Restoring mucosal tolerance by non-digestible oligosaccharides under inflammatory conditions

A key role for galectins

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Restoring mucosal tolerance by non-digestible oligosaccharides under inflammatory conditions

A key role for galectins

Een essentiële rol voor galectins bij herstel van mucosale tolerantie door niet-verteerbare oligosacchariden tijdens ontsteking

(met een samenvatting in het Nederlands)

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General introduction

The intestinal mucosa is constantly exposed to many antigens, which are derived from amongst others food antigens and microbial antigens from either the commensal microbiota or invaded pathogens. Nevertheless, the large amount of foreign antigens to which the intestine is exposed does not result in inflammatory responses in healthy individuals. This is the consequence of a key feature of the mucosal immune system present in the intestine, which is known as oral tolerance. The mucosal surface of the intestine contains the largest part of the immune system present in the human body. Approximately 70% of the cells of the immune system is present in the gut and is continuously discriminating between harmless and potential dangerous antigens and micro-organisms. In healthy individuals, an inflammatory response towards antigens derived from for example the microbiota or ingested food is avoided via the induction of oral tolerance. However, if oral tolerance towards harmless luminal antigens is broken, or in the case of a deregulated immune response, this may result in local or systemic pathological inflammation. This is seen for instance in food allergy, celiac disease or inflammatory bowel disease – diseases characterized by an inflammatory immune response towards either food antigens, gluten or the microbiota respectively.

The mucosal immune response in the intestine

Understanding the mucosal immune system of the intestine, known as the gut-associated lymphoid tissue (GALT), is essential to gain insight in both disease pathogenesis and to design new therapeutic strategies to prevent or cure inflammatory diseases of the intestine. The GALT is the largest and most complex immunological tissue of the human body. The immune cells are separated from luminal contents by a monolayer of intestinal epithelial cells (IEC). The GALT is subdivided in sites where immunological responses are induced – known as Peyer's patches – as well as sites where effector immune responses are carried out (Mowat, 2003). As an antigen ends up in the lumen of the intestine, it is generally recognized by dendritic cells (DC) present in Peyer's patches, after the antigen is transported into the Peyer's patch via specialized IEC known as M cells, or in the lamina propria via sampling through dendrites protruding the intestinal epithelium (Rescigno et al., 2001). Upon recognition, DC become activated and migrate towards the draining mesenteric lymph nodes (MLN). In the MLN, DC present the sampled antigen through MHC-II molecules to amongst others naïve CD4+ T cells. After antigen presentation, the naïve T cell is activated to become an effector T cell, which migrates back towards the intestinal lamina propria to carry out its effector functions (Agace, 2006). The outcome of the immune response is regulated at multiple levels, including the intestinal epithelium and the activation of DC (Artis, 2008; Iliev et al., 2009; Rescigno and Di Sabatino, 2009). Insights into the underlying mechanisms how IEC and DC maintain intestinal

homeostasis, while raising an inflammatory response in case of pathogen invasion is crucial for the development of prevention or treatment strategies for intestinal inflammatory diseases.

Induction of immunological tolerance has the attention of many scientists since a long time, and is important for maintaining homeostasis at mucosal surfaces. Mucosal tolerance is characterized by reduced immunological responsiveness towards antigens, as a consequence of prior oral exposure to the same antigen. Mucosal tolerance induced through protein feeding is generally known as oral tolerance. Abrogation of mucosal tolerance can result in for instance food hypersensitivity or chronic inflammatory responses. Induction of tolerance is associated with the induction of regulatory T (Treg) cells, which suppress adaptive immune responses through cell-cell contact dependent mechanisms or secretion of the anti-inflammatory cytokines IL-10 or TGF-β. Indeed, induction of Treg cells results in abrogation of food hypersensitivity responses (Adel-Patient et al., 2011; Yamashita et al., 2012). Furthermore, a higher frequency of allergen-specific Treg cells is observed in children that have outgrown cow's milk allergy (Shreffler et al., 2009). In addition, in a clinical setting, allergen-specific immunotherapy in order to desensitize patients with allergic disease has been shown to induce Treg cells (reviewed by Akdis and Akdis, 2011), implicating that induction of Treg cells is essential for mucosal tolerance.

IEC-DC crosstalk

IEC are the first cells that come in contact with luminal antigens. IEC create a physical and chemical barrier, which prevents direct contact of luminal antigens with the underlying GALT. Directly underneath the intestinal epithelium, DC create a close network resulting in a close interaction between IEC and DC. IEC are able to recognize luminal antigens through Toll-like receptors (TLR). TLR recognize microbial fragments, including bacterial cell wall fragments, bacterial endotoxins and bacterial or viral RNA or DNA and hence recognize both antigens derived from the microbiota as well as invading pathogens. Mainly under inflammatory conditions, IEC express TLR at the apical surface, which contribute to both inflammation as well as immune tolerance (Abreu et al., 2002; Singh et al., 2005; Ewaschuk et al., 2007). Increased epithelial surface TLR2 and TLR4 expression is associated with inflammatory bowel disease (IBD) (Frolova et al., 2008). In contrast, apical TLR9 stimulation contributes to intestinal homeostasis (Lee et al., 2006). In addition, many antigens are glycosylated as well, and IEC express proteins, including galectins, which are involved in recognizing glycan structures and regulating immune responses (see review in Chapter 2 of this thesis). Recognition of antigens via TLR as well as carbohydrate binding proteins results in a highly specific immune response. Upon stimulation of IEC, the intestinal epithelium becomes activated and

secretes various soluble mediators that communicate with the immune cells in the lamina propria (Rimoldi et al., 2005). Especially, DC conditioned by IEC show an anti-inflammatory phenotype and instruct Treg responses, resulting in immunological tolerance (Hawiger et al., 2001; Iliev et al., 2009a; Iliev et al., 2009b). Furthermore, DC co-cultured with IEC showed less expression of MHC-II and the co-stimulatory molecules CD80 and CD86, were unresponsive towards TLR stimulation and were not capable of inducing T cell proliferation (Butler et al., 2006). Likewise, DC present in Peyer's patches and lamina propria in mice are educated by IEC to produce high amounts of the immune regulatory cytokines IL-10 and TGF-β(Iwasaki and Kelsall, 1999). These DC may therefore induce less T cell activation and polarization into effector Th1, Th2 or Th17 cells. On the other hand, DC conditioned by IEC can promote differentiation of naïve T cells into Treg cells **(Figure 1)**.

