding an intracellular TLR4 inhibitor (Invivogen; CLI-095, 3µM) for 1 hour at 37 degrees Celsius prior to adding stimuli. After six days, immature DC were matured with 1 µg/ml LPS (*Escherichia coli*, Sigma, L2880) or 3 µg/ml R848 (Invivogen: tlrl-r848-5) and 20 µg/ml Poly I:C (Sigma, P1530) for 48 hours.

Isolation and staining of moDC

After 24 hours, 6 days (immature-) or 8 days (mature-) DC were incubated on ice (while shaking) for 30 minutes in ice cold FACS buffer (PBS (Lonza, BE17,516F) containing 0.5% BSA fraction V (Roche, 10735086002), 2.0 mM EDTA (Merck, 108418) and 0.05 NaN_3) to facilitate the detachment of DC from the surface. Surface marker expression was analyzed by using fluorochrome-conjugated antibodies directed against CD14 (FITC; BD Biosciences, 555397), CD86 (V450; BD Biosciences, 560357), CD83 (FITC; BD Biosciences, 556910), HLA-DR (APCef780; eBiosciences, 47-9956-42), CD80 (PE-Cy5; BD Biosciences, 559370), PD-L1 (PE-Cy7; BD Biosciences, 558017), CD1a (PerCP/Cy5-5; Biolegend, 300130) or ICOSL (PE; BD Biosciences, 552502). Matching isotype controls mIgG2a FITC (BD Biosciences, 555573), mIgG1 V450 (BD Biosciences, 560373), mIgG1 FITC (BD Biosciences, 555748), mIgG2b APC-ef780 (APCef780; eBiosciences 47-4732-80), mIgG1 PE-Cy5 (BD Biosciences, 555576), mIgG1 PE-Cy7 (BD Biosciences, 557872) were included by adding the –to the specific Ab panel corresponding– isotype mixture to cells. Fluorescence minus one (FMO) controls were included in channels with spectral overlap. Compensation beads (eBiosciences, 01-2222-41) stained with single antibodies were run for every experiment. Cells were washed with 200µl FACS buffer and stained by incubating the antibody mixture for 30 minutes in the dark at 4°C. Before measuring 7-AAD (BD Biosciences; 555815), DRAQ7 (Abcam; ab109202) or fixable viability dye ef506 (eBiosciences, 65-0866-14) was added to stain nonviable cells. The fixable viability dye requires two washing steps with PBS and 30 minutes of staining in the dark at 4°C. Thereafter cells were resuspended in 100µl FACS buffer and acquired on a BD FACS Canto II (BD Biosciences) and analyzed using the FlowJo software V10.

Quantification of cytokine levels in supernatants

Levels of IL-6, IL-10, TNF and IL-12p70 were measured in the supernatants of moDC cultures using cytometric bead array technique (BD biosciences). Individual flex-sets for IL-6 (558276), TNF (560112), IL-10 (558274) or IL-12p70 (558283) were run according to the manufacturer's instructions.

TLR signaling capacity of milk oligosaccharides

HEK-293 cells expressing human TLR2, TLR2-1 or TLR4 and harboring a pNIFTY construct (Invivogen, Toulouse, France) were grown on medium containing DMEM and Glutamax (Fisher Emergo, Landsmeer, the Netherlands) supplemented with

10% FCS, 100 ug/ml penicillin/streptomycin (Sigma, St Louis, MO), Zeozin (50 µg/ml) and Normocin (100 µg/ml) (Invitrogen, Carlsbad, USA) in an atmosphere of 5% CO_2 at 37ºC. TLR2, TLR2-1 and TLR4 expressing HEK cells were additionally supplemented with Blasticin (100 ug/ml), Puromcyin (100 ug/ml) or HygroGold (45 ug/ml) (Invitrogen, Carlsbad, USA), respectively. HEK-293 cells were seeded at $3*10^5$ cells/ml and cultured overnight before stimulation the next day. NF-κB activation was measured after 24-hour stimulation by adding Bright-Glo™ (Promega, Fitchburg, USA) substrate to cells. The plate was shaken and luminescence was measured using a spectramax M5 (Molecular Devices, Sunnyvale, USA).

LPS detection

3'SL was tested for LPS contamination by a recombinant factor C LAL assay that was performed according to the manufacturers recommendations (EndoZyme recombinant factor C assay, Hyglos; 609050).

LPS removal with Triton X-114

An optimized method of LPS removal by Triton X-114 (Amresco, cat. # M114) was used [210]. In short, 2 % v/v Triton X-114 was added to the sample and stirred for 30 minutes at 4˚C and transferred to a 37˚C water bath for 10 minutes. The micelles were spun down by centrifugation for 10 minutes at 20.000g at 25 ˚C. The upper layer was collected and treated with 10 mg/ml Bio-beads SM-2 (cat. # 152-8920) to remove triton traces.

Analysis of Sialyllactose and Sialic Acid by HPAEC-PAD

LPS-free 3'SL was loaded on a Dionex system to confirm that the Triton X-114 method only removed LPS and not 3'SL. 3'SL was separated using a CarboPac PA1 column (250x4 mm) and a CarboPac PA1 guard column (50x4 mm) with a flow rate of 1.0 ml/min. 3'SL was detected using an AU-electrode (2 Hz). The following eluents were respectively used; 200mM NaOH (carbonate free), 100mM NaOH (carbonate free) + 500mM NaAc and HPLC grade water. The percentage of eluent A, B and D fluctuated between 0-55 minutes. 3'SL was quantified using an external sialyllactose sodium salt standard (mixture of 3'SL and 6'SL Carbosynth; OS04397 and OS04398).

Statistics

Statistical differences between two groups were tested by paired student t-tests. A repeated measures ANOVA with a Dunnet's or Tukey's post-test was performed when multiple groups were compared to untreated cells (moDC) or between different groups, respectively. If the represented data was normalized to the moDC per donor, statistics was performed on the raw data, maintaining the variances of the groups[276]. Significant differences between conventional differentiated cells and a single treatment group or multiple groups were indicated by letters or asterisks, respectively; a/*** = P< 0.001, b/** = P< 0.01 and c/* = P< 0.05. All values are represented as mean +/- SEM. Graphpad Prism V. 5.0 was used for all statistical analyses.

Results

3'SL induces phenotypical and functional distinct DC

Monocytes require a differentiation time of 5-7 days in the presence of IL-4 and GM-CSF to become functional immature DC (iDC)[277]. Monocytes were cultured for six days in the presence of IL-4 + GM-CSF with or without 3'SL to investigate the effect of 3'SL on DC differentiation (Figure 1A). 3'SL concentrations used were based on physiological concentrations of 3'SL present in breast milk (76 - 350μg/ml)[78]. Addition of 3'SL to the monocytes resulted in an early increase in membranous PD-L1 compared to monocyte-derived DC (moDC) (Figure 1B), that was not further elevated during the differentiation period (Figure 1C). 3'SL also induced CD80 expression on immature DC (iDC; day 6), although it was not elevated after 24 hours compared to moDC (Figure 1C). iDC cultured in the presence of 3'SL showed upregulation of CD80 and PD-L1 in a dose-dependent fashion (Figure 1D). Since 3'SL was responsible for upregulation of specific activation markers, we investigated whether cytokines were produced during the differentiation phase. 3'SL induced the production of IL-6 and TNF. This peaked already after 24 hours and did not increase during prolonged incubation (Figure 1E). 3'SL induced cytokine production by monocytes in a dose-dependent fashion during DC differentiation (Figure 1F). Next, we investigated whether 3'SL altered the differentiation of monocytes into DC by determining the percentage of CD14+ and CD1a+ cells. CD14+CD1a- monocytes differentiated into both CD14-CD1a+ and CD14-CD1a- iDC. After incubation with 3'SL a significantly higher percentage of cells were CD14+ and a lower percentage was CD1a+ compared to moDC (Figure S1). This indicates that 3'SL halts DC differentiation, resulting in a monocyte-like DC phenotype.

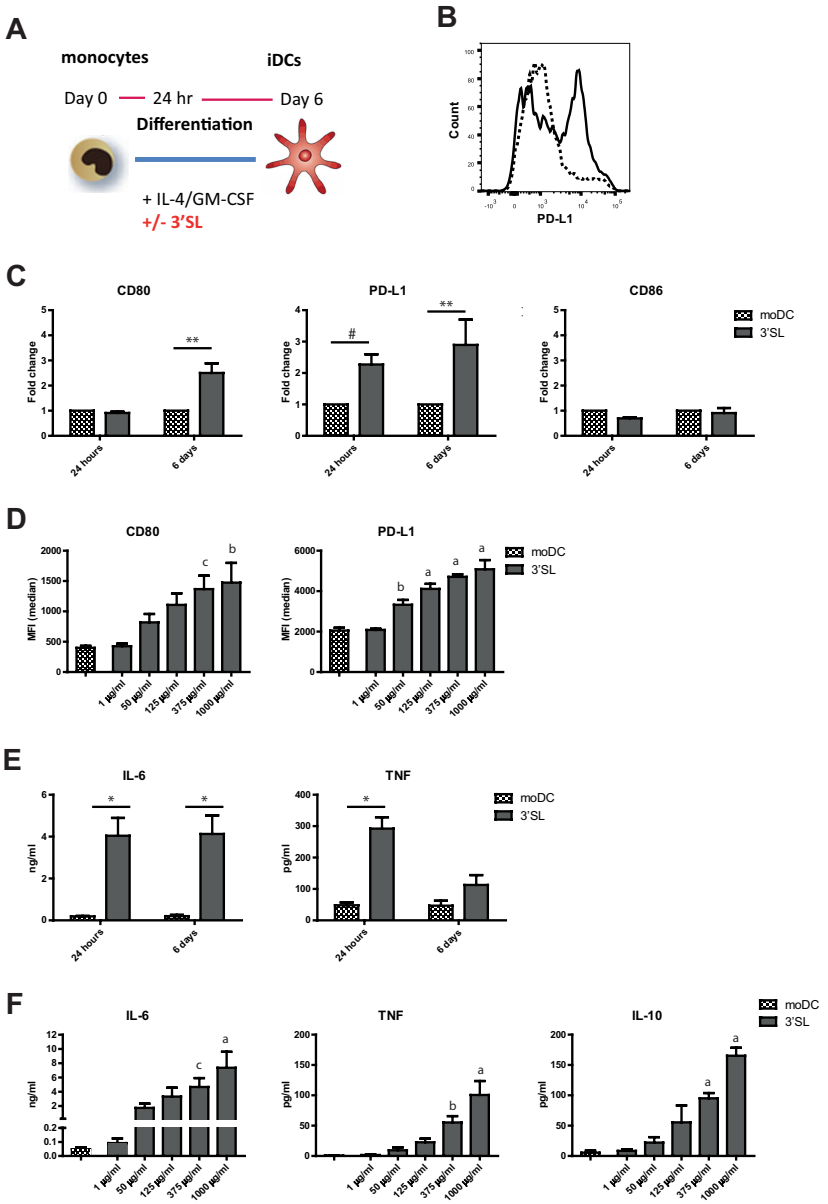

Figure 1. 3'SL induces phenotypical and functional distinct DC. (**A**) Monocytes were differentiated with IL-4 and GM-CSF into iDC for six days in presence or absence of 3'SL. Cells were harvested after 24 hours (during differentiation) or after six day of differentiation (end of differentiation period). (**B**) PD-L1 expression after 24 hours in the presence (solid line) or absence (dotted line) of 3'SL was measured on monocytes. Histogram shows one representative donor. (**C**) MFI of PD-L1, CD80 and CD86 of 3'SL exposed DC were compared to that of the moDC (n=3; 24 hours, n=9; six days). (**D**) MFI of PD-L1 and CD80 on iDC differentiated in the presence of 3'SL (1 – 1000 µg/ml) is shown (n=3-5). (**E**) A small fraction (1/10 of total volume) of supernatant was collected from the same culture on day 1 and day 6 and the cytokines IL-6 and TNF were quantified (n=3). (**F**) IL-10, TNF and IL-6 were measured in supernatants of iDC cultured in the presence of different doses of 3'SL (n=5).

3'SL induces the differentiation of monocytes into regulatory DC

CD1a$^-$ and CD1a+ DC were shown to be functionally different in their capacity to secrete cytokines and chemokines and activate T cells[213]. We therefore investigated whether 3'SL exposure during DC differentiation also affected the functionality of these DC when activated with different TLR ligands (Figure 2A). Tolerogenic DC are defined by their semi-mature (CD86^{int}CD83^{int}) phenotype after encountering a TLR ligand, which shapes the balance between tolerance and immunity[162]. DC differentiated in the presence of 3'SL showed a dose-dependent decrease of cells with a mature phenotype (CD83+CD86+) upon stimulation with LPS (Figure 2B) or Poly I:C (20 μg/ml) and R848 (3 μg/ml) (Figure 6C). 3'SL-exposed DC showed lower expression levels of CD86, CD83 and ICOSL in contrast to HLA-DR and PD-L1 that were not significantly lower expressed compared to moDC (Figure 2C). Moreover, 3'SL-exposed DC produced similar levels of IL-10 and fully abrogated IL-12p70 and TNF levels compared to moDC (Figure 2D). Thus the notion that differentiation in the presence of 3'SL induces regulatory DC was further supported by the cytokine production upon encountering different TLR ligands.

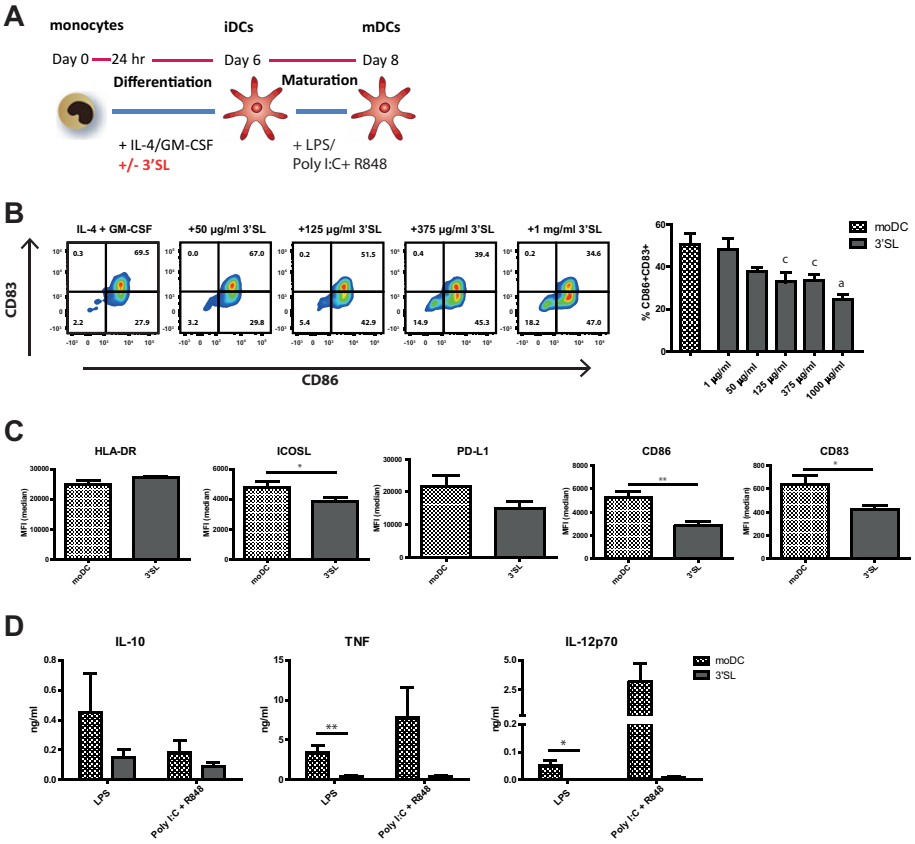

Figure 2. 3'SL induces differentiation into tolerogenic DC. (**A**) Monocytes were differentiated with IL-4 and GM-CSF into iDC for six days in the presence or absence of 3'SL. These different iDC types were stimulated with different TLR ligands for 48 hours. (**B**) The percentage of CD83+CD86+ DC was determined on DC differentiated in presence of 3'SL (1–1000 µg/ml) and subsequently stimulated with LPS. FACS plots depict one representative donor and the bar graphs show the mean of five donors. (**C**) Similarly, the MFI of various surface markers on LPS matured DC was compared between 3'SL (375 µg/ml) exposed DC and moDC (n=3-5). (**D**) Cytokine levels were measured in the supernatant (n=3; Poly I:C + R848, n=7; LPS).

3'SL induces TLR4 signaling

In mice, 3'SL was shown to directly activate TLR4 which resulted in activation of MLN DC[156]. We therefore investigated whether 3'SL could also function as a TLR4 ligand in humans. In line with the literature[156], 3'SL also showed NF-κB activation via the human TLR4 (Figure 3) and not via TLR2 or TLR2-1 (Figure S2). Comparing NF-κB activation via TLR4 of ultrapure LPS that was used as a control to that of 3'SL showed that 1 mg 3'SL induced a similar NF-κB activation as 3.64 EU of LPS (luminescence units = 2411.5 * ln[EU/ml LPS] + 5528.3; r^2 = 0.978).

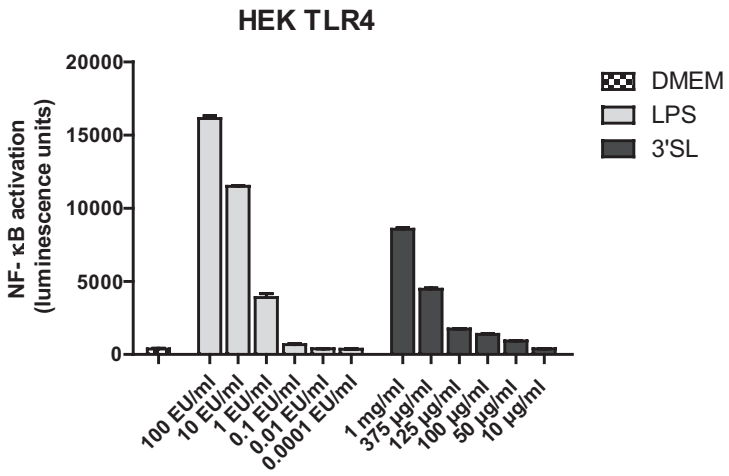

Figure 3. 3'SL induces hTLR4 mediated NF-κB activation. Commercial 3'SL (1 mg/ml – 10 µg/ml) and ultrapure LPS (100 EU/ml – 0.0001 EU/ml) were tested for their NF-κB activation via TLR4 (n=3).

5

Early activation during DC differentiation by 3'SL is mediated through TLR4 signaling

As we demonstrated that 3'SL activates NF-κB transcription via TLR4 we further investigated whether the early phenotypical changes by 3'SL were TLR4 mediated. TLR4 signaling was inhibited by adding an intracellular signaling inhibitor (CLI-095). CLI-095 (i.e. TAK-242) blocks the intracellular degradation of IRAK1[278]. Blocking TLR4 signaling was sufficient to completely abrogate upregulation of PD-L1 (Figure 4A), IL-6 (Figure 4B) and TNF production (Figure 4C) by 3'SL compared to that of moDC. 3'SL thus induces its phenotypical and functional effects during DC differentiation primarily via TLR4.

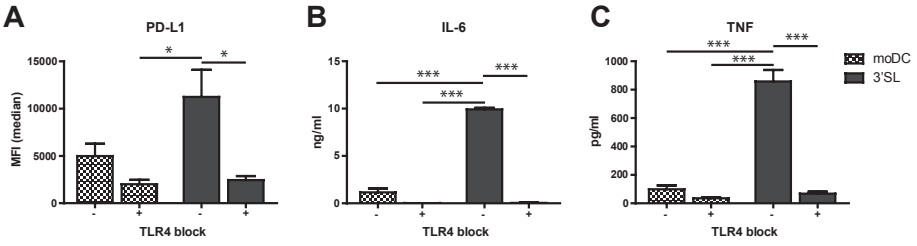

Figure 4. Early activation during DC differentiation by 3'SL is mediated through TLR4 signaling. Monocytes were cultured with IL-4 and GM-CSF with or without 3'SL (375 µg/ml). (**A**) PD-L1 expression and (**B**) IL-6 and (**C**) TNF production were measured after 24-hour differentiation. TLR4 signaling was blocked prior to adding stimuli by 1-hour pre-incubation with a TLR4 inhibitor (CLI-095; 3µM) (n=3).

TLR4 signaling is explained by LPS contamination

Since LPS is a major bacterial contaminant that signals via TLR4, we wanted to exclude that low level contamination with LPS could explain why 3'SL affected DC differentiation. Unexpectedly, two batches from different suppliers of commercial 3'SL showed to be positive in a recombinant factor C LAL assay, which resulted in TLR4 mediated NF-κB activation (Figure S3A-B). The 3'SL used for this study contained 10 ± 0.17 EU/mg 3'SL (Figure 5A) as measured by a recombinant factor C Endozyme assay. Therefore, 3'SL was treated with an optimized Triton X-114 method[210] to remove any trace of LPS. HPLC analysis confirmed that the concentration of 3'SL was unaltered after applying Triton X-114 and Bio-beads SM-2 were added to remove LPS and Triton traces, respectively (Figure S3C). This methodology decreased the LPS contamination drastically to 0.11 ± 0.08 EU/mg. (Figure 5A). Next, we investigated whether 3'SL, 3'SL devoid of LPS, and ultrapure LPS were able to activate NF-kB via TLR4. Indeed, all TLR4 signaling activity in 3'SL is abrogated by LPS removal by Triton X-114 treatment (Figure 5B).

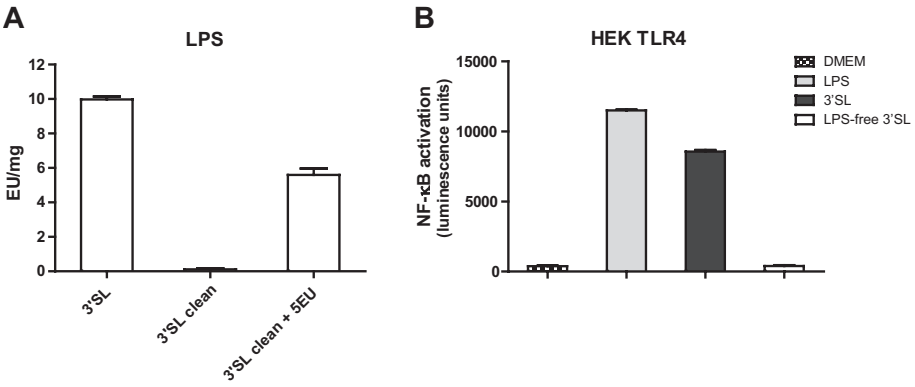

Figure 5. LPS in commercial 3'SL induces TLR4 mediated NF-κB activation. (**A**) 3'SL was tested for LPS contamination using a recombinant factor C LAL. LPS-free 3'SL was included with or without a 5 EU spiked control (n=2). (**B**) Commercial 3'SL (1 mg/ml) before and after Triton X-114 treatment and ultrapure LPS (10 EU/ml) were tested for their NF-κB activation via TLR4 (n=3).

Low levels of LPS induce regulatory DC

Having established that not 3'SL but LPS was responsible for TLR4 signaling and subsequent NF-κB activation, we compared the effect of 3'SL to LPS-free 3'SL on DC differentiation. Ultrapure LPS was used as a control with concentrations based on LPS levels measured in the LAL assay (Figure 5A; 10EU LPS/mg 3'SL). The phenotypical changes (higher CD80 and PD-L1) of 3'SL-exposed DC and cytokine production

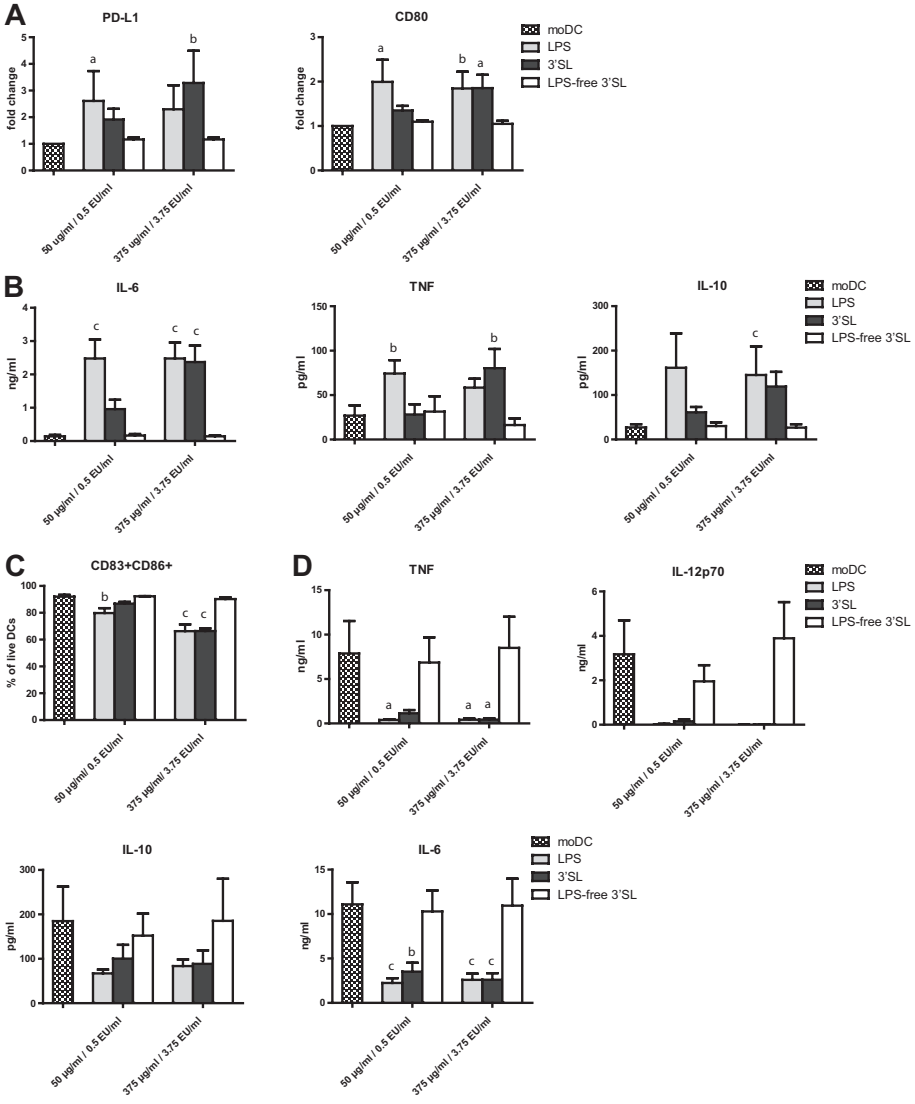

Figure 6. LPS induces tolerogenic DC. Monocytes were cultured with IL-4 and GM-CSF with or without LPS-free 3'SL, 3'SL (375 µg/ml) or ultrapure LPS. (**A**) CD80 and PD-L1 expression is shown on iDC. (**B**) the production of IL-6, TNF and IL-10 was measured in the supernatants (n=6). (**C**) Next, these iDC were stimulated with Poly I:C and R848. The percentage of CD86+CD83+ DC was assessed after 48 hours. (**D**) The cytokines TNF, IL-10, IL-6 and IL-12p70 were measured by CBA (n=3).

was restored to that of moDC when exposed to LPS-free 3'SL. LPS but not 3'SL was shown to induce higher expression of membranous CD80 and PD-L1 and cytokine production compared to moDC at a lower dose (Figures 6A-B). Our results

also indicate that DC were halted in their differentiation (remained CD14+CD1a-) solely due to the presence of LPS (Figure S1D). Thus phenotypical changes by 3'SL on iDC were shown to be fully driven by LPS. Next, we investigated the response to subsequent stimulations with different TLR ligands. Monocytes differentiated in the presence of 3'SL after LPS removal resulted in DC that showed a fully mature phenotype (CD86+CD83+) that were capable of producing IL-6, IL-12p70 and TNF in similar levels compared to moDC upon stimulation with Poly I:C + R848 (Figures 6C-D) or LPS (Figure S4). In contrast, IL-10 production was not significantly altered by the presence of LPS during DC differentiation (Figure 6D).

Discussion

Here we show that the human milk oligosaccharide 3'SL can induce regulatory DC via TLR4. However, in contrast to what was previously shown in literature for mice, not the oligosaccharide itself but LPS contamination of commercially available 3'SL was responsible for this effect. Moreover, removal of LPS from 3'SL resulted in an LPS free sample that did not induce NF-κB via hTLR4, and did not induce functional changes in DC.

HMOs are well known for their prebiotic effects by serving as an energy source for beneficial bacteria and preventing the adhesion of pathogenic bacteria[279]. In addition, HMOs can have direct effects on the immune system[40,78,155,267–272]. Since a fraction of the extremely high HMO concentrations in breast milk is taken up in the bloodstream, HMO may exert effects locally as well as systemically. Previous studies performed on human mononuclear cord blood cells showed that the acidic HMO fraction of breast milk – in contrast to the neutral HMO fraction – induced IFNγ+ and CD25+CD4+ T cells[274]. This effect was sufficient to restore the Th1/Th2 cytokine balance in PBMCs of peanut allergic patients stimulated with the major allergen Ara h1[273]. In line with these human *ex vivo* models, oral supplementation of the acidic 6'SL but also the neutral trisaccharide 2'FL attenuated allergic symptoms and boosted IL-10+ T cells in the Peyer's Patch in an OVA-induced feed allergic mouse model[155]. Despite these profound effects, no mechanistic evidence is currently available on how these HMOs exert their immunomodulation.

One specific feature of acidic HMOs is their sialic acid group that is known to bind sialic-acid-binding immunoglobulin-like lectins (Siglecs) on immune cells. The Siglec family is subdivided in two groups: evolutionary conserved receptors (e.g. Siglec-1, 2 and 4) and rapid changing CD33-related receptors[280]. Interestingly, the repertoire of CD33-related receptors is very differences between mice and man. Moreover, human but not mouse monocytes express Siglec-5, -7 and -9[281]. Siglecs contain immunoreceptor tyrosin-based inhibitory (ITIM) motifs that are responsible for dampening immune responses[280]. Nevertheless, we show here that exposure of LPS-free 3'SL does not change the phenotype and functional activity of monocytes and DC.

Both HMO and other prebiotic oligosaccharides are shown in literature to be immunogenic via interacting with TLR4 on myeloid[156,270,272] and epithelial cells[271]. TLR4 signaling is initiated by dimerization of MD-2 and TLR4, and results in activation of transcription factors that initiate the transcription of multiple genes including NF-κB, resulting in the production of pro-inflammatory cytokines[282]. Also other sources of natural polysaccharide structures are postulated to - depending on their structural confirmation - activate TLR4 signaling[283,284]. Lactose- or plant-derived oligosaccharides GOS, FOS and inulin were found to induce IL-10 production by human monocytes in a TLR4-dependent manner[270,272] and subsequently to induce regulatory T cells[272]. Similarly, these oligosaccharides induced TLR4-mediated production of chemokines by human colonic epithelial cell lines[271]. 3'SL was found to be the milk oligosaccharide that is capable of directly activating TLR4 on murine MLN DC in *ex vivo* stimulations[156]. However, here we found that 3'SL was not capable of activating NF-κB via human TLR4 after the removal of LPS. This suggests that either the specificity of mouse TLR4 is different from human TLR4, or that LPS is a confounding factor *in vitro* for HMO research.

Myeloid cells are particularly sensitive to LPS contamination[262]. Insufficient removal results in activation of human monocytes and DC; masking the true immunomodulatory effect of the molecule of interest. The data shown here demonstrate that the presence of LPS traces present in commercially available 3'SL preparations during DC differentiation results in regulatory DC. Cytokine production induced by LPS traces already peaked after 24 hours and did not further increase after prolonged exposure of the cells. This in line with previous findings that showed the induction of a negative feedback loop involving IL-6- and IL-10-mediated SOCS3

activation that dampens TLR signaling[285,286]. STAT3 was also shown to bind the PD-L1 promotor directly and to regulate its expression in TLR activated monocytes[287], explaining the early upregulation of membranous PD-L1 by LPS traces. In literature, it was shown that LPS triggers upregulation of SOCS1 that subsequently inhibits GM-CSF signaling[288]. This explains why monocytes exposed to LPS were halted in their differentiation into DC. Similarly, the IRAK1 degradation inhibitor (i.e. TLR4 inhibitor CLI-095) is known to interfere with GM-CSF signaling, resulting in lower cytokine and subsequent PD-L1 expression on TLR4 blocked- compared to IL-4 and GM-CSF exposed monocytes[289–291]. In contrast to fully mature CD1a+ DC, CD1a- DC do not produce IL-12p70 upon stimulation[212]. Indeed, we show that iDC exposed to LPS during the differentiation period produced abrogated IL-12p70 levels to multiple TLR ligands. Moreover, they showed a semi-mature phenotype, which is known to be involved in the polarization of naive T cells in regulatory T cells[145,162]. In literature, TLR4 signaling by oligosaccharides is also directly linked to the induction of regulatory responses[270,272]. Hence we stress the importance of the current findings, showing phenotypical changes and functional consequences of LPS contamination resulting in regulatory DC.

Specific glycan structures have previously been described to induce TLR4 signaling. Although this effect has also been described for the HMO 3'SL in mice, we show here that the effect of 3'SL on human monocytes and their differentiation into regulatory DC was driven by LPS contamination and not by 3'SL itself. Hence, it is an absolute necessity to remove LPS from glycan samples before studying their immunomodulatory capacity *in vitro*.

Acknowledgements
We thank Barry Schoemaker for conducting HPAEC-PAD analysis on Triton X-114 treated 3'SL and Prof. Wells of the Host-Microbe Interactomics group, Wageningen University, for providing us with the HEK TLR cell lines.

Supplementary files

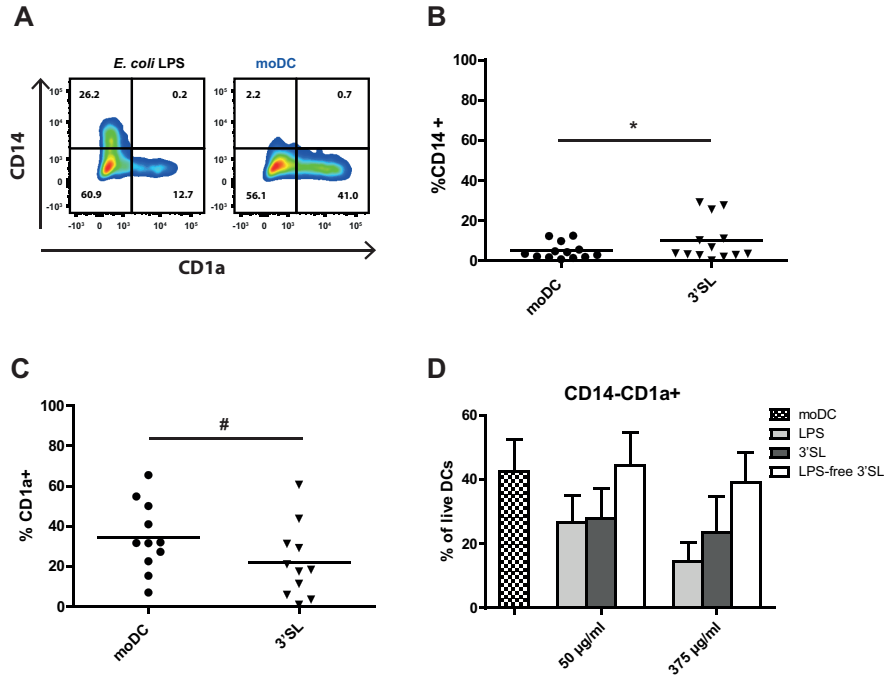

Figure S1. 3'SL halts DC differentiation. (**A**) CD14 and CD1a expression on iDC cultured with or without 200 ng/ml E. coli LPS. One representative donor is shown. iDC cultured in the presence of 3'SL was compared to moDC for their (**B**) CD14+ (n=13) and (**C**) CD1a+ (n=12) cells. (**D**) commercial-, LPS-free- 3'SL (375 µg/ml) and ultrapure LPS (0.375 EU/ml) were tested for their capacity to induce fully mature DC (CD14-CD1a+; n =3). #P<0.1.

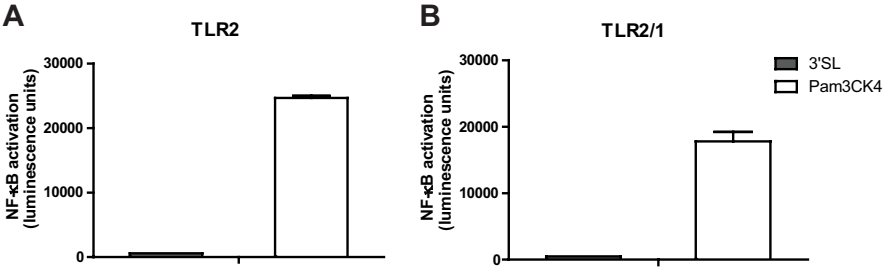

Figure S2. 3'SL does not induce hTLR2 or hTLR2-1 mediated NF-κB activation. 3'SL (375 µg/ml) showed no NF-κB activation via (**A**) TLR2 or (**B**) TLR2-1 (n=3).

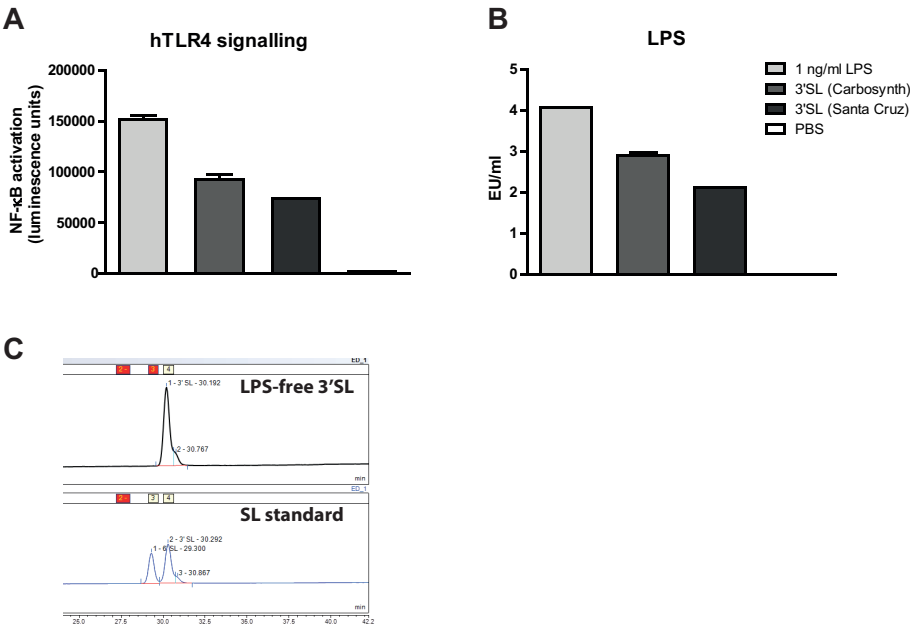

Figure S3. LPS in commercial 3'SL and 3'SL detection after LPS removal. Two different suppliers of 3'SL both show significant (**A**) levels of LPS contamination (n=3) and (**B**) subsequent TLR4 signaling (n=2). (**C**) Anion exchange chromatography showed that 3'SL was unaltered after LPS removal.

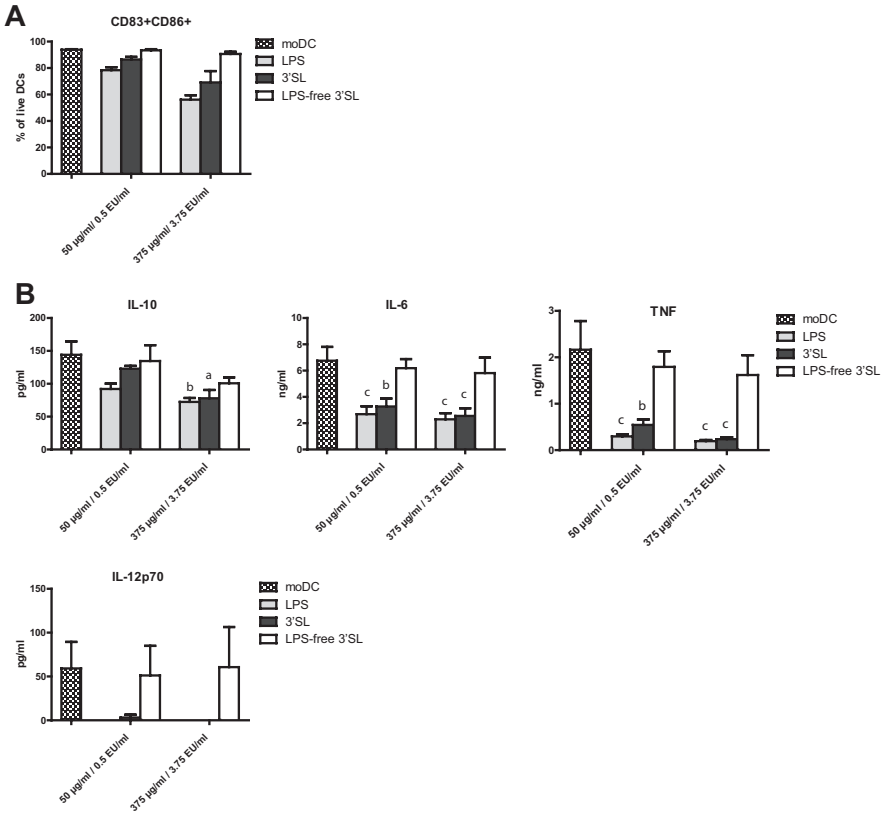

Figure S4. 3'SL induces tolerogenic DC. (**A**) commercial 3'SL, LPS-free 3'SL (375 µg/ml) and ultrapure LPS (0.375 EU/ml) exposed DC were compared for their capacity to induce mature CD83+CD86+ DC after stimulation with LPS. (**B**) The cytokines IL-12p70, IL-10, IL-6 and TNF were measured in the supernatant by CBA (n=3).

Chapter 6

The oligosaccharides 6'-sialyllactose, 2'-fucosyllactose or galactooligosaccharides do not directly modulate human dendritic cell differentiation or maturation

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Abstract

Breast milk plays an important role in immune development in early life and protects against diseases later in life. A wide range of the beneficial effects of breast milk are attributed to human milk oligosaccharides (HMOs) as well as components such as vitamin D3 (VitD3) or TGFβ. One mechanism by which HMOs might contribute to immune homeostasis and protection against disease is the induction of a local tolerogenic milieu. In this study we investigated the effect of the HMOs 6'-sialyllactose (6'SL) and 2'-fucosyllactose (2'FL) as well as prebiotic galactooligosaccharides (GOS) on DC differentiation and maturation. Isolated CD14+ monocytes were cultured for six days in the presence of GM-CSF and IL-4 with or without 6'SL, 2'FL, GOS, VitD3 or TGFβ. Additionally, immature VitD3DC, TGFβDC and moDC were used as different DC types to investigate the effect of 6'SL, 2'FL and GOS on DC maturation. Surface marker expression and cytokine production was measured by flow cytometry and cytometric bead array, respectively. Unlike TGFβ and vitD3, the oligosaccharides 6'SL, 2'FL and GOS did not influence DC differentiation. Next, we studied the effect of 6'SL, 2'FL and GOS on maturation of moDC, VitD3DC and TGFβDC that showed different profiles of HMO-binding receptors. 6'SL, 2'FL and GOS did not modulate LPS-induced maturation, even though their putative receptors were present on the different DCs types. Thus, whereas VitD3 and TGFβ halt DC differentiation, which results in phenotypically distinct tolerogenic DCs, 6'SL, 2'FL and GOS do not alter DC differentiation or maturation of *in vitro* differentiated DC types.

Introduction

Our mucosal surfaces are continuously exposed to foreign antigens that can be harmless or harmful. The mucosal immune system needs to distinguish between these antigens to mount regulatory or inflammatory responses. Proper development of the mucosal immune system in early life is therefore essential for health later in life. Breast milk plays an important role in immune development in early life[49]. It has become clear that breastfeeding is associated with lower child morbidity, higher cognitive abilities and reductions in overweight and diabetes[4]. Nevertheless, only a third of the infants are exclusively breastfed in the first 6 months of life worldwide[4]. Thus, the majority of the infants is currently depending on infant formula. Studying the functionality of breast milk components individually may identify components that aid in mucosal immune development and may improve infant formula.

Breast milk contains proteins, lactose, a wide variety of oligosaccharides, fatty acids and vitamins. These breast milk components contribute to the development of the mucosal immune system and maintain homeostasis in the neonate[34]. Moreover, breast milk components enhance epithelial gut maturation, reduce viral infections, influence the colonization of the GI tract and promote the development of tolerance[34]. A wide range of these beneficial effects are attributed to human milk oligosaccharides (HMOs)[40]. HMOs escape hydrolysis in the intestine and are metabolized by the microbiota in the colon - promoting the outgrowth of bacteria. Additionally, a small fraction of HMOs is taken up into the circulation[105,106,279]. HMOs can be subdivided into acidic and neutral HMOs. Acidic HMOs contain sialic acid, and neutral HMOs can be subdivided into fucosylated or non-fucosylated HMOs. All three groups of HMOs consist of a lactose backbone that can be further elongated into complex structures[40]. In contrast to the wide variety of oligosaccharides in breast milk, infant formulas nowadays often contain lactose- (e.g. galactooligosaccharides) or plant-derived (e.g. fructooligosaccharides) oligosaccharides, that are best known for their prebiotic effect to mimic the functionality of HMOs in breast milk[292]. In addition to the prebiotic effects of HMOs and oligosaccharides used in infant formulas, milk oligosaccharides may have direct effects on the immune system[155,156,269–274]. However, breast milk contains over 140 different HMOs and the immunomodulatory potential of only a few has been investigated to date, and detailed mechanistic evidence is currently lacking.

Dendritic cells (DC) are unique in their ability to induce antigen-specific T cell responses. Monocytes differentiate into monocyte-derived DC in the presence of inflammatory cytokines. Recent evidence suggests that our mucosal surface is in a state of "primed homeostasis", in which monocytes are recruited to the lamina propria[31]. Breast milk components such as TGFβ and the active form of VitD3 (i.e. 1α,25-dihydroxyvitamin D3) promote the differentiation of these recruited monocytes into tolerogenic DC[160,162]. Such tolerogenic monocyte-derived phagocytes are present in the GI tract and are essential to maintain oral tolerance in the lamina propria[30,293]. Additionally, components in breast milk may dampen inflammatory responses induced by immature DC upon activation by pathogens (i.e. maturation). As such, a complete pool of HMOs was shown to induce differentiation of bone marrow-derived cells into tolerogenic DC[294]. Immune regulation by such breast milk components may contribute to immune homeostasis.

To date no direct immunomodulatory effects of HMOs on human dendritic cell differentiation and maturation have been reported. We aimed to address this question by studying the effects of HMOs on DC differentiation and maturation. 6'sialyllactose (6'SL) and 2'fucosyllactose (2'FL) were used as representatives of acidic and neutral fucosylated HMOs and galacto-oligosaccharides (GOS) as neutral non-fucosylated oligosaccharides, which were compared to the known tolerogenic effects of TGFβ and VitD3 that were included as positive controls. First, the effect of 6'SL, 2'FL and GOS was investigated on differentiation by culturing monocytes with IL-4 and GM-CSF in the presence or absence of these oligosaccharides. Second, differentiated moDC, vitD3DC and TGFβDC were assessed for their expression of HMO-recognizing receptors. These DC types were used to study the effect of 6'SL, 2'FL and GOS on LPS-induced maturation.

Materials and Methods

Isolation and culturing of monocyte-derived DC types

Monocytes were isolated from PBMCs from healthy anonymous donors (Sanquin blood bank, Nijmegen) as described earlier[157]. Immature moDCs were generated by differentiating monocytes for 6 days in the presence of 20 ng/ml IL-4 (Peptrotech; 200-04) and GM-CSF (Peprotech; 300-03) in the presence or absence of TGFβ (3 ng/ml TGFβ1 + 10ng/ml TGFβ2, Peprotech; 100-21/100-35), 10nM VitD3 (Sigma-

Aldrich, D1530), 0.5 mg/ml 2'FL (Inalco S.p.A.) or GOS (FrieslandCampina), or 0.375 mg/ml 6'SL (Carbosynth, OS04398). These immature moDC, TGFβDC or VitD3DC were stimulated (i.e. maturated) with 1 μg/ml *E. coli* OIII:B4 LPS (Invivogen; tlrl-3pelps) with or without 0.5 mg/ml GOS, 2'FL, or 0.375 mg/ml 6'SL for 48 hours.

LPS detection

6'SL, 2'FL and GOS were tested for LPS contamination by a recombinant factor C LAL assay that was performed according to the manufacturers recommendations (EndoZyme recombinant factor C assay, Hyglos; 609050). Endotoxin levels of <0.05 EU/mg were found in GOS and 2'FL preparations. 6'SL contained trace amounts of LPS; 0.12 EU/mg.

Isolation and staining of moDCs

DCs were put on ice for 30 minutes under constant shaking. The collected cells were stained with a mixture of conjugated monoclonal antibodies or matched isotype controls (S1 Table). Cell viability was analyzed by staining the cells with DRAQ7 (Abcam; ab109202) or fixable viability dye ef506 (eBiosciences, 65-0866-14). Cells were acquired on a BD FACS Canto II (BD Biosciences) and analyzed using the FlowJo software V10.

Quantification of cytokine levels in supernatants

Levels of IL-8 IL-6, IL-10, TNF and IL-12p70 were measured in the supernatants using a cytometric bead array technique (BD Biosciences). Individual flex-sets for IL-8 (558277), IL-6 (558276), TNF (560112), IL-10 (558274) or IL-12p70 (558283) were run according to the manufacturer's instructions.

Statistics

The raw data was tested for normality with a Kolmogorov-Smirov test. Data with unequal distributions were transformed using the logarithm or square root of the raw data. A linear mixed model with a LSD test was used on the raw or transformed data to compare the various treatment groups to the control (moDC) as a reference group unless stated otherwise. Significant differences were indicated by: *** = P< 0.001, ** = P< 0.01 and * = P< 0.05. All values are represented as mean +/- SEM. IBM SPSS Statistics V23.0 was used for the statistical analysis.

Results

To investigate the effect of the breast milk components on DC differentiation, monocytes were differentiated into immature monocyte-derived DC (iDC) by culturing the cells for six days with IL-4 and GM-CSF in the presence or absence of individual breast milk component. 6'SL, 2'FL or GOS did not alter the expression of any of the measured surface markers on immature DC (Figure 1). Monocytes differentiated in the presence of VitD3 remained CD14+ and did not gain CD1a expression on their surface, showing that VitD3 was capable of halting DC differentiation (Figure 1A). Similarly, significantly less DC that were differentiated in the presence of TGFβ showed CD1a expression compared to moDC (Figure 1A). In contrast, 6'SL, 2'FL or GOS did not alter CD14 and CD1a expression (Figures 1B-C). Although fewer TGFβDC and VitD3DC showed to be CD1a+ compared to moDC, both DC types showed a different expression profile of activation markers. VitD3DC showed significantly lower HLA-DR (Figure 1D), CD80 (Figure 1E), CD86 expression (Figure 1F) and higher PD-L1 (Figure 1G) compared to moDCs. In contrast, TGFβDC expressed significantly lower levels of CD80 (Figure 1E) and PD-L1 (Figure 1G) on their surface compared to moDC.

To investigate whether breast milk components induced the production of cytokines during differentiation, we measured the cytokines in the supernatants that were produced during the differentiation period. Intriguingly, VitD3 induced the production of IL-6 (Figure 2A) and reduced the production of IL-8 (Figure 2B) during DC differentiation. TGFβ reduced the production of IL-8 and IL-6 (P=0.08) during DC differentiation compared to moDC. IL-10 and TNF production was below the detection limit of the assay (<10 pg/ml). 6'SL induced an approximate two-fold increase in IL-6 production compared to moDC (Figure 2A) and a trend (p=0.078) towards higher IL-8 levels (Figure 2B). However, since we measured trace amounts of 0.12EU/mg of LPS in this sample, we applied an optimized Triton X-114 method to remove LPS traces [210]. Triton X-114 treated 6'SL showed a similar production of IL-6 and IL-8, showing that the two-fold increase in IL-6 was caused by LPS traces (Figure 2C).

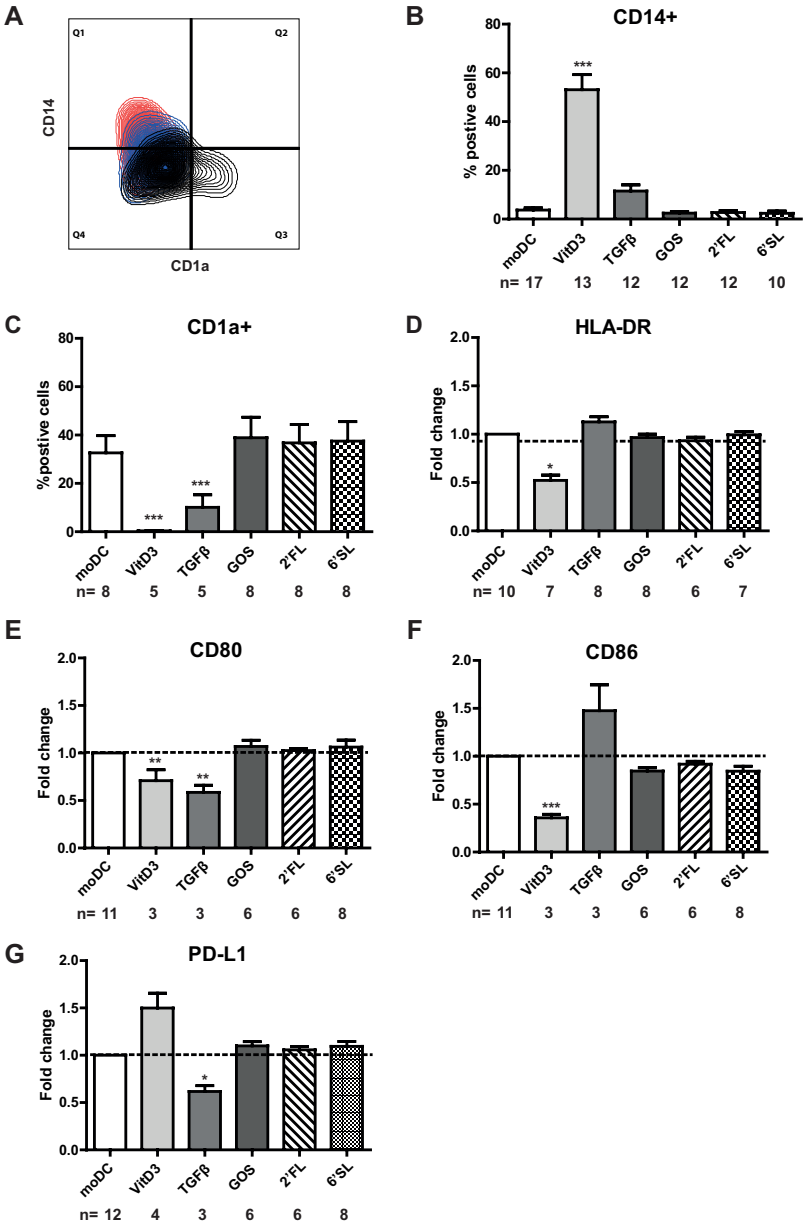

Figure 1. TGFβ and VitD3 induce phenotypic distinct DCs. CD14+ monocytes were cultured in the presence of IL-4 and GM-CSF for six days in the presence or absence of breast milk components. Surface marker expression was measured by flow cytometry. (**A**) A multi-color overlay of CD14 expression versus CD1a expression on moDC (black), TGFβDC (blue) or VitD3 (red) of one representative donor is shown. The percentage of (**B**) CD14+ and (**C**) CD1a+ DC and relative surface marker expression of (**D**) HLA-DR, (**E**) CD80, (**F**) CD86 or (**G**) PD-L1 on immature DC differentiated in the presence of TGFβ, VitD3, 6'SL, 2'FL or GOS is shown. Relative fold change was calculated by dividing the MFI (median fluorescence intensity) of DC differentiated in the presence of a breast milk component/MFI of moDC of each respective donor.

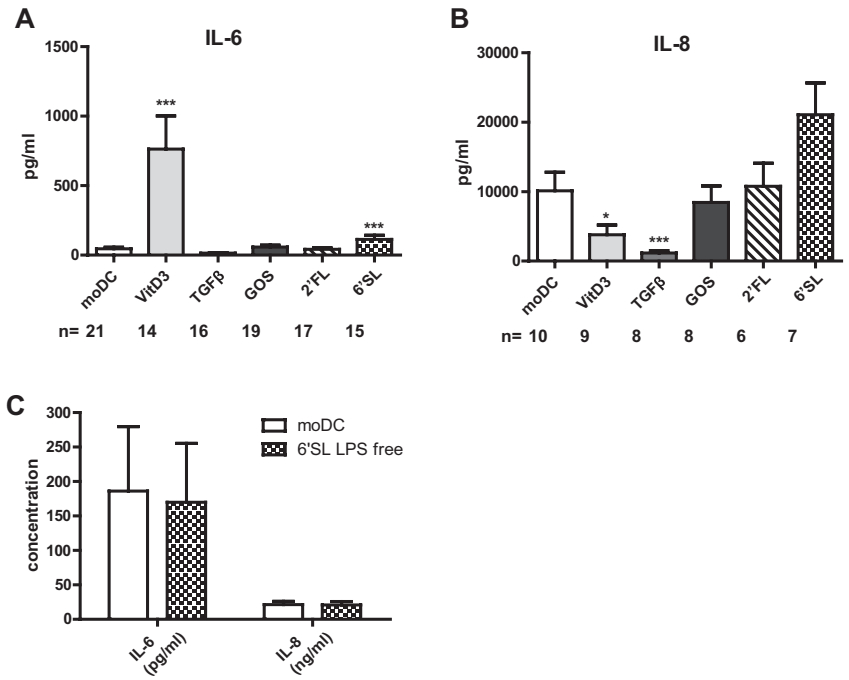

Figure 2. TGFβ and VitD3 differentially modulate the production of IL-6 and IL-8 during differentiation. 6'SL was treated with an optimized Triton X-114 method to remove LPS traces. Immature DC were cultured for six days in the presence or absence of VitD3, TGFβ, 6'SL, LPS-free 6'SL, 2'FL or GOS. (**A,C**) IL-6 and (**B,C**) IL-8 were measured in the supernatant by CBA.

After the differentiation period of six days, we assessed whether the DC types that were cultured in the presence of 6'SL, 2'FL, GOS, VitD3 or TGFβ were functionally different by stimulating these immature DC for 48 hours with LPS. moDC upregulated the expression of CD83, PD-L1, CD80 and CD86 upon LPS-induced maturation (Figure 3A). Immature dendritic cells that were generated in the presence of 6'SL, 2'FL or GOS did not show any phenotypic changes (Figures 3B-3E) or cytokine production (Figures 3F-3G) upon maturation compared to moDC. VitD3DC and TGFβDC showed to be hyporesponsive towards LPS as shown by a semi-mature phenotype (i.e. reduced expression of both CD83 and CD86) (Figures 3B-3D) and lower expression of CD80 (Figure 3E), HLA-DR and PD-L1 (Figures S1A-B). In addition, VitD3DC and TGFβDC produced lower amounts of IL-12p70 after LPS stimulation compared to moDC (Figures 3F). However, the ratio of IL-10/TNF and quantitative IL-10 and TNF levels (Figures S1 C-D) was not significant different compared to conventional DCs (Figures 3G). Thus we showed 6'SL, 2'FL and GOS, in contrast to vitD3 and TGFβ, did not induce differentiation of monocytes into tolerogenic DC.

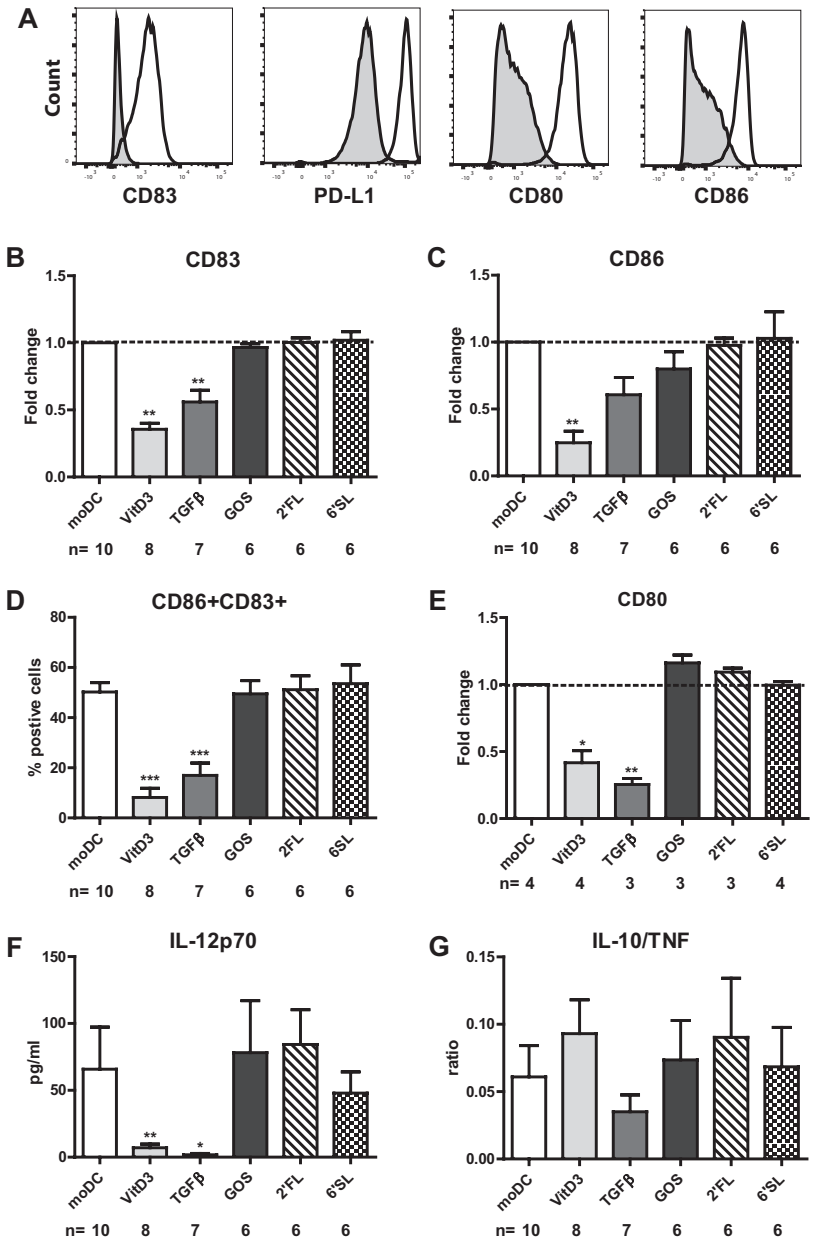

Figure 3. TGFβDC and VitD3DC induce tolerogenic DC. Immature DC were stimulated with LPS for 48 hours. (**A**) The expression of CD83, PD-L1, CD80 and CD86 on immature DC (shaded histograms) or mature DC (open histograms) of one donor is shown. The relative surface marker expression of (**B**) CD83, (**C**) CD86 and (**E**) CD80 are shown. Relative fold change was calculated by dividing the MFI (median fluorescence intensity) of DC differentiated in the presence of a breast milk component/MFI of moDC of each respective donor. (**D**) Percentage of CD86+CD83+ DCs. (**F**) IL-12p70 and IL-10 and TNF were measured in the supernatant by CBA. (**G**) The IL-10/TNF ratio is shown for the different mature DC that were differentiated in the presence of different breast milk components.

Next, we investigated the expression of HMO-recognizing receptors on moDC, TGFβDC and VitD3DC to assess their potential responsiveness towards 6'SL, 2'FL and GOS. Moreover, we investigated the expression of Siglec-5 and Siglec-7 as potential receptors for 6'SL. DC-SIGN and CD206 were included as potential receptors of 2'FL and galactin-3 as putative receptor for GOS. We also measured the expression of TLR4 since we used LPS to mature DC. Monocytes expressed high levels of Siglec-5, Siglec-7, and TLR4, no expression of DC-SIGN and CD206 and low expression of Galactin-3 (Figure 4A). The expression of CD206 (Figure 4B) and DC-SIGN (Figure 4C) was high after differentiation of the monocytes into moDC. In contrast, the expression of Siglec-5 was markedly lower after differentiation. Interestingly, VitD3DC showed significantly lower expression of Siglec-5, Siglec-7, DC-SIGN and TLR4 compared to moDC (Figures 4D-F). TGFβDC of all three tested donors showed a lower expression of DC-SIGN (Figure 4C) and significant lower expression of Siglec-7 (Figure 4F) compared to moDC. This data thus showed that moDC, TGFβDC and vitD3DC express different levels of receptors that are known to recognize HMOs. 6'SL, 2'FL or GOS may therefore differentially modulate moDC, TGFβDC and VitD-3DC.

After having shown that moDC, TGFβDC or vitD3DC express different levels of TLR4 and HMO-recognizing receptors, we used these DC types to investigate the effect of 6'SL, 2'FL or GOS on LPS-induced maturation. While we observed differences in receptor expression, none of the oligosaccharides tested affected the phenotype of TGFβDC, VitD3DC or moDCs in the absence or presence of LPS compared to the respective DC type that was matured in the absence of HMOs (Figure 5). In line with these findings, 6'SL, 2'FL or GOS did not modulate the production of IL-6, IL-8, IL-10, IL-12p70 or TNF production by moDC, TGFβDC or VitD3DC in the presence or absence of LPS (Figure S2). VitD3DCs showed a higher expression of PD-L1 compared to TGFβDC or moDC (Figure 5A), which was also seen before on day 6 of culture (Figure 1G). As shown in Figure 3 and Figure S1 LPS stimulated VitD3DC and TGFβDC showed a lower expression of PD-L1, CD83 and CD80 compared to moDC (Figures 5C-D).

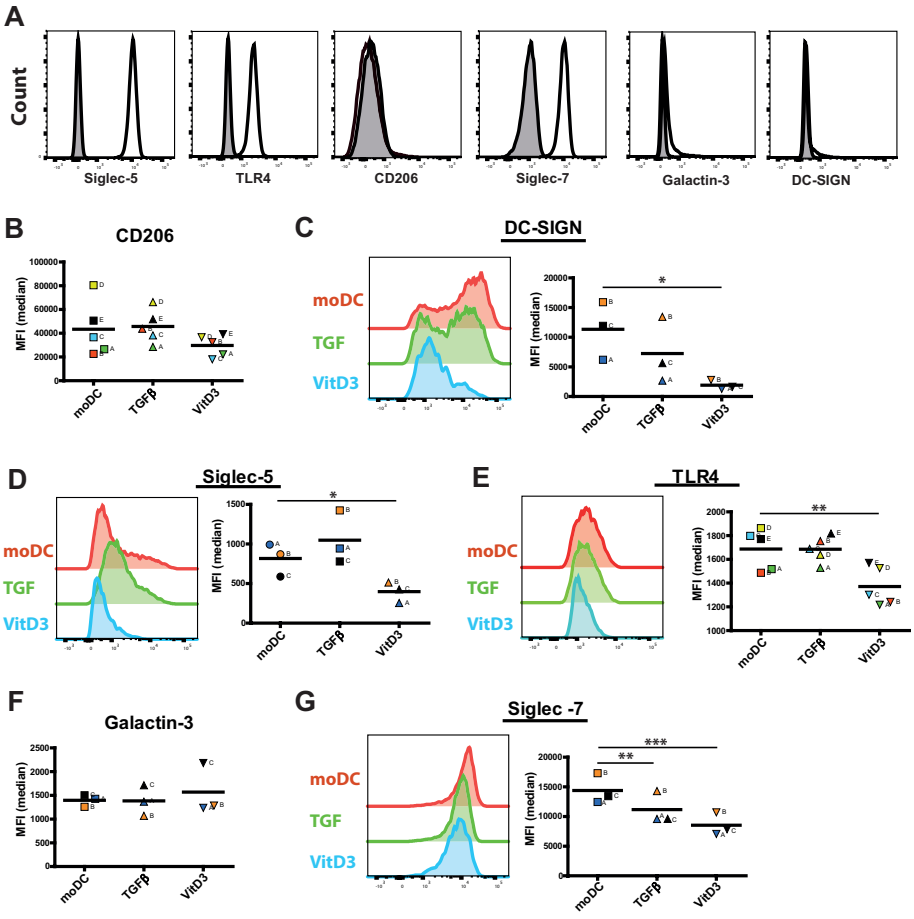

Figure 4. moDC, TGFβDC and VitD3DC express different levels of HMO-recognizing receptors. The expression of several receptors that are shown in literature to recognize HMOs were measured on (**A**) monocytes and (**B-G**) moDC, TGFβDC and vitD3DC. The expression of Siglec-5, Siglec-7, TLR4, CD206, Galactin-3 and DC-SIGN (open histograms) and their matching isotype control (shaded histograms) were measured on monocytes. The expression of (**B**) CD206, (**C**) DC-SIGN, (**D**) Siglec-5, (**E**) TLR4, (**F**) Galactin-3 and (**G**) Siglec-7 is shown by scatter plots (n=3-5) and histogram of one representative donor. Normal distribution was assumed due to the low sample size. Significance was tested by a repeated measures ANOVA with a Tukey's multiple comparison post-hoc test.

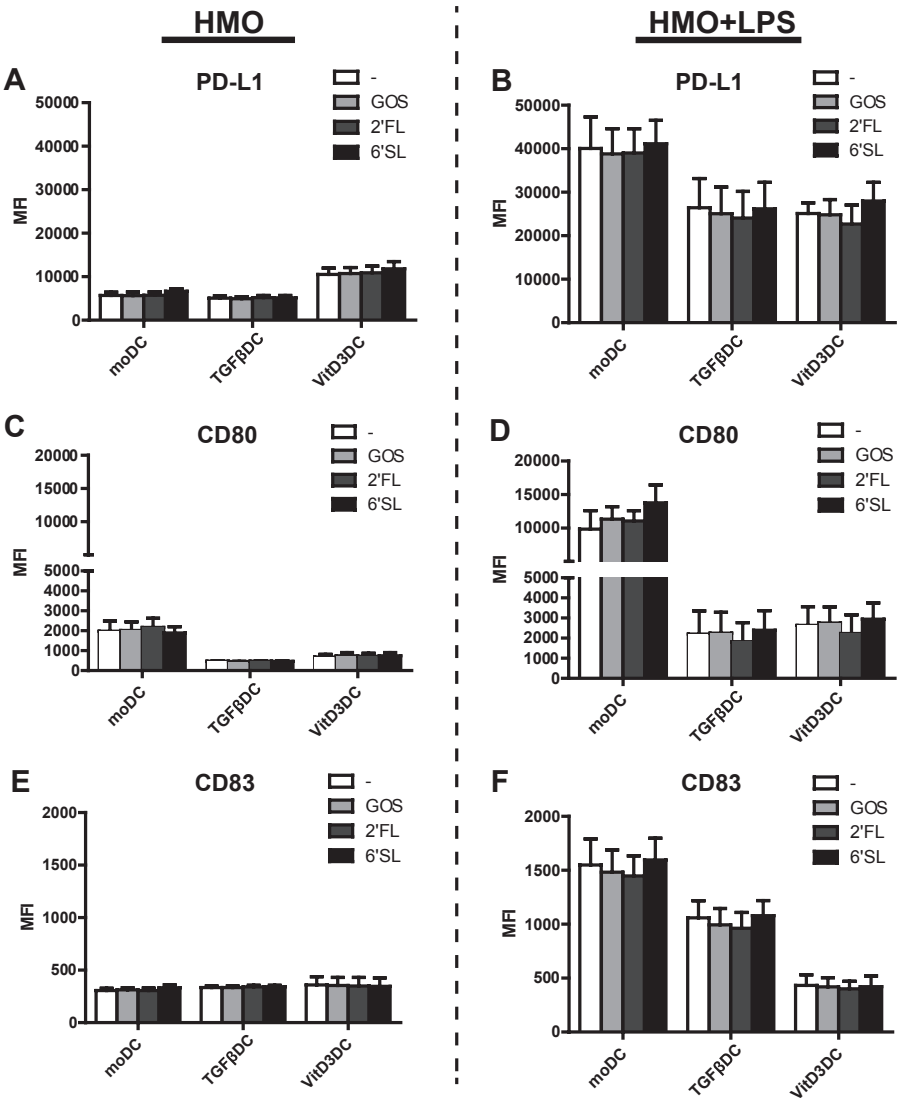

Figure 5. HMOs do not impact maturation of different in vitro generated DC. Immature moDC, TGFβDC and VitD3DC were stimulated with LPS with or without 6'SL, 2'FL or GOS for 48 hours. The effect of 6'SL, 2'FL and GOS was measured in the absence (**A, C, F**) or presence of LPS (**B,D,F**). The surface marker expression of (**A,B**) PD-L1 and (**C,D**) CD80 and (**E,F**) CD83 is shown as MFI (median fluorescence intensity) (two independent experiments, six donors).

Discussion

In this study we investigated the effect of 6'SL, 2'FL and GOS and the well-studied factors TGFβ and VitD3 on DC differentiation. None of the oligosaccharides tested affected DC differentiation. VitD3 and TGFβ induced phenotypical distinct immature DCs. These immature DC produced different levels of cytokines during differentiation compared to moDC. As shown by others, DCs differentiated in the presence of VitD3 or TGFβ showed a tolerogenic phenotype; showing hyporesponsiveness towards LPS and abrogated IL-12p70 production[160,295–297]. We showed that these moDC, TGFβDC and VitD3DC differentially express HMO-binding receptors and TLR4. However, 6'SL, 2'FL and GOS did not modulate LPS-induced maturation on any of these DC types.

The GI tract is constantly exposed to commensal microorganisms and their metabolites. As a result, monocytes are constantly recruited to the GI tract[31]. These monocytes differentiate into macrophages or dendritic cells in the presence of cytokines. Environmental factors (e.g. diet) can alter the differentiation of these monocytes into functionally distinct cells[160,162]. Oligosaccharides are largely fermented in the colon and a small fraction becomes systemically available[105,106]. In bone marrow-derived DC cultures, a complete fraction of HMOs was shown to induce regulatory responses[294]. It is therefore of interest to study the direct immunomodulatory effect of individual HMOs on monocytes and DC. The data presented here show that in contrast to TGFβ and vitD3, 6'SL, 2'FL and GOS have no immunomodulatory effect on the differentiation of monocytes into DC. Next, we used these immature moDC, TGFβDC and vitD3DC to measure the surface marker expression of HMO-recognizing receptors and subsequently test their responsiveness towards 6'SL, 2'FL or GOS. Receptors that are postulated to bind HMOs are CD206 and DC-SIGN, which are shown to bind 2'FL[298], while sialic-acid-binding immunoglobulin-like lectins (Siglecs) are known to bind sialic acid containing HMOs such as 6'SL. Siglecs contain intracellular tyrosin-based inhibitory (ITIM) motifs that dampen immune responses[280]. In line with literature, we showed that human monocytes express Siglec-5 and Siglec-7[281]. We showed that the expression of Siglec-5 and -7 was markedly downregulated on TGFβDC and vitD3DC compared to moDC. Nevertheless, we observed no difference in the immunomodulatory effect of 6'SL on both DC types, indicating that 6'SL does not induce DC functioning via Siglec-7. This could be explained by the low binding affinity of 6'SL to Siglec-7[280].

Similarly, activation of CD206, DC-SIGN and galactin-3 may result in dampening of inflammation[298–302]. GOS may bind galactin-3 - a β-galactoside-binding lectin that is expressed on monocytes[299]. Although galactin-3 signaling has been shown to induce differentiation of monocytes into anti-inflammatory alternative activated macrophages[300], we did not show any phenotypical changes if GOS was added during DC differentiation. Thus, we showed that although TGFβDC and vitD3DC express different levels of receptors that may recognize HMOs, 6'SL, 2'FL or GOS did not modulate DC maturation.

In contrast, others do show immunomodulation by HMOs *in vitro*[155,156,274,294]. For instance, the acidic HMO fraction of breast milk was - in contrast to the neutral HMO fraction - shown to induce the frequency IFNγ+ T cells and CD4+CD25+ regulatory T cells in a human mononuclear cord blood cell culture[274]. This immunoregulatory effect of the acid fraction was sufficient to restore the Th1/Th2 cytokine balance in PBMCs of peanut allergic patients stimulated with the major allergen Ara h1[273]. In mouse pups, the acidic trisaccharide 3'SL was shown to promote colitis by inducing inflammation in a TLR4 mediated manner[156]. The prebiotic oligosaccharides scGOS/lcFOS mixture, FOS and inulin were also shown to exert immunomodulation on monocytes via TLR4[270,272]. Here we show that 6'SL, which is abundantly present in breast- and cow's milk[79], induces the production of IL-6 and IL-8 during DC differentiation and did not alter the phenotype. However, after applying an optimized Triton X-114 method[210], we showed that this twofold increase was caused by trace amounts of LPS (0.12 EU/mg). We have previously shown that a 100-fold higher contamination of LPS in commercial 3'SL results in rapid IL-6 and IL-8 production and induces differentiation into tolerogenic DC[157]. Since LPS is an important contaminant that signals via TLR4, caution must be applied interpreting direct immunomodulation of HMOs via TLR4. Even though we could not demonstrate effects of 6'SL, 2'FL or GOS on monocytes and dendritic cells, it is possible that other HMOs or other prebiotic oligosaccharides can have immunomodulatory effects on monocytes and dendritic cells, since breast milk contains at least 140 different HMOs. Additionally, HMOs have been shown to have an important immunomodulatory role indirectly by altering the microbiota composition and enhancing the production of short chain fatty acids (SCFA). These SCFA produced by the microbiota are known to exert immunosuppressive effects on moDC[303].

VitD3 and TGFβ altered the differentiation into phenotypical distinct immature DCs. Both of these immature VitD3DC and TGFβDC types are hyporesponsive towards LPS. The notion that VitD3 and TGFβ induce phenotypical different DC types, which produce a very different cytokine profiles during differentiation, indicates that different pathways are responsible for the differentiation into regulatory DCs. This hypothesis is in line with literature showing distinct pathways involved in NF-κB regulation by VitD3 and TGFβ on human monocyte-derived DC[297]. Although TGFβ and VitD3 may act via different mechanisms, we show in this study that both are capable of halting DC differentiation. One possible mechanism by which VitD3 inhibits DC differentiation is by the inhibition of GM-CSF signaling via upregulation of SOCS1[288,304], which could halt DC differentiation. The mechanism by which TGFβ influences DC development is not well established. Since the SMAD-induced pathways was shown to be active in moDC[297], TGFβ may halt DC differentiation via this pathway. Interestingly, we show here that CD1a is expressed on fewer TGFβDCs compared to moDCs. CD1a+ DCs do in contrast to DCs produce IL-12p70 upon stimulation[212], which we also observed for VitD3DCs and TGFβDCs. Additionally, we showed that VitD3 and not TGFβ triggers the production of IL-6 during DC differentiation. IL-6 in turn can induce STAT3 activation *in vitro* and *in vivo* and halts DC differentiation[286,305]. STAT3 binds the PD-L1 promotor directly and regulates its expression[287]. Indeed, we showed that vitD3 triggers IL-6 production and that PD-L1 expression is higher on immature VitD3DCs compared to TGFDCs or moDC. Thus, VitD3DCs and TGFβDCs are less responsive to LPS (i.e. tolerogenic DC), as observed by their semi-mature phenotype and lower cytokine production, which is line with literature[160,162,295,306].

Conclusions

The GI tract is constantly repopulated by monocyte derived DC and macrophages due to the exposure of microbes and their metabolites. Breast milk contains components such as VitD3 and TGFβ that can alter the differentiation of monocytes into tolerogenic DCs. Even though oligosaccharides are thought to play an important role in immune development in early life, the HMOs and GOS tested here do not alter DC differentiation or maturation of *in vitro* differentiated mucosal DC types. Further unravelling the impact of these and other breast milk components on immune homeostasis will improve our understanding of how breastfeeding promotes

immune homeostasis and development. This knowledge can be applied to develop new strategies to protect infants against infections and allergies.