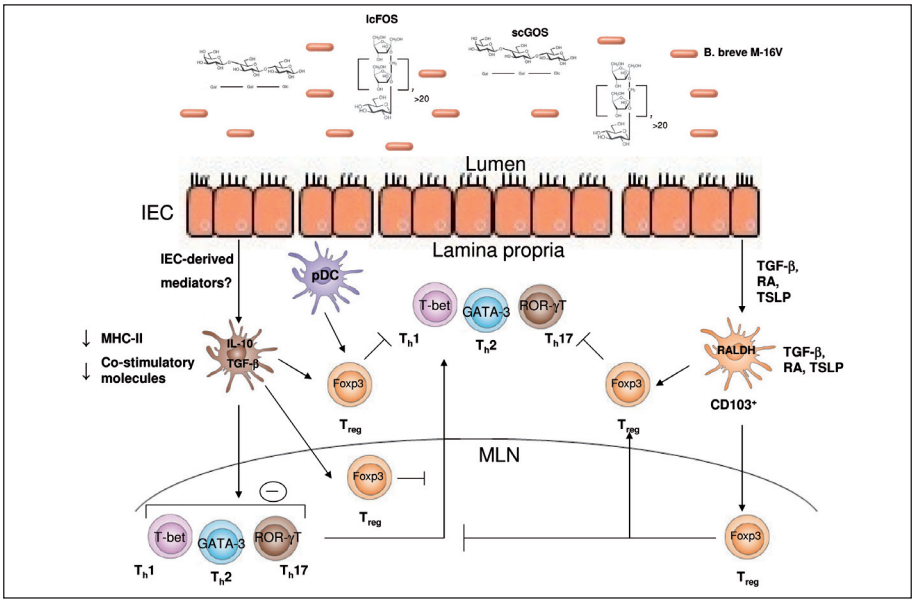

Figure 1 Conditioning of DC and regulation of mucosal immune responses by IEC. *IEC actively contribute to the generation of tolerogenic DC in the GALT. IEC have been described to suppress the expression of MHC-II and co-stimulatory molecule expression by DC in vitro (Butler et al., 2006). IEC-conditioned DC may induce Treg cell polarization in the lamina propria, or migrate to the MLN to generate less effector Th1, Th2 and Th17 but more Treg cells. In addition, IEC produce TGF-β and RA to generate CD103+ DC, which migrate to the MLN to induce Treg polarization (Coombes et al., 2007; Sun et al., 2007; Iliev et al., 2009a). In addition, plasmacytoid DC (pDC) have been described to contribute to the generation of Treg cells as well (Goubier et al., 2008). Treg cells and effector T cells generated in the MLN migrate back to the lamina propria to carry out their effector functions. Apical exposure of IEC to Bifidobacterium breve M-16V and scGOS/lcFOS may contribute to generation of tolerogenic DC and Treg cell polarization in the GALT.*

DC subsets in the intestine

Several unique DC subsets have been characterized in the murine intestine according to the expression of CD11c, CD11b and CD8α. Within murine Peyer's patches, CD11c+CD11b+CD8α- DC have Th2 and Treg polarizing capability and induce IgA class switching, while CD11c+CD11b-CD8α+ DC induce the differentiation of naïve CD4+ T cells towards Th1 cells (Iwasaki and Kelsall, 2001; Sato et al., 2003). In addition, Th1 polarization is induced by CD11c+CD11b-CD8α- DC as well (Iwasaki and Kelsall, 2001). Lamina propria DC can be subdivided in populations expressing CD103 or CX3CR1. While CX3CR1+ DC extend their dendrites between intestinal epithelial cells and do not migrate to the MLN (Niess et al., 2005), CD103+ DC are described to migrate towards the MLN to instruct Treg immune responses (Sun et al., 2007; Matteoli et al., 2010). Interestingly, the conversion of CD103- DC to CD103+ DC is dependent on the secretion of TGF-β and retinoic acid (RA), a metabolite of vitamin A, which induces the production of TGF-β, RA and TSLP by CD103+ DC in the MLN driving Treg polarization (Coombes et al., 2007; Sun et al., 2007; Iliev et al., 2009a; Spadoni et al., 2012). Finally, plasmacytoid dendritic cells (pDC), characterized as CD11c(int)B220+Ly6c+, may contribute to oral tolerance through induction of Treg differentiation through induction of Treg differentiation (Goubier et al., 2008; Gilliet and Liu, 2002) (**Figure 1**). Although these DC subsets have been characterized in mice, human intestinal DC subsets are less well characterized. However, a CD103+ DC population exists in the human intestine and intestinal DC in humans show less capacity to induce pro-inflammatory immune responses as well (Jaensson et al., 2008; Rimoldi et al., 2005). Interestingly, the cytokine production of human intestinal DC was found to be dependent on specific bacterial strains in the microbiota (Ng et al., 2010), indicating that DC function is affected by stimuli present in the intestinal lumen, which communicate to immune cells in the lamina propria, possibly via the intestinal epithelium.