Acknowledgements
We thank T.V.L. Berghof, PhD and A. Hagmayer, MSc for helping us with the statistical analysis.

Supplementary files

Table S1. Conjugated antibodies used for flow cytometry

Target	Fluorochrome	Company	Cat.nr. Ab	Cat. nr. Isotype control
CD14	FITC	BD Biosciences	555397	555573
CD83	FITC	BD Biosciences	556910	555573
CD1a	PerCP-Cy-5	Biolegend	300130	400149
CD86	V450	BD Biosciences	560357	560373
HLA-DR	APCef780	eBiosciences	47-9956-42	47-4732-80
CD80	PE-Cy-5	BD Biosciences	559370	555576
PD-L1	PE-Cy7	BD Biosciences	558017	557872
Galactin-3	Alexa Fluor 488	Biolegend	125410	553929
Siglec-5	PE	Biolegend	352003	555573
Siglec-7	APC	Biolegend	339205	17-4714-81
DC-SIGN	BV421	BD Biosciences	566278	562438
CD206	PE-Cy7	eBiosciences	25-2069-42	552868
TLR4	BV421	BD Biosciences	5167523	562438

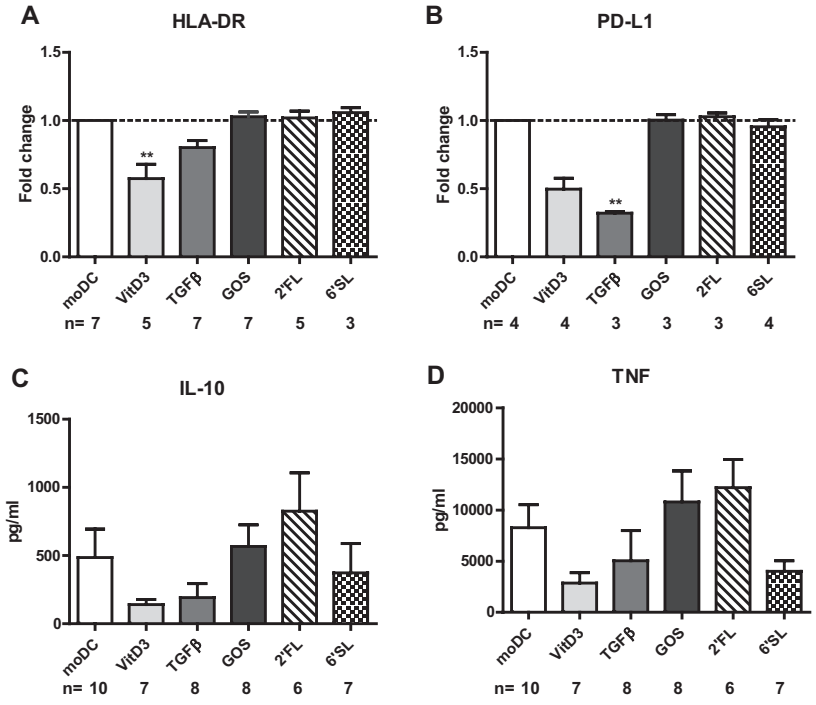

Figure S1. TGFβ and VitD3 induce tolerogenic DC. Immature DC differentiated in presence of 6'SL, 2'FL, GOS, TGFβ or VitD3 were stimulated with LPS for 48 hours. The relative surface marker expression of (**A**) HLA-DR and (**B**) PD-L1 are shown. Relative fold change was calculated by dividing the MFI (median fluorescence intensity) of DC differentiated in the presence of a breast milk component/MFI of moDC of each respective donor. (**C**) TNF and (**D**) IL-10 were measured in the supernatant by CBA.

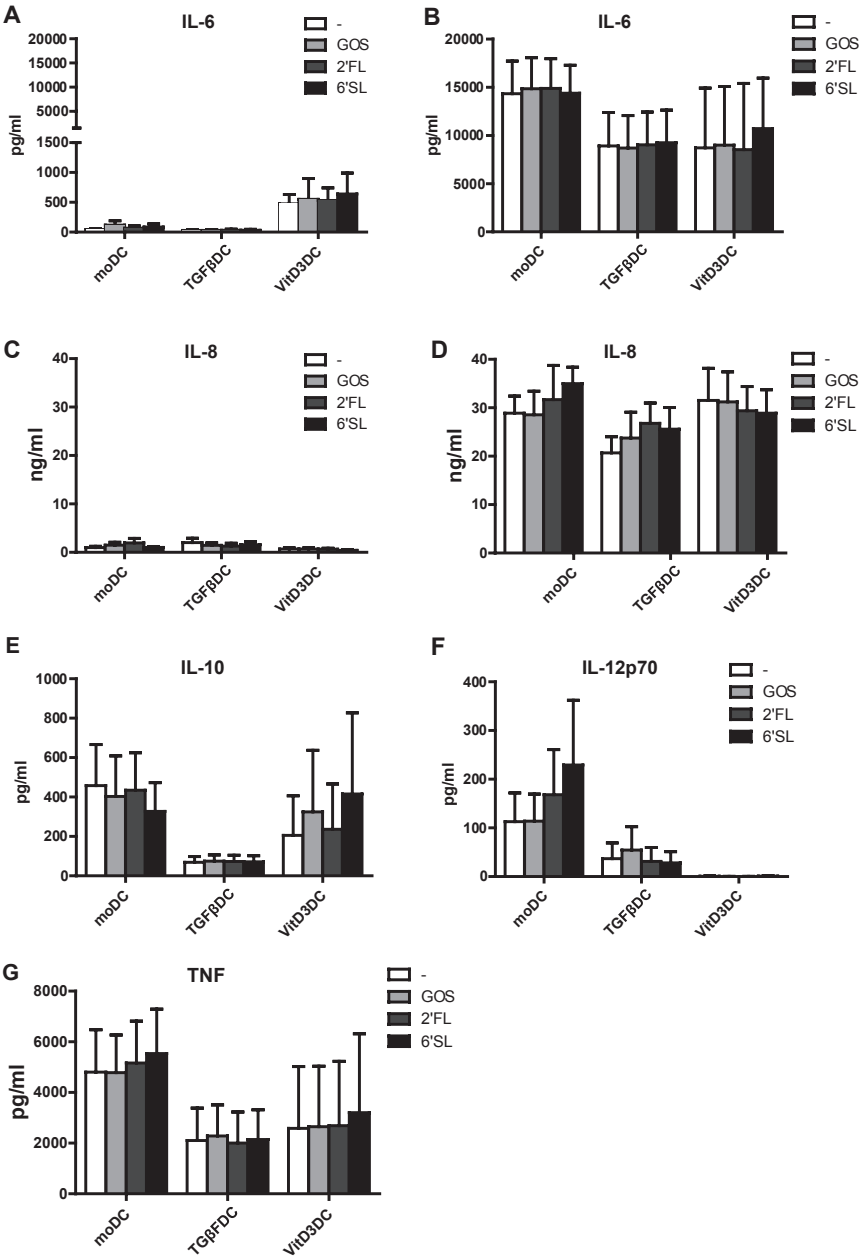

Figure S2. HMOs do not impact maturation of different in vitro generated DC. Immature moDC, TGFβDC and VitD3DC were stimulated with LPS with or without 6'SL, 2'FL or GOS for 48 hours. The effect of 6'SL, 2'FL and GOS on cytokine production was measured in the absence (**A,C**) or presence of LPS (**B,D-G**). (**A,B**) IL-8, (**C,D**) IL-6, (**E**) IL-10, (**F**) IL-12p70 and (**G**) TNF were measured by CBA.

Chapter 7

Sialyllactose and galactooligosaccharides promote epithelial barrier functioning and distinctly modulate microbiota composition and short chain fatty acid production

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Submitted

Abstract

Human milk oligosaccharides (HMO) and prebiotic oligosaccharides are proposed to confer several health benefits to the infant. They shape the microbiota, have anti-inflammatory properties, and support epithelial barrier functioning. However, in order to select the best oligosaccharides for inclusion in infant formulas, there is a need to increase our understanding of the specific effects of HMO and prebiotics on the host immune system. Therefore, we investigated the effects of the HMO sialyllactose (SL), and galactooligosaccharides (GOS) on epithelial barrier functioning, microbiota composition, and SCFA production. Transcriptome analysis showed that SL and GOS both induced pathways that regulate cell cycle control in fully polarized Caco-2 cells. This gene-expression profile translated to a phenotype of halted proliferation and included the induction of alkaline phosphatase (ALP) activity, a marker of epithelial cell differentiation. SL and GOS also promoted re-epithelialization in an *in vitro* epithelial wound repair assay. SL induced the outgrowth of members of the *Bacteroides* genus, including *B. fragilis* as well as *Feacalibacterium prausnitzii*, and led to elevated levels of propionate and butyrate production. Interestingly, these microbial species have both been associated with protection against colitis in animal models. GOS appeared to specifically support the outgrowth of the *Bifidobacterium* genus, which coincided with elevated levels of lactate and butyrate production. Our results show that SL and GOS can both modulate epithelial barrier function by inducing differentiation and epithelial wound repair, but differentially promote the growth of specific genera in the microbiota, which is associated with differential changes in SCFA profiles.

Introduction

Microbial colonization directly after birth and breastfeeding are crucial events that determine health in neonatal and adult life[48,49]. Breast milk is a unique mixture of immunoregulatory proteins, antimicrobial peptides, micronutrients, milk fat globular membrane, miRNA containing extracellular vesicles, and human milk oligosaccharides (HMO)[34,307]. It has been widely recognized that breast milk confers health benefits to the infant by shaping the microbiota, preventing infections and promoting cognitive abilities[4]. Breast milk serves as the golden standard for infant nutrition and exclusive breastfeeding for the first six months in life is advocated by the WHO[308]. Nevertheless, more than half of the infants is not exclusively breast-fed during the first six months of life worldwide[4] and is thus dependent on infant nutrition. At present, infant formulas are commonly supplemented with prebiotic oligosaccharides like galactooligosaccharides (GOS), fructooligosaccharides (lcFOS), or polydextrose, or a mixture of these. Among these polymeric glycans, GOS is the most widely used prebiotic oligosaccharide in infant nutrition.

Contrary to other mammals, human breast milk contains a very high amount and a structurally diverse set of oligosaccharides that even exceeds the protein content of breast milk[40,102,309]. HMO consist of a lactose backbone that can be elongated into polymeric glycan structures that can contain fucose or sialic acid moieties. The simple trisaccharides 3'-sialyllactose (3'SL) and 6'-sialyllactose (6'SL) are among the most abundant sialylated HMO, with estimated concentrations in mature breast milk ranging between 170-500 μg/ml 6'SL and 76-300 μg/ml for 3'SL[78]. Compared to human milk, mature bovine milk contains low levels of 3'SL (30-119 μg/ml) and 6'SL (17-88 μg/ml)[78].

Exogenous sialic acids have been suggested to be essential for brain development in early life[310]. Additionally, sialylated oligosaccharides may, although mostly investigated *in vitro* and animal models, exert other health effect via neutralization of pathogens, fermentation into anti-inflammatory metabolites, direct immunomodulation, and improved epithelial barrier functioning[78]. HMO and prebiotics are metabolized by the microbiota in the colon and upon their colonic fermentation, sialylated oligosaccharides may function as an essential exogenous source of sialic acid. Animal models have shown that endogenous production of sialic acid in the liver is low during the first weeks of life[311], suggesting that microbial production of sialic acid

in neonates could be relevant, and emphasizing the importance of proper colonic microbial colonization and fermentation.

It has been suggested that growth factors in human milk are important for epithelial barrier functioning. For instance, breast milk contains growth factors that promote the proliferation and differentiation of epithelial cells, thereby promoting gut maturation[96,312]. Improving gut maturation by means of nutrition in early life may be particularly relevant in the first weeks of life, for premature infants and infants suffering from intestinal infections or inflammatory bowel conditions[127,222]. Furthermore, nutrition may play a role in maintaining barrier function later in infancy during adverse episodes, e.g. when the epithelial layer is challenged by inflammation due to infection or physical damage.

The neonatal microbiota is shaped by colonization of bacteria from the mother during vaginal delivery and is further shaped by breastfeeding[39]. As reviewed by Mueller *et al.* (2015), introduction of infant formula instead of breastfeeding results in microbial changes. Breastfed children show a lower microbial diversity, decreased abundance of Clostridiales and Bacteroidetes members[313], a lower prevalence of *C. difficile* and *E. coli* and increased prevalence of bifidobacteria[39,314,315] and lactobacilli[39,316]. The capacity of bifidobacteria to digest HMO has been appreciated since the 1950s, which is the most likely explanation of the expansion of this bacterial group in breastfed infants.

The best described and most abundant group of metabolites are short-chain fatty acids (SCFA). SCFA such as acetate, propionate and butyrate are produced in the colon and reach high concentrations (20-100 mM) locally and much lower levels systemically (in the μM range)[317]. The increase in bifidobacteria was correlated with an increase in fecal lactate and acetate concentrations and lower pH[318]. SCFA are shown to exert direct anti-inflammatory effects on colonocytes and restore epithelial barrier functioning, which results in suppression of colitis in animal models[319,320]. Additionally, SCFA may exert systemic responses via inducing epigenetic changes and directly modulating gene transcription in immune cells. For instance acetate was shown to enhance regulatory T cell functioning by epigenetic modification of the FOXP3 gene[45]. This study by Thornburn *et al.* (2015) and comparable studies in other mouse models show that the diet can, via microbial-derived SCFA, alleviate respiratory diseases[44–46]. Although largely unexplored, fermentation of HMO or prebiotics in the colon may thereby serve as an important carbohydrate source that may impact the physiology and health of the infant[321–323].

To introduce part of the functionality of HMO into infant nutrition, prebiotic oligosaccharides like GOS have been added to infant formula to support the outgrowth of bifidobacteria. Prebiotics have no detrimental effect on growth of the infant and may reduce the pH and increase the softness of the stool, increase stool frequency and increase the fecal *Bifidobacterium* and *Lactobacillus* counts[324–328]. Fecal SCFA levels of neonates fed infant formula supplemented with a 9:1 GOS:FOS mixture are more similar to breast-fed neonates compared to infants that are bottle-fed without prebiotic supplementation[329]. Sialyllactose (SL) is an easily accessible oligosaccharide present in bovine and human milk that could be of interest for early life nutrition. Identification of such HMO for use in infant nutrition may be of interest to improve health of infants. However, a better mechanistic understanding of individual HMO and currently used prebiotics is required. The aim of our study was therefore to investigate the effects of SL and GOS on epithelial barrier function (i.e. re-epithelialization, proliferation and differentiation of Caco-2 cells; supported by underlying mechanisms using transcriptomics), and their effects on microbiota composition and subsequent SCFA production.

Materials and Methods

Cell culturing

Ca9-22 (JCRB0625) gingival epithelial cells and Caco-2 cells (HTB-37) colon epithelial cell lines were purchased from the National Institute of Biomedical Innovation JCRB Cell Bank (Osaka, JP) and ATCC (Manassas, USA), respectively. Both cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing Glutamax (GIBCO, Invitrogen, Paisley, UK) and 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, MO, USA). The cell lines were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 and passaged every second day. Experiments were performed using cell-passage numbers 9-20 and 12-41 for Ca9-22 and Caco-2, respectively.

Scratch Assay and automated image acquisition and segmentation

The scratch assay and analysis was performed as previously described[330]. In short, approximately 35.000 cells/well were seeded in 96-wells plates in DMEM + 10% FCS and grown overnight into a confluent monolayer. The next day, cells were starved for two hours by replacing the medium with DMEM without FCS. The cellular

cytoplasm was labelled with 2 µM CellTracker™ Red CMTPX (Molecular Probes, OR, USA) and the nuclei were stained with 2 µg/ml Hoechst 33342 (Molecular Probes, OR, USA). Longitudinal scratches (0.3 × 2 mm) were introduced in the monolayers using the HTSScratcher (Peira, Antwerp, BE). After washing away the cell debris, 100 µl of a mixture of 3'SL and 6'SL, as present in cow's milk (25 µg/ml 3'SL + 75 µg/ml 6'SL), human breast milk (125 µg/ml 3'SL + 375 µg/ml 6'SL) or human colostrum (750 µg/ml 3'SL + 2250 µg/ml 6'SL)[78] or respectively matching concentrations of GOS of 100, 500 and 3000 µg/ml, 4 ng/ml TGFα (R&D Systems, MN, USA) or 10 µM of p38 inhibitor (SB203580; Cell Signaling Technology, MA, USA) + 10 µM MEK1/2 inhibitor (U0126, Cell Signaling Technology) in DMEM without FCS were added into the wells in a randomized manner. The BD Pathway 855 Bioimaging System (BD Biosciences, CA, USA) was programmed to acquire fluorescent and bright-field images every 20 minutes for five hours. Image segmentation was performed using CellProfiler 2.1.1 and visualized in FCS Express 4 Plus (De Novo Software, CA, USA) software. The lag time (λ in minutes), repair rate (μ_m in cells minute^{-1}) and the maximum number of cells (A) within the scratch area of each well were calculated in R by fitting the modified Gompertz equation through the re-epithelialization measurements.

Caco-2 stimulation and RNA extraction for microarray and qPCR

One million cells were seeded and polarized for two weeks in each well of a 6-wells Transwell system or on flat bottom plates, for microarray and qPCR analysis, respectively. The polarized cell layer was incubated with 10 mg/ml sialyllactose isolated from bovine milk (FrieslandCampina) or GOS (FrieslandCampina) for transcriptome analysis and 10 mg/ml SL [mixture of 5 mg/ml 3'SL (OS04397, Carbosynth) + 5'SL 6'SL (OS04398, Carbosynth)] for verification by qPCR. Cells were grown in DMEM + 10% FCS and refreshed every second day. All treatments and controls were performed in triplicate. Medium was aspirated from the inserts and TRIzol or RLT buffer was added to lyse the cells for transcriptome and qPCR analyses, respectively. The samples for qPCR analysis were thawed and 1:1 diluted with 70% ethanol RNA and homogenized using a syringe. A RNeasy Mini Kit was used according to the manufacturer's recommendations to isolate RNA for qPCRs (ref 74106, Qiagen). Cells were stored at -80°C until further use. RNA was extracted by first adding 200 µl of chloroform to 1 ml of the thawed cell lysate. Next, samples were vortexed for 15 seconds, incubated for 2-3 minutes at room temperature and centrifuged for 15 minutes at 11.000 rpm at 4°C. The upper aqueous phase was

transferred to a new tube and 500 µl isopropanol was mixed with the sample and incubated for 5-10 minutes at room temperature to precipitate the RNA. The samples were then centrifuged for 10 minutes at 11.000 rpm at 4ºC. Supernatants were discarded and the pellet was washed with 1 ml 75% ethanol and centrifuged for 10 minutes at 4ºC. The supernatant was discarded and the pellet was dried for 5 minutes. 100 µl MQ was added and RNA was further purified using RNeasy Mini Kit, according to the manufacturer's recommendations (ref 74106, Qiagen).

Gene expression profiling

RNA quality control using Nanodrop and Bioanalyzer, RNA labelling and array hybridizations were performed by Jenny Jansen and Mechteld Grootte Bromhaar, (Nutrition, Metabolism & Genomics (HNE), Division of Human Nutrition, Wageningen University;). RNA quantity and quality was assessed spectrophotometrically via a Nanodrop device (ND-1000, NanoDrop Technologies, Wilmington, DE, USA) and with 6000 Nano chips via a Bioanalyzer 2100 device (Agilent, Santa Clara, CA, USA), respectively. RNA was judged as being suitable for array hybridization only if samples showed intact bands corresponding to the 18S and 28S ribosomal RNA subunits, displayed no chromosomal peaks or RNA degradation products, and had a RIN (RNA integrity number) above 8.0. The Ambion WT Expression kit (Life Technologies, cat. no. 4411974) in conjunction with the Affymetrix GeneChip WT Terminal Labelling kit (Affymetrix, Santa Clara, CA; cat. no. 900671) was used for the preparation of labelled cDNA from 100ng of total RNA without rRNA reduction. Labelled samples were hybridized on Affymetrix GeneChip Human Gene 1.1 ST arrays that contain 30.000 coding transcripts and over 11.000 long intergenic non-coding transcripts, provided in plate format. Hybridisation, washing and scanning of the array plates was performed on an Affymetrix GeneTitan Instrument, according to the manufacturer's recommendations. Detailed protocols can be found in the Affymetrix WT Terminal Labelling and Hybridisation User Manual (part no. 702808 revision 4), and are also available upon request. Quality control of the hybridizations to the Human Gene 1.1 ST array and primary data analysis were performed according to strict criteria to ensure that the array data were of the highest possible quality.

Statistical and functional analysis of microarray data

Packages from the Bioconductor project[331] (www.bioconductor.org) were used for analyzing the scanned Affymetrix arrays. Arrays were normalized using quantile

normalization, and expression estimates were compiled using the pre-processing algorithm Robust Multiarray Analysis (RMA), applying the empirical Bayes approach available in the Bioconductor library affyPLM using default settings. Gene functional annotations, gene ontology (GO) enrichment and differential expression calculations were carried out using Bioconductor[331] packages and third-party software modules (see below). The Bioconductor packages were integrated in the automated on-line MADMAX pipeline[332]. Various advanced quality metrics, diagnostic plots, pseudo-images and classification methods were applied to ascertain that only arrays that passed the most rigorous quality controls were used in the subsequent analyses[333]. Arrays were considered of sufficient quality when they showed not more than 10% of specks in fitPLM model images, were not deviating in RNA degradation and density plots, were not significantly deviating in NUSE and RLE plots, and were within each other's range in boxplots. For a more extensive description of quality criteria, please contact the authors. Probe sets were redefined according to Dai *et al.* (2005)[334] utilizing current genome information. In this study, probes were reorganized based on the Entrez Gene database (remapped CDF v13 (November 2010), http://brainarray.mbni.med.umich.edu). Differentially expressed probe sets were identified using linear models, applying moderated t-statistics that implement empirical Bayes regularization of standard errors using Bioconductor's limma package[335]. A Bayesian hierarchical model was used to define an intensity-based moderated T-statistic (IBMT), which takes into account the degree of independence of variances relative to the degree of identity and the relationship between variance and signal intensity[336]. When gene expression was low and just below significance, results of the limma analysis were compared to the IBMT test. P-values were corrected for multiple testing using a false discovery rate (FDR) method[337]; the quality of the data was such that FDR *p*-values (Q values) of < 0.01 to < 0.0001 yielded thousands of differentials, depending on the specific comparison. For pathway analysis comparisons, it is not recommended to use different numbers of input genes (several hundred compared to several thousand), which would occur if a fixed FDR *p*-value were used for all datasets submitted to pathway analysis. Therefore, FDR values between $p < 10^{-7}$ and $p < 10^{-8}$ were chosen so that the number of genes included in Ingenuity Pathway Analysis (IPA) and Cytoscape were about 800 genes.

Data analysis

The open-source Bioconductor (www.bioconductor.org) packages were used for array quality control analyses and statistics. These packages are part of the MADMAX

pipeline where the microarray data were stored. For array annotations, we used the following software and database versions: R-version 2.11.1, Bioconductor version 2.6; Custom CDF library: hugene11stv1hsentrezgcdf, and hugene11stv1hsentrezg.db version 13.0.0.

Biological interpretation of transcriptome datasets

To identify pathways and processes among regulated genes activated or suppressed in response to saccharides (the "compounds", Ingenuity Pathways Analysis (IPA) (Ingenuity Systems, Redwood City, CA, USA) was used (see below). The output was used to prioritize differentially regulated pathways that reflect Caco-2 responses to compounds, to identify cascades of upstream transcriptional regulators that could explain the observed gene expression changes, and to reconstruct protein-protein networks that could be used to overlay gene expression data and identify central regulatory proteins that were most likely to have driven differential gene expression following compound exposure.

We used three complementary methods for functional analysis of microarray expression data: ErmineJ (GO annotation enrichment or overrepresentation), Gene Set Enrichment Analysis (GSEA) and Ingenuity Pathway Analysis (IPA) (see below). Using these, we performed: (i) identification of statistically supported overrepresentation of functional gene ontology (GO) annotation (ErmineJ and GSEA), (ii) mapping of expression data onto pathways to determine their up- or downregulation in a statistical meaningful way (IPA), (iii) projection of transcript fold-change values of co-expressed genes onto interaction maps of the corresponding proteins (IPA), and (iv) the reconstruction of networks from the interactions of proteins expressed by all gene differentials per dataset to identify central regulators (IPA).

Two complementary methods were employed to relate changes in gene expression to functional changes. One method, ErmineJ, is based on overrepresentation of Gene Ontology (GO) terms[338]. As input all t-test *p*-values from the probe set comparisons across the respective conditions were used. Another approach, gene set enrichment analysis (GSEA) takes into account the broader context in which gene products function, namely in physically interacting networks, such as biochemical, metabolic or signal transduction routes[339]. This method aids the identification of up- or downregulated processes. Both applied methods have the advantage that they are

unbiased, because no gene selection step is used, and a score is computed based on all genes in a particular GO term or gene set.

Pathway analysis

All listed or reconstructed cellular pathways were derived from the expert-annotated pathways that are provided by the Ingenuity Knowledge Base (www.ingenuity.com) that includes protein-protein and protein-DNA interaction information that is present in on-line databases including NCBI, Ensembl, BIND, KEGG and MIPS and cross-checked in the Human Protein Resource Database (www.hprd.org). As a consequence, the data interpretation used curated information. Biological cellular functions and transcriptional networks altered after compound exposure were identified using Ingenuity Pathways Analysis (IPA; Ingenuity Systems, Redwood City, CA). IPA annotations follow the GO annotation principle, based on a proprietary knowledge base of over 1,000,000 protein-protein interactions. For IPA analysis, gene expression ratios x between 0 and 1 were transformed to negative fold-changes using the formula fc =-1/(ratio x). We also performed an IPA upstream regulator analysis to identify the cascade of upstream transcriptional regulators that can explain the observed gene expression changes in a user's dataset, which can provide insights into the biological activities occurring in the tissues or cells being studied. The IPA output includes metabolic and signaling pathways with statistical assessment of the significance of their representation being based on Fisher's Exact Test. This test calculates the probability that genes participate in a given pathway relative to their occurrence in all other pathway annotations. Input gene lists included the differentially regulated genes (FDR p-values (Q values) < 10^{-7} or < 10^{-8} where appropriate; we aimed to include similar amounts, 780-800 genes for IPA analysis of the different HMOs, since the statistical output of the Fisher's Exact Test that IPA uses is sensitive to highly diverse numbers of input genes. IPA computes networks and ranks these according to a statistical likelihood approach. All networks with a score of at least 10 focus genes were considered to be biologically relevant and representative to show part of the underlying biology of the responses of Caco-2 cells to saccharide compounds. Every interaction between gene products in the network was supported by published information that was directly retrieved from within the Ingenuity software suite.

cDNA synthesis

Caco-2 cells were stimulated with 10 mg/ml sialyllactose (5 mg/ml 3'SL + 5 mg/ml 6'SL) for verification of the transcriptome analysis by quantitative PCR (qPCR

analysis). 1 µg of RNA was treated with 1U DNase (Qiagen, Germany) for 15 minutes at room temperature. DNase activity was inhibited with 25 mM EDTA (Invitrogen) followed by incubation for 10 minutes at 65ºC. Next, 300 ng of random primers and 0.5 mM dNTPs were added and incubated for 10 minutes at 65ºC followed by 5 minutes at 4ºC for annealing. Thereafter a mixture of 200 U of Superscript III (Invitrogen), 0.1M DTT (Invitrogen) and 40 of RNAse OUT inhibitor in first strand buffer was incubated for 10 minutes at 21°C. The reaction was performed for 50 minutes at 50ºC using a Biometra T3 Thermocycler (Westburg, The Netherlands). The reverse transcriptase reaction was inactivated by incubation at 70 °C for 15 min. cDNA was stored at -20°C.

Fecal sample preparation and batch cultures

A pooled preparation of infant and adult fecal samples was prepared to test the prebiotic effect of SL and GOS in a batch culture system. All participants had no known gastrointestinal disorders and did not consume prebiotic products three months prior to sampling. Infant feces were collected from 13 infants from 1-6 months of age who did not receive solid food, antibiotics, prebiotics or probiotics in the last month. 6 grams per infants was pooled in 300 ml dilution medium containing lactose (1% w/v) as a carbon source as a replacement for the carbohydrates (e.g. starch, pectin) used in adult fecal cultures[340]. Adult fecal slurry was prepared from fecal samples collected from 16 adult volunteers. Individual fecal samples were diluted to a 10% w/v mixture using 0.1M, pH7, reduced PBS and pooled. This fecal slurry was homogenized for 120 seconds at normal speed (Seward Stomacher 80 Biomaster), aliquoted and frozen until further use. Culture medium was reduced overnight in an anaerobic cabinet (10% H2, 10% CO2, 80%N2) and 10% w/v fecal slurry was added the next day. Cultures were kept at 37ºC for 24 hours. Autoclaved anaerobic chemostat nutrient medium, according to the preparation described in[341] was used. The chemostat vessels were equipped with magnetic stirrers and pH meters that were set to maintain pH between pH6.8 and pH7 by adding 0.5M NaOH or 0.5HCl. 10g/L of GOS or SL were added to the vessels just before adding 10% w/v of the fecal slurry.

Detection of SCFA by HPLC

SCFA levels were measured in fecal samples from the batch cultures using a method developed by Guerrant *et al.*[342].

qPCR

A mixture containing 7µl SYBR Green Mix, 1 µg cDNA template and 2.1 µM of forward and reverse primers per sample was run on the Rotor Gene Real-timer Cycler (Corbett Research, the Netherlands). Primers amplifying genes that were highly modulated by SL in the microarray (Table I) were designed by the authors and synthesized by BaseClear. In total, 35 reaction cycles consisting of 95°C for 15 s, 60°C for 30 s and 72°C for 45 s were run followed by a final hold of 60°C for 1 min. Data was analyzed using RotorGene Q series software (Qiagen). Relative gene-expression was calculated using the Pfaffl method[343]. To assess the abundance of bacterial genera from the different batch cultures by qPCR, fecal slurry was thawed and DNA was isolated using DNA Stool Mini Kit (Qiagen) according to the manufacturer's recommendations. The differential bacterial counts were quantified using quantitative PCR. The bacterial count was calculated based on a dilution series of DNA that was isolated from bacterial strains with predetermined CFU. Primers and probes were based on 16S rDNA gene sequences retrieved by http://greengenes.lbl.gov. Primer Express software was used to design the primers.

Table I. list of qPCR primers for verification of commercial SL

Gene		primer sequence
IFIT1	FW	GTGTCCAGAAATAGACTGTGA
	RV	CCATCCAGGCGATAGGCA
CDK1	FW	TCAACTCTTCAGGATTTTCAG
	RV	GGATGATTCAGTGCCATTT
EDNRB	FW	CTTGGCTCTGGGAGACC
	RV	CACGGAGGCTTTCTGTAT
MARCH3	FW	CTGTCGCACTCTTCACTATTTA
	RV	CAGACTTTGGAATGAGGAGAATC
TNFRSF9	FW	GTAAACAAGGTCAAGAACTGA
	RV	CCATTCACAAGCACAGAC
GAPDH	FW	TGC ACC ACC AAC TGC TTA GC
	RV	GGC ATG GAC TGT GGT CAT GAG

Intestinal microbiota chip

Microbiota samples from infant and adult incubations were analyzed by microarray analysis according to Ladirat *et al.* (2013)[344]. Adult samples were analyzed with the I-chip which was based on adult microbiota composition, whereas the II-chip was used for analyzing the infant samples. The II-chip was based on infant microbiota composition and therefore partly includes different micro-organisms as compared to the I-chip, but all experimental protocols for working with both chips are identical[344].

Caco-2 cell counts and alkaline phosphatase measurements

Caco-2 cells were seeded at a density of 200.000 cells/well in flat bottom 24-wells plates. GOS or SL were added to the wells for four days. Physiological relevant mixtures of SL as present in cow's milk (25 µg/ml 3'SL + 75 µg/ml 6'SL), human milk (125 µg/ml 3'SL + 375 µg/ml 6'SL) or human colostrum (750 µg/ml 3'SL + 2250 µg/ml 6'SL) were included[78] as well as higher concentrations (5 and 10 mg/ml) of equal amounts of 3'SL and 6'SL to match the concentrations of GOS we used. The supernatant was collected and quantified for secreted ALP by QUANTI-Blue (Invivogen, rep-qb2) as a marker of differentiation. QUANTI-Blue powder was reconstituted according to the manufacturer's instructions. 60µl supernatant was added to 190µl QUANTI-Blue and incubated for 3 hours at 37°C. The optical density (OD) at 625µm was measured using a FilerMAX F5 (Molecular Devices, Nederman, Germany). To count the cells, trypsin was added to the cells. The cells were spun down at 1,800 rpm for 5 minutes and washed twice with PBS. 150 µl Trypsin-EDTA (0.25%) (ThermoFisher, 25200) was added to detach the cell monolayer. Trypsin was inactivated by resuspending the cells in 150 µl PBS + 4% FCS + 0.02% EDTA. Dead cells were stained with DRAQ7 (Abcam; ab109202) and 50 µl of 0.975E6 beads/ml (Fluoresbrite YG Carboxylate microspheres 10µm, 18142) were added to count cells (Figure S2; gating strategy). Cells were acquired on a BD FACS Canto II (BD Biosciences) and analyzed using the FlowJo software V10.

Results

Intestinal epithelium

Since the majority of oligosaccharides are considered to be digested in the large intestine, undigested prebiotics may directly affect epithelial cells in the proximal regions of the intestinal tract. To investigate the effect of GOS and SL on barrier functioning, a fully polarized monolayer of Caco-2 cells that has the biochemical characteristics of a small intestinal epithelial cell line[128] was exposed for six hours to 10 mg/ml SL or GOS. We performed a microarray to investigate what pathways were modulated by GOS or SL. IPA identified cellular pathways in Caco-2 cells that had been differentially modulated by the oligosaccharides compared to the medium control. In total, 28 pathways were significantly modulated by SL, of which 3 were predicted to be repressed and one to be induced (Table S1). Of the 25 other significantly modulated pathways, the gene expression profiles did not enable IPA to

predict whether a modulated pathway was induced or repressed. Cell cycle: G2/M damage checkpoint regulation, ATM signaling and role of CHK proteins in cell cycle checkpoint control were all predicted to be downregulated (Figure 1A). The mitotic role of polo-like kinases and ERK5 were predicted to be upregulated by SL (Figure 1A). Next, we investigated the effect of GOS on epithelial barrier transcriptomes. GOS induced the modulation of 63 pathways of which 8 were predicted to be activated and 13 to be repressed according to IPA (Table S1). Interestingly, 4 out of the top 5 most significantly modulated pathways by SL compared to medium control were also significantly modulated in the same direction by GOS (Figure 1B). Additionally, GOS was predicted to induce estrogen-mediated S phase entry and to modulate ATM signaling and role of CHK proteins, pathways that participate in DNA replication checkpoints, among others. Additionally, GOS repressed cell cycle: G2/M DNA damage checkpoint regulation and strongly induction of mitotic roles of polo-like kinases modulation Taken together, IPA analysis showed that both oligosaccharides modulate pathways involved in cell cycle progression and mitosis (supplementary text). Since the SL (FC) used for the transcriptome studies on Caco-2 was isolated from bovine milk and may contain salts, we verified the transcriptome data with qPCR using commercially available, synthesized SL (Carbosynth) (Figure S1). To investigate whether these transcriptional effects were also translated to a cell cycle progression and (or) cell proliferation phenotype, we cultured Caco-2 cells for four days in the presence of SL or GOS in complete medium. The number of cells and the fraction of live cells was assessed using flow cytometry (Figure S2A). We cultured the Caco-2 cells for four days in the presence of SL or GOS the cell number was more than doubled and the monolayer was still non-confluent, suggesting little contact-inhibition (Figure S2B). Caco-2 cells grown for four days in the presence of high concentrations (10 mg/ml or 20 mg/ml) of SL or GOS showed a lower cell count (Figure 1C), which could partially be explained by cell death (Figure S2C). Nevertheless, GOS showed more cell death in higher concentrations (> 3 mg/ml) as compared to SL (Figure S2C). This decrease in cell counts was accompanied by an increase in ALP activity at high concentrations of GOS and SL (Figure 1D), which suggests that SL and GOS modulate the balance between cell proliferation and cell differentiation and may thus influence intestinal homeostasis.

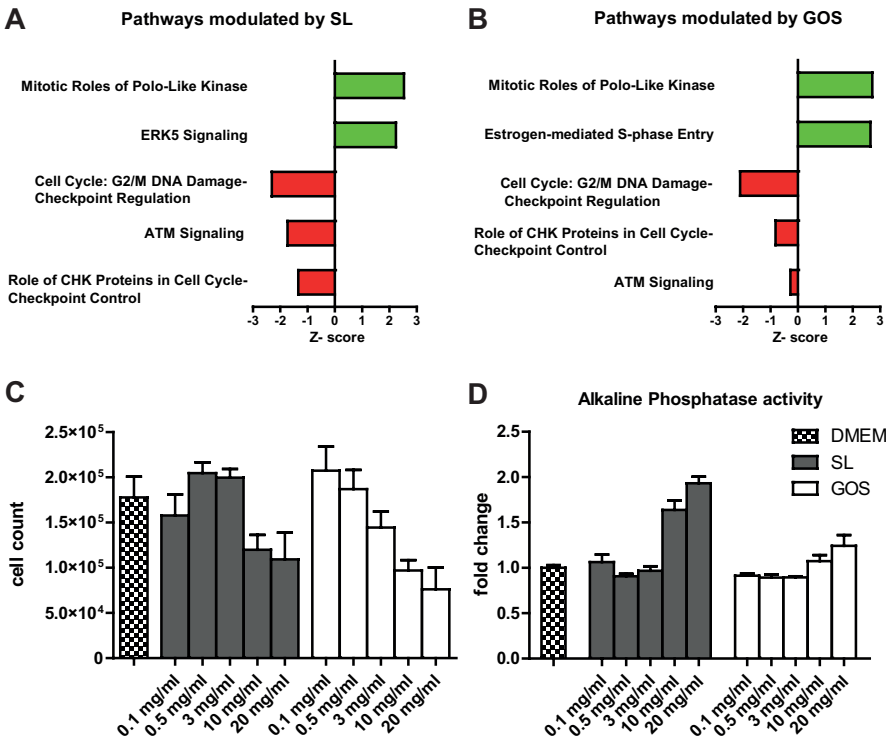


Figure 1. SL and GOS modulate cell proliferation and induce differentiation. A fully polarized epithelial layer of Caco-2 cells cultured in DMEM + 10% FCS was exposed to 10 mg/ml SL or GOS for 6 hours. After 6 hours the cells were lysed and RNA was isolated for microarray analysis and subsequent IPA analysis. The top five most significantly, excluding ERK5 (p=0.07), regulated pathways by (**A**) SL and (**B**) GOS (IPA analysis results). Next, phenotypic changes were assessed by culturing Caco-2 cells for 4 days in the presence of different concentrations of GOS and SL (0.1- 20 mg/ml), followed by (**C**) cell counting by flow cytometry and (**D**) ALP activity determination in culture supernatants.

We thus showed that GOS and SL alter cell cycle progression and, at higher concentrations, halt proliferation and induce differentiation of Caco-2 cells. We therefore investigated the effect of SL and GOS on re-epithelialization, an important phase in wound-healing where control of epithelial cell proliferation and differentiation is tightly controlled by intrinsic pathways. We used a scratch assay that tracks the presence (in terms of numbers and location) of epithelial cells in the scratch area over time. Caco-2 cells are tightly connected to neighboring cells, which makes this cell line unsuitable for scratch assays. We therefore used Ca9-22 cells (immortalized gingival-) epithelial cells[330]. We tested a mixture of SL as present in cow's milk (0.1 mg/ml), human breast milk (0.5 mg/ml) and human colostrum (3 mg/ml) and the same concentrations of GOS. Strikingly, GOS and SL induced a significant increase

in the maximum number of cells present in the scratch area five hours after applying the oligosaccharides when compared to medium control (Figure 2A). The repair rate, as a non-linear measurement of cellular influx over time, also showed an increase at the lowest dose used although not significantly different when compared to the medium control (Figure 2B). The positive control TGFα, stimulated a significant increase in the repair rate (Figure 2B) whereas the maximum number of cells present in the scratch area was not significantly higher (Figure 2A). The negative control consisting of p38 mitogen-activated protein kinase- and MEK1/2 phosphorylation inhibitors resulted in a significantly lower maximum number of cells present in the scratch area and repair rate (Figures 2A-B). Thus, SL and GOS in concentrations present in cow's milk and breast milk both stimulated re-epithelialization of Ca9-22 in terms of a more complete closure of the scratch area within 5 hours after applying the oligosaccharide mixture (Figure 2C).