Origin of lamina propria DC

The maintenance of the pool of lamina propria DC is the result of continuous migration of precursor cells from the circulation, derived from the bone marrow, into mucosal tissues. Lamina propria DC arise from distinct precursor cells. As discussed previously, CD103+ and CX3CR1+ DC play a crucial role in mucosal immune responses. In the bone marrow, hematopoietic stem cells give rise to myeloid and lymphoid precursor cells. Especially, myeloid precursor cells differentiate in the bone marrow into monocytes and common DC precursor cells, which enter the circulation. Whereas monocytes give rise to macrophages and CX3CR1+ lamina propria DC, CD103+ DC arise from common DC precursor cells derived from the bone marrow, indicating that lamina propria DC arise from

different lineages (Bogunovic et al., 2009; reviewed by Geissman et al., 2010). During an inflammatory response, monocytes and DC precursor cells as well as T cells actively migrate into the inflamed tissue resulting in an immune response. Interestingly, monocytes have recently been described to take up β-lactoglobulin, a major allergen involved in cow's milk allergy (Marengo et al., 2011). In addition, generating monocyte-derived DC from asthmatic patients in presence of the house dust mite allergen Der p 3 showed that these DC expressed lower levels of DC-SIGN, accompanied with increased phagocytosis of the allergen, indicating the importance of glycan binding receptors in presentation of allergens by DC. Furthermore, stimulation of DC from asthmatic individuals produce less IL-12p70 and more IL-6, resulting in Th2-polarization by naïve CD4+ T cells in allogeneic DC-T cell interaction studies (Huang et al., 2011). The characteristics of monocyte-derived DC depend on the cytokines monocytes encounter during differentiation (reviewed by Banchereau and Palucka, 2005). Especially in allergy, it has been shown that TSLP produced by epithelial cells of the skin, lungs and intestine induces monocyte-derived DC that induce pro-inflammatory Th2 responses (Soumelis et al., 2002; Shikotra et al., 2012; Taylor et al., 2009). These data suggest that monocytes may play a crucial role in allergy and that mediators secreted by amongst others IEC can play a crucial role in DC differentiation from precursor cells, affecting the outcome of the effector immune response.

Dendritic cells in disease

Food allergy

Food allergy is in majority a type I hypersensitivity response, which is characterized by a strong Th2 response to generally harmless antigens. The induction of a Th2 type effector response to an allergen results in the production of IgE by plasma cells. IgE binds to the high affinity Fcε receptors on mast cells. This phase comprises the sensitization phase of the allergic response. Upon subsequent exposure to the allergen, the allergen binds to IgE bound to the mast cell, resulting in cross-linking of the Fcε receptors. Cross-linking of the Fcε receptors triggers degranulation of mast cells. This phase is also known as the challenge phase. Mast cells contain preformed vasoactive amines, such as histamine, stored in granules and upon degranulation, histamine is released in the intestinal mucosa and causes contraction of smooth muscles and transepithelial fluid loss in the gut lumen (vomiting and diarrhea). In severe cases, allergens that enter the circulation can cause mast cell degranulation in the connective tissue in the periphery, resulting in urticaria and atopic dermatitis, airway constriction and even anaphylactic shock (Sicherer and Sampson, 2010) **(Figure 2)**. Although much work is conducted aiming at the challenge phase and mast cells, less is known about the processes underlying allergic sensitization.

The role of DC in food allergy has not been studied extensively. A few studies have been conducted, showing the importance of DC in food allergy. Transfer of Peyer's patch CD11c+B220- DC isolated from cow's milk allergic mice into wild type recipient mice induces a cow's milk specific IgE response in recipients and these DC produce less IL-10 compared to control DC (Chambers et al., 2004). Recently it has been shown that in a murine model for peanut allergy a shift towards a decreased frequency of CD103+ DC is observed, whereas pDC depletion during expansion of DC *in vivo* prevented the induction of tolerance (Smit et al., 2011) (**Figure 2**). Furthermore, T-cell immunoglobulin-domain mucin (TIM)-4 is expressed by mucosal DC and has been reported to play a critical role in the induction of food allergy. Only recently, galectin-9 secretion by IEC was found to enhance TIM-4 expression by DC to sustain food allergy (Yang et al., 2007; Feng et al., 2008; Chen et al., 2011). However, galectin-9 has been described to be protective in allergic disease as well (see review Chapter 2 of this thesis). Targeting TLR expressed by DC has been shown to suppress the production of Th2-associated cytokines by T cells in DC-T cell cultures, indicating that DC are interesting targets to prevent or treat allergic disease (Pochard et al., 2010). Hence, understanding of the crosstalk between IEC and DC provides insights in the induction of allergic responses and how to prevent allergic responses via targeting of the mucosal immune system of the intestine.

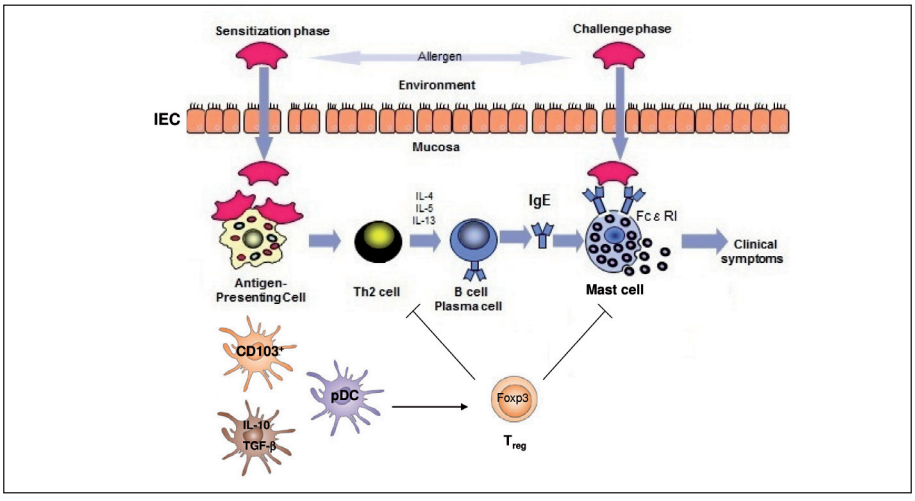

Figure 2 Potential prevention of food allergic responses. *During allergic sensitization, antigen presenting cells take up the allergen resulting in a strong Th2 polarized effector response. This leads to the production of IgE by B cells, which binds to the FcεRI expressed by mast cells. Subsequent exposure (challenge) to the allergen causes mast cell degranulation through cross-linking of the FcεRI, resulting in clinical symptoms. Generation of tolerogenic DC and consequently Treg induction may suppress Th2 polarization as well as mast cell degranulation.*

Inflammatory bowel disease

Many DC functions have been studied using murine models for IBD. IBD is characterized by a destructive inflammatory response in the intestine. Crohn's disease (CD) and ulcerative colitis (UC) are two major types of IBD. Although the exact pathogenesis of IBD is not completely understood, it has been described that genetic as well as environmental factors are involved (reviewed by Xavier and Podolsky, 2007). In IBD, it is believed that tolerance towards the normal gut microbiota is lost. This results in a disturbed homeostasis within the intestinal mucosa, which is maintained by a complex interaction between the microbiota, the intestinal epithelium and mucosal DC (reviewed by Maloy and Powrie, 2011). Although many different DC subsets have been described, the exact role of DC in inflammatory diseases in the intestine, for example food allergy, IBD and celiac disease, is not known yet. Several mouse models as well as data obtained from human studies indicate an important role for DC in driving exaggerated Th1/Th17 responses towards microbiota constituents in IBD or gluten peptide in celiac disease (reviewed by Rescigno and Di Sabatino, 2009). Both innate as well as adaptive immunity and disruption of the epithelial barrier play an important role in the inflammatory response characteristic in IBD. In IBD lesions, it has been observed that IEC as well as antigen presenting cells (APC), including macrophages and DC, secrete high amounts of IL-6, IL-8 and TNF-α. Especially, anti-TNF-α has been proven to be effective in alleviating IBD symptoms (Melmed et al., 2010). Furthermore, neutralization of IL-6 prevented TNF-α production in the intestinal mucosa as shown in a murine model for colitis (Noguchi et al., 2007). In IBD, expression of TLR2 and TLR4 on IEC is increased and activation of these receptors results in production of IL-6, IL-8 and TNF-α by IEC (Abreu et al., 2002; Frolova et al., 2008). Secretion of IL-8 by IEC results in influx of neutrophils, which produce high amounts of IL-6, IL-8 and TNF-α as well, resulting in tissue damage and destruction of tight junctions between IEC (Kucharzik et al., 2001).

As a consequence of the tissue damage, mucosal lymphocytes are directly exposed to the microbiota potentially leading to changes in the immune response in the gut. Although it was accepted that UC results from Th2/NK T cell-associated IL-13 secretion and CD is caused by an inflammatory Th1 polarized response (Kaser et al., 2010), activation of pathogen recognition receptors, including TLR, on mucosal DC and macrophages results in high production of IL-23 driving a destructive Th17 response in both UC and CD (Maloy and Kullberg., 2008). Treg and Th17 cell differentiation is reciprocally regulated and requires both TGF-β production by APC. However, Th17 cell differentiation is promoted in presence of high IL-6 production, while conversion of Treg is suppressed by high levels of IL-6. In IBD, APC strongly induce Th17 cells, while Treg differentiation is suppressed (Littman

and Rudensky, 2010). Interestingly, aberrant DC distribution and activation is observed in murine models for IBD as well as in human tissue. In both CD and UC, increased numbers of pDC were observed that produced high amounts of TNF-α and IL-6, but low concentrations of IFN-α (Baumgart et al., 2011). In addition, in a rat model for colitis CD103+ DC and CD11b+ DC were absent in acute inflammation, but increased when the inflammation was resolved, whereas increased CD83 expression was only observed in inflamed tissue (Silva et al., 2006). Likewise, increased numbers of activated DC were observed in human IBD tissue as well (te Velde et al., 2002).

Specific non-digestible oligosaccharides and probiotic bacterial strains

The microbiota is the largest source of microbial stimulation in the gut and it has been shown that the microbiota is necessary for development of the intestinal immune system (Artis, 2008). Disturbances in the commensal bacterial composition in the gut, reflected by increased colonization with *E. coli* or *C. difficile*, is associated with an increased risk with respect to the development of allergic disease and IBD (Penders et al., 2007; Loh and Blaut, 2012). One of the most well-known hypotheses by which microbial stimulation suppresses allergic diseases is known as the 'hygiene hypothesis', which states that microbial stimulation polarizes the immune response towards Th1, while lack of microbial stimulation results in a Th2 polarized immune response, which is characteristic for atopy (Romagnani, 2004). Likewise, the 'IBD hygiene hypothesis' states that an extreme hygienic environment increases the risk of developing IBD later in life. Absence of exposure to intestinal helminths appears to be an important environmental factor contributing to the development of IBD. Helminths have been described to regulate the expression of interleukin and interleukin receptor genes (Fumagalli et al., 2009). Hence, a promising strategy to suppress food allergy or IBD is dietary intervention with specific probiotic bacterial strains, which are live micro-organisms that alter the enteric microbiota and have a beneficial effect on health. Indeed, both *Lactobacillus* and *Bifidobacterium* strains – either the whole organisms or a microbial fragment – have been shown to induce Treg type immune responses, thereby suppressing allergy (von der Weid et al., 2001; Smits et al., 2005; Lammers et al., 2003; Zhang et al., 2010). In IBD, a multitude of *Bifidobacterium* species have shown to exert protective effects in murine models for colitis. Prior administration of a probiotic mixture including four *Bifidobacterium* strains to the induction of DSS colitis in mice has been shown to attenuate colitis (Kumar et al., 2008). However, the exact mechanisms by which probiotics prevent the development of allergic and inflammatory disease in the intestine remains to be elucidated. Breast feeding also affects the microbiota composition by increasing the amount of *Bifidobacteria* as shown by higher fecal *Bifidobacteria* counts