Microbiota

The microbiota plays an important role in gut homeostasis and barrier functioning. We therefore investigated the effects of SL and GOS on microbiota composition. Pooled adult or infant fecal cultures were spiked with 10 mg/ml SL or GOS. Fecal slurry was collected after 3, 6, 9 and 24 hours for microbiota analysis. Microbiota analysis was first performed on genus level by performing qPCRs using generic or specific 16S rRNA primers to detect the numbers of total bacteria, *Bifidobacterium*, *Bacteroides*, *Lactobacillus* and *Escherichia coli*. The starting amount of bacteria in the fecal slurry of the pooled adult samples (Figure 3A) was higher compared to those of infants (Figure 3D). Nevertheless, the total bacterial numbers in infant and adult cultures increased in the first 6 hours of the culture, independent of the inoculum (Figures 3A,D). Notably, infants showed relatively to the low bacteria numbers a high amount of bifidobacteria compared to adults. In infant batch cultures, SL and GOS both induced an increase in bifidobacteria (Figure 3F) while lactobacilli numbers were not markedly increased compared to control (Figure S3A,D). Also in infant batch cultures, the number of *Bacteroides* bacteria had increased after spiking by GOS (Figure 3E). After spiking of fecal batch cultures of adults by SL, the numbers of *Bacteroides* bacteria had increased; this increase had not been induced by GOS (Figure 3B). However, again in the adult fecal batch cultures, GOS substantially promoted the outgrowth of bifidobacteria (Figure 3C). No differences were observed in the numbers of *Clostridium* and *Escherichia* (Figure S3).

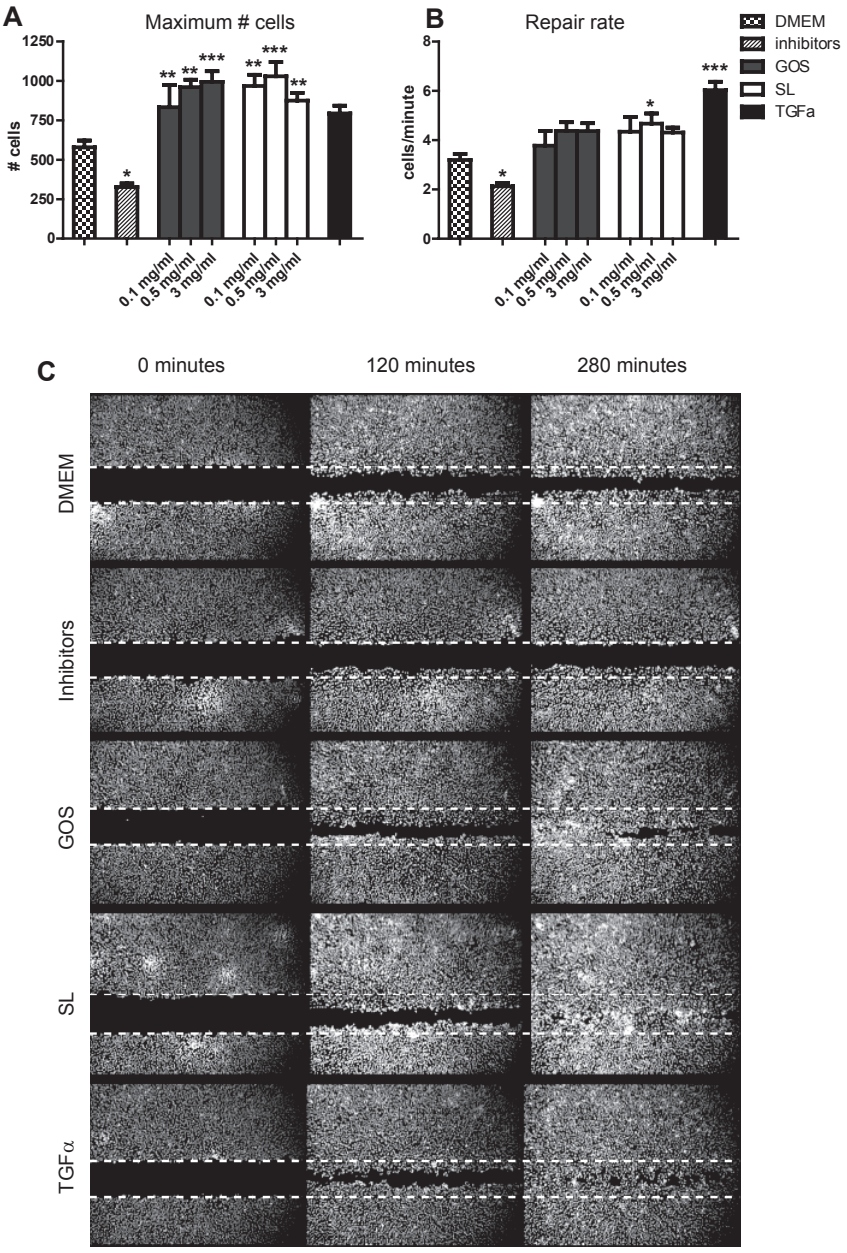


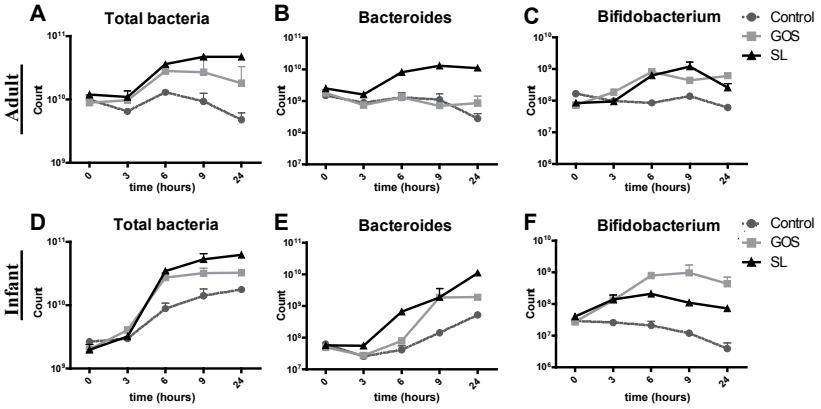
Figure 2. SL and GOS promote re-epithelialization, one aspect of wound healing. Longitudinal scratches were applied to a confluent layer of labelled Ca9-22 epithelial cells. The medium with cell debris was replaced by fresh medium containing mixtures of 3'SL and 6'SL as present in cow's milk (0.1 mg/ml), human milk (0.5 mg/ml) and human colostrum (3 mg/ml) or matching concentrations of GOS or inhibitors or TGFα that were used as negative and positive controls, respectively. (**A**) The total number of cells at the end of the measurement (t = 280 minutes) and (**B**) the increase in cell numbers over time (i.e. repair rate) was calculated using a non-linear model using the Gompertz equation. (**C**) An image of one representative well per stimulus is shown.

Next, fecal slurries from time point 0 and 24 hours were analyzed at species level using an intestinal microflora chip (I/II-chip; TNO). Of the 400 species quantified on the chip, changes in bifidobacteria, Bacteroides and several other species were markedly changed by the prebiotic supplements. Overall, the chip analysis confirmed the 16S rRNA qPCR data, showing that Bacteroides and bifidobacteria were markedly increased in adult batch cultures in the presence of SL and GOS, respectively (Figure 3G), and that this distinct pattern was less pronounced in infant fecal batch cultures (Figure 3F). In adult batch cultures, SL induced a specific increase of Faecalibacterium prausnitzii, Ruminococcus obeum, Collinsella aerofaciens, Eubacterium halii and Ruminococcus productus (syn. Blautia producta, comp. nov.) (Figure 3G). GOS increased the abundance of several Bifidobacterium species, including B. bifidum, B. infantis and B. longum in adult cultures (Figure 3G). In infant cultures, SL induced the specific outgrowth of members from the B. prevotella group and B. thetaiotaomicron (Figure 3H). In line with the qPCR data, this increase in abundance of Bacteroides species (e.g. B. fragilis, B. thetaiomicron, B. ovatus) induced by SL was also seen in if infant batch cultures were supplemented with GOS (Figure 3H). The specific outgrowth of Bifidobacterium species GOS was less pronounced in batch cultures from infants compared to adults (Figure 3H). Thus, we showed that SL and GOS distinctly modulate microbiota composition as shown by the outgrowth of Bacteroides and bifidobacteria species, respectively. Several of the abovementioned taxa belong to the class of Clostridia (i.e. Rumincococcus species), well-known producers of SCFAs, suggesting that supplementation of SL and (or) GOS in fecal batch cultures might alter SCFA production.

Short chain fatty acids

Next, we assessed whether the microbial changes induced by SL or GOS altered SCFA production in the fecal batch cultures. SL and GOS both boosted the total production of SCFA including the production of acetate in adult- (Figure 4B) and infant batch cultures (Figure 4H). Strikingly, in contrast to the control batch culture that showed no butyrate production, butyrate was produced after 9 hours of the batch culture, only if GOS or SL were present (Figures 4C, I). In adult batch cultures, GOS (Figure 4D) and SL (Figure 4E) induced the production of lactate and propionate, respectively. The concentration of lactate declined after 6-9 hour of batch culturing, indicating that secondary producers may have used lactate as a carbon source (Figure 4D ,J). This decline in lactate coincided with the increase of butyrate if growth medium was supplemented with GOS or SL (Figures 4C,I). These

secondary producers include *Eubacterium halii* and *Faecalibacterium prausnitzii* and *Clostridium butyricum* as quantified in adult (Figure 3G) and infant (Figure 3H) batch cultures, respectively. Butyrate was also produced in batch cultures inoculated with SL in absence of lactate production (Figures 4C and D). In infant fecal cultures, lactate (Figure 4G) and propionate (Figure 4K) were produced in the presence of SL or GOS. The concentration of formate, acetate and total SCFA was higher in SL and GOS-containing cultures compared to batch cultures in baseline medium (Figures 4A,B,F,G,H,L). Thus in line with the associated changes in microbiota composition, SL and GOS induce a distinct SCFA profile that is dominated by propionate and lactate, respectively.



G Adult

strain	Control		GOS		SL	
Bacteroides distasonis	4	8	16	9	29	26
Bacteroides fragilis	18	36	104	146	144	242
Bacteroides ovatus	27	25	106	150	111	201
Bacteroides prevotella group	11	16	29	52	50	142
Bacteroides thetaiotaomicron	31	52	92	81	137	232
Bacteroides vulgatus	11	20	66	37	127	240
Bacteriodes species	28	82	75	110	114	220
Bacteroides group	8	17	26	35	70	83
Bifidobacterium Catenulatum	9	9	59	32	42	38
Bifidobacterium catenulatum group	7	11	10	26	15	43
Bifidobacterium species	77	45	110	212	168	167
Bifidobacterium group 02	5	7	9	13	15	33
Clostridium butyricum	1	1	21	22	35	19
Prevotella group 03	8	4	28	2	55	13
Ruminococcus albus	19	6	20	1	90	6
Streptococcus agalactiae	2	2	11	30	1	1
Streptococcus anginosus	1	1	4	9	2	1
Streptococcus bovis	4	2	59	80	3	6
Veillonella species	4	8	59	1	78	5

H Infant

strain	Control		GOS		SL	
Bacteroides fragilis	8	13	37	13	134	111
Bacteroides ovatus	201	173	144	87	107	104
Bacteroides vulgatus	116	135	129	193	179	216
Bacteroides prevotella group	35	20	40	49	99	66
Bacteroides thetaiotamicron	10	10	10	4	2	2
Bifidobacterium Adolescentis / B. Angulatum	4	9	87	18	8	6
Bifidobacterium angulatum	1	1	39	12	1	1
Bifidobacterium bifidum	3	4	40	45	6	7
Bifidobacterium Breve	1	1	5	15	1	1
Bifidobacterium catenulatum group	3	4	67	11	4	3
Bifidobacterium infantis	1	1	13	46	2	2
Bifidobacterium longum	2	2	14	74	2	2
Bifidobacterium group 02	4	6	100	40	4	4
Bifidobacteriaceae	6	11	123	72	13	13
Collinsella aerofaciens	3	45	89	86	173	215
Eubacterium halii	1	3	39	6	20	33
Faecalibacterium prausnitzii	1	4	4	3	52	86
Ruminococcus albus	6	41	73	12	131	97
Ruminococcus obeum	2	2	2	3	10	19
Ruminococcus productus	1	1	1	1	26	25

Figure 3. SL and GOS differentially modulate microbiota composition. Batch cultures of adult and infant pooled fecal samples cultured in growth medium were supplemented with or without SL or GOS in duplo. Fecal samples were collected at the start of the batch culture and after 3, 6, 9 and 24 hours. Microbiota composition on genus level (**A–F**) and on species level (**G,H**) was determined by qPCR and chip analysis, respectively. Bacterial numbers were shown as mean ±SEM of two independent batch. Raw fluorescence data are shown for both individual runs for chip analysis.

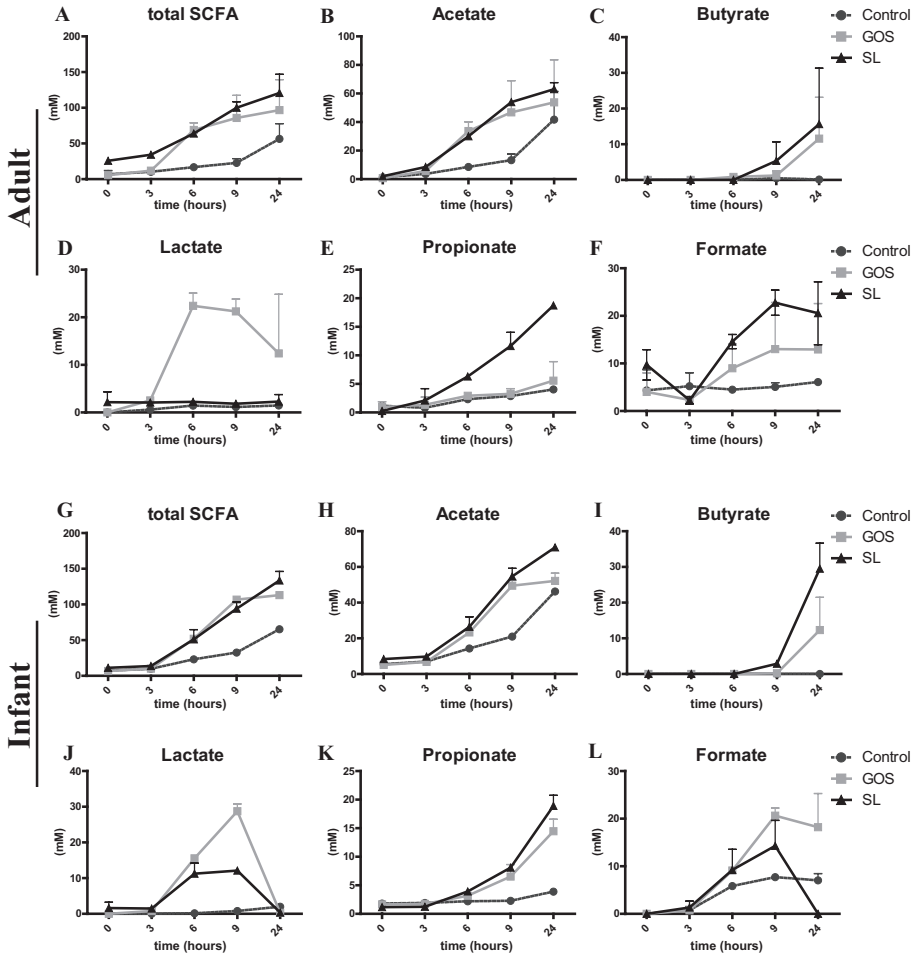


Figure 4. SL and GOS differentially stimulate SCFA production. Batch cultures of (**A-F**) adults and (**G-L**) infants pooled fecal samples were inoculated with SL or GOS. Fecal samples were collected at the start (0 hours) and after a culture time of 3, 6, 9 and 24 hours. SCFA levels were measured in the fecal samples by HPLC. SCFA levels of two independent batch cultures were represented as mean ±SEM.

Discussion

Dietary fibers are widely considered to contribute to infant and adult intestinal health, via largely unexplored biological mechanisms that are thought to result from microbial fermentation of fibers into short-chain fatty acids (SCFAs). In this paper we show that SL and GOS both directly positively contribute to regulation of epithelial cell proliferation and differentiation *in vitro.* Transcriptome analysis of

Caco-2 intestinal epithelial cells suggested that cell cycle pathways were modulated by SL and GOS; this could be corroborated in bioassays where phenotypes showing less proliferation and more differentiation were obtained. *In vitro* epithelial wound healing assays showed that SL and GOS oligosaccharides also promoted re-epithelialization of a scratch area introduced in a confluent epithelium monolayer, an important aspect of wound healing. Additionally, our data showed that SL and GOS distinctly modulate microbiota composition, by promoting the outgrowth of *Bacteroides* and bifidobacteria, respectively, which resulted in distinct changes in SCFA profiles, including increased propionate and lactate levels, respectively, and increased levels of butyrate for both SL and GOS.

The epithelial lining along the GI tract is the physical barrier that separates the intestinal lumen from the underlying tissue and protects against harmful antigens. Of note, gut closure (i.e. the state in which no macromolecules leak through the barrier) occurs in humans, in contrast to mice, already a few days after birth[127]. Later in infancy, infection may temporarily disrupt barrier function, so continuous barrier function support is important to appropriately protect underlying tissues from invasion and to prevent inflammation. Nutritional intervention to support barrier function is especially relevant in premature infants whose gut function is characterized by immature mucosal and immune function and high level epithelial leakage, increasing the risk of uncontrolled inflammatory responses and the detrimental consequences of such response. Microbiota dysbiosis in combination with barrier dysfunction, and unbalanced immune responses can aggravate these detrimental consequences[222]. HMO have been shown to improve intestinal homeostasis *in vitro*[135,345,346] and provide systemic anti-inflammatory effects in animal models[294]. It is thus essential to translate these *in vitro* and animal studies to human nutritional intervention studies.

We assessed the effect of GOS and SL on epithelial barrier functioning using *in vitro* assays investigating proliferation, differentiation and re-epithelialization. Our transcriptome analysis showed that GOS and SL both modulated the expression of genes involved in regulating stages of the cell cycle (e.g. cyclins and cyclin-dependent kinases; see Supplementary text). To investigate how this transcriptome profile showing modulation of pathways regulating cell cycle control translated into an epithelial phenotype in Caco-2 cells, we cultured the Caco-2 cells for four days in the presence of HMO. Cell counts after 4 days showed that at higher concentrations (>10 mg/ml) SL and GOS suppressed proliferation and induced differentiation, as shown

by increased activity of ALP, a well-established differentiation marker for enterocytes. ALP was shown to modulate microbiota composition and inactivate different serotypes of LPS[347]. The effects of SL that we observed are in line with previous findings by other groups that also reported suppressed proliferation and induced differentiation[135,346]. Thus, SL and GOS halt proliferation and induce production of ALP, which is a marker for intestinal homeostasis and epithelial differentiation. Importantly we show in this study that this effect was not restricted to SL, but was also observed when Caco-2 cells had been incubated for 4 days with GOS.

Since SL and GOS modulated very basic cellular mechanisms involved in epithelial differentiation, we questioned whether they are also capable of promoting re-epithelialization of epithelial cells, a process that involves tight regulation of proliferation and differentiation of epithelial cells. In the re-epithelialization phase of the "wound healing" process, cells migrate into the wound area, which involves reorganization of cellular cytoskeleton and cellular differentiation after the wound is closed[348,349]. HMO were previously shown to indirectly contribute to the wound healing process in the colon by modulating microbiota composition[349] and boosting SCFA production[350]. Here, we observed that GOS and SL in concentrations present in cow's milk and breast milk can directly induce closure of the scratch area representing the wound in an *in vitro* model of wound healing. A well-known signaling cascade that induces re-epithelization is activation of the epidermal growth-factor receptor (EGFR) by ligands such as TGFα[351,352]. Interestingly, acidic HMO fraction and to a lesser extent, neutral HMO induced EGFR signaling in HT-29 cells[345], and we observed a similar boosting effect of the acidic SL and neutral GOS in our re-epithelialization model. Thus, we show here that oligosaccharides such as GOS and SL may promote epithelial barrier function and thus contribute to intestinal homeostasis. Future studies could investigate these effects on barrier function in early life using animal (e.g. allergy or colitis) models.

Since the early 1980's, research unraveled that breastfed infants show higher numbers of bifidobacteria and a lower pH compared to bottle-fed infants supplemented without prebiotics[267,353]. This lower pH of the stool in breastfed infants is caused by higher lactate and acetate levels[318,354]. Infant formulae supplemented with a mixture of GOS/FOS promote a more bifidogenic microbiota composition[324–328] and a SCFA profile more similar to breast-fed infants[329,354]. In line with these findings, our batch cultures showed that GOS induced the outgrowth of bifidobacteria and an increased

production of lactate. Although to a lesser extent, SL also boosted the abundance of bifidobacteria, which is in line with earlier reports showing that different strains of bifidobacteria are capable of metabolizing both neutral and acidic HMO[102,103,355].

We showed that GOS induced the production of acetate and lactate in fecal batch cultures of adults and, to a lesser extent, in infants. Animal models have shown that acetate can protect against *E.coli* infections in the gut[356] and systemic diseases such as asthma[45]. The concentration of lactate declined rapidly after 9 hours of the culture, indicating utilization by other bacteria which may include secondary butyrate producers (e.g. *Eubacterium halii* and *F. prausnitzii*)[357]. Butyrate is known to induce anti-inflammatory response via binding G-protein coupled receptor (GPR) 109A and downregulation of NF-κB activity[358].

Multiple pathogenic bacteria and several commensal bacteria from different phyla encode genes that may participate in biochemical pathways that utilize sialic acid[359]. Commensal bacteria that express pathways to utilize SL are *Bacteroides* members such as *B. fragilis*, *B ovatus* and *B. vulgatus*. Our data shows that *Bacteroides* increase in abundance upon culturing in the presence of SL. SL also increased the abundance of *Ruminococcus obeum* that was shown to switch to propionate production if fed with fucose or rhamnose[360]. Propionate has been shown to exert systemic anti-inflammatory responses in allergic animal models for instance, suppression of allergic inflammation[44]. Recent evidence shows that purified sialylated bovine milk oligosaccharides are mainly metabolized by *B. fragilis*[158], which is in line with *in vitro* cultures[361]. The authors showed that sialylated oligosaccharides may be essential for optimal growth in early life. *B. fragilis* was also shown to contribute to immune homeostasis by producing polysaccharide A, a molecule that was shown to induce differentiation of CD4+ T cells into regulatory T cells in the gut[362].

Importantly, our results show that SL and not GOS promotes the outgrowth of *F. prausnitizii* in adult batch cultures. GOS supplementation to elderly[363] or healthy adults did also not show a marked increase in fecal *F. prausnitizii* abundance[364]. The infant pooled samples did not show the presence of *F. prausnitzii* at the start of the batch culture that might explain its absence after the incubation period with the inoculum (data not shown). Interestingly, at least some *F. prausnitzii* strains produce proteins that degrade sialic acid[359]. *F. prausnitizii* induces anti-inflammatory responses which is shown to protect against colitis in animal models[365] and its prevalence has

been reversely associated with the prevalence of ulcerative colitis in adults[366]. However, increased *F. prausnitizii* numbers were observed in pediatric Crohn's disease[367].

Conclusions

In conclusion, SL and GOS directly interact with the epithelial lining where they can support differentiation and wound repair. In addition, these compounds may distinctly modulate intestinal microbiota composition and activity and change the corresponding SCFA profiles in the gut lumen. The consequences of the effects in terms of the host health in infants and adults remain to be determined.

Acknowledgements
We thank Ellen Looijensteijn, Stefanie Oude Elferink, Alfred Bonte and Helmie van Dijck of FrieslandCampina for conducting the fecal batch culture experiments and quantification of bacteria by qPCR.

Supplementary files

Table S1. Number of pathways predicted to be induced or repressed by GOS or SL.

	GOS	SL
total	63	28
NaN	39	24
positive Z-score	8	1
negative Z-score	13	3
Z-score = 0	3	0

SL vs medium	
(log2 based) Fold Change	gene name
-7.91	IFIT1
2.32	FOXM1
2.70	CDK1
5.43	TNFRSF9
6.36	EDNRB
6.69	MARCH3

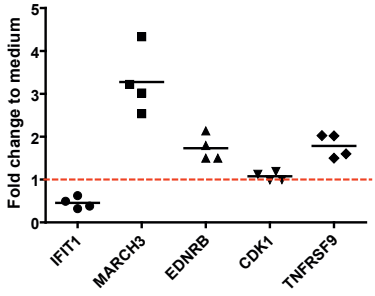


Figure S1. Comparison gene-expression induced by SL as quantified by transcriptome and qPCR.

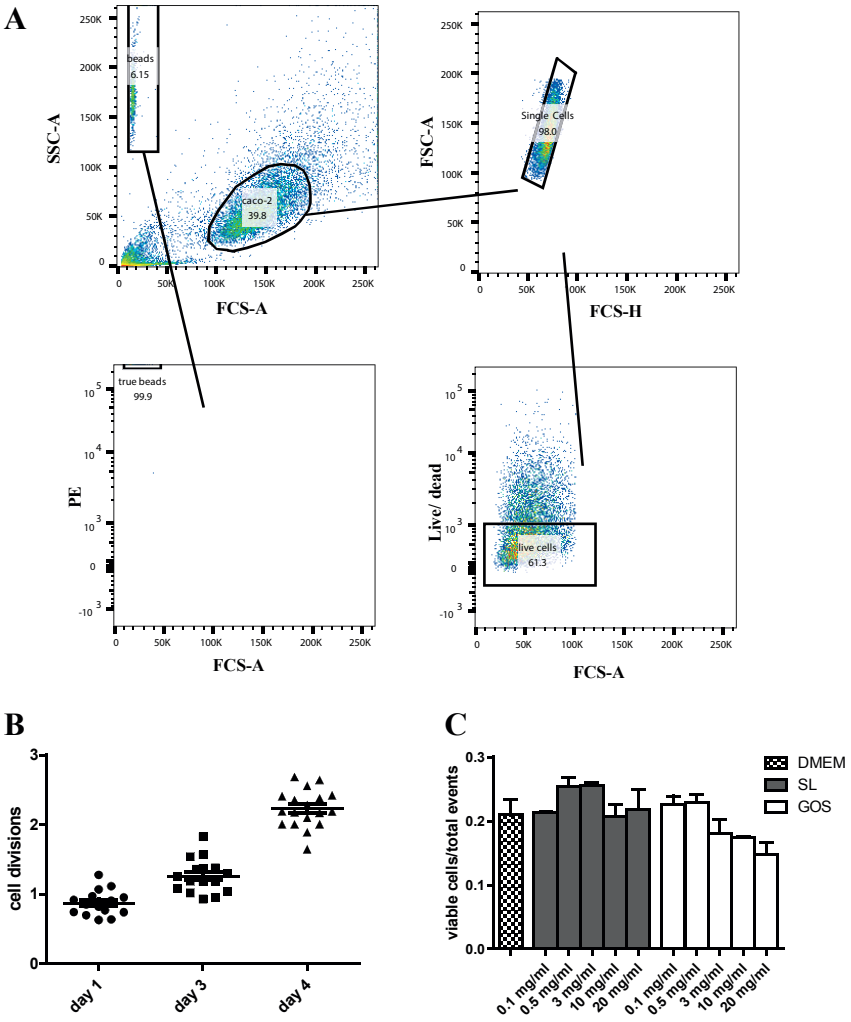


Figure S2. Counting of viable Caco-2 cells by flow cytometry. (**A**) Counting beads were added to the trypsinized cell suspension of Caco-2 cells to count the number of viable cells. Caco-2 cells were gated based on size (FSC-A) and granularity (SSC-A). Single cells were selected based on plotting FSC-A versus FSC-H. These single cells were then gated on live cells. The number of viable cells/ml was calculated by: viable cell count/ (volume of beads * beads count) * bead concentration. (**B**) the number of Caco-2 cells grown in DMEM + 10% FCS was counted at different days of culture. (**C**) viable Caco-2 cell counts if cultured in the presence of SL or GOS.

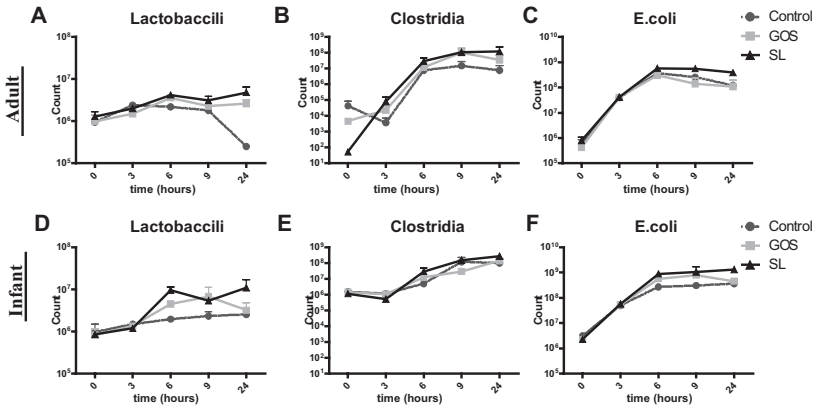


Figure S3. SL and GOS do not modulate the numbers of lactobacilli, *Clostria* or *E. coli*. Batch cultures of (**A-C**) adults (**D-F**) and infants pooled fecal samples were inoculated with SL or GOS. Fecal samples were collected at the start and after a culture time of 3, 6, 9 and 24 hours and determined for the numbers of bacteria by qPCR.

Supplementary text

In this transcriptome study, we found, using Ingenuity Pathway Analysis (IPA), that cell cycle control stands out among the significantly modulated pathways. Intriguingly, our transcriptome analysis shows that GOS and SL modulate cell cycle control in a similar fashion in Caco-2 cells. Here, we provide a more extensive analysis of cell cycle genes that were significantly differentially regulated.

Cell cycle control is regulated by two key classes of molecules, cyclin-dependent kinases (CDKs) and their binding partners, the cyclins. In mammals there are a wide variety of CDK that form complexes with the corresponding stage-specific cyclins during different phases of the cell cycle. In the first phase of the cell cycle, the G1 phase of cell division, the cell increases in size and ensures it is ready for DNA synthesis. Cdk4 are activated by cyclin D proteins, resulting in the transcription of E2F transcription factors and cell cycle progression. E2F responsive genes are CCNA and CCNE, coding for cyclin A and E, respectively. Cyclin E activates Cdk2, resulting in E2F transcription, which results in entry into the S-phase. Our transcriptome data shows that GOS and SL upregulate genes involved in G1/S phase progression; these genes can also be regulated by estrogen. This is in agreement with the predicted (IPA analysis) induction of the estrogen-mediated S-phase entry pathway by GOS. Our IPA output shows that this pathway is only induced by GOS and not by SL, which can be explained by the strict statistical cut-off value (i.e. FDR 3^{-8}) that we used for the analysis of the transcriptome of SL stimulated Caco-2 cells. Nevertheless, SL also

induced these genes involved in G1/S progression (Figure 2C).

At the beginning of the S-phase, cyclin A that phosphorylates proteins involved in DNA replication, is synthesized. Finally, cyclin B binds CDK1 to enter prophase and drive mitosis (Peters 2002). Our results showed that CDK1, CCNB1 and CCNB2 are upregulated by SL and GOS. Yet additional proteins regulate cell cycle. PLK activates CDC25 and together with CDK1 initiate the entry of G2 to mitosis phase. PLK and CDC25A and CDC25C are upregulated by SL and GOS. Cyclin B:CDK1 complexes are involved in the final stage of the cell cycle, inducing cell proliferation. PLK and MKLP1 are also needed for the formation of mitotic spindles (Sumara et al. 2004) and midzone formation (Zhu et al. 2005). In line with the upregulation of genes coding for the Cyclin B:CDK complex, these mitosis progression genes were upregulated as well by SL and GOS treatment. Proteins of the Cip/Kip family (e.g. $p21^{Cip1}$ and $p27^{Kip1}$) inhibit the activity of cyclin A, B and E activity thus blocking multiple stages of cell division. Interestingly, we found that $p21^{Cip1}$, coded by CDK1NA had been 3 fold downregulated in SL and GOS treated Caco-2 cells. A table listing the differential expression of cell cycle control genes is provided below (Table S2). In summary, our IPA results showed that at least some concentrations of SL and GOS induce the transcription of genes that control multiple stages of cell division.

Table S2: Genes involved in cell cycle control as fold change compared to medium control. Genes were selected based on the SL dataset with a FDR cut-off value of 10^{-8}.

	fold change	
	SL	GOS
AURKA	4.1	3.3
BLM	2.0	3.7
CCNA2	3.3	4.8
CCNB1	4.5	3.6
CCNB2	3.2	3.2
CCNE2	4.5	5.5
CDC25A	4.8	4.4
CDC25C	5.9	4.4
CDK1	6.5	6.5
CDKN1A	0.4	0.3
CHEK1	2.6	3.5
CKS2	7.6	4.4
CREB3L3	0.4	0.3
E2F1	5.6	3.8
E2F7	3.4	5.9
E2F8	3.9	5.2
FANCD2	0.5	4.2
FBXO5	4.0	3.7
HIPK2	2.9	3.1
KIF11	3.7	4.4
KIF23	7.3	5.5
MDM2	3.3	3.7
PCNA	3.0	3.8
PLK1	5.4	4.3
PLK4	6.1	4.9
PPM1D	6.6	2.7
PRC1	7.4	4.0
PTTG1	4.4	3.1
RPS6KA1	3.2	2.5
SFN	10.1	3.2
SMC2	3.7	3.8
SMC4	4.1	3.5
TOPB1	2.8	2.6
TP73	3.3	3.2
FOS	2.2	3.2
FOSL1	6.1	4.9

Chapter 8

3'SL increases Akkermansia prevalence in mice fed a low-fiber diet

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Manuscript in preparation

Abstract

Epidemiological studies show that the consumption of cow's milk in early life is associated with reduced prevalence of asthma, hay fever and allergic sensitization. Since causality cannot be proven in controlled studies due to safety risk of raw cow's milk consumption in infants, mechanistic studies are limited to *in vitro* work and animal models. Mouse models show that allergies and asthma can be prevented by a high fiber diet, boosting the production of microbial-derived short chain fatty acid (SCFA) that have local and systemic anti-inflammatory effects. Cow's milk is rich in immune related proteins and contains sialylated oligosaccharides such as 3'-sialyllactose (3'SL) and 6'-sialyllactose (6'SL). Here we investigated whether cow's milk, 3'SL or 6'SL can protect against asthma via effects on the microbiota. To this aim we used an ovalbumin (OVA)-induced asthma model using female BALB/c mice. Mice were switched from normal chow to a low-fiber diet at the start of the study and had *ad libitum* excess to drinking water supplemented with 100 μg/ml 3'SL, 100 μg/ml 6'SL or lactose-depleted raw cow's milk. After sensitization with OVA, airway inflammation was measured by airway hypersensitivity measurements after metacholine challenges, and white blood cell numbers in bronchioalveolar lavage fluid were quantified. Fecal and cecal samples were collected and analyzed for microbiota composition and SCFA levels, respectively. No significant differences were found between mice receiving raw cow's milk or sialyllactose in methacholine-induced airway hyper-responsiveness or white blood cell counts in bronchioalveloar lavage fluid. A gradual decrease in Bacteroidetes and increase in Firmicutes was observed during the complete course of the study. Most of the variation in microbiota composition was explained by the day of collection, rather than the dietary supplementation with raw milk or sialyllactose. Nevertheless, mice that received 3'SL or raw cow's milk showed a higher abundance of the genus *Akkermansia* or *Corynebacterium*, respectively at the day of sacrifice. No differences in SCFA levels in the caecum were found. We conclude that the microbial changes are most likely driven by the transition of normal chow to low-fiber diet. It is therefore possible that the immunomodulatory effects of the dietary intervention are masked by the effect of the low-fiber diet. Future studies should therefore include a longer run-in period (of at least four weeks) before studying the effect of a dietary intervention.

Introduction

Children growing up on a farm have a lower risk of developing asthma, hay fever and allergic sensitization[83]. Interestingly, epidemiological studies show inverse correlations between the consumption of cow's milk in early life and allergy and asthma prevalence[66,84,86,88,368,369]. A few epidemiological studies show that the thermosensitive fraction of cow's milk is responsible for the protective effect[66,88], while others do not specify the heating status of the milk or only look at unprocessed cow's milk[84–86,368–370]. However, causality cannot be proven since controlled studies with unprocessed cow's milk in infants are ethically not possible due to safety reasons. Hence, the effects of cow's milk on the mucosal immune system has mostly been investigated *in vitro*. Nevertheless, a recent study in mice showed that raw cow's milk and not heated cow's milk prevents against house dust mite (HDM) induced asthma[67]. Several mechanisms (e.g. improved epithelial barrier functioning and induction of regulatory immune cells) have been proposed by which cow's milk contributes to immune homeostasis in the respiratory tract[371].

It is appreciated that there is a critical window in early life in which the microbiota composition and diversity, driven by environmental factors such as diet, is essential for appropriate immune functioning later in life[49,163]. The microbiota is essential for the host for energy harvest and fermentation[323]. In the last decade a wealth of information is emerging showing that the microbiota and microbial-derived metabolites have a central role in human physiology and health[321–323]. Fermentation in the colon of non-digestible carbohydrates by the microbiota results in the production of metabolites that become available to the host[372]. When these non-digestible carbohydrates confer physiological effects to the host they may be defined as functional fibers[372].

One group of metabolites produced in large amounts upon fiber fermentation in the colon are short-chain fatty acids (SCFA). SCFA as well as other metabolites (e.g. medium-chain fatty acids) bind specific G-protein coupled receptors that induce directly (e.g. on colon epithelium and immune cells) or indirectly (e.g. epigenetics) anti-inflammatory responses in the host[321]. Interestingly, two recent mouse studies show mechanistic evidence for the consumption of fibers, which resulted in the production of SCFA that protected against asthma[44,45]. Trompette *et al.* (2014) showed a higher abundance of *Bifidobacteria* and a higher production of SCFA in mice fed with pectin[44]. The SCFA propionate showed to alleviate HDM-induced allergic symptoms

in a GPR41-dependent manner. Interestingly, the authors showed that propionate induced hematopoiesis of dendritic cell precursors. These dendritic cells were found in the lungs and showed an impaired ability to induce Th2 responses[44]. The second study showed that acetate can inhibit allergic airway diseases by enhancing regulatory T cell numbers and function, which was induced by epigenetic changes promoting transcription of the FOXP3 promoter[45].

In contrast to breast milk, cow's milk contains only a limited amount of non-digestible oligosaccharides that are dominated by sialylated oligosaccharides of which the trisaccharides 3'-sialyllactose (3'SL) and 6'-sialyllactose (6'SL)[78]. 3'SL and 6'SL are utilized by several bifidobacteria strains *in vitro*, resulting in the production of SCFA[103].

Thus, cow's milk might protect against allergies and asthma, although the relative contribution of milk proteins and sialyllactose is not known. One protective mechanism against upper airway inflammation would be the prebiotic effect of SL resulting in an enhanced production of SCFA. To this aim, we investigated the effect of raw cow's milk, 3'SL and 6'SL in an OVA-induced asthma model of BALB/c mice fed a low-fiber diet.

Materials and Methods

Mice

Ten week old in-house bred BALB/c female mice (n=8 per group) were housed at the animal facility of ALK-Abelló, Hørsholm, Denmark. Mice were fed normal chow, and were switched to a low-fiber chow at the start of the study (C1013, Altromin). Drinking water was available *ad libitum*. Animal experiments were conducted in accordance to the Danish animal ethical committee (2016-15-0201-01059).