(Haarman and Knol, 2005). Human milk contains a high amount of non-digestible oligosaccharides with over 1000 different oligosaccharide structures and it has been shown that human milk, as well as specific dietary fibres like chicory-derived inulin and lactose-derived short-chain galacto-oligosaccharides selectively support the growth of *Lactobacillus* and *Bifidobacterium* strains (Boehm et al., 2005). Therefore, these oligosaccharides have prebiotic effects in the intestine. Based on the basic structure and size of neutral non-digestible oligosaccharides present in human milk, a specific prebiotic mixture consisting of short chain galacto-oligosaccharides (scGOS) and long-chain fructo-oligosaccharides (lcFOS) in a 9:1 ratio has been developed. Oral supplementation of scGOS/lcFOS has been shown to reduce allergic symptoms in mice and humans (Schouten et al., 2010; Arslanoglu et al., 2008; van Hoffen et al., 2009). Especially dietary supplementation with a combination of scGOS/lcFOS and *Bifidobacterium breve* M-16V (GF/Bb), are effective in reducing allergic symptoms (Schouten et al., 2009; van der Aa et al., 2010). With respect to IBD, not many studies have been performed to evaluate the effects of non-digestible oligosaccharides on the prevention of intestinal inflammation. In a colitis model in rats, inulin and FOS reduced colitis, which was associated with increased *Bifidobacterium* species and reduced *Enterobacteriaceae* and *C. difficile* in the faeces (Koleva et al., 2012). The underlying mechanisms are not known. However, exposure of IEC to GF/Bb may result in the generation of tolerogenic DC and consequently Treg polarization in the GALT (Schouten et al., 2010) **(Figure 1)**. Furthermore, induction of Treg cells suppresses the degranulation of mast cells as well (Gri et al., 2008)**(Figure 2).**

Outline and scope of this thesis

The prevalence of inflammatory diseases, including allergy and IBD, are rapidly increasing in Western society. There is a strong need to develop novel preventive and therapeutic strategies to manage these diseases as there is currently no cure. Understanding the immune system and selecting appropriate immune modulators is crucial for generating safe, preventive and potentially therapeutic treatment strategies. This thesis aims at understanding the mucosal immune system of the intestine and modulation of the GALT through observations on changes in immune cell subsets and function resulting from dietary intervention using specific non-digestible oligosaccharides to induce immunological tolerance.

Major aims of the thesis:

1 To set up an *in vitro* model, studying the interaction between IEC and immune cells. Using this *in vitro* co-culture model, the immune modulatory effects of apical TLR ligand or *Bifidobacterium breve* M-16V exposure of IEC on the underlying effector immune response are investigated. In addition, the effects of scGOS/lcFOS or a combination of scGOS/lcFOS and TLR ligands, in the absence and presence of IEC, on the effector immune response are addressed as well.

2 To verify the results obtained from the *in vitro* co-culture model in well-described *in vivo* models for food allergy and colitis.

3 To investigate the molecular and cellular mechanisms by which dietary intervention with GF/Bb exert their protective effects in the human cell co-culture model and in murine models for food allergy and colitis.

Chapter 1 serves as a general introduction to describe the scientific rationale behind the studies performed in this thesis. In the general introduction, special attention is given to the complexity of the mucosal immune response and the different cell subsets involved. The current knowledge and hypotheses on the molecular mechanisms by which dietary intervention with specific non-digestible oligosaccharides modulate immune responses are described in **chapter 2**. In addition, an overview of glycan-binding receptors and their involvement in health and disease is provided, especially in food allergy and IBD. In **chapter 3** a co-culture model is described, in which IEC were grown on transwell inserts and co-cultured with human peripheral blood mononuclear cells. Interestingly, during an ongoing effector response, IEC differentially respond to TLR stimuli, driving regulatory or inflammatory responses. **Chapter 4** further elaborates on the *in vitro* co-culture model to address the effects of scGOS/lcFOS. The underlying cellular mechanisms by which scGOS/lcFOS modulate the mucosal immune response are investigated. In these *in vitro* studies it was observed that scGOS/lcFOS support apical TLR9-induced immune regulatory effects through secretion of galectin-9 by IEC. The results presented in **chapter 4** are confirmed *in vivo* using a murine model for whey-induced cow's milk allergy in **chapter 5** and a murine model for hen's egg allergy in **chapter 6**. In **chapter 5**, the effects of dietary intervention with GF/Bb on T cell polarization and mast cell degranulation is investigated. It is shown that dietary intervention with GF/Bb enhanced galectin-9 expression and secretion in mice and humans, resulting in suppressed mast cell degranulation. In **chapter 6** further insights in the capacity of GF/Bb to modulate DC phenotype and functionality using ovalbumin (OVA) as antigen are described. It is shown that galectin-9 contributes to generation of

tolerogenic DC, with increased capacity to induce functional Treg cells. Furthermore, galectin-9 suppresses pro-inflammatory cytokine production by DC and exogenous galectin-9 inhibits OVA-specific Th2 responses by lamina propria lymphocytes *ex vivo*. In **chapter 7** it is evaluated whether GF/Bb could prevent the development of DSS-induced colitis. Dietary intervention with scGOS/lcFOS and GF/Bb delays the onset of DSS-induced colitis, and enhances the percentage of tolerogenic DC and Treg cells in MLN. Furthermore, in **chapter 8**, preliminary data regarding potential protective effects of scGOS/lcFOS on the barrier integrity of IEC upon co-culture with CD3/28-activated PBMC are described. In conclusion, **chapter 9** provides a general overview and discussion of the results demonstrated in this thesis.

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Glycan recognition at the interface of the intestinal immune system: target for immune modulation via dietary components

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Abstract

The intestinal mucosa is constantly exposed to the luminal content, which includes micro-organisms and dietary components. Prebiotic non-digestible oligosaccharides may be supplemented to the diet to exert modulation of immune responses in the intestine. Short chain galacto- and long chain fructo-oligosaccharides (scGOS/lcFOS), functionally mimicking oligosaccharides present in human milk, have been reported to reduce the development of allergy through modulation of the intestinal microbiota and immune system. Nonetheless, the underlying working mechanisms of scGOS/lcFOS are unclear. Intestinal epithelial cells lining the mucosa are known to express carbohydrate (glycan)-binding receptors that may be involved in modulation of the mucosal immune response. This review aims to provide an overview of glycan-binding receptors, in particular galectins, which are expressed by intestinal epithelial cells and immune cells. In addition, their involvement in health and disease will be addressed, especially in food allergy and inflammatory bowel disease, diseases originating from the gastrointestinal tract. Insight in the recognition of glycans in the intestinal tract may open new avenues for the treatment of intestinal inflammatory diseases by either nutritional concepts or pharmacological intervention.

Introduction

The gastrointestinal immune system is the largest and most complex immunological tissue present in the human body and is constantly discriminating between harmless and potentially dangerous antigens and micro-organisms. A key feature of the mucosal immune system in the intestine is therefore to protect against infection, while avoiding an inflammatory response towards the normal microflora or harmless food antigens. However, if tolerance towards dietary antigens or the microflora is broken or in case of dysregulated intestinal immune responses, this may result in local pathological inflammation as seen for instance in food allergy, celiac disease or inflammatory bowel disease (Cardoso et al., 2008; Rescigno and Di Sabatino, 2009; Sartor, 2008).

The gut-associated lymphoid tissue is covered by a single layer of intestinal epithelial cells, which creates a barrier between the intestinal lumen and the immunological compartments in the lamina propria (Mowat, 2003). Furthermore, specialized intestinal epithelial cells that produce mucus (goblet cells) or antimicrobial peptides (Paneth cells) as well as IgA secreted by plasma cells contribute to the mucosal barrier (Artis, 2008; Fagarasan and Honjo, 2003). Recent advances show that intestinal epithelial cells are also crucial in maintaining homeostasis and directing mucosal immune responses (Artis, 2008; Iliev et al., 2009b). Intestinal epithelial cells express several receptors that recognize antigens present in the intestinal lumen, including pathogen recognition receptors engaging pathogen-associated molecular patterns. These receptors include Toll-like receptors (TLR)(Abreu, 2010) and Nod-like (Franchi et al., 2009) receptors, which recognize bacterial fragments such as peptidoglycans (TLR2), lipopolysaccharide (TLR4), flagellin (TLR5), unmethylated CpG DNA sequences (TLR9) and muramyl di- and tripeptides (Nod1 and Nod2). Upon activation of Toll-like receptors, especially under inflammatory conditions, intestinal epithelial cells contribute to the modulation of immune responses by secreting soluble mediators that bridge luminal signals with the immunological tissues. In the intestine, this implies that intestinal epithelial cells can have interaction with highly organized lymphoid structures (e.g. Peyer's patches) where immune responses are induced, as well as with the effector site where lymphoid cells are scattered in the lamina propria and adaptive immune responses are carried out. Soluble mediators produced by the intestinal epithelium may therefore interact with antigen presenting cells, such as dendritic cells or macrophages, or effector T cells, shaping the immune response in the intestinal mucosa towards a more tolerogenic phenotype (Artis, 2008a; Iliev et al., 2009a; Iliev et al., 2009b).

Many extracellular proteins and bacterial proteins are glycosylated and the recognition of these carbohydrate or glycan structures is possibly important in the fine-tuning of the mucosal immune response (van Kooyk and Rabinovich, 2008). Interestingly, glycosylation of Arah1, a major allergen in peanut allergy, has been shown to activate dendritic cells through dendritic cell-specific intercellular adhesion molecule (ICAM)-3-grabbing non-integrin (DC-SIGN), thereby inducing Th2 maturation (Shreffler et al., 2006). This indicates that carbohydrate structures are, at least in allergy, involved in disease pathogenesis. Both antigen presenting cells and intestinal epithelial cells express proteins involved in the recognition of carbohydrate (glycan) structures present on for instance bacterial products, dietary components or glycosylated membrane proteins expressed by different cell types. Glycan-binding receptors, also called lectins, include the family of the C-type lectin receptors, galectins and siglecs (Crocker et al., 2007; Leffler et al., 2002; Weis et al., 1998), which recognize different glycan structures (**Table 1**). Hence, antigen presenting cells and intestinal epithelial cells are important sensors and regulators of potential danger signals within the intestine and are crucial in the cross-talk between luminal antigens and immune cells that reside in the lamina propria.

Nowadays, the prevalence of atopic and inflammatory diseases of the intestine is increasing. Although active inflammation in inflammatory bowel disease -in many cases can be effectively reduced using immunosuppressants or corticosteroids, no therapy is currently available for patients affected with food allergy. Dietary supplementation with non-digestible oligosaccharides has been shown to reduce the risk of developing allergic disease and suppresses acute allergic symptoms, such as acute allergic skin responses in animal and clinical studies (Arslanoglu et al., 2008; Moro et al., 2006; Schouten et al., 2009; van der Aa et al., 2010). Underlying mechanisms of the observation that non-digestible oligosaccharides suppress acute allergic symptoms include improved microbiota (Bakker-Zierikzee et al., 2005), higher counts of *Bifidobacteria* similar as in breast-fed neonates (Haarman and Knol, 2005) and increased fecal IgA (Scholtens et al., 2008). Although dietary supplementation with a specific prebiotic mixture of short-chain galacto-oligosachharides (scGOS) and long chain fructo-oligosaccharides (lcFOS) in a 9:1 ratio has been shown to suppress the development of acute allergic symptoms, possibly involving the induction of regulatory T cells (Schouten et al., 2010 ; van der Aa et al., 2010), the exact mechanisms remain to be unravelled. Understanding how prebiotics affect the mucosal immune response in the intestine is crucial in developing effective strategies to treat immunological disorders such as food allergy. This review focuses on the possible mechanisms by which mucosal immune responses are regulated by both antigen presenting cells and intestinal epithelial cells. First we will discuss which receptors expressed by antigen presenting cells and intestinal epithelial cells are involved in recognition of glycans. As intestinal epithelial cells are in direct contact with luminal

contents, non-digestible oligosaccharides may affect the mucosal immune system via direct interaction with the intestinal epithelium and dendritic cells are in close proximity to the epithelium. We will highlight the role of galectins in disease, from the perspective of treatment via dietary supplementation of non-digestible oligosaccharides.