Raw milk preparation

Raw milk from a local farm was stored at <6°C until used. The protein, fat and lactose content of the milk was measured in duplicate by the Milkoscan (Foss Electric, Hillerod). Milk samples were sent to internal lab (LQS FrieslandCampina, Leeuwarden) for detailed compositional and microbiological analysis. Diafiltration together with concentration was performed using an ultrafiltration system with a

hydrophilic Poly Ether Sulfone membrane (model TUF3838 N1) with a molecular weight cut-off of 10 kDa in a spiral-wound UF format (Toray Membrane Europe AG). During UF concentration, the retentate was recycled and the permeate was continuously removed from the system until a mean concentration factor (CF) of 2, as determined by the volume of permeate collected, reached at constant transmembrane pressure (167 kPa) and temperature(15°C). At a concentration factor 2, diafiltration water was added to the balance tank and the same volume of permeate was collected. This procedure was repeated two more times. After the final water addition, permeate was removed till the final CF of 3 and desired lactose to protein ratio was achieved. The final retentate was fed in multiple disinfected metal trays and kept in a freeze dryer (Virtis Genesis 25XL) to produce raw milk powder. The produced lactose depleted powder samples from each tray were collected into sterilized bags and vacuum sealed. The powder was frozen at -20°C. To improve the solubility, the raw cow's milk powder was freshly dissolved at 50% of the protein concentration found in the milk.

OVA-induced asthma model

A schematic overview of the experimental set-up is shown in Figure 1. Mice were group housed from the start of the study. Mice had *ad libitum* access to 100 μg/ml 3'SL (Carbosynth, OS04397), 100 μg/ml 6'SL (Carbosynth, OS04398) or 50% of the protein content found in the raw cow's milk (FrieslandCampina) in the drinking water. Raw cow's milk was depleted for lactose, freeze-dried and the powder was kept at -20°C until further use. Drinking bottles were refreshed each day to limit bacterial growth. The cow's milk was tested negative for mouse pathogens. Mice were weighed at day 0, 14, 20 and 30 and individual fecal samples were collected at day 0, 14 and 30. A parallel experiment with 5 mice per group was conducted for airway hyperresponsiveness measurements. Mice were sensitized intra peritoneal (i.p.) with 250 μg OVA and 300 μg 1.3% Alhydrogel (batch INMA-RM0001870975-ZLI4-01) at day 14 and 20 of the study. Intranasal challenges with 25 μg OVA were performed at three consecutive days, one day prior to sacrificing the mice (Figure 1).

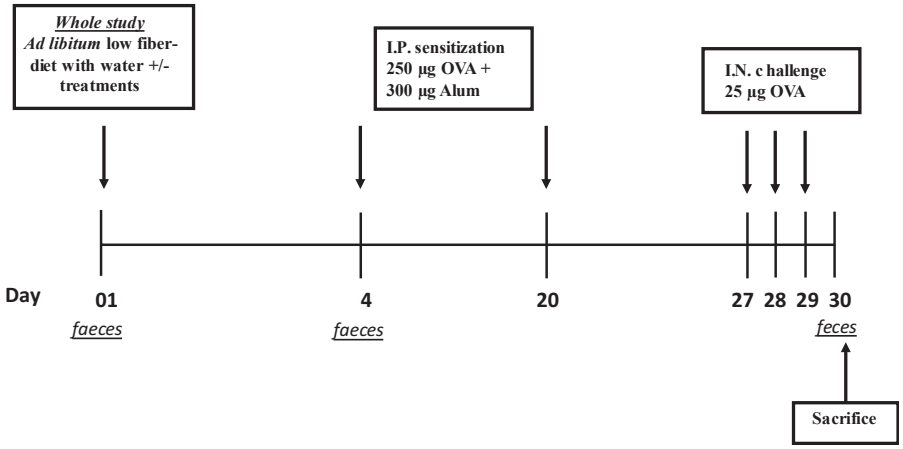

Figure 1. Schematic study set-up. From day 0 onwards, mice were fed a low-fiber diet and cow's milk, 3'SL or 6'SL were supplemented ad libitum via the drinking water. After an adjustment time of 14 days, mice were sensitized at day 14 and day 20. Mice were challenged intra-nasally on three consecutive days prior to sacrifice.

Airway-responsiveness

Anaesthetized mice were intubated with tracheal cannula and exposed to ascending concentrations of methacholine (Mch) (0, 0.1, 0,3, 1.0, 3.0 and 10 MCh/kg) (Sigma-Aldrich, A2251). Bronchoconstriction was measured in real-time with the Flexivent apparatus (Scireq, Montreal, Canada).

White blood cell counts in bronchoalveolar lavage fluid

Bronchoalveolar lavage (BAL) was performed by washing the lungs of the mice with 750 µl Hank's balanced salt solution. The collected fluid was kept on ice and spun down at 1900 rpm for 8 minutes at 4°C. Supernatants were frozen and kept at -20°C. The pellet was resuspended in 100 µl PBS containing 0.5% FCS and white blood cell counts were performed with Sysmex 1800i apparatus.

ELISA: cytokine measurements BALF and OVA specific IgE levels

IL-5 and IL-13 were quantified in BALF using a mouse IL-5 (R&D Systems DY405-05) or IL-13 Duoset ELISA (R&D Systems DY413-05) according to the manufacture's recommendations, respectively. OVA specific IgE was measured in serum. Serum was diluted 30X times in PBS and measured by a mouse OVA-IgE ELISA kit (M036005N, MDBiosciences) according to the manufacture's recommendations.

Microbiota analysis

Bacterial DNA was extracted by using a Maxwell® 16 Total RNA system (Promega) with Stool Transport and Recovery Buffer (STAR; Roche Diagnostics Corporation, Indianapolis, IN, USA). Approximately 0.1 g of fecal sample was homogenized with 0.25 g of sterilized 0.1 mm zirconia beads and three glass beads (2.5 mm) in 350 µL STAR buffer for 3 min at 5.5 m/s. The samples were incubated for 15 min at 95°C and pelleted by 5 min centrifugation at 4°C and 14000 g. The supernatant was stored and the pellets were processed again with 200 µL STAR buffer. The obtained supernatants were pooled and 250 µL was loaded for purification with Maxwell® 16 Tissue LEV Total RNA Purification Kit (AS1220) following manufacturer's instructions. DNA was cleaned using magnetic racks and eluted with 50 µL of DNAse and RNAse free water (Qiagen, Hilden, Germany). DNA concentrations were measured with NanoDrop ND-1000 (NanoDrop® Technologies, Wilmington, DE, USA) and adjusted to 20 ng/µL with DNAse and RNAse free water for downstream steps. The V5-V6 region of 16S ribosomal RNA (rRNA) gene was amplified in duplicate PCR reactions using 20 ng of template DNA. Each sample was amplified with a unique barcoded primer 784F-n and 1064R-n (10 µM each/reaction; Supporting information), 1x HF buffer (Finnzymes, Vantaa, Finland), 1 µL dNTP Mix (10mM each, Roche Diagnostics GmbH, Mannheim, Germany), 1 U Phusion® Hot Start II High Fidelity DNA Polymerase (Finnzymes, Vantaa, Finland) and 36.5 µL of DNAse and RNAse free water in a total volume of 50 µL per reaction. The amplification program included 30 sec initial denaturation step at 98°C, following by 25 cycles of denaturation at 98°C for 10 sec, annealing at 42°C for 10 sec and elongation at 72°C for 10 sec, and a final extension at 72°C for 7 min. The PCR product was tested qualitatively for correct amplification with gel electrophoresis 1% size (~290 bp). PCR products were purified with CleanPCR kit (CleanNA, Alphen aan den Rijn, Netherlands), and DNA concentrations were measured with Qubit® dsDNA BR Assay Kit (Life Technologies, Leusden, Netherlands). For the construction of the libraries, 100 ng from each barcoded and purified sample was used. Each library contained 70 unique barcode tags, thus 70 different samples were included. The final amplicon pool was concentrated with CleanPCR kit to 25 µL volume and 1 µL was used for DNA quantification using Qubit® dsDNA BR Assay Kit. Mock communities, with known microbial composition were included as sequencing control within each library. Finally, the libraries were sent for adapter ligation and HiSeq sequencing (GATC-Biotech, Konstanz, Germany). Raw data was processed using NG-Tax pipeline[373]. Analyses of alpha and beta diversity along with PCoA

generation were conducted using QIIME. Diversity index group comparisons were done in GraphPad. Finally, multivariate redundancy analysis was performed with Canoco5 using the microbial composition data in phylum and genus level.

Statistics

Normality was tested with a Kolomogorov-Smirnov test. Abundances on genus level were log transformed to fit a normal distribution. A Kruskal-Wallis test was used to compare groups within each time point and to assess differences between time points. A repeated measures linear mixed model with LSD post-hoc test was used to compare microbial changes between different time points with each other. Treatment groups were compared within each time point with a Tukey post-hoc test or non-parametric Dunn's multiple comparisons test. Graphpad Prism 5.0 and IBM SPSS Statistics 23 were used.

Results

Mouse characteristics in OVA-induced asthma model

During the study no signs of discomfort or changes in stool consistency were observed in any of the experimental groups. Mice were weighed at the start of the study, before sensitization and before sacrifice. The mice gained weight in the first 20 days of the study, which stabilized in the last week of the study as seen by the significant increase in weight (Figure S1A). Mice in the non-challenged group were significantly lighter at the start and the end of the study compared to mice in the 3'SL and challenged group (Figure S1B-C). Mice receiving raw cow's milk drank approximately twice the amount of those in the control groups (data not shown) and ate slightly less during the first 20 days of the study (Table S1). Nevertheless, no signs of discomfort (e.g. diarrhea) or weight loss were observed in any of the groups.

Airway hyper responsiveness and bronchoalveolar lavage

Mice were sensitized with OVA intraperitoneally twice during the study and intra-nasally challenged with OVA (challenged mice) or PBS (non-challenged mice) on three consecutive days before sacrifice (Figure 1). All treatment groups were compared to the challenged control mice to test whether the consumption of raw cow's milk or SL alleviated airway inflammation. Airway hyperresponsiveness upon metacholine challenge was measured as an indicator for bronchoconstriction. The respiratory system resistance (Rrs) increased in all groups receiving OVA challenge compared to

the non-challenged mice. No differences were observed between the treatment groups and challenged control mice (Figure 2A). Next, we performed a bronchoalveolar lavage (BAL) and measured differential white blood cell counts (WBC) and cytokines in the bronchoalveolar lavage fluid (BALF). The WBC in the challenged mouse group was $2.7*10^5$ with a 95% CI of [2.0-3.4*10^5]. In the 3'SL group the mean was lower showing a mean WBC of $1.9*10^5$ with a 95% CI of [1.4-2.4*10^5]. However, only the non-challenged mice and not the treatment groups showed lower WBC compared to challenged control mice (Figure 2B). In line with these non-significant changes between groups, eosinophilia was observed in all treatment groups except from non-challenged mice (Figure 2C). The frequency of monocytes and neutrophils in the BAL in challenged mice compared to non-challenged mice was therefore lower (Figures 2C,S2A-C). Unexpectedly non-challenged mice showed significantly higher IL-13 levels in BALF (Figure 2D), which was not observed for IL-5 (Figure S2E). No significant differences were found systemically in OVA-specific IgE levels, indicating that these serum levels are elevated by the sensitization (Figure S2F).

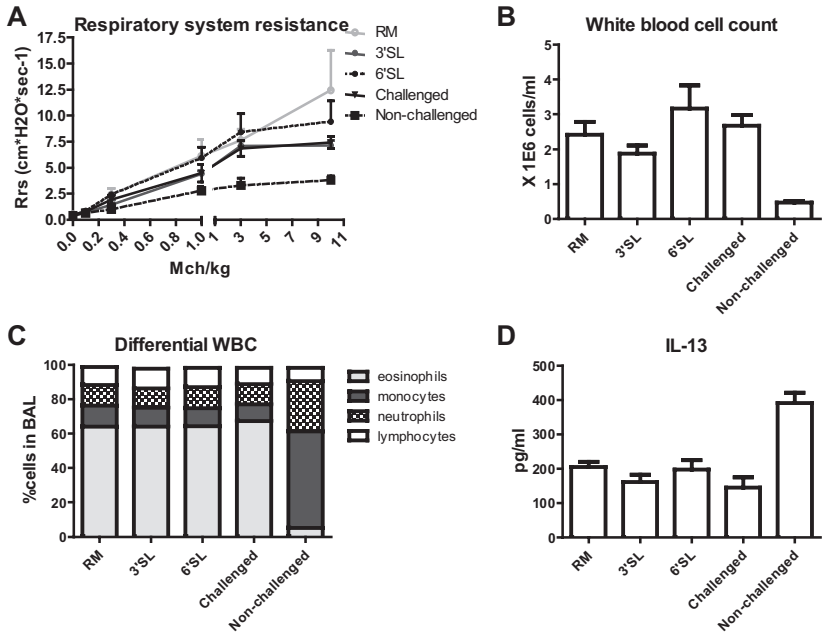

Figure 2. Asthmatic and immunological read outs in the lungs. (**A**) Bronchoconstriction measured with flexivent (Rrs). Raw milk (n=3), 3'SL (n=4), 6'SL (n=4), OVA/OVA (n=5) and OVA/PBS (n=4). (**B**) Total white blood cell counts as found in the BALF were shown per treatment group. (**C**) The mean counts of different white blood cell types is shown. (**D**) IL-13 was measured by ELISA in the BALF. Bar graphs are represented as mean +/- SEM (n=8/group).

Microbiota

At the start of the study, mice were switched from a normal chow diet to a low-fiber diet with or without supplementation in the drinking water. First we analyzed whether the variables time and treatment could explain changes in microbiota composition. We therefore performed principal component analysis (PCA) and redundancy analysis (RDA) on genus level. The phylogenetic diversity decreased between first sensitization (day 14) and sacrifice (day 30) of the study, and not between the start of the study and first sensitization (Figure 3A). Next, we performed PCA of beta-diversity, using the weighted and unweighted distances. Strikingly, the samples clustered together based on the time point of collection in the unweighted distance matrix and not in the weighted distance matrix, showing that the separation is driven by change in microbial taxa over time (Figures S3A-B). In line with these findings, RDA plots showed separation that revealed 11.06% of the total variation that was mainly driven by the microbial change over time (Figure 3B). As shown by PCA, time point 0 showed to account for the highest variation by RDA. This suggests that the changes in microbiota composition of mice are mostly driven by deprivation of fiber (Figure 3B). We therefore further assessed the changes in microbiota over time. On phylum level we showed that the mean abundance of Bacteroidetes decreased and Firmicutes and Actinobacteria increased (Figure 3C). The decrease in Bacteriodetes was mainly driven by a decrease in the genus *Bacteriodales* and increase in the genus *Faecalibaculum* (Figure 3D) of the family Bacteroidales and Erysipelotrichacaea, respectively (Figures S3C-D). Next we performed pairwise analysis on bacterial groups that showed significant changes over time. On phylum level, the decrease in Bacteroidetes and increase in Firmicutes (Figure 3E) was significant between all-time points. On genus level this was represented by a significant decrease of *Bacteriodales* (Figure 3F), while *Faecalibaculum* was not significantly increased (pairwise analysis not shown). However, the genus *Blautia,* also a member of the Firmicutes, was significantly increased over time, showing the most drastic increase after 14 days (Figure 3F). The frequency of *Lactobaccilus* was significantly increased in the first 14 days and reduced to initial levels between day 14 and the end of the study, respectively (Figure 3F). Despite these dramatic changes over time, RDA showed that the treatment groups accounted for minor (2.0-2.8% of the total variation), but significant ($P<0.05$) variation in the total data set as well. Thus this shows that the dietary supplementation of raw cow's milk or SL did induce significant microbial changes, although the biggest variation in microbiota composition driven by the change over time.

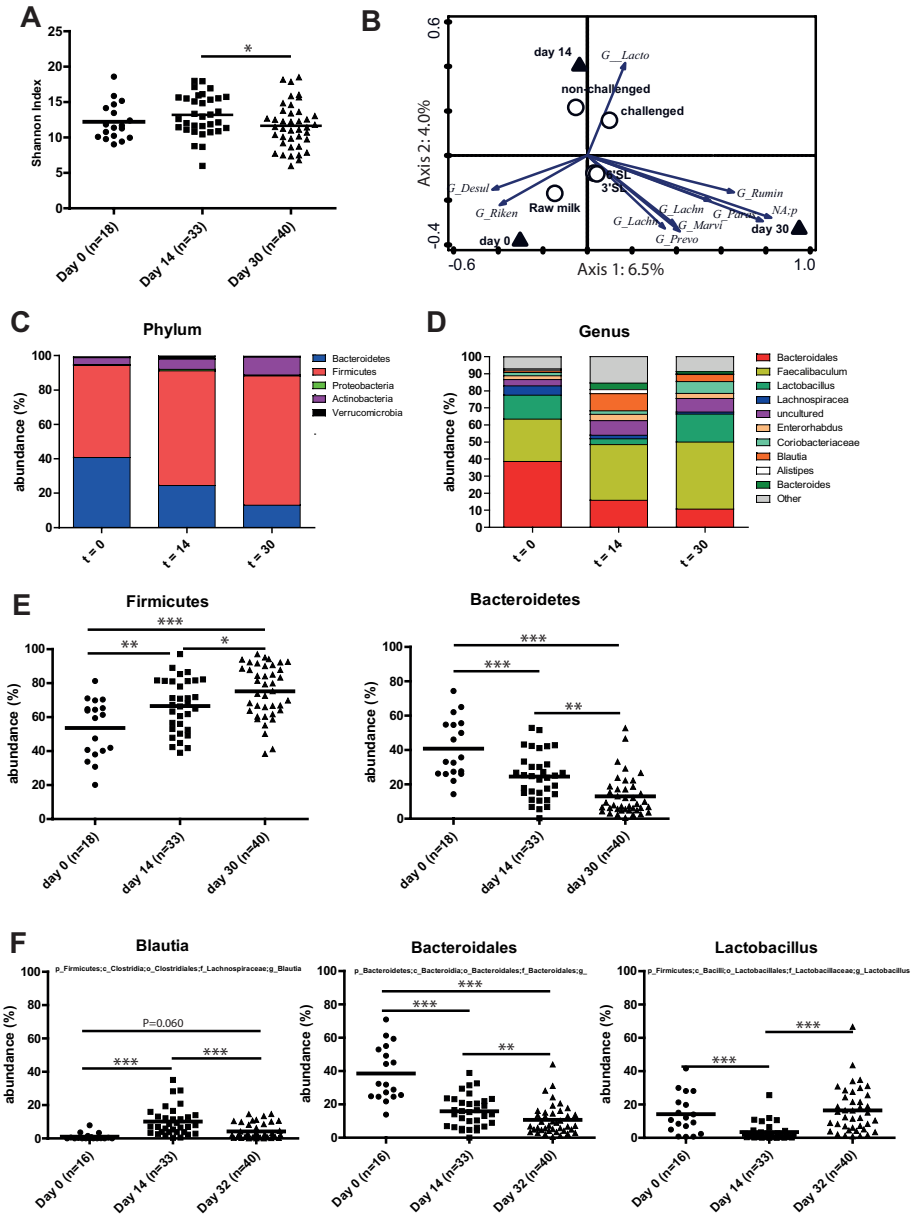

Figure 3. Microbial changes over time. (**A**) the phylogenetic diversity is shown over time. () Redundancy analysis **B** on genus level using as explanatory variables the time points and the treatment. Explanatory variables account for the 11.06% of the total variation. The mean abundances of (**C**) phyla and (**D**) genus per time point were shown by stacked bar graphs. Pairwise comparison on significantly changing (**E**) phyla and (**F**) genus over time were performed.

Next, we compared whether the microbiota was significantly different between treatment groups. At the day of the first sensitization (day 14 of the study) no significant differences were found between treatment groups. At the day of sacrifice however, the abundance of Verrucomicrobia (P=0.007) and Actinobacteria (P=0.06) tended to be different between the groups, showing a higher prevalence of both phyla in the 3'SL group (Figure 4A). On genus level the treatment groups clustered together deviating from the control groups who received water without supplements (i.e. 3'SL, 6'SL or raw cow's milk) (Figure 4B). No differences between the treatment groups was measured in phylogenetic diversity (Figure S4). The mice that received 3'SL showed a different microbiota profile on genus level compared to the other groups (Figure 4C). For instance, they showed a lower mean abundance of *Faecalibaculum* and higher mean abundance of *Coriobacteriacaea* compared to the other groups (Figure 4C). However, these changes were not significantly different (pairwise analysis not shown). The significant change in Verrucomicrobia was completely driven by the genus *Akkermansia* that was significantly different between the treatment groups. Moreover, significant higher abundances of *Akkermansia* (Figure 4D) and *Corynobacteria* (Figure 4E) were found, in the 3'SL and raw milk groups, respectively. Both genera of bacteria are present in their respective treatment group in 6/8 mice compared to 0/8 or 1/8 in the other groups. To investigate whether these microbial changes correlated with an increase in local SCFA levels, we measured SCFA in the caecum. No significant differences were found between the treatment groups in caecum levels of acetate, propionate and lactate (Figure S5).

Discussion

Controlled studies with unprocessed cow's milk in infants are not ethically possible due to safety risks. Therefore, causal links of cow's milk consumption and protection against asthma and allergies in infants are limited to epidemiology, *in vitro* studies and animal models. The mechanism by which raw cow's milk affects lung health remains largely unknown. We postulated that, among others, modulation of the gut-lung axis via fermentation of milk proteins or sialylated oligosaccharides promotes immune homeostasis in the respiratory tract[371]. Although no animal model can represent the complex clinical episodes in asthma patients and even various factors within the model (e.g. dosage, mouse strain, duration of challenge) can alter the response drastically, they may provide us with information about potential mechanisms[374]. An important

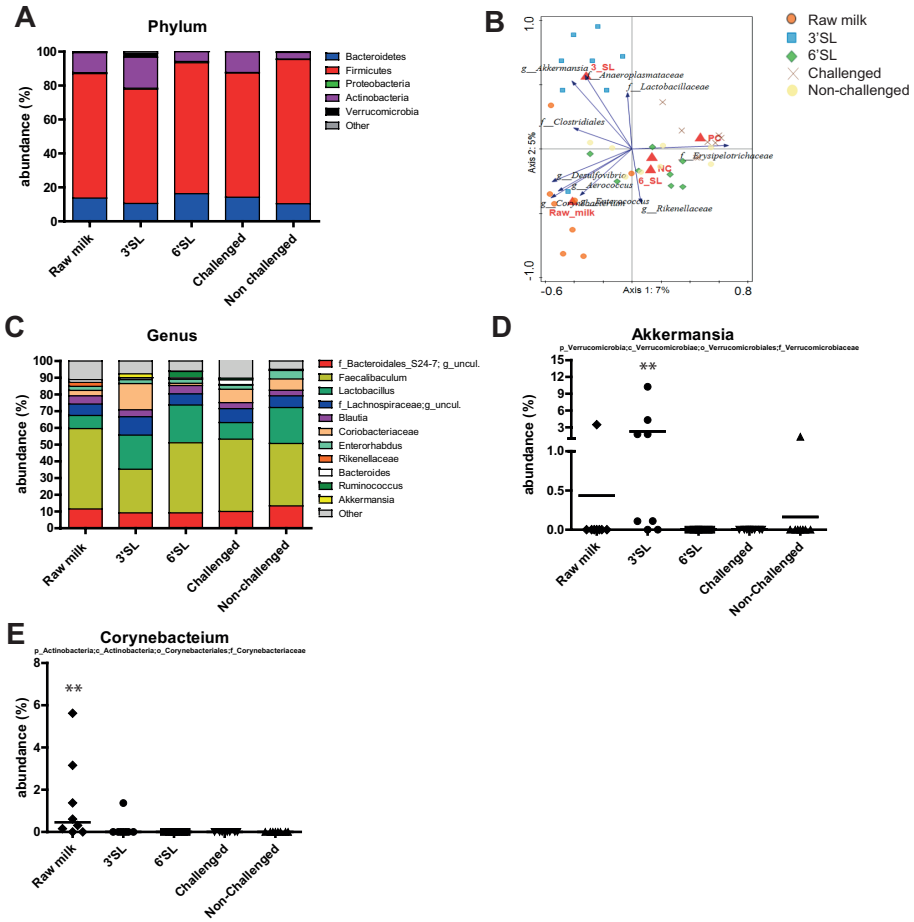

Figure 4. Microbial changes per treatment group at day 30. (**A**) the phylogenetic diversity is shown over time. (**B**) Redundancy Analysis (RDA) was performed to investigate the effect of the different treatments on genus level. (**C**) The mean abundance per group on genus level is shown by stacked bar graphs. (**D,E**) pairwise comparisons on genus level were performed.

limitation of the OVA-induced asthma model that was used here is that mice are sensitized via the intra-peritoneal route, making it impossible for cow's milk proteins to directly interact with the allergen. For instance, one of the postulated mechanisms involves complex formation of bovine IgG with allergens in the oropharynx that could alter immune responses. Indeed, bIgG was shown to bind allergens[112] and human FcγRII *in vitro*[111]. Interestingly, a recent study showed, that gavages with raw cow's milk and not with heated milk protected against house dust mite-induced asthma in mice[67]. The authors showed that raw milk reduced the production of IL-5 and IL-13 by lung homogenates upon HDM restimulation. The authors supplemented

0.5 ml of cow's milk via oral gavage three times per week to the mice. Since the mice in this HDM model are sensitized intra-nasally, it is not likely that these protective effects are mediated by a direct interplay of milk proteins (e.g. bIgG) and HDM. However, they did not assess the microbiota composition or production of SCFA[67]. Our model aimed to investigate indirect effects of raw cow's milk or SL consumption on respiratory health via the microbiota.

In this study we show in an OVA-induced mouse model that the microbiota changed dramatically during the complete length of the study, which is most likely driven by the dietary change at the start of the study from normal chow to low-fiber diet. Therefore, it is possible that this transition masked the effect of our treatments given via the drinking water on microbiota composition and SCFA production. Hence, we cannot draw firm conclusions on the effects of the ingredients tested due to the study set-up. Future studies investigating the effect of a dietary intervention on respiratory health should include a run-in period of at least a month for the microbiota to adjust to the low-fiber diet. Nevertheless, a strong increase in *Akkermansia* and *Corynebacteria* in mice receiving 3'SL and raw cow's milk, respectively was noted at the day of sacrifice, which is considered as a beneficial microbe[375].

The microbiota changed drastically during the first two weeks of the study due to the switch from normal chow to a low-fiber diet at the start of the study. In line with literature, the microbiota consisted at the start of this study mainly of Firmicutes and Bacteroidetes, which shifted towards dominance of the Firmicutes over Bacteroidetes after feeding the low-fiber diet for two weeks[44]. However, we show a continued decrease of Bacteroidetes during the entire duration of the study. This is indicative of adaptation to the low-fiber diet during the complete length of the study, but might theoretically also be driven by the OVA-induced asthma model. However, a very similar microbial shift was seen between day 0 and day 14 (i.e. just before first i.p. sensitization), suggesting that the low-fiber diet is the main driving factor of these changes. Hence, follow-up studies should include a run-in period of at least four weeks before investigating the effects of a dietary intervention in mice fed a low-fiber diet. Interestingly, a similar switch towards dominance of Firmicutes was seen in germ free mice inoculated with human microbiota from a low-fat polysaccharide-rich diet to a high-fat, high-sugar "Western" diet, which is also low in fiber[376]. These mice showed higher adiposity, which could be transferred to recipient gnotobiotic mice via the microbiota. In the present study we observed an increase in weight in the first 3

weeks of the study. However, this weight gain is exactly in line with the normal weight gain of 10 weeks old BALB/c mice[377] and thus is likely not related to the low-fiber diet.

We further noted that mice fed with 3'SL have a lower abundance of Firmicutes and a higher abundance of Verrucomicrobia. The Verrucomicrobia found in this study are all from the genus *Akkermansia*. The genus *Akkermansia* consists of several species of which only one has been cultivated and which is suggested to contribute to various anti-inflammatory mechanisms[375]. The specific microbial changes between mice fed 3'SL or 6'SL has been shown before in C57/BL6 mice, showing a significant increase of Verrucomicrobia and *Akkermansia* after receiving 3'SL-fortified chow compared to control chow[378]. Indeed, supplementation of non-fermentable carbohydrates to animal models have been shown to induce the abundance of fecal *Akkermansia*[379,380]. An increase in fecal *Akkermansia* after prebiotic supplementation in rats was shown to be indicative of higher mucin levels in the colon and postulated to improve gut health[380]. Others show improved growth in animal models fed with sialylated oligosaccharides[158] and the authors showed that the primary consumer of sialylated oligosaccharides in animal models is *B. fragilis*. This commensal bacterium was shown to induce the conversion of CD4+ T cells into FoxP3+ T cells by producing polysaccharide A (PSA) that promoted mucosal tolerance[362]. In contrast, *in vitro* batch cultures showed that 3'SL and 6'SL are most efficiently metabolized by *Bifidobacterium longum*, resulting in the production of SCFA[103]. However, in this study no differences between treatment groups were found for the genus *Bacteroides* and Bifidobacteria were not detected. Nevertheless, a higher prevalence of *Akkermansia* in the colon and feces after prebiotic supplementation in rats was shown to correspond with higher colonic mucin levels and SCFA levels[380]. Despite the higher prevalence of *Akkermansia* in mice receiving 3'SL, we did not find elevated SCFA levels in the caecum. We also observed significant microbial changes in mice fed with raw milk. However, this increase in *Corynobacteria* might be derived of the milk itself, which is present in cow's milk[381,382] and breast milk[383]. Even though these bacteria may be derived from the milk, it is of interest to note that breastfed children who were better protected against respiratory infections and wheezing in early infancy showed a higher abundance of Corynebacterium in the nasopharynx[38].

Conclusions

In this study we show a major shift in microbiota composition during the complete length of the study, which is most likely driven by the low-fiber diet. Therefore, the effects of dietary intervention might be masked by the microbial changes induced by the low-fiber diet. Nevertheless, mice that received 3'SL showed a significant increase in *Akkermansia*, that has been shown to contribute to immune homeostasis. Future studies should include a run-in period for at least four weeks if shifting mice from normal chow to a low-fiber diet before testing any dietary intervention.

Supplementary files

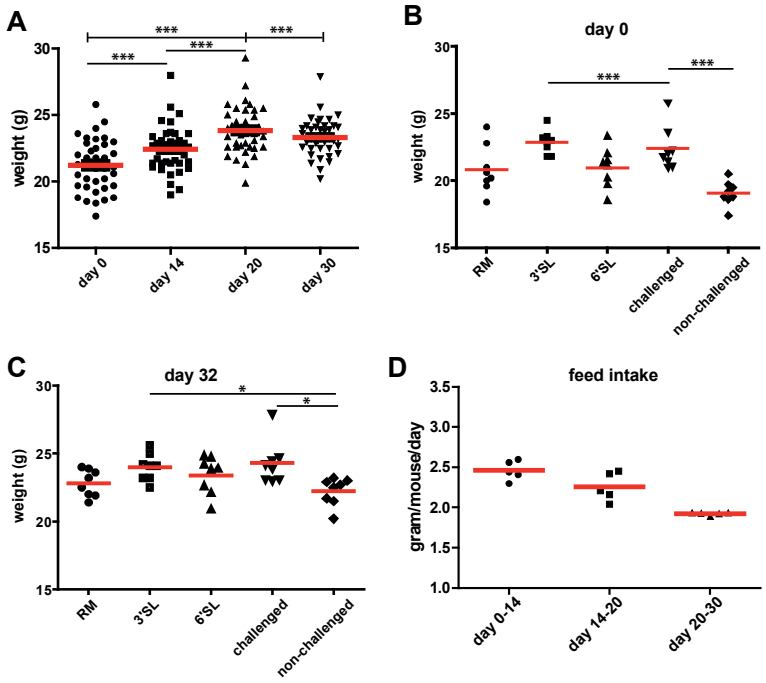

Figure S1. Feed intake and weight gain declines during the course of the study. (**A**) All mice were weighted at the start of the study, before the first and second sensitization and before sacrifice. Mouse weight at (**B**) the start of the study (t=0) and (**C**) at the end of the study. (**D**) Feed intake per cage.

Table S1. Feed consumption per mouse/day (grams).

Day	0-14	14-20	20-30
Raw milk	2.3	2.0	1.9
3'SL	2.4	2.2	1.9
6'SL	2.6	2.4	1.9
challenged	2.6	2.5	1.9
non-challenged	2.4	2.2	1.9

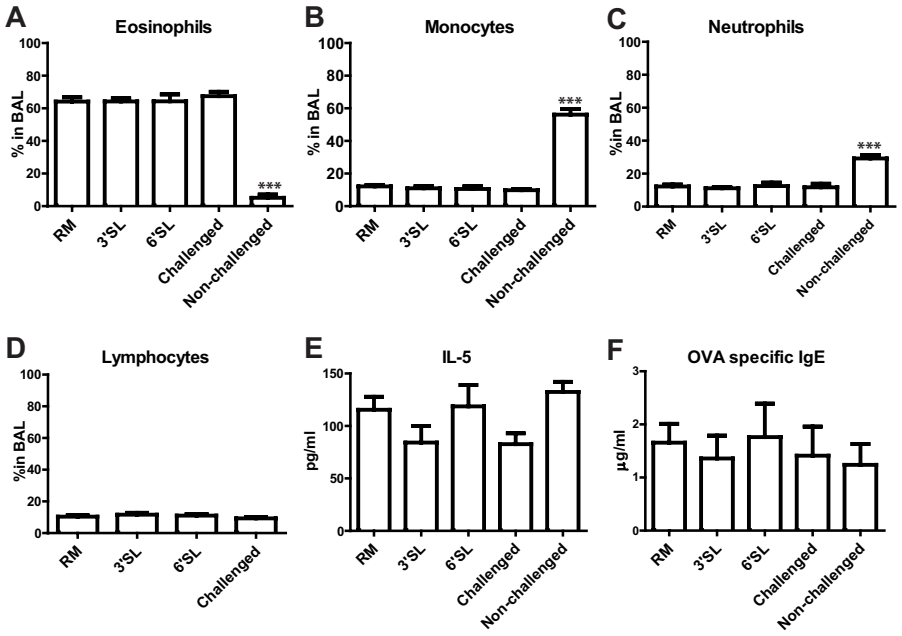

Figure S2. Immunological parameters in OVA-induced asthma model. The percentage of (**A**) eosinophils, (**B**) monocytes, (**C**) neutrophils, (**D**) lymphocytes were determined BAL. (**E**) IL-5 was measured in the BALF. (**F**) OVA-specific IgE levels were measured in the serum.

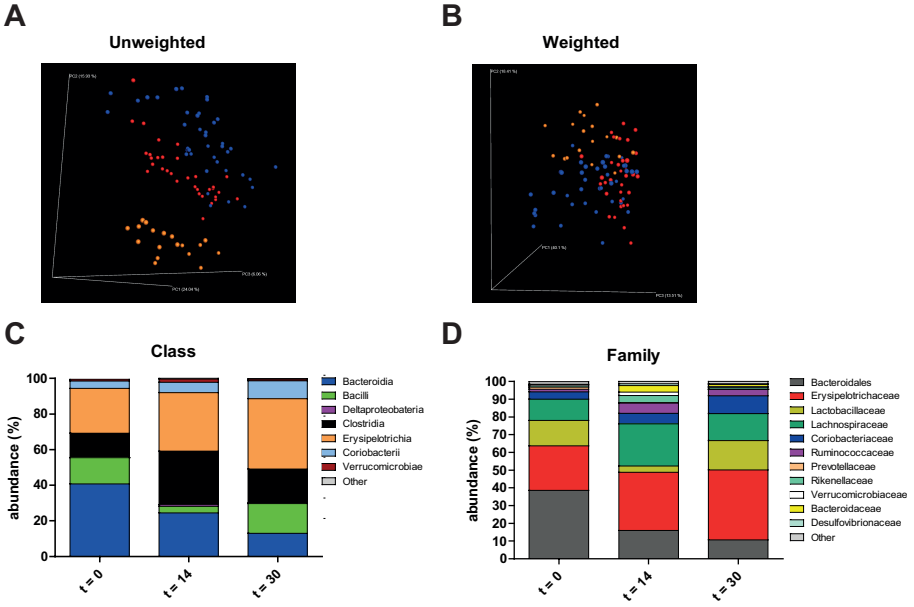

Figure S3. Microbial changes over time. Beta-diversity is shown by plotting (**A**) unweighted and (**B**) weighted unifraq scatter plots. Mean changes on microbial (**C**) class and (**D**) family are shown.

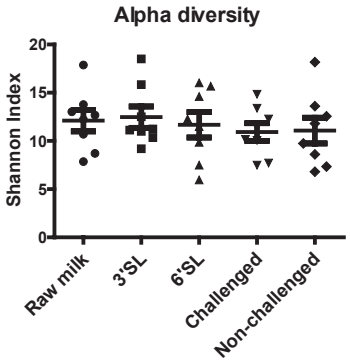

Figure S4. Microbial diversity at day of sacrifice per treatment group. The microbial diversity per treatment group at the day of sacrifice was represented by the Shannon-index.

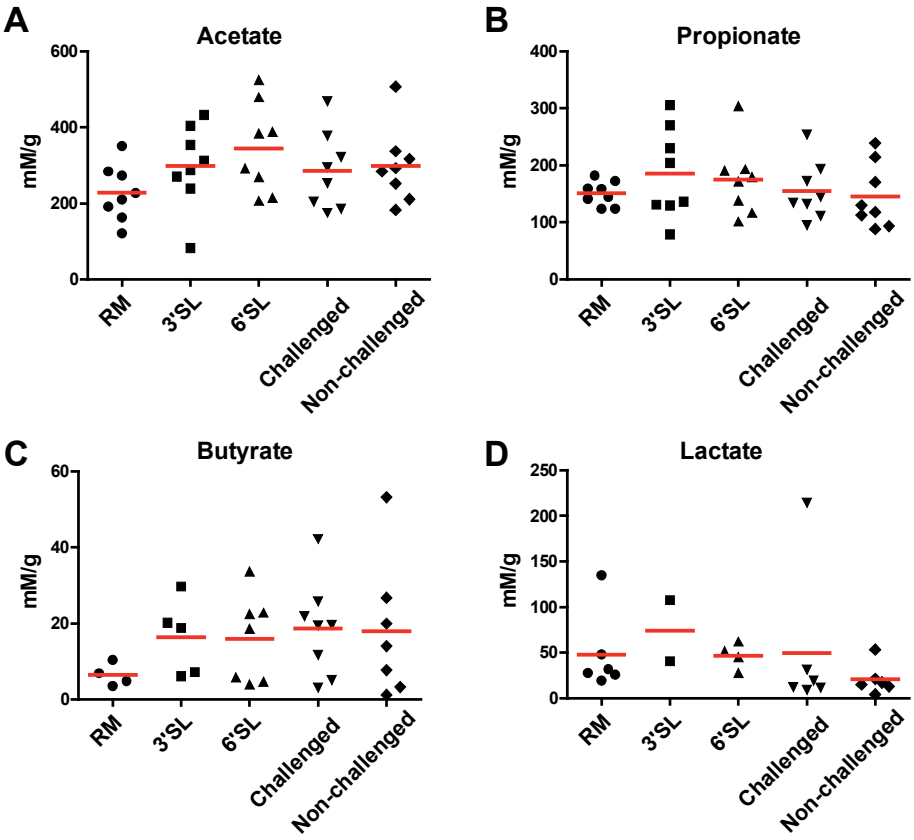

Figure S5. SCFA levels in caecum samples. (**A**) Acetate, (**B**) propionate, (**C**) butyrate and (**D**) lactate were quantified in the cecum and normalized for the weight of the cecal content.

Chapter 9

General Discussion

In a growing world population in which chronic inflammatory diseases are on the rise and allergies and asthma affect 30-40% of the population[384], there is a pressing need to understand underlying mechanisms to develop successful preventive strategies for these diseases. Recent advances in translational, clinical and mechanistic research provide evidence for the modulation of immune responses by dietary ingredients and natural products.