Glycan recognition

Much focus has been on the contribution of dendritic cells within the intestine in regulating intestinal immune responses, which are present in highly organized lymphoid tissues known as Peyer's patches or are scattered in the lamina propria. Many different subsets of intestinal dendritic cells have been described and can induce Th1, Th2, Th17 or a regulatory T cell type effector responses upon activation (Coombes and Powrie, 2008; Rescigno and Di Sabatino, 2009). Dendritic cells are in close contact with the intestinal epithelium and create

Table 1 - Glycan receptor families expressed in the intestine and their carbohydrate specificity

Glycan-binding receptor	Membrane-bound / soluble	Specificity	Expression	Refs
C-type lectins	Membrane-bound	Mannose and Fucose	Dendritic cells, Macrophages	van Kooyk and Rabinovich, 2008 Robinson et al., 2006 Figdor et al., 2002
		Galactose and N-acetyl-galactosamine	Dendritic cells, Macrophages	van Kooyk and Rabinovich, 2008 van Vliet et al., 2006
Galectins	Soluble and bound to cell surface glycoproteins	N-acetyllactosamine (Gal-β1,4-GlcNAc)	Dendritic cells, Macrophages, Granulocytes, IEC	van Kooyk and Rabinovich, 2008 Leffler et al., 2002 Lahm et al., 2001 Lippert et al., 2007 Nio-Kobayashi et al., 2008 Rapoport et al., 2008 Hirabayashi et al., 2002
Siglecs	Membrane-bound	N-acetyl-neuraminic acid	Resting T cells Dendritic cells, Macrophages	van Kooyk and Rabinovich, 2008 Crocker et al., 2007 Avril et al., 2006

(DC, dendritic cell; Gal, galactose; GlcNAc, N-acetylglucosamine; IEC, intestinal epithelial cell)

a close network directly present beneath the epithelial monolayer and dendrites from dendritic cells can directly sample luminal antigen by protruding the epithelial monolayer (Chehade and Mayer, 2005; Rescigno et al., 2001). Upon antigen recognition, dendritic cells migrate to the draining mesenteric lymph nodes, where naïve T cells are activated. A characteristic feature of dendritic cells in the mesenteric lymph nodes is to induce gut-homing molecules on T cells, including α4β7 integrin and CCR9, resulting in migration of T cells into the intestinal lamina propria, which was shown to be essential in the induction of oral tolerance (Agace, 2006; Hadis et al., 2011). However, to induce an immune response or oral tolerance, antigens must be recognized and dendritic cells as well as intestinal epithelial cells express proteins that recognize glycan structures.

C-type lectins

Expression of membrane-bound C-type lectins is mainly restricted to antigen presenting cells, including dendritic cells and macrophages (Figdor et al., 2002; Robinson et al., 2006). C-type lectins are divided in proteins containing one or more carbohydrate recognition domain (CRD) and proteins that contain a C-type lectin-like domain without the CRD. The latter subgroup is mainly found on natural killer cells and as it does not contain a CRD this subgroup may not be involved in glycan interactions (Weis et al., 1998). Most of the CRD-containing C-type lectins are Ca2+-dependent (C-type), while the presence of a specific amino acid motif determines the specificity of the C-type lectins for certain glycans. C-type lectins containing an EPN-motif (Glu-Pro-Asn) have high specificity for mannose- and fucose-terminating glycans, whereas the presence of a QPD-motif (Gln-Pro-Asp) is important for galactose- or N-acetylgalactosamine (GalNAc)-terminating glycans (van Kooyk and Rabinovich, 2008).

Dendritic cells express a wide variety of receptors that are important to initiate an immune response, including Toll-like receptors. C-type lectins, recognizing specific carbohydrate antigens, are mainly associated with antigen uptake and processing (Figdor et al., 2002; Granucci and Ricciardi-Castagnoli, 2003; van Kooyk and Rabinovich, 2008). Several studies have already shown that glycan recognition may be essential as activation of a specific C-type lectin, DC-SIGN, on dendritic cells integrates Toll-like receptor signaling, resulting in an IL-10-mediated immune response and induction of regulatory T cells (Gringhuis et al., 2007; Smits et al., 2005). In addition, C-type lectins are also involved in the interactions between antigen presenting cells and T cells. Ligation of macrophage galactose- specific lectin on the cell surface of macrophages or immature dendritic cells with GalNAc epitopes on CD45 expressed on effector T cells inhibits T cell receptor signaling, thereby decreasing cytokine production (van Vliet et al., 2006).

Siglecs

Sialic acid binding immunoglobulin-like lectins, siglecs, are type I transmembrane proteins and based on evolutionary conservation siglecs can be divided into CD22- or CD33-related groups. Like the membrane-bound C-type lectins, siglecs are expressed on dendritic cells and macrophages, but also other immune cell types – except resting T cells – express siglecs. Siglecs recognize sialylated glycans of which N-acetylneuraminic acid is the most common (Crocker et al., 2007). Whereas it is not known what signalling pathways can be activated by C-type lectins (Robinson et al., 2006), siglecs are known to have immunoreceptor tyrosine based inhibitory motifs (ITIM) (Crocker et al., 2007). Activation of receptors containing ITIM results in inhibition of activating signals and thereby inhibit leukocyte function (Avril et al., 2006).

Galectins

Although antigen presenting cells can respond to carbohydrate structures directly via amongst others C-type lectins in the intestinal tract, antigens first encounter a monolayer of epithelial cells. Like antigen presenting cells, intestinal epithelial cells express various Toll-like receptors – including TLR2, TLR4, TLR5 and TLR9 – and interestingly it has been shown that the expression of Toll-like receptors at the apical surface is increased under inflammatory conditions (Abreu et al., 2002; Ewaschuk et al., 2007; Singh et al., 2005). Moreover, we and others have shown that intestinal epithelial cells stimulate pro-inflammatory responses or tolerogenic responses depending whether Toll-like receptors are activated on the apical or basolateral membrane (de Kivit et al., 2011 ; Lee et al., 2006). Although intestinal epithelial cells do not express C-type lectins or siglecs, intestinal epithelial cells express various galectins. It has been proposed, like for the Toll-like receptors, that the functionality of galectins depends whether it is expressed at the apical or basolateral membrane of intestinal epithelial where they are secreted, in contrast to C-type lectins, which are transmembrane proteins (Delacour et al., 2008; Huflejt et al., 1997; Wasano and Hirakawa, 1997). After secretion, galectins bind to glycoproteins or receptors at cell surfaces and hence can regulate cell functions.