It is widely accepted that breast milk confers health benefits to the infant (e.g. increased cognitive ability, reduced gastrointestinal (GI) and respiratory infections, and reduced diabetes prevalence). Although there is some discrepancy in the current literature[5] that may be caused by individual differences in breast milk composition, most studies show that breast milk protects against the development of allergies and asthma. Raw cow's milk consumption in early life has been associated with a significant decrease in the prevalence of allergies and asthma. Raw cow's milk contains components (e.g. tumor growth factor (TGF) β, lactoferrin (LF), immunoglobulins (Igs), and vitamin D3 (VitD3)) that are also present in breast milk. These bovine components are highly homologous to their human counterpart and might be functionally active on human immune cells. Hence, raw cow's milk may be an important source to deliver functional components that can potentially be applied in infant nutrition, and that may aid in inducing and maintaining immune homeostasis. The *aims* of this thesis were therefore:

1. To address the effect of components that are present in raw cow's milk on human immune function
2. To investigate the effect of sialyllactose on barrier functioning and microbiota composition
3. To describe implications of these findings for respiratory health

In this discussion, I will discuss the implications of our findings and provide considerations for future research to continue with this line of research.

Which factors in raw cow's milk protect against allergies and asthma?

In chapter 2 we described potential mechanisms by which raw cow's milk components aid in mucosal immune homeostasis and immune functioning in the upper respiratory tract

and intestine[371]. We did not aim to describe the full array of potential immunomodulatory components in bovine milk since excellent reviews are already available describing the immunomodulatory effects of milk proteins and peptides[73,385], exosomes[307,386], fatty acids[387], microbes[388], and sialylated oligosaccharides[78]. The basis for the proposed mechanisms of cow's milk mediating immune homeostasis in the respiratory tract as described in chapter 2, are based on epidemiological studies. We here further elaborate on these studies to discuss the background of this thesis. Several epidemiological studies showed inverse associations between farm milk consumption in early life and the prevalence of allergies and asthma[85,86,88,368,369] or respiratory tract infections[66]. Most of these studies specify that the consumed cow's milk was essentially unprocessed[66,85,86,88,368,369], while one study defined it as "farm milk"[85]. Only two studies compared the consumption of different heated and processed milks in early life on respiratory health[66,88]. Loss *et al.* (2011) collected cow's milk that was consumed on the farm itself and specified the heating status of the milk by reporting the residual enzyme activity and whey and fat concentrations in the milk. This study allowed for the first time to associate individual milk fractions with the risk of developing asthma or allergies. Importantly, the authors showed that the whey fraction and not the total protein concentration, the viable bacterial count or total fat content was associated with the risk of developing asthma or allergies[88]. These epidemiological findings thus exclude the possibility that heat-stable bacterial-derived components such as Toll-like receptor (TLR) ligands (e.g. lipopolysaccharide (LPS)) or total bacterial counts are protective factors in the cow's milk. Of note, although pathogenic bacteria (e.g. *Listeria monocytogenes*) are reduced after heating of the milk, it is also plausible that probiotic bacteria are killed by the heat treatment[388]. It is therefore possible that, although the reduction in total bacterial counts did not correlate with allergy and asthma prevalence, the decline of specific probiotic bacterial species does correlate with allergy and asthma prevalence. A second study was based on weekly diaries in which parents specified the quantity and the type of milk (i.e. ultra-heat treated (UHT), pasteurized or boiled) that was consumed by their neonate. This study confirmed that raw cow's milk consumption in early life improved respiratory health later in life as observed by a reduced odds ratio's for rhinitis, respiratory tract infections, otitis and fever[66]. Thus, based on these epidemiological findings, heat instable components and particular whey proteins are of interest for mechanistic studies. The top five most abundant whey proteins are β-lactoglobulin, α-lactalbumin, bovine serum albumin, Igs and LF[389]. Hence, due to their high abundance and known immunomodulatory effects Igs and LF, as well as TGFβ (present in lower amounts), are of special interest as functional proteins for infant formulas.

Bovine milk ingredients and homeostasis in the upper airways

The upper airways of infants are constantly exposed to allergens and viral antigens. Since viral infections and allergen exposure are risk factors for asthma development, we speculated in chapter 2 that bovine milk components can contribute to maintaining local immune homeostasis in mucosal tissues. The upper and lower airways are clearly linked, as was shown by a high percentage of asthma patients (i.e. 88%) suffering from allergic rhinitis[117]. In our review, we proposed that bovine Igs can neutralize allergens or viruses. We also speculated that bovine IgG (bIgG):allergen complexes may induce regulatory immune responses, as was shown for human IgG[15,16] and murine IgG[14]. Although bIgG is reported to bind house-dust mite (HDM) extract[112], we measured a very low specificity of bIgG to the two main allergens of HDM. The low specificity of bIgG to recombinant Der-p1 and Der-p2 (data not shown), indicates that bIgG recognizes a different HDM allergen. Ingested antigens can come into contact with the palatine and lingual tonsils in the upper airways. Interestingly, a novel mechanism involving a tightly regulated interplay between innate group III lymphoid cells (ILC3) and B cells was discovered in the palatine tonsils[36]. The authors showed that ILC3 produced B cell activating factor (BAFF) that activated interleukin (IL) 15 production by B cells. IL-15 in turn upregulated CD40 ligand (CD40L) expression on tonsillar ILC3 that stimulated survival, IL-10 production and programmed death (PD) L1 expression by B cells[36]. Since allergic patients showed a reduced ILC3 frequency in palatine tonsils, the authors proposed that these cells are essential for maintaining immune homeostasis. These ILC3 also promoted barrier functioning in the gut[20]. Their role in upper airway epithelium is however unknown. It is an interesting thought that dietary ingredients or immune complexes may impact the induction of regulatory B cells in the tonsils. A recent study by Byars *et al* indicated that surgical removal of tonsils and adenoids early in life are associated with long-term risk of upper airway infections and allergic diseases[390]. Nevertheless, the role of the tonsils in oral tolerance induction in early life remains poorly understood and requires more research.

Sialylated oligosaccharides, LF and TGFβ in the GI tract

In chapter 3 and chapter 6 we investigated the effects of bovine LF and TGFβ on monocyte differentiation. Since most proteins are degraded in the small intestine,

we will initially discuss the bioavailability of these proteins in infants. LF is poorly digested by infants and is found back in their urine and stool[197], and in blood and lymphatics of mice and piglets after oral supplementation[201,391]. In the intestine of cows, a higher binding affinity of LF was found at the epithelium overlaying Peyer's Patches, indicating uptake of LF via microfold cells[392]. LF was shown to bind to human LF receptor (intelectin-1) on human epithelial cells *in vitro* and induced the production of TGFβ[77].

Early studies in TGFβ1 knock-out mice showed that exogenous milk-derived TGFβ becomes systemically available and is essential for normal development[393]. Since TGFβ derived from human and cow's milk is structurally identical[73], cow's milk might be an important source of TGFβ. TGFβ binds to its receptor that is expressed on intestinal epithelial cells. The TGFβ receptor (TGFβR) family consists of two structurally different transmembrane serine/threonine kinases TGFβR (type I and II). Upon binding of TGFβ to these receptors, it binds to accessory proteins and initiates intracellular SMAD (in)dependent signaling that subsequently induces regulation of several genes[394]. Human epithelial cells express TGFβRI, which was also found on duodenal biopsies of human infants (aged from 7-120 days) with food protein-induced enterocolitis[395]. Thus, milk-derived LF and TGFβ are actively taken up into the mucosa of the intestine and may even become systemically available in infants.

Unlike humans, cows produce very low amounts of milk oligosaccharides. The oligosaccharides that are present in cow's milk are sialylated oligosaccharides of which the structurally simple trisaccharides 3'-sialyllactose (3'SL) and 6'-sialyllactose (6'SL) are predominantly present[78]. A small fraction of these oligosaccharides is taken up in the circulation and the majority is fermented in the colon to short-chain fatty acids (SCFA) where they may modulate immune cells and microbiota composition, respectively[40].

Dendritic cells and oral tolerance

To date, immunotherapy to treat allergies consists of oral tolerance induction by administering different dosages of allergens. Although the exact mechanism is poorly understood, allergen immunotherapy results in the expansion of allergen-specific regulatory T cells (Tregs) that are essential for the suppression of allergen-specific IgE

production and allergic inflammation[396]. In addition, the IgG4/IgE ratio is increased upon immunotherapy. This may further reduce IgE-receptor mediated T cell activation and mast cell degranulation, since IgG4 blocks binding of IgE to FcεR[397]. In early life, it is thought that oral tolerance induction to breast milk components is essential to maintain immune homeostasis and prevent allergen sensitization. This could be mediated by the induction of a regulatory milieu by breast milk components that allows the suppressive responses towards allergens[34]. Such a form of aspecific tolerance induction was effective to suppress allergic inflammation, as shown in mouse models using *in vitro* generated IL-10-treated dendritic cells (DC)[398,399]. Recent evidence implicates that a local microenvironment consisting of dietary and microbial factors are essential to instruct DC to maintain immune homeostasis in the GI tract. Hence, the induction of tolerogenic DC may be a promising strategy to prevent inflammation. In chapters 3, 5, and 6 we therefore investigated the effect of bovine milk components on human DC functioning.

To study this immunoregulatory effect of bovine milk components on DC, we used monocyte-derived DC (moDC). moDC are often used as a model for DC functioning, since the numbers of CD34+ pre-classical (pre-DC) or mature DC in adult human blood are limited. Conventional DC (cDC; i.e. all DC, except plasmacytoid DC or pDC) arise from monocytes or pre-DC. cDC express high levels of CD11c and major histocompatibility complex (MHC) class II molecules. Monocytes and pre-DC originate from a common macrophage-DC progenitor that differentiates into a monocyte progenitor or a common DC progenitor that gives rise to pDC and pre-DC[23,400]. These pre-DC differentiate into two distinct circulating DC types (i.e. CD141+ and CD1c+) or tissue-specific DC types[23]. Differentiation of DC is highly dependent on environmental signals (e.g. cytokines like FMS tyrosine kinase 3 ligand or Flt3L) that drive expression of selective transcription factors. cDC thus originate from monocytes or pre-DC that may respond differently upon environmental exposure. In chapter 3, 5 and 6 moDC were used as a model study the effect of bovine milk components on human DC differentiation and function. It is therefore essential to first discuss the relevance of these cells in the GI tract.

Tolerogenic CD103+ DC in the gut – Induction of Tregs

Most of our knowledge of DC biology in the GI tract is derived from experiments performed in mice. In the gut, a local microenvironment comprised of dietary components and microbial- or epithelial-derived molecules induce differentiation

of pre-DC into DC expressing CD103[25]. These CD103+ DC express retinal dehydrogenase (RALDH) that is capable of converting dietary vitamin A into retinoic acid (RA). This production of RA and local TGFβ aids in the capacity of CD103+ DC to induce polarization of naive T cells into Forkhead box P3+ (FoxP3+) Tregs[180]. Although CD103+ and CD103- DC can both migrate to the mesenteric lymph node (MLN) to prime naïve T cells[28], only CD103+ DC have the unique capacity to induce polarization into tolerogenic T cells that express the gut-homing receptors α4β7 and C-C chemokine receptor (CCR) 9 (Figure 1)[25,26]. In mice, these CD103+ DC derived from pre-DC and were essential for oral tolerance induction[401]. However, human moDC that were cultured in the presence of RA expressed CD103 and were capable of inducing Tregs[27,160]. In humans, it is therefore possible that also monocytes contribute to the pool of CD103+ DC that prime naive T cells to become tolerogenic T cells. Additionally, although recent studies performed in mice indicate that monocytes are less important in inducing oral tolerance, in the next section we discuss the importance of monocytes in maintaining oral tolerance.

Monocytes and moDC maintain oral tolerance

Monocytes have a short half-life of 22 days in the periphery and were originally merely seen as precursors for macrophages and DC[402]. However, recent evidence shows that monocytes do not always differentiate after extravasation and are capable of sampling antigens in the tissue and recirculate to draining lymph nodes[403]. Additionally, monocytes give rise to CX3C chemokine receptor 1+ (CX3CR1+) macrophages that are essential to suppress inflammation in the gut[404]. These CX3CR1+ cells are distinct from CD103+ DC as shown by their capacity to sample antigens directly from the gut lumen and their poor T cell stimulatory capacity[405]. Compromising 80% of all MHC class II-expressing cells in the colon, CX3CR1+ macrophages are the dominant antigen presenting cell that release high levels of IL-10 in response to microbiota[30,293]. A similar distribution of these monocyte-derived cells was suggested to reside in the human and mouse ileum[30]. Interestingly, these CX3CR1+ macrophages were shown to induce expansion of Tregs in the lamina propria by presenting antigens in this local tolerogenic microenvironment that aided in oral tolerance induction[406]. CD103-CX3CR1+ mouse DC are similar to a population of CD103-Sirpα+ DC in the human small intestine, which were transcriptionally related to blood monocytes[407]. Although these cells expressed lower levels of CCR7 compared to CD103+Sirpα+ DC, these monocyte-like DC might be able to migrate to the MLN as well[407]. Collectively, these studies show that the local microenvironment

in the GI tract, driven by microbial and dietary products, allows a constant state of primed homeostasis or sterile inflammation, which induces monocyte migration into tissues. Under homeostatic conditions, these monocytes may thereby contribute to the maintenance of regulatory responses by inducing Treg expansion in the lamina propria[30,31] (Figure 1).

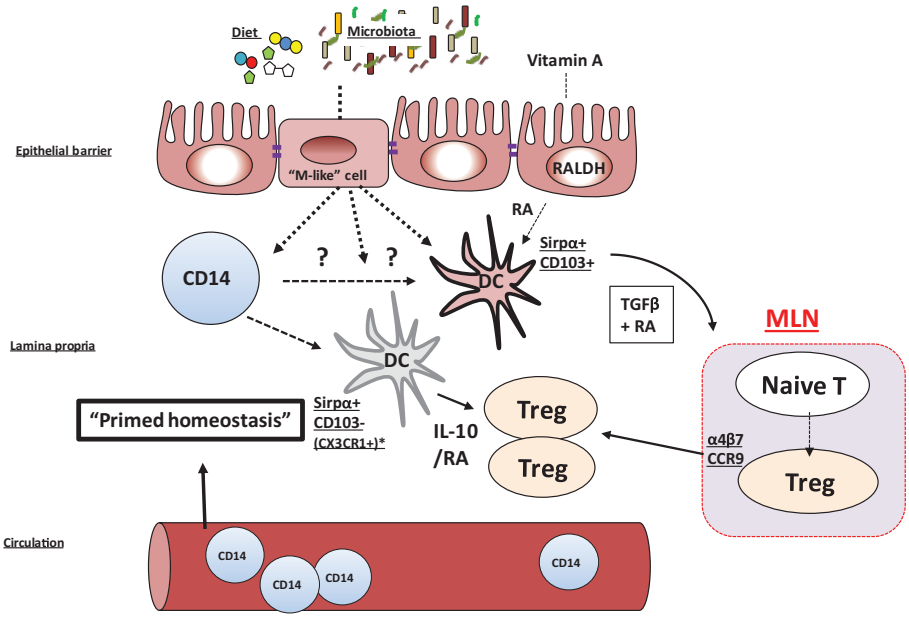

Figure 1. Distinct roles for monocytes and DC in oral tolerance. In homeostasis, the intestine is in a constant state of low-grade inflammation ("primed homeostasis") that is driven by exposure to microbial compounds. This results in a constant influx of monocytes that differentiate by unknown factors into distinct subsets of monocyte-derived cells. The subpopulation of monocyte-derived CX3CR1+ macrophages are shown to be essential in maintaining homeostasis by secreting IL-10 and RA. It is to date unknown whether monocytes give rise to CD103+ DC. These DC migrate to the MLN to prime naive T cells to polarize into Tregs expressing gut homing markers (e.g. α4β7 and CCR9). *Sirpα+CD103- in human intestine are transcriptionally related to CX3CR1+ phagocytes in the lamina propria of mice.

Bovine milk components and human DC differentiation

In chapter 3, 5 and 6 we investigated the effect of milk components on the differentiation of monocytes into moDC and their responsiveness upon exposure to different TLR ligands. We aimed to investigate whether bovine milk ingredients are capable of inducing the differentiation of monocytes into tolerogenic DC.

Human monocytes can differentiate in the presence of RA into CD103+ DC, which were shown to induce Tregs[27,160]. None of the bovine components tested induced differentiation into CD103+ DC (data not shown). The moDC differentiated in the presence of bovine milk components expressed CD11c, human leukocyte antigen (HLA) DR and similar expression levels of CCR7, showing that the monocytes differentiated into moDC. Nevertheless, the functionality of these moDC largely depends on environmental factors, as these "moDC" may also express more classical monocyte or macrophage markers. Hence, classification of monocyte-derived cells into DC or macrophages in remains controversial. Hence, these monocyte-derived cells are to date often termed as monocyte-derived phagocytes. In this thesis, we referred to our monocytes cultured in the presence of IL-4 and granulocyte-macrophage colony stimulating factor (GM-CSF) as DC. However, even in this *in vitro* system it is challenging to define a true DC. For instance, VitD3-induced DC (VitD3DC) show low expression of DC-specific intracellular adhesion molecule (ICAM)-grabbing non-integrin (SIGN), retain CD14 expression and show lower HLA-DR expression (chapter 6), indicating that these VitD3DC are more 'monocyte-like cells' than moDC generated in the presence of only IL-4 and GM-CSF.

Bovine milk components halt CD1a expression: a marker of DC differentiation

Monocytes differentiated in the presence of IL-4 and GM-CSF lose expression of their pan monocyte marker CD14 and gain expression of CD1a during differentiation. CD1a was initially described as a marker for Langerhans cells and is now known to be also expressed on DC in different tissues, including the human colon[408]. However, the functionality and expression of CD1a on DC subsets *in vivo* remains largely unknown. Nevertheless, we will discuss here that CD1a expression marks DC differentiation and determines the responsiveness of DC to TLR stimulation, which may consequently alter T cell polarization. Interestingly, in chapter 3, 5 and 6 we showed that LPS, TGFβ, VitD3, and LF inhibited CD1a expression that results in a DC phenotype that is hyporesponsive towards LPS or polyinosinic-polycytidylic acid (Poly:IC) + resiquimod (R848) stimulation. Moreover, in chapter 3 and chapter 6 we showed that LF and TGFβ respectively inhibit CD1a expression, while we showed in chapter 5 and 6 that DC differentiated in the presence of LPS or VitD3 remain CD14+ and do no gain CD1a expression.

Milk-induced modulation of DC differentiation via distinct pathways

Several mechanisms may underlie the altered DC differentiation pattern induced by selected bovine milk components. In chapter 5 and 6 we observed that, besides exposure to VitD3, LPS contamination also halts DC differentiation as shown by their CD14+CD1a- phenotype on LPS-DC and VitD3-DC. In literature it was shown that a common factor upon exposure of monocytes to TLR ligands and VitD3 is the production of suppressor of cytokine signaling (SOCS) 1[288,304]. SOCS1 production was boosted by VitD3 via the suppression of microRNA (miRNA) 155[304]. Suppression of miRNA-155 in turn hampered IL-12p70 production by DC[409]. Indeed, SOCS1 inhibits the generation of a pro-inflammatory response and halts DC differentiation by loss of sensitivity to GM-CSF[285,288]. In chapter 6 we showed, in line with others, that VitD3 induces specifically high levels of IL-6 that induce PD-L1 expression[295]. Although, we observed a non-specific production of IL-6 by moDC that were differentiated in the presence of LPS, IL-6, signal transducer and activator of transcription (STAT) 3 dependent expression of PD-L1 has also been described for these TLR exposed DC[287]. However, IL-6 production is abrogated early during DC differentiation (as shown in chapter 5), which may be regulated by SOCS3 that induces a negative feedback loop dampening autocrine IL-6 production[285]. It has been shown that the selective production of IL-6 may induce DC differentiation into tolerogenic DC[286]. Indeed, an early study showed that IL-6 activates STAT3 and halts DC differentiation, promoting the number of resting immature DC that are hyporesponsive upon TLR stimulation (i.e. tolerogenic) in lymph nodes and spleen[410]. However, more recent studies show that blocking STAT3 by SOCS1 is an important therapeutic target for halting DC differentiation and inducing tolerogenic DC[411–413]. Thus, a complex interplay between cytokine production, STAT3 activation and SOCS proteins regulates DC differentiation. The underlying mechanism by which VitD3 and LPS may block DC differentiation and induce tolerogenic DC involves IL-6 production by monocytes. This results in STAT3 activation, PD-L1 expression and SOCS3 production. Subsequently, SOCS3-incuded dampening of IL-6 production may repress STAT3 expression and allow SOCS1 to suppress GM-CSF signaling. Suppression of GM-CSF signaling by TLR ligands and VitD3 thereby halts differentiation as shown by increased CD14 and low CD1a expression.

In contrast to DC cultured in the presence of VitD3 or LPS that do not completely lose CD14 expression, we demonstrated in chapter 3 and 6 that DC cultured in the presence of bovine LF or TGFβ do lose CD14 expression. Although all these bovine

milk components have in common that they halt CD1a expression on immature DC, their differential expression of other surface markers (e.g. CD14) indicates that their effect is mediated via distinct pathways. We showed that 10 μg/ml LF did not induce the production of tumor necrosis factor α (TNF), IL-10 or IL-6 and yet halted CD1a expression, indicating that IL-6 production was not the driving factor. Although we cannot exclude that other cytokines (e.g. IL-8) are responsible for halting CD1a expression, it seems that there is a different mechanism responsible. We postulate that LF may directly modulate gene transcription since LF can be taken up and internalized into the nucleus of monocytes[147]. Transfection experiments of the LF gene in a human embryonic cell line showed that LF inhibits GM-CSF expression[216]. Future research should investigate the role of SOCS1 in the LF-induced differentiation of monocytes into tolerogenic DC. Similarly, TGFβ modulates DC differentiation that were initially described to be morphologically and functionally similar to Langerhans cells[306]. In line with literature[297], we showed in chapter 6 that TGFβDC express a distinct array of phenotypic markers (e.g. CD80, PD-L1) compared to VitD3DC, indicating that differentiation is modulated via distinct pathways. However, the exact mechanism by which TGFβ halts DC differentiation is unknown. Importantly, monocytes cultured solely in the presence of IL-4 differentiated into a DC type expressing low CD1a levels[215]. Similarly, moDC cultured in the presence of components present in bovine milk such as VitD3, TGFβ and LF express lower CD1a levels compared to conventional moDC, indicating that these bovine milk components inhibit GM-CSF signaling. Interestingly, novel evidence implies that the microbiota also modulates GM-CSF signaling, which was essential for the induction of oral tolerance[414] and protection against respiratory infections[415]. ILC2 are now known to play an essential role in lung immunity[416]. Microbial stimuli were shown to enhance the production of GM-CSF via macrophage-derived IL-1β that triggers GM-CSF production by ILC2, which enhanced mononuclear phagocyte effector functioning and increased Tregs numbers[414]. This is in line with earlier findings showing that monocyte-derived cells are essential for oral tolerance induction[406] (Figure 1). Thus, based on our *in vitro* data, we postulated that not only microbial products but also dietary ingredients modulate GM-CSF signaling (Figure 2). Future research should elucidate the relationship between dietary and microbial-induced GM-CSF production and its effect on monocyte differentiation.

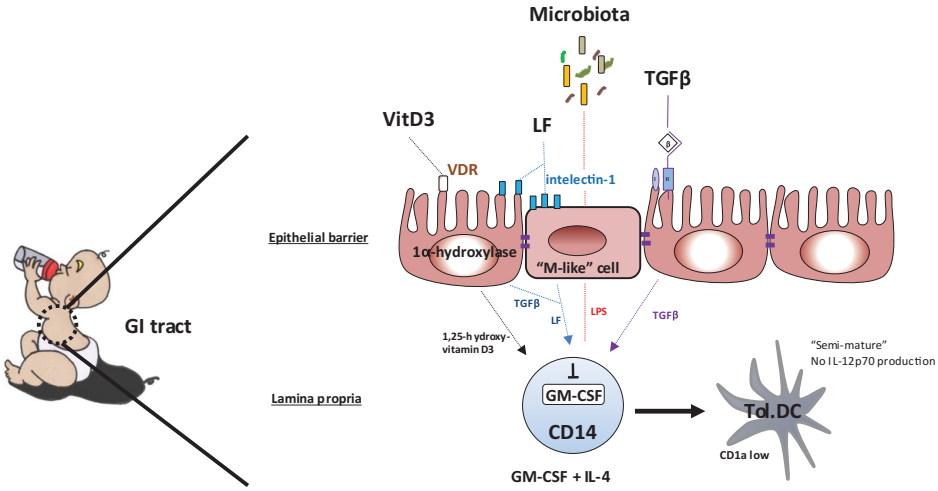

Figure 2. Bovine milk components halt DC differentiation by blocking GM-CSF signaling. In this thesis, we showed that VitD3, TGFβ, LF, and LPS halt differentiation of monocytes into moDC. We suggest that these components block GM-CSF signaling that results in hyporesponsiveness of the moDC towards TLR ligands, which could be important in immune homeostasis.

CD1a as a marker for tolerogenic DC

Thus, LF, TGFβ, VitD3 and LPS all hamper CD1a expression, which might be caused by reduced activity of GM-CSF. After six days of differentiation, we stimulated these distinct DC types with TLR ligands and assessed their responsiveness. Interestingly, we showed that these DC were hyporesponsive to LPS or Poly:IC and R848, as was most profoundly observed by abrogated production of IL-12p70. This is in line with earlier studies that showed an abrogated production of IL-12p70 by CD1a- cells[212,213,417]. Additionally, other reports showed that CD1a- DC were capable of decreasing IFNγ production[212,213,417] and increasing IL-10[213] by T cells compared to T cells that were polarized by CD1a+ DC. This suggests that CD1a- DC are prone to induce T cells with regulatory functions. CD1a- DC are also found *in vivo* in the circulation and secondary lymphoid organs. These DC produce IL-10 and are CD14+HLA-DR+CD11c+, which shows that they relate to monocytes and also have regulatory functions *in vivo*[144].

Other markers of tolerogenic DC

DC that were differentiated in the presence of bovine milk components (i.e. VitD3, TGFβ, LF and LPS) showed to be hyporesponsive towards TLR ligands. These DC showed a reduced expression of all costimulatory molecules measured (e.g. CD80,

CD86), but also inhibitory molecules (e.g. PD-L1 and inducible T cell costimulator ligand) and production of all cytokines measured (e.g. not only IL-12p70 but also IL-10, TNF, IL-6) compared to moDC cultured in the absence of bovine milk ingredients. Hence, the question is whether these DC are truly tolerogenic. Previous work showed that DC that have a lower expression of costimulatory molecules (i.e. showing a semi-mature phenotype) are associated with tolerance induction *in vivo* and *in vitro*[418,419]. Indeed, semi-mature DC and soluble factors such as TGFβ and RA are required for the polarization of naïve T cells into Tregs[162,420]. In line with our findings, Unger *et al.* (2009) showed that VitD3 induced PD-L1 expression on immature DC. However, after maturation VitD3-DC show lower expression of PD-L1 compared to moDC. Nevertheless, this PD-L1 expression on VitD3-DC was essential to induce Tregs. The authors proposed that the ratio of PD-L1/CD86 expression determined the potential of the DC to induce tolerogenic responses[295]. However, human moDC cultured in the presence of RA expressed CD103 and fully matured upon stimulation[27]. Yet, these RA-DC were capable of inducing differentiation of naïve T cells into IL-10 producing T cells and FoxP3+ T cells when cultured in the presence of TGFβ. Indeed, DC expressing high levels of costimulatory molecules were also shown to be capable of expanding CD4+CD25+ T cells[421]. Thus other signals such as indoleamine 2,3-dioxygenase expression, the capacity to activate cytotoxic T lymphocyte antigen (CTLA) 4 on T cells[422] and the production of anti-inflammatory soluble mediators such as RA, TGFβ and IL-10 also determine the tolerogenic nature of the DC[145,162,398]. Hence, it is essential for future studies to follow-up on our work investigating the effect of our DC phenotype on the polarization and chemokine receptor expression of T cells.

Nutritional immunology or endotoxin tolerance?

In nutritional immunology, many researchers work with naturally derived food or feed products. One of the major challenges is to isolate the components of interest without endotoxins. These endotoxins can mask the true immunogenicity of the food compound or give false positive results. The gram-negative bacterial cell-wall constituent LPS is one of the major contaminants that can be responsible for false positive results due to its high immunogenicity on myeloid cells. Hence, in chapter 4 we optimized a method using Triton X-114 to remove LPS from food proteins. Triton X-114 is an amphiphilic (i.e. contains hydrophilic and lipophilic traits) molecule that

can be used to remove LPS from aqueous solutions by a simple bilayer separation. Since we showed that the introduction of Triton X-114 inhibits cell viability, gives false positive results in LPS detection (limulus amebocyte lysate) assays and immunogenicity[210], it is essential to remove the Triton X-114 traces after removing LPS from the sample. We showed that Triton-binding beads are the most efficient method and that the protein structure of bovine β-lactoglobulin and soy proteins remains identical after applying this method[210]. In chapter 3, we applied this method to remove LPS from a LF concentrate. Although we observed a fivefold reduction in LPS in the sample, complete removal of LPS was unsuccessful. We hypothesized that the Triton X-114 method was not able to remove all LPS due to the high binding affinity of LPS to LF[208,423] and that the method only removed non-bound LPS in aqueous solutions. The LF concentration was unaltered after applying the method, showing that the LF is not caught in the lipophilic fraction, possibly because its interaction with LPS is based on charge and not on hydrophobicity[424]. In contrast, molecules carrying highly hydrophobic domains will be caught into the hydrophobic layer. Indeed, Triton X-114 has been successfully used to extract membrane proteins (that have highly hydrophobic domains) from total cell lysates[261]. As such, we showed that the Triton X-114 method removed major HDM allergen Der-p2 from HDM extract (Figure 3). The Triton X-114 method is therefore unsuitable to remove LPS from proteins with highly hydrophobic domains. On the other hand, it is an efficient method to remove specific hydrophobic molecules such as Der p 2. Der p 2 is a lipid-binding protein highly homologous to myeloid differentiation 2 (MD2) that binds hydrophobic molecules such as LPS[425,426]. Our optimized method is thus not applicable to remove all LPS from proteins with hydrophobic domains (e.g. membrane proteins) or proteins that bind LPS (e.g. LF). Therefore, in chapter 3, we applied proteinase K treatment to degrade LF instead of aiming to further remove LPS from LF. Of note, proteinase K treatment did not affect the capacity of LPS to activate NF-κB signaling via TLR4. Hereby we showed that the LF-induced effects on DC functioning were completely abrogated after proteinase K-mediated degradation of LF, showing that the protein structure is essential for its immunomodulatory effect.

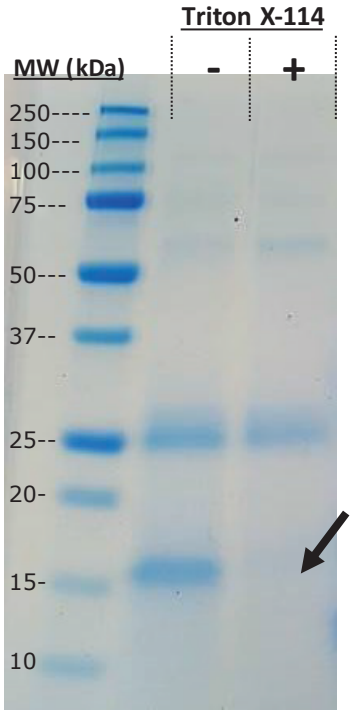

Figure 3. Triton X-114 method applied to HDM extract removes Der p 2. HDM extract (ALK-Abello) was treated with Triton X-114 to remove LPS. This HDM extract was loaded on SDS-PAGE gel before and after application of the Triton X-114 method. The arrow points toward the missing band of Der p 2 (15.6kDa).

LPS in sialyllactose

In chapter 5 we showed that LPS contamination in commercially available 3'SL results in differentiation of monocytes into tolerogenic DC. The concept of endotoxin tolerance upon exposure to LPS is a well-known and accepted phenomenon. In particular myeloid cells are highly sensitive to LPS[262]. In line with an earlier study performed in mice[156], we showed that LPS contaminated 3'SL and not 6'SL induced signaling via TLR4 that induced the differentiation into tolerogenic DC[157]. In chapter 5 we showed that 0.5 EU/ml of LPS induced the differentiation into tolerogenic DC. In chapter 6 we showed that even small traces of 0.17 EU/ml as measured in 6'SL, did not induce phenotypical changes on DC, and induced the production IL-6 and IL-8 during DC differentiation. The in chapter 4 optimized Triton X-114 method showed to be a valuable method to remove these LPS traces in oligosaccharide preparations. Removal of LPS showed that 3'SL, 6'SL, galactooligosaccharide (GOS) and 2-fucosylalactose (2'FL) do not modulate DC differentiation or functionality.

Prebiotics, human milk oligosaccharides and TLR4 signaling

Infant nutrition is nowadays often supplemented with synthetic or plant-derived prebiotic oligosaccharides. Due to the wide variety of health effects attributed to the presence of human milk oligosaccharides (HMO)[40], it is of interest to discover functional HMO that can be isolated from cow's milk. HMO and prebiotic oligosaccharides are suggested to directly modulate immunity since a small fraction is absorbed in the intestine into the bloodstream where they are postulated to directly affect the immune system.

One of the receptors that has been described in literature to mediate both anti- and pro-inflammatory immune responses is TLR4. Several studies show that the prebiotics inulin and fructooligosaccharides (FOS)[270,271], 3'SL[156], a 9:1 mixture of short-chain GOS/long-chain FOS[272] and GOS[271] induce immune responses via interacting with TLR4. In Chinese medicine a variety of other natural polysaccharides are used that - depending on their structural composition – are shown to induce TLR4 signaling[283]. However, in chapter 5 we showed that LPS contamination could result in false conclusions drawn with regard to the immunomodulatory capacity of 3'SL. Hence, an important question that was raised is whether it is possible that simple or structurally more complex oligosaccharides can actually induce TLR4 signaling.

Although TLR4 signaling is triggered by exposure to a wide variety of molecules, the exact interaction of TLR4 and its ligand remains largely unknown. However, the interaction of TLR4 with LPS has been extensively researched. LPS initially interacts with several proteins including the LPS binding protein (LBP) that shuttles insoluble lipids such as LPS or phospholipids[427] to CD14, which in turn facilitates binding to the MD2:TLR4 complex[228]. This complex formation results in recruitment of its downstream adaptor proteins (including MyD88 and TRIF) that induce downstream signaling resulting in nuclear factor (NF) κB activation[228]. The interaction of LPS with MD2:TLR4 has been studied in great detail. LPS contains a highly immunogenic part, which is the amphipathic lipid A tail. The lipid A part of *Escherichia coli*-derived LPS consists of two phosphorylated glucosamines that are connected to six lipid chains[428]. MD2 has two anti-parallel β-sheets that form a large hydrophobic binding pocket for hydrophobic molecules such as LPS. These long lipid chains of LPS are essential for the interaction with the hydrophobic binding pocket of LPS[428].

Although conventional chemical models are capable of predicting specificity of antibodies to antigens and peptides that fit into the MHC groove, it remains difficult to predict ligand:TLR interactions due to difficulties defining the crystal structural of the ligand-binding domain of TLRs[429,430]. A wide range of self (damage associated molecular patterns; DAMPS) and exogenous molecules (pathogen associated molecular patterns; PAMPS) have been shown to activate TLR4[431]. Although there is evidence showing evolutionary pressure on the ligand-recognition domain of TLRs[430], TLR4 signaling is very conserved between species. It was therefore suggested that there must be a very basic mechanism that allows recognition of this wide range of ligands. Seong and Matzinger (2004) proposed that the common factor of the ligands that bind TLR4 is the hydrophobic portions that docks into the MD2:TLR4 pocket. They propose that hydrophobicity is the common evolutionary driver for TLR4 signaling. On the one hand the hydrophobic nature of phospholipids is essential to dissociate between the interior milieu of cells and the outside world and is thus crucial for life. On the other hand, hydrophobic domains tend to cluster together, forming aggregates that are harmful for the cell. Hence, humans have evolved to express a wide array of chaperone proteins (e.g. heat-shock proteins (HSP)) that shield hydrophobic groups of e.g. LPS[432]. Intracellular release of HSP thereby signals to the cell that cell-damage is being controlled, which results in induction of anti-inflammatory responses[433]. Sudden release of hydrophobic molecules is therefore a sign of cellular damage that the immune system should react upon[429]. Pathogens such as viruses use fusion proteins with highly hydrophobic domains to enter host cells and PAMPs (e.g. LPS, peptidoglycans, lipoteichoic acid and even the supermolecular organization of RNA) have large hydrophobic structures[429]. Thus, TLR4 signaling is triggered by molecules with hydrophobic domains that can dock into the MD2:TLR4 pocket. Therefore, there is structurally no grounded reason to assume that hydrophilic components such as HMO and prebiotic oligosaccharides should interact with TLR4 on immune cells.

Sialyllactose and GOS affect microbiota composition and SCFA production

The establishment of a stable microbiome runs in parallel to immune maturation in early life. This microbial community remains stable later in life and alteration are shown to increase the risk of several diseases[434]. Microbial colonization in early life is

driven by a few crucial events such as vaginal delivery and breast milk. The microbial composition is shaped upon vaginal delivery that enriches bacteria of the genera *Bifidobacterium* and *Bacteroides*[39]. In the first weeks, the microbiota gradually changes from a community with facultative anaerobic bacteria to a community comprised of obligate anaerobic bacteria (e.g. *Bacteroides fragilis* and bifidobacteria)[435]. Thereafter in the first months of life, breast milk is essential to further shape the microbiota composition. We already know now for almost fifty years that the high amounts of HMO in breast milk drive the dominance of bifidobacteria[40]. Since HMO are postulated to exert a wide range of beneficial effects, our hypothesis was that finding HMO that aid in establishing a microbial community most similar to breast milk may be essential for improving the health of the infant. Moreover, breastfeeding results in the outgrowth of bifidobacteria, lactobacilli and *B. fragilis* that are thought be important in achieving tolerance in early life and are associated with lower allergy prevalence[37,434]. Perturbation during the important colonization phase in early life may result in different bacterial compositions (i.e. enterotypes) that are associated with allergic disease. As such, a microbial colonization pattern consisting of *Akkermansia, Ruminococcus gnavus,* and *Lachnospiracaea* are associated with lower atopic dermatitis incidence[436]. In line with this finding, another recent study showed that the abundance of *Akkermansia* in the gut is negatively associated with asthma risk[437].

Recently, SL gained a lot of interest because of its postulated health effects and due to the fact that it can be easily isolated from bovine milk[78]. Infant formulas are nowadays often supplied with prebiotic oligosaccharides such as GOS and FOS, which are synthetically-derived polymers of lactose and plant-derived oligosaccharides, respectively. This prebiotic mixture of GOS/FOS in a 9:1 ratio is often used and was shown to induce the outgrowth of *Bifidobacteria* compared to infant nutrition not supplemented with these prebiotics[324–328]. Nevertheless, compared to breastfed infants, formula-fed infants show a more diverse microbial profile than breastfed infants and a decrease in the genera *Bifidobacterium* (e.g. *B. longum*) and *Lactobacillus* while the genus *Bacteroides* (e.g. *B. adolescenentis*) and *Clostridium* species are more abundant[39,438]. In chapter 7 we investigated the effect of SL and GOS on microbiota composition using fecal batch cultures. Interestingly, we showed that GOS predominantly induced the outgrowth of *Bifidobacteria*, while SL induced the outgrowth of *Bacteroides,* including *Bacteroides fragilis* but also other bacteria such as *Feacalibacterium prausnitzii. Bacteroides* is a genus of bacteria that contains many species capable of fermenting

plant-derived polysaccharides[439]. The fact that *Bacteroides* are main fermenters of plant-derived fibers is in line with reports showing that infants of four months of age that are not exclusively breastfed show a higher abundance of *Bacteroides* in the feces compared to exclusively breastfed infants[39]. A recent study showed that LPS derived from *Bacteroides* induced immune silencing in early life rather than immune priming (e.g. as induced by *E. coli*-derived LPS). The authors show that this immune silencing may induce the development of early onset auto-immune diseases[275]. Additionally, sialic acid becomes available upon fermentation of SL that can be potentially utilized by pathogenic bacteria[359]. On the other hand, we showed that SL induced the outgrowth of species such as *Fecalibacterium prausnitizii* and *B. fragilis* that have anti-inflammatory effects[362,365,366]. Additionally, SL was also essential for growth in stunted infants mediated via microbiota-derived metabolites[158]. Thus, future research should further investigate the effect of SL on microbiota (e.g. risk of outgrowth of pathogenic and probiotic bacteria), subsequent metabolite production and immune functioning in animal models before moving to clinical trials. We confirm the bifidogenic effect of GOS. This supports the current line of though and previous research showing prebiotic effects of GOS supplementation to infant nutrition, since bifidobacteria are the signature bacteria of breast-fed infants.

Interestingly, upon fermentation of carbohydrates *Bacteroidetes* produce metabolites of which SCFA are shown to be essential for the hosts physiology[321–323]. Recent evidence shows that SCFA can alleviate allergic symptoms via distinct mechanisms. For instance, SCFA promote the induction of oral tolerance via inducing RALDH expression in epithelial cells[159,440]. Recently, it was shown that SCFA also directly dampen DC activation and T helper (Th) 2 cell skewing[46]. Additionally, propionate was shown to induce hematopoiesis of tolerogenic DC that prevented allergic inflammation in the lung[44]. A high fiber diet, acetate or propionate supplementation to mice was also capable of AAD via epigenetic modifications[45]. The authors showed that acetate induced epigenetic changes that regulated the accessibility to the FOXP3 promoter, resulting in systemic anti-inflammatory that prevented allergic airway inflammation. Additionally, although the mechanism is still unknown, SCFA are also shown to directly dampen Th2-mediated airway inflammation through DC and T cells[46].

In this line of thought, enhanced SCFA production in the colon in early life may be essential for allergy prevention. It has been recognized for decades that a higher

prevalence of bifidobacteria results in a lower pH. Interestingly, a recent review highlighted that the pH of infant stool increased in the last century[441]. This increase in pH and thus decrease in bifidobacteria prevalence may be caused by disturbances in microbiota colonization in early life (e.g. delivery by caesarean section, formula feeding and antibiotic use)[441]. Indeed, formula fed infants without prebiotic supplementation are shown to have higher pH compared to breastfed infants[442]. Moreover, breastfed infants show higher lactate- and lower propionate and butyrate levels compared to formula fed infants. Infants receiving infant formula that contains a 9:1 GOS:FOS ratio show a SCFA profile that resembles that of breastfed infants[329]. Similarly, GOS supplementation to infant formula resulted in lower butyrate levels and lower stool pH compared to non-supplemented infant formula[327]. Of note, in contrast to normal breastfed infants, exclusive breastfeeding resulted in an increase in total SCFA levels in very-low birth weight infants[444]. Thus, HMOs in breast milk maintain a specific microbial profile comprised of *Bifidobacterium* and *Lactobacilli.* These bacteria produce high levels of lactate and low levels of other SCFA, which results in a low fecal pH. Prebiotic supplementation to infants have been shown to boost the abundance of bifidobacteria and mimic the SCFA profile of breastfed infants. Although bifidobacteria are shown to protect against GI infections, SCFA (i.e. acetate, propionate and butyrate) are shown to have anti-inflammatory effects and dampen Th2 cell immunity in animal models[44–46]. Therefore, there is a pressing need to investigate the role of metabolites such as SCFA on immune cell maturation during early life. This knowledge is required to interpret the relevance of SCFA production by prebiotics (e.g. the SL-induced propionate production as described in **chapter 7**).

Microbial-derived SCFA alleviates allergic airway inflammation

In chapter 8, we aimed to investigate the effect of raw cow's milk and its predominant oligosaccharides 3'SL and 6'SL in concentrations present in cow's milk in a murine ovalbumin (OVA)-induced asthma model. Of note, this OVA-induced asthma model with acute intranasal challenges cannot fully mimic the chronic inflammatory symptoms of asthma in humans[445]. Nevertheless, it allowed us to study the *in vivo* effect of diet, microbiota composition and SCFA production on lung health in particular. Based on previous studies[44,45,446], we chose to feed the mice a low fiber diet, since we hypothesized that this would augment microbial-derived SCFA production induced upon fermentation of SL. However, there were several technical issues in this pilot study revealed by the microbiota analysis. We aimed to run a two-week run-in

period with raw cow's milk or SL before introducing the first sensitization. This two-week run-in period was in line with an earlier study by Trompette *et al*, who studied the effect of fibers on HDM-induced allergy in mice[44]. This preventive strategy was shown to be effective if mice were fed a high fiber diet or were supplemented with 200 mM acetate or propionate while receiving normal chow for three weeks. This has been shown to protect against HDM-induced allergy[45]. However, the low-fiber diet was introduced in the two-week run-in period in which the supplements were given to the mice. The microbiota analysis revealed that the microbial changes might be purely driven by the dietary transition and that this change lasted throughout the total study period of 30 days, which makes it difficult to draw conclusions of the effect of raw cow's milk and SL in this model. Hence, in follow-up studies mice should be fed a low-fiber diet for at least four weeks before testing specific dietary effects on airway-induced inflammation. This follow-up study ideally should use a more physiological relevant allergy model such as a HDM-induced model. Additionally, supplementing the dietary ingredients via oral gavages would reduce inter-individual variation between intake volume. Nevertheless, it is interesting to note that 3'SL in our pilot study induced the outgrowth of the probiotic genus *Akkermansia* that has been negatively associated with development of atopic dermatitis and asthma. Unfortunately, the intestinal microbiota chip results provided in chapter 7 in the human batch cultures treated with SL do not allow to detect this genus of bacteria. Chapter 7 and 8 indicate that it would be very interesting to test different dosages of SL in HDM-induced allergy model (e.g. GPR41 and GPR43 knock-out mice).

Epithelial barrier functioning

The term "epithelial barrier functioning" is often used to describe an epithelial barrier that does not allow leakage of macromolecules[447]. However, the definition is poorly defined and can encompass several underlying mechanisms such as differentiation and proliferation of epithelial cells, expression of tight junction proteins and even anti-inflammatory responses induced by the epithelium. Early life is often referred to as a period in life in which barrier integrity is suboptimal, which could result in inflammation and exposure of allergens. However, this vulnerable period in humans lasts only for a few weeks in homeostasis[127] and a mild dysfunctional barrier has been shown to promote the formation of Tregs that protect the GI tract against colitis[448]. Thus, the first week of life may actually give a window of opportunity to

induce regulatory responses to environmental antigens and breast milk components. However, breastfed neonates show a lower permeability, as measured by the lactulose/mannitol ratio in urine, compared to infants fed infant formula, indicating that certain breast milk components aid in gut maturation[96]. From an evolutionary point of view, breast milk is the golden standard. Hence, promoting gut closure would thus be beneficial. Later in life, stress factors such as infections may temporarily disrupt barrier function. It is therefore possible that continuous barrier function supported by nutrition may be important to suppress the inflammatory response.

Intestinal epithelial cells are at the frontline to discriminate harmful bacteria from commensals. The highly polarized organization of epithelial cell allows discrimination between signals coming from the commensal microbes and pathogenic bacteria infiltrating the intestine[20,449]. The microbiota express TLR ligands[450] and SCFA[451] that signal via TLRs, thereby inducing epithelial cell differentiation. We showed in chapter 7 that SL and GOS modulate cell cycle control and induce differentiation of Caco-2 cells, which is in line with earlier reports showing enhanced alkaline phosphatase (ALP) production by intestinal cell lines[135,345,346]. Overexpression of ALP in Caco-2 cells was shown to induce tight junction expression[452]. ALP is a well-established differentiation marker for enterocytes, and was shown to modulate microbiota composition and inactivate different serotypes of LPS[347]. Together, increased ALP levels are known to increase barrier function. HMO or prebiotic-oligosaccharides may thus improve barrier functioning directly and indirectly via modulation of microbiota composition that in turn produce SCFA and directly via microbe associated molecular patterns (MAMPs) that enhance barrier functioning (Figure 4). Additionally, we showed in chapter 7, using a different human *in vitro* assay, that SL and GOS also induced re-epithelization of epithelial cells. Re-epithelization is a phase in the "wound healing" process in which epithelial cells migrate into the wound area, reorganize their cytoskeleton and differentiate after the wound is closed[348,349]. Previous research showed that HMO may also indirectly modulate this process via the microbiota[349] and SCFA production[350]. Future studies should identify the relevance of these findings using epithelial barrier studies on human organoids or colitis animal models.

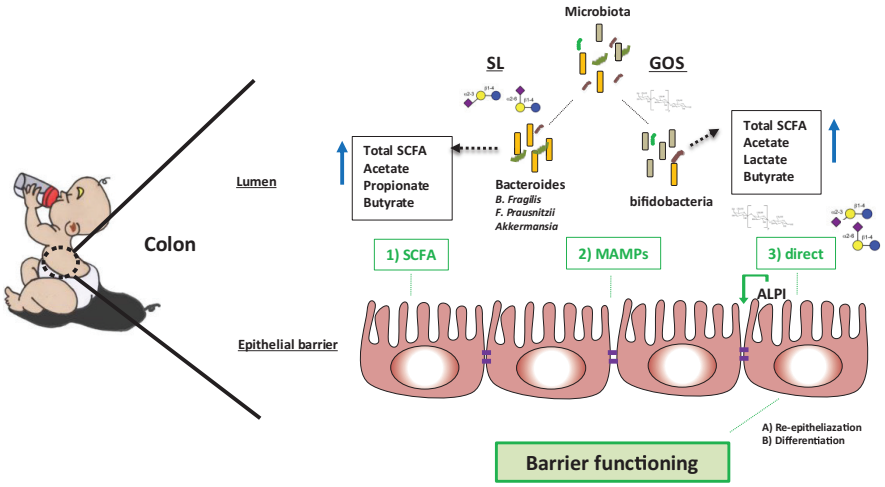

Figure 4. SL and GOS directly and indirectly modulate epithelial barrier functioning. In *in vitro* batch cultures, we showed that SL and GOS induce the outgrowth of *Bacteroides* and bifidobacteria that produce propionate and lactate, respectively. Oligosaccharides may thereby indirectly promote barrier functioning indirectly via the microbiota via the production of 1) SCFA, 2) MAMPs (e.g. TLR ligands), and directly via production of 3) ALP.

Concluding remarks

There is a pressing need to better understand the mechanisms underlying the worldwide rise in chronic inflammatory diseases. Since allergic sensitization and predisposition to childhood asthma already occur early in life, preventive strategies should aim at this period. Various environmental factors have been shown to trigger the regulatory compartment of our immune system. Exposure to these factors may be essential to maintain immune homeostasis and induce tolerance towards harmless antigens such as allergens. Breast milk has been suggested to consist of a unique mixture of tolerogenic components that could prevent allergen sensitization. As such, it is interesting that epidemiological studies show an inverse correlation between raw cow's milk consumption in early life and allergy and asthma prevalence. Since some components are identical between breast milk and cow's milk and others are highly homologous, raw cow's milk may be a useful source for functional ingredients for infant nutrition.

The overall aim of this thesis was therefore to investigate the effect of raw cow's milk and its components on human mucosal immune homeostasis. We found a

novel role for LF in inducing the differentiation of monocytes into tolerogenic DC. **Chapter 3** shows that LF alters the differentiation of monocytes into tolerogenic DC. This shows that LF is not just an anti-microbial or anti-viral protein but that it has immunoregulatory effects. Follow-up studies should therefore investigate whether fortification of infant formula with LF could, in addition to prevention of GI infections and inflammation, potentially also result in a reduction of allergy or asthma prevalence. From a mechanistic point of view, it would be very interesting to further investigate the mechanism by which LF halts DC differentiation. In **chapter 4** we optimized an existing method to remove LPS, which we applied in **chapter 3, 5, and 6**. However, we also warrant that this method does not work for removing LPS from proteins with highly hydrophobic domains (e.g. membrane proteins) or proteins that strongly interact with LPS such as LF (**chapter 3**). Thus, in line with other commercially available methods to extract LPS, the suitable method is based on the structure of the protein of interest. Additionally, in **chapter 5** we challenged the current thought that HMO or prebiotic oligosaccharides can activate TLR4 signaling by showing that in contrast to mice, 3'SL does not induce human TLR4 signaling. In this discussion we further elaborated why, based on biochemical features of these milk oligosaccharides, it is not likely that any HMO would activate TLR4 signaling. Further attempts to predict TLR:ligand interactions *in silico* are needed to efficiently develop therapeutics and screen for immunomodulatory food components. Additionally, we showed in **chapter 6** that the trisaccharides 2'FL, 3'SL, and 6'SL and the prebiotic GOS do not modulate human DC differentiation or maturation. Instead, we showed in **chapter 7** that high concentrations of SL and GOS are capable of inducing epithelial cell differentiation and distinctly modulate microbiota composition and SCFA production. In line with literature, GOS induced the outgrowth of bifidobacteria and boosted lactate production, while SL induced the outgrowth of *Bacteroides* and produced propionate in fecal batch cultures from adults. Nevertheless, these changes were not this black and white in infant fecal batch cultures. Of note, our knowledge on the role of SCFA in early life is too limited to interpret these results. Hence, future studies should investigate the role of host metabolome in early life to understand the implications of these findings. In **chapter 8**, we aimed to measure the effect of raw cow's milk or SL supplementation on microbiota composition and systemic anti-inflammatory effects by SCFA in a murine model. However, our data showed that effect of the raw cow's milk and SL are probably masked due to fact that the microbiota of the mice was not stabilized to the low-fiber diet. Nevertheless, 3'SL induced the outgrowth of *Akkermansia*, which

has been shown to contribute to immune homeostasis and is reduced in children with atopic dermatitis[436] and asthma[437]. Hence, it is essential to repeat the current pilot study, preferably in a different allergic model such as a HDM-induced model with mice fully adapted to the low-fiber diet (i.e. fed for at least four weeks) and SL or raw milk fed via oral gavage.

In this thesis we show that prebiotic oligosaccharides (i.e. GOS) and HMO such as sialylated oligosaccharides induce differentiation of intestinal epithelium and modulate microbiota composition and their production of SCFA upon fermentation. Further deciphering these microbiota-dependent mechanisms are essential to understand the impact of milk and prebiotic oligosaccharides on the physiology of the infant. We could not demonstrate direct immunomodulatory effects of these oligosaccharides on monocytes and DC. In contrast, bovine milk-derived proteins such as LF and TGFβ do contribute to immune homeostasis by inducing differentiation of monocytes into tolerogenic DC. Finally, we also highlighted the necessity of endotoxin removal in nutritional immunology, and discuss the strength and weaknesses of our optimized LPS-removal method. This thesis thereby gives novel insights into regulation of mucosal immune responses by cow's milk components. Future studies should investigate the effects of these components in *in vivo* allergy and respiratory infection models, which ultimately could result in novel approaches to improve respiratory health.

Appendix I

Mucosal immune development in early life: setting the stage

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Abstract

Our environment poses a constant threat to our health. In order to survive all organisms must be able to discriminate between good (food ingredients and microbes that help digest our food) and bad (pathogenic microbes, viruses and toxins). In vertebrates, discrimination between beneficial and harmful antigens mainly occurs at the mucosal surfaces of the respiratory, digestive, urinary and genital tract. Here, an extensive network of cells and organs form the basis of what we have come to know as the mucosal immune system. The mucosal immune system is composed of a single epithelial cell layer protected by a mucus layer. Different immune cells monitor the baso-lateral side of the epithelial cells and dispersed secondary lymphoid organs, such as Peyer's patches and isolated lymphoid follicles are equipped with immune cells able to mount appropriate and specific responses. This review will focus on the current knowledge on host, dietary and bacterial-derived factors that shape the mucosal immune system before and after birth. We will discuss current knowledge on fetal immunity (both responsiveness and lymphoid organ development) as well as the impact of diet and microbial colonization on neonatal immunity and disease susceptibility (summarized in tables 1 and 2). Lastly, Inflammatory Bowel Disease will be discussed as an example of how the composition of the microbiota might predispose to disease later in life. A fundamental understanding of the mechanisms involved in mucosal immune development and tolerance will aid nutritional intervention strategies to improve health in neonatal and adult life.

Fetal life

Sterile or not?

Previously it was thought that the fetal environment in the uterus was sterile and the fetal immune system was immature and inactive. However, in recent years more and more evidence has emerged that the fetus is actually exposed to environmental antigens prior to birth and that the contact between the immune system of mother and child is far more intimate than previously thought.

For example, bacteria belonging to the genus of *Enterococcus, Streptococcus, Staphylococcus*, and *Propionibacterium* could be cultured from umbilical cord blood of healthy neonates born by caesarian section (table 1)[453]. Additionally, while cultivation of the placental samples did not reveal the presence of viable bacteria, *Bifidobacterium* and *Lactobacillus* DNA could be detected in 33 and 31 of 34 placenta samples respectively[454]. In a recent study, 320 placental samples were analyzed by comparative 16S ribosomal DNA–based and whole-genome shotgun metagenomics. Here, the authors report that the placenta harbors a unique microbiome consisting of several non-pathogenic bacteria. This placental microbiome mostly resembled the mother's oral microbiome[455] The placenta, therefore, might harbor several antigens to which the fetus needs to develop tolerance[456]. Furthermore, lactic acid bacteria and enteric bacteria have been found in the meconium - the first fecal discharge of neonates - that was thought to be sterile[457]. These data suggest that bacteria or at least bacterial DNA can come in contact with fetal tissues and this does not automatically lead to premature birth or spontaneous abortion. Thus, during fetal life overt inflammatory responses towards environmental or maternal (commensal) bacteria must be prevented, to forestall premature birth or death of the fetus.

Table 1. Environmental factors influencing host immunity during fetal and neonatal life

	Factor	Specific substance	Immunological mechanism/clinical effect on host	Model	Ref.
Fetal life	**Placental microbiota**		APCs epigenetically regulate RORγt expression in umbilical cord T cells	human	458,459
		microbial derived riboflavins	fetal intestinal MAIT cells produce IFN and IL-22	human	460–464
	Amniotic fluid	AMPs	bacterial lytic effects	human	465–467
		endotoxin-neutralizing AMPs	preventing TLR signaling	human	467
		EGF	preventing TLR signaling	human	468
	Maternal factors	cells that cross the placenta	induction Tregs in secondary lymphoid tissue	human	469
		consumed vegetables	less intra-epithelial lymphocytes and RORγt+ ILCs	mice	470–472
		probiotics (*B.lactis* and/or *L. rhamnosus* GG)	altered TLR expression in exfoliated cells	human	473
*	**Microbial colonization**	**(Table 2)**			
Neonatal life	**breast milk**	growth factors	increased epithelial barrier functioning	human	96
		lactoferrin	antimicrobial	human	474,475
		oligosaccharides	improve diversity and microbial metabolism	human/mice	476–478
		milk glycans	protection from enteric pathogens	human/mice	479,480
		Insulin like growth factors	wound healing and tissue repair	rats	481,482
		epidermal growth factors	anti-inflammatory and induced mucus production	rats	481,482
		commensal bacteria	inhibition pathogens?	human	383,483,484
		IgA	humoral immunity/modulates microbiota composition	human	485–488
	raw cow milk/	bIgG	recognizes pathogens that can also infect humans (e.g. RSV)	human	111
	colostrum	bIgG	reduces recurrent diarrhea in AIDS patients	human	489
		lactoferrin, lactoperoxidase and lysozyme	protects low birth weight infants from necrotizing enterocolitis	human	195
	vitamin A		establishes normal levels of type 3 (RORγT+) intestinal lymphoid cells	mice	490
		retinoic acid (+ TGF-β)	promotion of Tregs via CD103+ DCs	human/mice	160,491
			inhibits Th17/converts Tregs to T follicular helper cells/upreg. CCR9 and α4β7	mice	492–497
		retinoic acid			
		retinoic acid	induce IgA secreting B cells	human/mice	184
		miR-10a induced by retinoic acid	T-bet expression/Th1 immunity	mice	497
	vitamin D		increase CD8αα+ intraepithelial T cells	human	498
			Treg induction by binding of VDR-RXR to enhancer of Foxp3 gene	mice	499
	fermentation products	SCFAs	recruitment of leukocytes and T cell activation	mice	500,501
	starch	butyrate and acetate	Treg differentiation via colonic DCs and macrophages (via GPR109A receptor)	mice	502
		butyrate	anti-inflammatory: epigenetically (HDAC, FOXP3)/reduced chemotaxis of monocytes	human/mice	503–506
		acetate or propionate	reduce LPS-induced TNF release from neutrophils	human/mice	507
	vegetables	glucosinolates (e.g. TCDD)	epigenetic modulation of FoxP3 and RORγT+ genes (via aryl hydrocarbon receptor)	mice/rats	508,509

Underdeveloped or repressed immunity?

Stoppelenburg and colleagues have shown that umbilical cord blood T cells fail to differentiate toward the pro-inflammatory Th17 lineage in the presence of autologous antigen-presenting cells[459]. In a separate study, they also showed that neonatal T cells have an intrinsic mechanism that prevents Th17 differentiation through the regulation of RORγt expression, possible via DNA methylation and histone acetylation[458]. This again indicates that overt inflammatory responses are actively repressed in the fetus and neonate. At the same time, this might pose a risk to mother and child. Indeed, it has been shown that pregnant women have a 20-fold increased risk of developing listeriosis; infection with Listeria bacteria that causes infections of the central nervous system of the unborn, such as meningitis[510]. This is probably due to repressed Th1 cell proliferation and IFNγ production during pregnancy[510].

To further prevent pro-inflammatory responses, the fetus is surrounded by amniotic fluid. This amniotic fluid contains antimicrobial peptides such as defensins and lactoferrin. Furthermore, it contains endotoxin-neutralizing histones and lipopolysaccharide (LPS)-binding protein that might prevent Toll-like receptor (TLR) signaling and possibly fatal immune responses for the unborn child[465,466]. Recently, it was shown in mice that epidermal growth factor (EGF) in the amniotic fluid inhibits fetal TLR signaling through binding to the EGF-receptor on fetal intestinal epithelial cells[468]. So instead of being underdeveloped and unresponsive, the fetus can respond to antigens, however, these responses are actively prevented.

Development of mucosal lymphoid tissue during fetal life

Meanwhile in the gut of the fetus, interspersed Peyer's patches develop around 11 weeks of gestation and functional B and T cells can be found from 12 and 16 weeks respectively (Figure 1)[469,511–515]. Both the Gut Associated Lymphoid Tissue (GALT) and the intestinal epithelium mature during the gestational period. Specialized epithelial cells called Paneth cells develop in the colon and small intestine at 13.5 weeks of gestation. After 17 weeks, Paneth cells are confined to the small intestine[516]. Paneth cells reside at the bottom of the crypts, secrete anti-microbial peptides and are important in protecting the intestinal stem cells and maintaining intestinal homeostasis[19,517,518]. In the human fetal intestine Goblet cells appear around 9-10 weeks of gestation[519]. Goblet cells produce mucins, that serves as a first line of defense against luminal antigens. In addition to mucins (large glycoproteins), mucus consists of water, ions and immune mediators such as immunoglobulin A (IgA) and anti-

microbial peptides, which help clear pathogens[520,521]. Early during development lymphoid precursor cells are present and spread to Peyer's patches and mesenteric lymph nodes[522]. Memory T cells were found to be relatively abundant in fetal spleen and in cord blood samples from premature births. These cells comprised about 25% and 10% of the T cells, respectively, expressed CD25 and were anergic[523]. Later, it was shown that (at least part) of these cells are regulatory T cells (Tregs) that are already present in the second trimester of the pregnancy. At that time, 15-20% of CD4+ T cells in the fetus' secondary lymphoid tissues are comprised of Tregs. Murine studies suggest that these Tregs are largely induced by maternal cells that cross the placenta and reside in fetal lymph nodes[469]. In this way, regulation of fetal anti-maternal immunity is established. The authors also suggest that this form of in utero induced antigen-specific tolerance, might also be active in regulating immune responses after birth[469]. Next to the GALT, the nasopharynx-associated lymphoid tissue (NALT), and bronchus-associated lymphoid tissue (BALT) are also part of the mucosal-associated lymphoid tissue (MALT). The NALT (named Waldeyer's ring in humans), consists of the nasopharyngeal tonsil, tubal tonsils, palatine tonsils and lingual tonsils[524]. Its appearance is similar to Peyer's patches; follicles underneath follicle-associated epithelium (FAE) containing interspersed microfold (M) cells that can sample antigens[525]. Tonsils are secondary lymphoid organs. The tonsillar subepithelial space is formed by several lymphoid follicles containing B and T cell areas. Tonsils are not encapsulated like the spleen, but are lined by tonsillar epithelium that invaginates forming crypts [524]. From the 14th week of gestation B and T cells populate the area under the tonsillar epithelium and primary follicles develop from 16 weeks of gestation (earlier than any other secondary lymphoid tissue). The tonsils will keep growing until 7 years of age after which they slowly involute[526]. While NALT is present at birth, BALT develops from 3-4 days of age[525,527,528]. It isn't until 3 to 4 weeks of age until B and T cell areas are formed in the BALT[525,528].

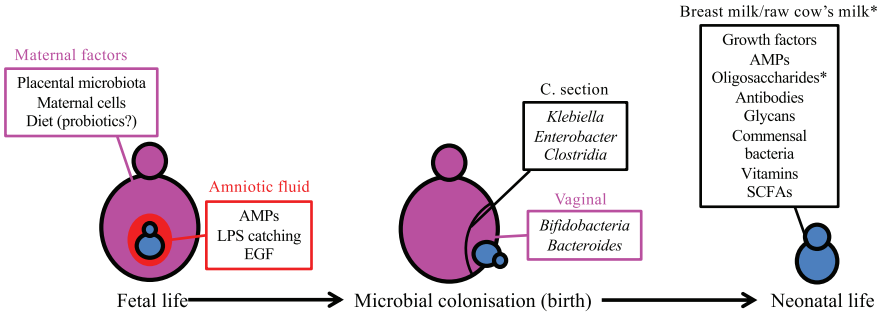

Figure 1. Important factors in early life affecting mucosal immune development. During the fetal life stage there is a direct interaction between maternally derived environmental factors (e.g. diet and microbes) and the fetus. Additionally, the amniotic fluid contains antimicrobial peptides (AMPs) and epidermal growth factors (EGF) and endotoxin-neutralizing proteins that protect against pathogenic bacteria and possible fatal immune responses, respectively. Birth, and the way of delivery, is a critical point in immune development that determines which types of microbes will colonize the GI-tract. In the neonatal life stage, breast milk (or alternatively infant formula) provides the infant with proteins, short-chain fatty acids (SCFAs) and vitamins that are critical for immune cell differentiation and development. Environmental factors such as diet and microbes early in life set an immunological stage that impact the hosts susceptibility towards disease.

Immune modulation via dietary or bacterial factors during fetal life?

Recently, a specific subset of T cells with an invariant receptor (mucosa-associated invariant T cells (MAIT)) was also found to be present in the second trimester of human fetal tissues[463]. MAIT cells are innate-like T cells that recognize antigens in complex with the MHCIb-like protein MR1[464]. MAIT cells recognize microbial-derived riboflavin metabolites and can subsequently produce IFNγ, TNF and IL-17[460–462]. Interestingly, these cells are present at high frequency in fetal lung, liver and small intestine, and display a mature phenotype (i.e. they express IL-18Rα(+) and CD8αα)[463]. Compared to adult MAIT cells fetal small intestinal MAIT cells have an increased proliferative capacity and can respond to bacterial stimulation with production of IFNγ and IL-22[463]. The factors that drive this fetal MAIT maturation are currently unknown, but also might suggest that the human fetal environment is not devoid of external or environmental stimuli.

The fact that environmental factors can reach the fetal immune system via the placenta, suggests that fetal immunity might be altered or enhanced by dietary or microbial intervention in pregnant women. However, scientific evidence on the effect of dietary intervention in pregnant women on fetal immunity is limited. Rautava and colleagues (2012) report that women that received either *Bifidobacterium lactis* or *Bifidobacterium lactis* together with *Lactobacillus rhamnosus* GG (LGG) 14 weeks

prior to elective caesarean section showed altered TLR expression in the exfoliated cells present in the meconium of the newborn as compared to the placebo group[473]. However, others have shown that dietary supplementation with probiotics during late pregnancy might alter maternal immune parameters, but does not alter fetal immune responses[529,530]. Additionally, while supplementation with galacto-oligosaccharides (GOS) and long-chain fructo-oligosaccharides (lcFOS) alters maternal fecal microbiota (increase of bifidobacteria), it did not affect fetal immunity as measured by cord blood cell stimulation assays[531]. However, experiments performed with pregnant mice suggest that live bacteria can transfer from the mother to the fetus. Labeled *Enterococcus faecium* that were orally given to pregnant mice, could be cultured from the amniotic fluid as well as from the mammary glands of the mothers[453]. Interestingly, in mice treated with a diet devoid of vegetable material decreased numbers of intraepithelial lymphocytes are seen as well as a reduction in type three innate lymphoid cells (RORγt+ ILC) in the intestines[470–472]. Additionally, in a recent paper van de Pavert and colleagues (2014) have shown in mice that maternal diet derived vitamin A induces lymph nodes in the unborn pups. Pups derived from mice fed vitamin A-deficient diets had markedly reduced lymph node size and decreased efficiency of immune responses. In this paper, van der Pavert showed that retinoic acid (RA; the metabolite of vitamin A) is necessary for differentiation of lymphotoxin inducer cells (LTi) that play a crucial role in lymph node formation[532].

In conclusion, while increasing evidence suggests a direct interaction between the maternally derived environmental factors (such as diet and microbes) and the fetus, more research is warranted to investigate the mechanisms by which these factors might (beneficially) alter fetal and subsequent neonatal immunity.

Neonatal life

Caesarean section versus vaginal birth - Effect on the microbial composition

During birth the amniotic membranes will rupture and the unborn child will passage through the birth canal. This birth canal is not sterile and during labor the child will get exposed to vaginal bacteria, maternal skin and feces followed by exposure to environmental antigens[533]. This exposure has a profound impact on the host. Here, we summarize what is known in this interesting research field (see also Table 2).

Studies comparing children born vaginally or by caesarean section have shown differences in microbial community and immune responses. For example, a Venezuelan cohort showed that most vaginally delivered infants acquired a bacterial composition dominated by *Lactobacillus, Prevotella*, or *Sneathia*; species that are found in their mothers vaginal microbiota[534]. In contrast, infants born by caesarean section displayed a bacterial community dominated by *Staphylococcus, Corynebacterium,* and *Propionibacterium,* typical skin bacteria[534]. A Finnish study compared the microbiota and antibody production at 1 month after birth and showed that children delivered by caesarean section harbored fewer bifidobacteria and were shown to mount a stronger humoral immune response. During the first year of life, infants born vaginally displayed lower total IgA-, IgG- and IgM-secreting B cells in peripheral blood[535]. The mode of delivery also has been reported to affect serum cytokine levels. Malamitsi-Puchner *et al.* (2005) reported that soluble IL-2 receptor, IL-1β and TNF were significantly higher in cases of vaginal delivery than in cases of elective caesarean section in neonates at day 1 (IL-1β , IL-2Receptor and TNF) and day 4 (IL-2R, TNF) of life[536]. These two studies might suggest that children born vaginally have lower humoral and higher cellular immunity in early life, compared to children born by caesarean section. However, more data will be necessary to support this hypothesis. Several studies report increased abundance of bifidobacteria and *Bacteroides* in vaginal-delivered children compared to children born by caesarean section[535,537]. Additionally, analysis of bacterial colonization from birth to 12 months of age in a cohort of Swedish, Italian and British infants using culturing techniques showed that children delivered by caesarean section displayed more *Klebsiella, Enterobacter*, and *Clostridia*, including the pathobiont *Clostridium difficile* compared to vaginally delivered babies[538–540]. Interestingly, studies performed in Western countries revealed that children born by caesarean section take 6 months to a year to acquire the same levels of *Bacteroides,* bifidobacteria and *Escherichia coli* colonization as vaginally born children display directly after birth[538,540,541]. In contrast, children born by caesarean section in the developing world catch up much quicker indicating that the environment is an important factor in colonization patterns after birth[542].

Caesarean section versus vaginal birth - Effect on allergic diseases

Thus, from these studies it seems that vaginally born children harbor bacterial species that have been considered beneficial (bifidobacteria), while children born by caesarean section are more prone to harbor species that are associated with, but do

not necessarily lead to, disease (*Escherichia coli* and *Clostridia*). Indeed, colonization with *Clostridium difficile* has been associated with a higher risk of a diagnosis of atopic dermatitis[543,544]. Several meta-analyses have shown that babies born by caesarean section are at higher risk to develop allergy, including food allergies. Interestingly, in a Norwegian birth cohort it was shown that children of allergic mothers that were born by caesarean section had a seven fold increased risk of developing food allergy to egg, fish or nuts[545]. This effect was not seen in children whose mothers were not allergic, indicating that a predisposition exists that together with birth by caesarean section can lead to food allergy. Likewise, in a German cohort, babies with a family history of allergy and born by caesarean section also showed an increased risk of allergic sensitization to food allergens compared with babies at risk born vaginally[546]. Finally, a large meta-analysis in which 26 studies on the effect of delivery by caesarean section on one or more allergies were described showed that caesarean section was associated food allergy, atopy, allergic rhinitis, asthma, and hospitalization for asthma. However, they found no association with inhalant atopy and eczema/atopic dermatitis[547]. Since children born by caesarean section have an altered bacterial community, it is generally thought that this altered microbiota can lead to differences in mucosal immune tolerance which can predispose to the development of allergies[548]. Indeed in Dutch cohort, colonization by *Clostridium difficile* (associated with caesarean section) at an age of 1 month was associated with wheeze and eczema in the first 6 years of life and with asthma from age 6[549]. Although the associations exist, reports on the mechanisms how these changes early in life lead to disease are understandingly scarce. However, from animal studies we do know that exposure to certain bacterial species has an important impact on host immunity. In the next section, we will discuss the current knowledge of microbial modulation of host immunity generated using animal models.

Table 2. Effect of microbial colonization on host immunity

	Factor	Microbial composition	Immunological mechanism/ clinical effect on host	Model	Ref.
Birth	Vaginal birth	more *Bifidobacteria* and *Bacteroides*	stronger humoral response (higher levels of IgA, IgG and IgM secreting B cells)	human	[535,537]
			higher serum levels of sIL-2r and TNF	human	[536]
	Caesarean section	more *Klebsiella*, *Enterobacter* and *Clostridia*	higher risk of allergies (excl. inhalant atopy and eczema)	human	[538–540,543,544,547]
	Bottle feeding	more intestinal *Bacteriodes* and *Clostridia*	might predispose to development of autoimmunity, and childhood infections, atopy and asthma	human	[533,550]
		oral microbiome without *Lactobacillus*		human	[551,552]
	Breast feeding	more intestinal *Bifidobacteria*	associated with protection from autoimmune disease, and childhood infections, atopy and asthma	human	[533,550,553]
		oral microbiome with *Lactobacillus*		human	[551,552]
		Segmented filamentous bacteria (SFB)	IgA plasma cells are restored to normal levels	mice	[554,555]
		Bacteria from conventional raised mice	increased FoxP3 expression in colitis model	mice	[556]
		Autologous bacteria	tolerance induction that protects against IBD	mice	[557]
		Altered Schaedler flora (ASF)	Treg induction	mice	[558–560]
		Bacteriodes fragilis	Treg induction in a polysaccharide A-TLR2 dependent manner	mice	[362]
		Faecalibacterium prausnitzii	enhances anti-inflammatory responses	mice	[365,561]
		Cluster IV, XIVa and XVIII of *Clostridia*	induce Treg frequency and inducible T-cell co-stimulator	mice	[562]
		Segmented filamentous bacteria (SFB)	more Th17 cells in small intestinal lamina propria, less in colon	mice	[563,564]

How do colonizing microbes influence host immunity?

The last decades it has become clear that the composition of the microbial communities that colonize our bodies have a profound influence on our health. Most if this knowledge derives from studies using gnotobiotic experimental animals. These studies show that colonization by different microbial species early in life has clear effects on the development of the intestinal mucosal immune system. Interestingly, host responses to microbial colonization are highly conserved between species. A

study investigating zebrafish responses towards colonization revealed 59 responses that are conserved between mouse and zebrafish. These responses included pathways involved in epithelial proliferation, promotion of nutrient metabolism, and innate immune responses[565]. Several immune cells and mediators are influenced by the microbiota, for example, germfree mice that are devoid of bacteria have almost no IgA-secreting plasma cells. Only upon colonization with specific subtypes of bacteria IgA plasma cells are restored to levels seen in conventionally raised mice[554,555]. IgA is the predominant antibody secreted by plasma cells in the mucosal tissues[566]. Low-affinity, poly-specific IgA is believed to prevent adhesion of commensal bacteria to epithelial cells, while high-affinity, mono-specific IgA neutralizes toxins and pathogens[558–560].

Studies have also shown that germ-free animals have altered Treg frequency. In a transfer model of colitis, it was shown that co-transfer of CD4+CD62L- cells into SCID mice prevented colitis induced by CD4+CD62L+ cells only when those cells were derived from conventionally-raised mice. The CD4+CD62L- cells from germfree animals were not able to suppress the colitis. This associated with a low expression of regulatory T cell marker FoxP3 in this population form germfree mice[556]. Already in 1995, Duchmann *et al.* reported that hyporesponsiveness exists towards the hosts' autologous bacteria[557]. Lamina propria mononuclear cells (LPMCs) and peripheral blood mononuclear cells (PBMCs) did respond towards heterologous intestinal microbes. In patients with inflammatory bowel disease this tolerance towards autologous bacteria was lost[557]. Together these studies clearly indicated that Tregs are directly or indirectly induced by the intestinal microbiota.

Using the altered Schaedler flora (ASF), a mixture of eight bacterial species including Lactobacilli, Bacteroides, Eubactrium, Mucispirillum, Fusiform and Clostridial species, MacPherson and colleagues demonstrated that ASF colonization of germfree mice increased the inducible Treg frequency in the colonic lamina propria by twofold[558–560]. Likewise, it was shown that Bacteroides fragilis was able to induce Tregs upon colonization. Interestingly, when germfree mice were given B. fragilis devoid of polysaccharide A (*B. fragilis* ΔPSA), Tregs were not induced[362]. Further experiments showed that PSA induction of FoxP3 in CD4+ T cells required TLR2 activation[362]. Likewise, Faecalibacterium prausnitzii has also been demonstrated to enhance anti-inflammatory responses[365,561]. This indicates that bacteria and their cell wall components are important mediators of immune cell differentiation. Recently, Atarashi and colleagues (2013) inoculated mice with a healthy human fecal sample,

and selected for mice enriched in Treg-inducing species[562]. From these selected mice, they isolated 17 strains of bacteria that were able to enhance Treg frequency and induce interleukin-10 and inducible T-cell co-stimulator (ICOS) upon inoculation into germ-free mice. Identification of these 17 strains revealed that these bacteria were members of the clusters IV, XIVa and XVIII of Clostridia, which lack prominent toxins and virulence factors[562].

More evidence for the bacterial specific effects on immune development was reported by Ivanov and co-workers who have shown that the ability to increase the number of Th17 cells in the small intestinal lamina propria associated with the presence of Segmented Filamentous Bacteria (SFB) in mice[563,564]. Th17 cells are T cells that produce IL-17A, Il-17F and IL-22 and have been shown to play a role in inflammatory responses and host defense against bacterial and fungal pathogens[567–569]. Conversely, in the colon lamina propria it was shown that germfree mice harbor more Th17 cells than conventionally raised mice. Upon microbial colonization epithelial cells produce IL-25, which in turn inhibits (either directly or indirectly) the expression of IL-23 by antigen presenting cells[570]. IL-23 is a cytokine that is described to be necessary for Th17 pool maintenance[571]. Reduction of IL-23 therefore results in decreased numbers of Th17 cells in the colon. Likewise, Corbett and colleagues (2014) reported that bacteria with an active vitamin B2 (riboflavin) pathway generate epitopes that (in conjunction with host metabolites) can be recognized by the MAIT cells via MR1[460]. This finding again illustrates that colonization by (specific subsets of) bacteria can give rise to different mucosal immune environments.

Recently, much attention has been directed towards a newly discovered cell subset: innate lymphoid cells (ILCs). Three types of ILCs have been identified: T-bet+ ILCs (including NK cells, ILC1), GATA3+ ILCs (ILC2) and RORγt+ ILCs (ILC3)[572]. These ILCs are in close contact with the microbes since they reside in between the epithelial cells[573–577]. While Gata3^{+} and T-bet+ ILC development does not seem to depend on microbial colonization, this is not completely clear for the RORγt+ ILCs. Some studies show normal development, while other show reduced frequency of RORγt ILCs in germfree mice[572,574,578–581]. RORγt ILCs express TLR2 and can therefore directly be activated by bacterial ligands[582].

In conclusion, colonization is an important process during which the immune system develops to a certain set-point in each individual. Therefore, colonization by Bifidobacteria or Bacteroides species (vaginally-delivered children), might result in a different immune cell-repertoire (for example T cell subsets) and distribution than colonization by Escherichia coli (Caesarean section), thereby leading to a different immunological set-point that may or may not predispose (in combination with host genetic susceptibility) towards certain diseases.

Dietary exposure and host immunity in early life

Bottle feeding versus breastfeeding

Next to bacteria, the newborn encounters several new environmental antigens of which most will be derived from the diet. Therefore, children that will be breastfed will be exposed to different dietary antigens than those that will be bottle-fed. Human breast milk contains immunoglobulins, cytokines, growth factors, lysozyme, lactoferrin and a complex mix of milk oligosaccharides[73,81,583]. Breast milk and colostrum contain large amounts of IgA, but also immune cells and cytokines, and soluble TLR2 that might help restrict innate immune activation by microbes[584,585]

The structure of breast milk oligosaccharides has been shown to be very diverse and depend on several factors including diet, lifestyle, and ethnicity[589]. Oligosaccharides can improve diversity and rate of metabolism of the microbiota[476–478]. Also breastfeeding has an impact on the composition of the microbiota. Breastfeeding is associated with high numbers of Bifidobacteria in the gastrointestinal tract of the newborns, whereas bottle feeding resulted in more intestinal Bacteroides and Clostridia[550,553,590]. Recently, it was shown that Lactobacilli could be cultured from saliva in 27.8% of exclusively and partially breast-fed infants, but not from formula-fed infants[551,552], indicating that the oral microbiome is also influenced by infant feeding[456]. Furthermore, it has been shown that human milk glycans can protect infants from enteric pathogens[479,480]. Insulin like growth factor is important for wound healing and tissue repair and epidermal growth factor plays a role in cell proliferation and differentiation, induces mucus production by intestinal Goblet cells and can suppresses pro-inflammatory cytokines[481,482]. Interestingly, human milk also contains bacteria. Culture-dependent mechanisms have shown the presence of Staphylococcus, Streptococcus and Bifidobacterium species[483,484]. Subsequently, sequence analysis has

identified the presence of DNA from 9 different bacterial genera[383]. Interestingly, recently it was reported that house dust mite allergen, DerP1, is present in human breast milk. Subsequent testing of breast milk containing DerP1 in a mouse model revealed that instead of protecting these mice from allergic responses, they were sensitized[591]. This suggests that not only are neonates exposed to dietary antigens early in life via breast milk, they are also exposed to respiratory allergens via breast milk, and this does not always lead to tolerance to the antigens but may well result in sensitization.

Maternal IgA is reflective of the environment of mother and child and therefore can protect the newborn against possible pathogens that he or she might encounter right after birth. Maturation of the IgA-producing plasma cells slowly develops after birth. While, IgA H chain transcripts are found in cord blood as early as 27 weeks of gestation, at 60 weeks of age, somatic mutation frequency of IgA H chain transcripts only reaches 25% of the adult values, with little evidence of Ag-driven selection[486]. Therefore, maternal IgA from the milk will equip the newborn with antigen-specific humoral immunity at the time the child itself does not have a fully developed repertoire. Interestingly, recently it was shown in mice that breast milk-derived IgA modulates the composition of the microbial community in the gastrointestinal tract [485]. Next to preventing bacterial infections, maternal IgA can also reduce the oxidative burst and represses TNF and IL-6 production by human monocytes[487,488].

Protection from disease?

There is a long debate in the literature about the possible beneficial effect of (prolonged and/or exclusive) breastfeeding for children at risk for type 1 diabetes. Already in 1984, Borch-Johnson reported an inverse correlation between breastfeeding and incidence rates of childhood type 1 diabetes[592]. Several other studies confirmed this correlation[593,594], while others did not[595,596]. Animal studies using the spontaneous diabetic rat model (the BB-DP rat) showed that prolonged exclusive breastfeeding decreased diabetes incidence by 40-50% and associated with increased frequency of Treg cells and less pro-inflammatory cytokine secretion in the mesenteric lymph nodes[597]. Furthermore, antibiotic treatment reduces the incidence in both the BB-DP rat and the NOD mouse model for spontaneous diabetes[598,599]. Interestingly, in the BB-DP rat the composition of the microbiota before onset of disease differed between BB-DP rats that did and rats that did not develop diabetes, suggesting that microbial dysbiosis occurs prior to disease onset[598]. Likewise, several studies

report an association between breast milk and protection against infection such as diarrhea, atopic diseases and asthma during childhood[600–603]. Interestingly, a meta-analysis of twelve human studies showed that the protective effect in most studies correlated with the (high) concentrations of TGF-β1 or TGF-β2 in the milk[71]. A recent meta-analysis of studies published between 1983 and 2012 on breastfeeding and asthma in children reported a strong protective association at ages 0-2 years between breastfeeding and asthma, which diminished over time[604]. The availability of nutrients, and especially of milk oligosaccharides, in the intestinal tract of newborns also has a profound influence on the microbial species that are able to survive there. Indeed it has been shown that breastfeeding and bottle-feeding result in different microbial colonization patterns, which results in different host immune responses[605].

To improve the composition of infant formulas for mothers that cannot provide breastfeeding to their child, investigators try to develop formulas that resemble the composition of human breast milk. Recent developments include the use of prebiotics to provide non-digestible oligosaccharides and probiotics. Like breast milk, bovine milk also contains several proteins that have an immunomodulatory function such as large quantities of immunoglobulins, lactoferrin, caseins and cytokines like TGFβ, but only very low levels of oligosaccharides[65]. Many of these proteins are, surprisingly, active across the species barrier. The active form of bovine TGFβ2 (the predominant cytokine in milk) is even 100% identical to human TGF-β2, and bovine IL-10 is fully comparable to human IL-10 in its anti-inflammatory effects of human monocytes and DC[50,73]. Bovine IgG can bind to human Fc gamma receptors on monocytes and neutrophils[111,606,607], and bovine IgG recognizes a wide range of pathogens that can also infect humans such as RSV[111,607]. Bovine colostrum, that is extremely rich in bovine IgG has been shown to significantly reduce recurrent diarrhea in AIDS patients, showing that bovine IgG can have an anti-pathogenic effect in humans[489]. Milk also contains anti-microbial proteins, most prominently lactoferrin, lactoperoxidase and lysozyme. Lactoferrin was shown to protect low birth weight infants against necrotizing enterocolitis[195]. In line with this, it has already been known for a long time that growing up in a farm environment lowers the risk of developing allergies[608]. Next to exposure to farm animals, drinking farm milk has also been implicated as a factor that might reduce allergy risk[65,609,610]. A recent study showed that consumption of raw milk inversely associated with development of rhinitis, respiratory tract infections, otitis, and fever in infants[66]. However, since bovine milk is heated and homogenized, a substantial proportion of these protective

proteins will be denatured in milk products[110]. New insights into how dietary components influence host immunity, continuously promotes development of health stimulating or disease preventing (infant) nutrition.

Fermentation products: how bacterial products influence host immunity

The microbes that are present in the intestinal tract of mammals are important for digestion of foods that would otherwise not be available to the host. The products of bacterial fermentation, such as butyrate, are readily taken up by colonocytes for energy, but also have important immunological effects. Most of the bacteria that reside in the mammalian gastrointestinal tract are saccharolytic, meaning that they mainly feed on carbohydrates[611]. Human milk oligosaccharides are complex glycan molecules that are present in very high concentrations in breast milk. Several studies have shown that milk oligosaccharides influence the composition of the intestinal microbiota[279,612,613]. Human milk oligosaccharides promote the growth of Bifidobacteria[612,613], and prevent pathogenic bacterial adherence to epithelial cells by acting as a soluble ligand for glycan receptors[412,614–616]. Next to effects on the microbiota milk oligosaccharides and non-digestible carbohydrates have also been show to directly influence host immunity and epithelial cell biology (reviewed in [553]).

Short chain fatty acids (SCFAs) are the end products generated by the colonic microbiota[617]. The type of SCFA formed is dependent on the substrate provided. Acetate and butyrate are mainly the result of starch fermentation, while acetate is the end product from the fermentation of pectin and xylan[618]. The succinate and acrylate pathways have been shown to lead to propionate production[619–622], and some bacteria can produce propionate from deoxy sugars such as fucose and rhamnose or lactate[623]. SCFAs can interact with G protein coupled receptors (GPR43, GPR41 and GPR109a)[500]. GPR43 is mainly located on neutrophils, and at lower levels on PBMCs and monocytes, while GPR41 is expressed on PBMCs but not on neutrophils, monocytes and dendritic cells. Both receptors have also been found on intestinal epithelial cells, and recently it has been shown that binding of SCFAs to these G protein coupled receptors can promote inflammatory responses in mice. Binding of SCFAs to GPR43 and GPR41 induced colon epithelial cell production of chemokines, recruited leukocytes and activated effector T cells[501]. Niacin receptor GPR109A has recently been shown to also be a receptor for butyrate in the colon. Singh and colleagues (2014) reported that Gpr109a signaling induced differentiation of Tregs and IL-10 producing T cells through effects on colonic macrophages and

dendritic cells[502]. Both propionate and acetate can reduce LPS-induced TNF release from human neutrophils[507], and butyrate seems to inhibit chemotactic effects on human monocytes[504]. Furthermore, SCFAs have been shown to reduce cell adhesion thereby preventing immune cell infiltration[624,625]. Interestingly, butyrate can inhibit histone deacetylase (HDAC). HDACs prevent gene transcription by keeping the chromatin in a closed form, so transcription is prevented. Butyrate inhibits this effect leading to hyper-acetylation and open chromatin[626]. Butyrate has been reported to have anti-inflammatory effect through its HDAC activity on the NF-κB pathway, IL-5 expression and COX-2 expression[410,503,506]. Another interesting example of the effect of butyrate on host immunity comes from the study by Atarashi and colleagues (2013)[562]. They isolated 17 strains Clostridial species that were able to enhance Treg frequency and induce IL-10 and ICOS upon inoculation into germ-free mice[562]. In a follow up study of the same research group they showed that these Clostridiales (indirectly or directly) induced butyrate that subsequently induced functional colonic Treg cells, via epigenetic modification of the FoxP3 gene in T cells[627].

In conclusion, SCFAs are able to modify host immunity directly by binding to receptors on host cells or indirectly through epigenetic changes of host DNA. These modifications result in activation or repression of host immune genes and the outcome will depend on the type of SCFA and host (immune) cell type studied. Whether SCFAs can induce epigenetic changes in the host throughout life, or whether a specific window (early in life) exists is currently unknown.

Vitamin A and D

Vitamin D deficiency, together with vitamin A deficiency are two of the most common food related medical conditions worldwide. As vitamin A and D are conveyed to the newborn via breast milk, vitamin A and D status of the mother is very important for the developing child. Vitamin D deficiency leads to poor skeletal development and bone and joint deterioration, while vitamin A deficiency is one of the important causes of blindness in children[628,629]. Appropriate vitamin D status has been reported to convey protection against several cancers, bacterial infections and autoimmune diseases such as rheumatoid arthritis and multiple sclerosis[630]. Also, low vitamin D levels during pregnancy associates with increased risk for type 1 diabetes in the offspring. However, too much vitamin D (especially D2) might lead to local tissue intoxication (reviewed in [630]). In recent years, vitamin A and D have received a lot of attention from immunologists. Vitamin A can be converted to retinal and subsequently

into retinoic acid by dendritic cells and epithelial cells. In an elegant paper, Coombes and colleagues (2007) showed that in mice retinoic acid together with TGF-β are essential for promotion of Tregs by CD103(+) DCs[180]. Recently, it was also shown that retinoic acid can promote the development of human CD103+ dendritic cells from monocytes[160]. The CD103+ intestinal DC subset can convert retinal into retinoic acid because it expresses the retinal dehydrogenase enzymes (RALDH1 and RALDH2)[180]. Retinoic acid has been shown to inhibit Th17 and the conversion of Tregs to T follicular helper (Tfh) cells, and induce intestinal mucosal homing molecules CCR9 and $\alpha_4\beta_7$[179,492,494,495,497]. Also, retinoic acid is important for IgA-secreting cells, since mice deficient for vitamin A lack these cells in the small intestine[184]. There have been reports that miR-10a, a microRNA induced by retinoic acid in Th17 cells can induce expression of T-bet (associated with Th1 cells)[497]. This indicates that next to Tregs retinoic acid might also induce Th1 cells. Indeed, in an inflammatory environment retinoic acid could induce Th1 immunity[631]. Not only has vitamin A update via the diet an effect on the host, vitamin A uptake by pregnant mothers also influences the development of the neonatal immune system. As shown by van de Pavert and colleagues (2014) pups derived from mice fed vitamin A-deficient diets had markedly reduced lymph node size and decreased efficiency of immune responses[532].

Vitamin D has been reported to enhance regulatory T cell induction via binding of the VDR-RXR (vitamin D receptor-retinoic X receptor) binding to an enhancer in the FoxP3 gene[498]. While vitamin D deficiency causes a reduction in CD8α+ intraepithelial T cells[499]. Recently, Spencer and colleagues (2014) showed that vitamin A deficiency leads to severely diminished type 3 innate lymphoid cells (ILC3s), which results in compromised immunity to acute bacterial infection. Additionally, vitamin A deprivation resulted in increased IL-13-producing ILC2s and resistance to nematode infection in mice[490]. Since vitamins A and D can have several direct and indirect effects on cells and signaling pathways, further research is necessary to understand their complete role in immune modulation. These findings, however, suggest that exposure to certain dietary factors (both in mother and child) can have profound influence on the development and effectiveness of the immune response. As with many multi-factorial diseases the interplay between host, microbes and dietary exposure might be different in each individual patient, making it extremely difficult to find causal relations rather than incidental associations. This is very well illustrated by what is known for Inflammatory Bowel Disease (IBD).

When homeostasis between host and microbes is lost – the case of Inflammatory Bowel Disease

In recent years, genome wide association studies have revealed many single nucleotide polymorphisms (SNPs) in host genes that are associated with multi-factorial diseases. For example, in Inflammatory Bowel Disease (IBD) >160 genes are found to be associated with either ulcerative colitis and Crohn's disease or both[632]. Each and every patient therefore, can have a unique combination of these SNPs. Interestingly, several of these associated genes has a role in bacterial-host interaction. Studies performed using experimental animals showed that knock-outs of these genes (such as Nod2 or enteric defensins) can change the intestinal microbial community[518,633]. Subsequently, changes in microbial community can influence disease susceptibility. The IL-10 knock-out mice for example, does not develop colitis under germfree conditions. Interestingly, narrow and broad spectrum antibiotics can prevent disease in IL-$10^{-/-}$ mice under specific pathogen free (SPF) conditions[634]. Furthermore, we have shown that that the composition of zebrafish intestinal microbiota can determine recruitment of different immune cells, enterocolitis susceptibility and severity[635].

An illustration of influence of gene alterations on microbial dysbiosis and disease susceptibility comes from the studies performed by Garrett and colleagues[636]. Mice deficient for transcription factor T-bet and Rag2 (TRUC mice) showed increased TNF production by colonic dendritic cells leading to increased apoptosis of colonic epithelial cells and spontaneous colitis. This colitis was dependent on the intestinal microbiota since treatment of TRUC mice with a combination of antibiotics cured the mice from colitis. Later studies confirmed that TRUC mice have an altered microbiota (presence of *Klebsiella pneumoniae* and *Proteus mirabilis*)[637]. This colitis was also transmissible via the microbiota, since co-housing adult TRUC mice and WT mice (3:1) rendered WT mice more susceptible to develop colitis. Likewise, when a TRUC mother fostered pups of $Rag2^{-/-}$ or wild type mice, these mice-pups were also more susceptible and developed colitis that was histologically similar to colitis in TRUC mice[637].

Another study that illustrates the importance of a functioning adaptive immune system was performed using zebrafish. In zebrafish, lymphocytes deficiency leads to failure to suppress bacteria of the order *Vibrionales* (that contains known fish pathogens) (our own unpublished observations). Adoptive transfer of T lymphocytes

could actively suppress outgrowth of these *Vibrionales*. Additionally, zebrafish T lymphocytes are able to induce epithelial *Cxcl8-l1* expression, thereby augmenting mucosal immune responses[638]. In summary, these studies emphasize that genetic deficiencies (of genes involved in mucosal immunity) can modify the mucosal environment and allow for modulation of the microbiota which in turn can alter susceptibility towards disease. This clearly illustrates that modulation of the gut microbiota might be beneficial for IBD patients. Indeed, Sokol and colleagues (2008) identified *Faecalibacterium prausnitzii* as an anti-inflammatory commensal bacterium, which was severely reduced in Crohn disease patients[365,639]. These studies have encouraged fecal transplantation as a therapy for IBD patients, which results in remission in some but not all patients[640–642]. Clearly, future research to elucidate the complex interaction between host, diet and microbes in the context of chronic intestinal inflammation and during health is dearly needed.

Timing of exposure, does a window of opportunity exist?

Next to investigating the different pathways by which food and microbes alter host immunity, investigation on the concept of timing will be crucial. It has been suggested, that a window exists early in life when microbes alter host immunity, after which a set point is reached and homeostasis is established. There is indication that some processes might indeed take place in a specific time window, where after they cannot be changed again. For example, iNKT cells, a subset of invariant T cells that recognize glycolipids in the context of MHC-like molecule CD1d, were found to be more abundant in the colon (and lungs) of germfree mice[643]. These germ-free mice displayed increased morbidity in models of IBD and allergic asthma. The increased number of iNKT cells in the colon (and lungs) of germfree mice was shown to be the result of high expression of the chemokine CXCL16. Colonization of neonatal - but not adult - germfree mice protected the animals from this mucosal iNKT accumulation and related pathology[643]. This difference in iNKT accumulation associated with epigenetic modifications that enabled modification of CXCL16 expression early in life, but not at adult age. This suggests that a host developmental (epigenetic) program exists that allows for environmental agents to shape immune responses only at certain time points of life. However, other studies suggest microbial and dietary modulation can also affect host immunity in later life. The success of fecal transplants in obese people and inflammatory bowel disease patients suggests

that lifelong modification of diet and microbes might be beneficial[644]. Likewise, it has been shown that glucosinolates derived from vegetables in the diet, such as cabbage and broccoli, can activate the aryl hydrocarbon receptor (AhR) and modulate immune responses[508]. AhR ligand TCDD can induce differentiation of Tregs while inhibiting development of Th17 cells, which correlates with increased methylation and demethylation of the respective promotors for FoxP3 and IL-17, indicating that epigenetic modification can occur upon AhR activation[509]. Thus, whereas host epigenetic changes might be induced by bacteria or nutrients it is not clear whether a specific window (early) in life exists or whether it can take place throughout life.

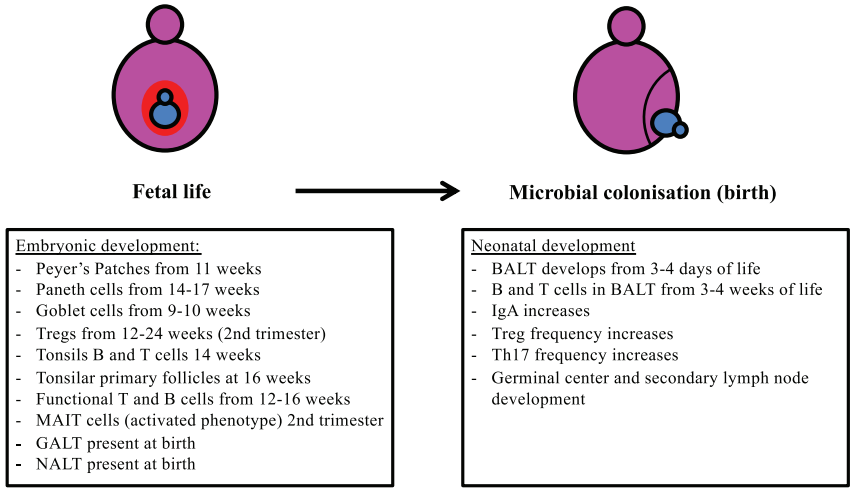

Figure 2. Development of mucosal immunity before and after birth. Contrary to what was believed, the fetal immune system contains mature T and B cells, that are actively repressed by regulatory T cells. Of note, the gut-associated lymphoid tissue (GALT) and the nasal-associated lymphoid tissue (NALT) are present before birth, while the bronchial-associated lymphoid tissue (BALT) develops after birth.

Future perspectives

In the last decade, through the development of large-scale metagenomic technologies, we have gained access to enormous datasets containing information on microbial and host genes in health and disease. The future challenge will be to make sense of these large datasets and to stratify patient groups according to their genomic or metabolomic profiles. In addition, modification of the mucosal immune system through dietary interventions (in both mothers and infants) requires more in depth

knowledge on how dietary nutrients or microbial patterns can alter host immunity (Figure 2). The fact that fetal life might not be as devoid of environmental stimulation as previously thought suggests that modification of the environment during pregnancy and early life might be able to (beneficially) alter immunity. Furthermore, epigenetic modification of the host by bacteria or dietary components might be time dependent. Future research should focus on the question whether host epigenetic modification can only be achieved in a specific window (early) in life or whether changes can be induced lifelong. Rapid technological advances in this field as evidenced by large metagenomic screens and epigenetic sequencing platforms will soon provide more answers on these questions.

In conclusion, environmental factors, such as dietary components and microbes can shape the mucosal immune system by influencing differentiation and development of immune cells and tissues. This in turn influences host susceptibility towards disease. By using model systems that can be easily manipulated both genetically and environmentally (i.e. zebrafish and mice) novel pathways can be discovered that control host responses towards environmental antigens. Elucidation of these conserved pathways will yield novel targets for nutritional interventions that will benefit human health.

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Summary

The increasing prevalence of allergies and asthma results in major health and socio-economic burden. Globally 300 million people suffer from asthma, which is the cause of an estimated 250.000 annual deaths. Important risk factors for asthma are allergic sensitization and respiratory viral infections in early life. Allergic sensitization occurs upon the induction of an aberrant T helper (Th) 2-mediated response towards an – under homeostatic conditions - harmless antigen. The hygiene hypothesis that was already postulated in 1989, suggested that our more hygienic lifestyle caused a misbalance in our immune system. Now we know that exposure to several environmental factors may in fact trigger the regulatory compartment of our immune system that is essential for immune homeostasis. Furthermore, several epidemiological studies show a negative association between raw cow's milk consumption in early life and allergy and asthma prevalence, indicating that raw cow's milk induces immune regulation. Hence, studying the immunomodulatory effect of raw cow's milk may improve our understanding of which components are relevant, and how we can apply these components to ultimately prevent allergic diseases and asthma.

In **chapter 2**, we reviewed the literature and suggested several mechanisms by which raw cow's milk might promote immune homeostasis in the respiratory tract. In **chapter 3**, we showed that bovine lactoferrin (LF) modulates differentiation of human monocytes into dendritic cells (DC) that are hyporesponsive towards lipopolysaccharide (LPS) stimulation. This phenotype is suggestive for tolerogenic DC. However, LF binds LPS with high affinity. Therefore, it was of interest to investigate whether the immunomodulatory effect was mediated by LF or bound LPS. We showed for the first time that the protein structure of bovine LF was essential for its immunomodulatory effect, showing that the effect of LF was not solely mediated by endotoxin tolerance. LPS is a gram-negative cell wall constituent that often contaminates biological samples. LPS is highly immunogenic on myeloid cells, which gives false positive results in immunological assays. Hence, we optimized an existing Triton X-114 method to ensure complete removal of LPS (**chapter 4**). Importantly, the tested LPS-depleted food proteins maintain their correct folding after applying this method, which is essential for their effect on the immune system. In **chapter 5**, we showed that in contrast to results from studies in mice, 3'-sialyllactose (3'SL) does not signal via human Toll-like receptor (TLR) 4. By applying the optimized Triton X-114 method, we concluded that TLR4 signaling was driven by LPS contamination

that modulated differentiation of IL-4+GM-CSF-cultured monocytes into tolerogenic DC. In **chapter 6** we showed that the milk saccharides 2'-fucosyllactose (2'FL), 6'-sialyllactose (6'SL) and galactooligosaccharides (GOS) do not modulate human DC differentiation. In contrast, vitamin D3 (VitD3) and TGFβ induced differentiation of monocytes into tolerogenic DC. Using these immature TGFβDC and VitD3-DC, which were characterized for their expression of receptors that recognize milk saccharides, we revealed that 2'FL, 6'SL and GOS do not modulate DC maturation. Instead, we showed in **chapter 7** that SL and GOS differentially modulate microbiota composition and short chain fatty acid (SCFA) production. Moreover, SL and GOS induced the outgrowth of *Bacteroides* and *Bifidobacterium* and increased the production of the SCFAs propionate and lactate, respectively. Additionally, SL and GOS induced the expression of genes involved in cell cycle control of the stimulated epithelial cells. These epithelial cells produced more alkaline phosphatase (ALP) that is indicative for enhanced differentiation. SL and GOS also modulated barrier functioning by enhancing the re-epithelization rate in an *in vitro* scratch assay. In **chapter 8**, an ovalbumin (OVA)-induced asthma model was used and we showed that intervention with a low-fiber diet resulted in a gradual change in microbiota composition along the complete duration of the study. Moreover, the abundance of *Firmicutes* increased while the abundance of *Bacteroides* decreased over time. Despite this pronounced impact of diet on microbiota composition, supplementation of 3'SL to the drinking water induced the outgrowth the genus *Akkermansia*. *Akkermansia* has been suggested to contribute to gastrointestinal homeostasis and has been associated with a lower allergy prevalence. In **chapter 9**, key findings of this research are summarized and discussed, and possibilities for future research are given. This thesis shows that bovine milk components (i.e. LF, TGFβ and vitD3 - but not SL) modulate DC differentiation and functioning. Additionally, we showed that SL and GOS both enhance differentiation of epithelial cells, and promote re-epithelization, while they induce the outgrowth of a distinct microbial- and SCFA profile. In an *in vivo* pilot study, we showed that 3'SL induces the outgrowth of *Akkermansia*. The impact of these microbial and metabolomic changes should be further investigated in allergic and asthma models. Additionally, there is a pressing need to reveal underlying mechanisms to fully understand the effect of bovine milk-derived components in early life on microbiota composition, metabolomics and its consequence for host physiology.

Nederlandse Samenvatting

De toename in allergieën en astma zorgt voor een verslechtering van de volksgezondheid en heeft negatieve gevolgen op sociaaleconomisch vlak. Wereldwijd lijden ongeveer 300 miljoen mensen aan astma en is de oorzaak van 250.000 doden per jaar. Belangrijke riscofactoren voor de ontwikkeling van astma zijn allergische sensibilisatie en luchtweginfecties op jonge leeftijd. Allergische sensibilisatie vindt plaats als het afweersysteem een afwijkende Th2 gemedieerde afweerreactie tot stand brengt tegen, onder normale omstandigheden, een ongevaarlijk antigeen. De hygiëne hypothese werd al geformuleerd in 1989 en suggereert dat onze hygiënische levensstijl een disbalans veroorzaakt in het afweersysteem. Nu weten we dat de blootstelling aan bepaalde omgevingsfactoren essentieel is om het regulerende gedeelte van ons afweersysteem te activeren, hetgeen essentieel is voor immuunhomeostase. Daarbij laten meerdere epidemiologische studies zien dat het drinken van rauwe koemelk op jonge leeftijd negatief gecorreleerd is aan het voorkomen van allergieën en astma, wat suggereert dat rauwe koemelk het regulerende gedeelte van ons afweersysteem activeert. Vandaar is het mogelijk dat wij door het bestuderen van het effect van rauwe koemelk op het afweersysteem uit kunnen vinden welke componenten uit rauwe koemelk relevant zijn en hoe deze componenten uiteindelijk toegepast kunnen worden om allergieën en astma te voorkomen.

In hoofdstuk 2 hebben wij de literatuur opnieuw bekeken en zijn op basis hiervan meerdere mechanismen voorgesteld hoe rauwe koemelk bijdraagt aan homeostase van het afweersysteem. In hoofdstuk 3 laten wij zien dat koemelk lactoferrine (LF) de differentiatie van menselijke monocyten naar dendritische cellen (DC) moduleert. Deze DCs reageren minder op blootstelling aan lipopolysacchariden (LPS) wat suggestief is voor tolerogene DC. Echter, LF bindt LPS met hoge bindingsaffiniteit. Het was daarom belangrijk om te onderzoeken of LF, of LPS gebonden aan LF, verantwoordelijk was voor het effect op het afweersysteem. Wij bewezen voor de eerste keer dat de eiwitstructuur van LF essentieel is voor het effect op het afweersysteem en dat het effect van LF dus niet alleen veroorzaakt werd door endotoxine tolerantie. LPS is een derivaat van de celwand van Gram-negatieve bacteriën en zorgt vaak voor vervuiling in biologische preparaten. LPS is zeer immunogeen voor myeloïde cellen, wat voor fout-positieve resultaten zorgt in immunologische testen. Daarom hebben wij een bestaande Triton X-114 methode geoptimaliseerd om LPS volledig te verwijderen uit biologische preparaten (hoofdstuk 4). Belangrijker nog, de geteste

LPS-vrije voedingseiwitten behielden hun correcte vouwing na het toepassen van deze methode, wat essentieel is voor hun effect op het afweersysteem. In hoofdstuk 5 hebben wij laten zien dat 3'-sialyllactose humane Toll-like receptor (TLR)4 signalering niet activeert, wat in tegenstelling is tot wat anderen hebben laten zien in muizen. Door het toepassen van de Triton X-114 methode bewezen wij dat TLR4 signalering gedreven werd door LPS vervuiling in het 3'SL preparaat. Deze vervuiling, en niet 3'SL, induceerde de differentiatie van monocyten in tolerogene DC. In hoofdstuk 6 onderzochten wij de melksuikers 2'-fucosyllactose (2'FL), 6'-sialyllactose (6'SL) en galactooligosaccharides (GOS) en hun effect op DCs. 2'FL, 6'SL en GOS waren niet in staat om humane DC differentiatie te moduleren. In tegenstelling, vitamine D3 (VitD3) en TGFβ induceerden wel de differentiatie van monocyten naar tolerogene DC. Door gebruik te maken van deze vitD3DC en TGFβDC, die gekarakteriseerd werden voor hun expressie van receptoren die mogelijk de melksuikers herkennen, lieten wij zien dat 2'FL, 6'SL en GOS ook niet in staat waren om DC maturatie te beïnvloeden. Daarentegen lieten wij in hoofdstuk 7 zien dat SL en GOS wel een differentieel effect hebben op de microbiële samenstelling en productie van kortketenige vetzuren. Daarnaast bevorderden SL en GOS de groei van respectievelijk *Bacteroides* en *Bifidobacterium* en de bacteriële productie van propionaat en lactaat. Ook induceerden SL en GOS de expressie van genen betrokken bij de regulatie van de celcyclus. SL en GOS hadden ook invloed op de barrierefunctie door het versnellen van de re-epithelisatieprocess in een *in vitro* krasassay. De epitheelcellen produceerde meer alkaline fosfatase, wat duidt op verhoogde differentiatie. In hoofdstuk 8 gebruikten wij een ovalbumine (OVA)-geïnduceerd astma model en lieten zien dat de interventie met een laag vezeldieet een geleidelijke verandering in microbiotasamenstelling veroorzaakte; de *Firmicutes* namen toe en *Bacteroides* namen af gedurende de duur van de studie. Ondanks dit grote effect van het dieet op de microbiële samenstelling, resulteerde de toevoeging van 3'SL in het drinkwater in een groei van het genus *Akkermansia*. *Akkermansia* kan bijdragen aan homeostase in het maagdarmkanaal en zijn aanwezigheid is negatief gecorreleerd met het voorkomen van allergieën. In hoofdstuk 9 worden de hoofbevindingen bediscussieerd en speculeren wij over de mogelijkheden voor vervolgonderzoek.

Dit proefschrift liet zien dat koemelkcomponenten (d.w.z. LF, TGFβ and VitD3 – maar niet SL) DC differentiatie en functie moduleerden. Daarbij bevorderden SL en GOS beiden de differentiatie en re-epitheliasatie van epitheelcellen, terwijl de toevoeging van SL en GOS aan bacteriele culturen een andere uitgroei van bacterien

en productie van kortketenige vetzuren induceerde. In een *in vivo* pilot studie, bevorderde 3'SL de groei van *Akkermansia*. De invloed van deze veranderingen in microbiële samenstelling en metabolieten moeten verder onderzocht worden in allergie en astma modellen. Daarbij is het noodzakelijk om de onderliggende mechanismen te ontrafelen om zo het effect van koemelkcomponenten in het vroege leven op microbiële samenstelling, verandering in metabolieten en de consequentie voor de fysiologie van de gastheer beter te begrijpen.

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Many thanks and much love to you all,
Olaf

About the author

Ólafur IJsbrand (Olaf) Perdijk was born in Lekkerkerk on the 20th of May, 1991. He was raised surrounded by several extraordinary pets and spent many vacations in nature. Therefore, it was no surprise that his interest for biology was already triggered early in life. He started with a year of polytechnic studies in Biology and Medical Laboratory research in Rotterdam, to continue with a BSc in Biology at Wageningen University.

During the three-years of his BSc studies, Olaf became interested in the molecular regulation of developmental and immunological processes, in particular in relation to disease. Hence, he chose to write his BSc thesis (18 ECTS) about the role of haptoglobin phenotypes in malaria infection. He continued his MSc studies in this direction, choosing the specialization "Health and Disease". He worked in the group of Prof. Troye-Blomberg, Stockholm University, Sweden to continue his malaria research (36 ECTS). Here, he was presented with the unique opportunity to independently conduct research, thereby applying the acquired theoretical knowledge during his BSc thesis. This research resulted in a first author paper that was published in 2013. He conducted his second MSc thesis at the Host-Microbe Interactomics group (36 ECTS) at the Wageningen University. He presented this work on immunomodulatory effects of probiotic bacteria at the spring meeting of the Dutch Society for Medical Microbiology in 2014. These three BSc and MSc research projects were all marked with a 9/10.

After his studies, Olaf started his PhD project at the Cell Biology and Immunology Group at the Wageningen University. The aim was to investigate the immunomodulatory effect of cow's milk components and its implications for respiratory health. His PhD thesis was an applied research project funded by STW with FrieslandCampina and ALK-Abelló as third parties. He visited ALK-Abelló, Denmark for a two-month work visit, conducting *in vivo* and *in vitro* studies with allergy models. These opportunities, together with collaborations with the Host-Microbe Interactomics and Microbiology group within the Wageningen University, resulted in the research as presented in this thesis.

After his PhD project, Olaf was awarded a three-month postdoc fellowship from the Wageningen Institute of Animal Sciences to write a research grant that might enable him to conduct research abroad. He pursues a research career as mucosal immunologist investigating the interplay between the microbiome and the host physiology.

List of publications

Perdijk, O., van Neerven, R.J.J., van den Brink, E., Savelkoul, H.F.J., & Brugman, S**.** Bovine lactoferrin modulates dendritic cell differentiation and function. *Nutrients,* **10** (7), 848 (2018).

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Perdijk, O., van Neerven, R.J.J., van den Brink, E., Savelkoul, H.F.J. & Brugman, S. The oligosaccharides 6'-sialyllactose, 2'-fucosyllactose or galactooligosaccharides do not directly modulate affect human dendritic cell differentiation and function. *PLoS ONE,* **13** (7), e0200356 (2018).

Perdijk, O., van Neerven, R.J.J., Meijer, B., Savelkoul, H.F.J. & Brugman, S. Induction of human tolerogenic dendritic cells by 3'-sialyllactose via TLR4 is explained by LPS contamination. *Glycobiology,* **28** (3), 126-130 (2017).

Teodorowicz, M., **Perdijk, O**., Verhoek, I., Govers, C., Savelkoul, H.F.K., Tang, Y., ... & Broersen, K. Optimized Triton X-114 assisted lipopolysaccharide (LPS) removal method reveals the immunomodulatory effect of food proteins. *PLoS ONE*, **12** (3), e0173778 (2017).

van Beek, A.A., Hugenholtz, F., Meijer, B., Sovran, B., **Perdijk, O**., Vermeij, W.P., ... & Leenen, P.J. Tryptophan restriction arrests B cell development and enhances microbial diversity in WT and prematurely aging Ercc1−/Δ7 mice. *Journal of Leukocyte Biology*, **101** (4), 811-821 (2017).

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Perdijk, O., van Baarlen, P. Fernandez Gutierrez, M.M. van den Brink, … & van Neerven, R.J.J. Sialyllactose and galactooligosaccharides promote epithelial barrier functioning and distinctly modulate microbiota composition and short chain fatty acid production. (*submitted*)

van Splunter, M., **Perdijk, O.**, Fick-Brinkhof, H., Feitsma, A., ... & van Neerven, R.J.J. Bovine lactoferrin enhances TLR-7 mediated responses in plasmacytoid Dendritic Cells in elderly women. (*submitted*)

van Splunter, M., **Perdijk, O.**, Fick-Brinkhof, H., Feitsma, A., ... & van Neerven, R.J.J. pDC and mDC functioning in ageing: a comparison between elderly and adult women. (*submitted*)

Perdijk, O., Fuchs, B., Konstanti, P., Hartman, J., van den Brink, E., Brimnes, J., Smidt, H., Savelkoul, H.F.J., van Neerven, R.J.J., & Brugman, S. 3'-sialyllactose increases Akkermansia prevalence in mice fed a low fiber diet but does not influence classical OVA-induced asthma symptoms. (*manuscript in preparation*)

Overview of completed training activities

The Basic Package	**3 ECTS**
WIAS Introduction Day	2015
Course on Essential Skills	2015
Research Integrity, Ethics and Animal Science	2016

Disciplinary Competences	**10 ECTS**
Writing a literature review	2015
Flow cytometry bootcamp	2015
Advanced course immunology UMC Utrecht	2015
6th Symposium & Master classes on Mucosal Immunology	2015
Two month traineeship at ALK-Abelló	2016

Professional Competences	**9 ECTS**
Project and time management	2015
Career assessment	2016
Interpersonal communication for PhD students	2016
WIAS science day committee 2016	2016
PhD carousel	2016
Biobusiness Summer school, Amsterdam	2017
Teaching and supervising thesis students	2017

Presentation Skills	**5 ECTS**
World Immune Regulation Meeting - oral and poster presentation	2016
Joint Symposium BSI and NVVI, Liverpool - poster presentation	2016
WIAS science day - oral presentation	2018
World Immune Regulation Meeting- poster presentation	2018

Teaching competences	**17 ECTS**
4x supervision Major MSc thesis (36 ECTS)	2014-2017
BSc course: “Cell biology” (theoretical and practical supervision)	2015, 2017
MSc course: “Development and healthy ageing” (lecturing and practical’s)	2016, 2018
Scientific writing	2017

Colophon

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Photographer cover photo; Wouter van Veen, starring Waldemar Berg
Lay-out; Ferdinand van Nispen, *my*-thesis.nl
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Propositions

1. Human milk oligosaccharides do not signal via Toll-like receptor 4 (*this thesis*).

2. Bovine milk components show immunomodulatory activity by halting monocyte-derived dendritic cell differentiation (*this thesis*).

3. Multi-disciplinary research is essential to understand human biology.

4. Science benefits the most if you take an adventurous approach.

5. Mistakes are of key importance for learning, so make them while doing a PhD.

6. Accepting what we do not know is key for novel ideas.

7. Writing at home increases productivity.

8. Gaming improves the ability to concentrate.

Propositions belong to the thesis, entitled

Immune modulation by cow's milk components:
Implications for respiratory health

Olaf Perdijk

Wageningen, October 12th, 2018