Galectins are secreted by intestinal epithelial cells (Lahm et al., 2001; Lippert et al., 2007; Nio-Kobayashi et al., 2008), but also various immune cells, including dendritic cells, macrophages and granulocytes, are known to express galectins (Rapoport et al., 2008). Galectins can be divided into three groups: (i) prototype galectins, containing a single CRD (galectin-1, -2, -5, -7, -10, -11, -13, -14), (ii) tandem-repeat galectins containing two CRD in a single polypeptide chain (galectin-4, -6, -8, -9, -12) and (iii) chimera type galectins (galectin-3) which contains a CRD and a non-lectin domain. Prototype galectins containing one CRD can form dimers via non-covalent dimerization, whereas galectin-3 can oligomerize as it contains a N-terminal collagen-

like sequence (chimeric type) (Leffler et al., 2002; Rapoport et al., 2008). Galectins recognize glycans containing the disaccharide N-acetyllactosamine (LacNAc; Gal-β(1-4)-GlcNAc) and lactose (Hirabayashi et al., 2002; Leffler et al., 2002; Rapoport et al., 2008). Interestingly, the binding specificity is not only determined by recognizing LacNAc-terminating glycans, but also depends on interactions of substitutions on galactose residues (i.e. sulfated, sialylated or fucosylated galactose moieties) (Rapoport et al., 2008). Hence, changes in the glycosylation pattern of glycoproteins, for example during inflammation, may affect binding of galectins to membrane glycoproteins on cells.

Modulation of the intestinal immune response by C-type lectins and galectins

Glycans can thus be recognized by different families of receptors, which are expressed on both antigen presenting cells and intestinal epithelial cells. Therefore, targeting these receptors may be interesting for pharmacological intervention in for instance food allergy or inflammatory bowel disease. In particular, human milk is an important source of oligosaccharides with known immunomodulatory properties and therefore non-digestible carbohydrates in breast milk are being studied for their immune modulating capacities (Field, 2005). Non-digestible oligosaccharides are known to be involved in maturation of the immune response of young infants and oral tolerance induction and are able to reduce the risk of developing allergic disease (Field, 2005; Newburg, 2000). These oligosaccharides are known to function as prebiotics, stimulating the growth of lactic acid bacteria and *Bifidobacteria* in the colon of the host (Boehm and Moro, 2008). Carbohydrates in human breast milk contain monomeric carbohydrates, including D-glucose, D-galactose, N-acetylglucosamine, N-acetylgalactosamine, L-fucose and sialic acid, and non-digestible oligosaccharides containing a core molecule characterized by repeats of galactose and N-acetylglucosamine via β-glycosidic linkages bound to lactose (Boehm and Moro, 2008) (**Figure 1A**). Based on the structures of non-digestible oligosaccharides present in human breast milk a specific 9:1 mixture of scGOS ([Gal- β(1,4)]3-8-Glc; Gal, galactose; Glc, glucose) and lcFOS ([Frc-β(2,1)]>20-Glc; Frc, fructose) was formulated (Boehm et al., 2003) (**Figure 1B**). The prebiotic mixture scGOS/lcFOS has been shown to be involved in maturation of the immune response of young infants and oral tolerance induction and are able to reduce the risk of developing allergic disease as shown in recently performed clinical trials, especially when combined with a probiotic bacterial strain (Arslanoglu et al., 2008; Grüber et al., 2010 ; van Hoffen et al., 2009; Schouten et al., 2009; van der Aa et al., 2010 ; van der Aa et al., 2011).

Although the beneficial effects of prebiotic oligosaccharides are known, the exact mechanisms by which non-digestible oligosaccharides exert their effects are not known. It has been described that human milk oligosaccharides are able to bind glycan-binding receptors including C-type lectin receptors (Naarding et al., 2005) and galectins (Di Virgilio et al., 1999). When applied orally, non-digestible oligosaccharides reach the intestinal lumen intact and may modulate immune responses initiated in the intestinal mucosa via antigen presenting cells and intestinal epithelial cells that express glycan binding receptors. All these families require one or more CRD for glycan binding. Whereas C-type lectins and siglecs are membrane-bound proteins, galectins are mostly secreted and subsequently may bind to glycoproteins or receptors on cell membranes. It is known that Toll-like receptors play an essential role in the recognition of pathogens. Ligation of Toll-like receptors activates the innate immune system leading to an appropriate effector immune response in case of pathogen invasion (Barton and Medzhitov, 2003). Likewise, recognition of glycans by C-type lectins or galectins may regulate immune responses (van Kooyk and Rabinovich, 2008). Although Toll-like receptors and glycan-binding receptors are widely expressed by different cell types present in the intestine, communication and interactions between these molecules in the intestine are beginning to be understood.

C-type lectins and dendritic cells

Within the intestine, non-digestible carbohydrates need to cross the epithelium or have to interact with dendrites of dendritic cells protruding the epithelium in order to bind C-type lectins, since intestinal epithelial cells are not known to express these receptors. The major antigen presenting cells present in the intestine are dendritic cells and macrophages. Dendritic cells and macrophages express various C-type lectins, including DC-SIGN (CD209), the mannose receptor (CD206) and macrophage galactose-specific lectin (MGL; CD301) (Robinson et al., 2006). Moreover, different subsets of dendritic cells may be characterized by the specific expression of Toll-like receptors (Kadowaki et al., 2001) and C-type lectins (Dzionek et al., 2000; Figdor et al., 2002) and activation of dendritic cells may result in up- or down regulation of C-type lectins (Kato et al., 2000). C-type lectins are important in recognizing antigens and antigen presentation, cell trafficking, T cell interactions and signaling, but also function as targets for several pathogens, including human immunodeficiency virus and mycobacteria, for survival within the host (van Kooyk and Geijtenbeek, 2003).

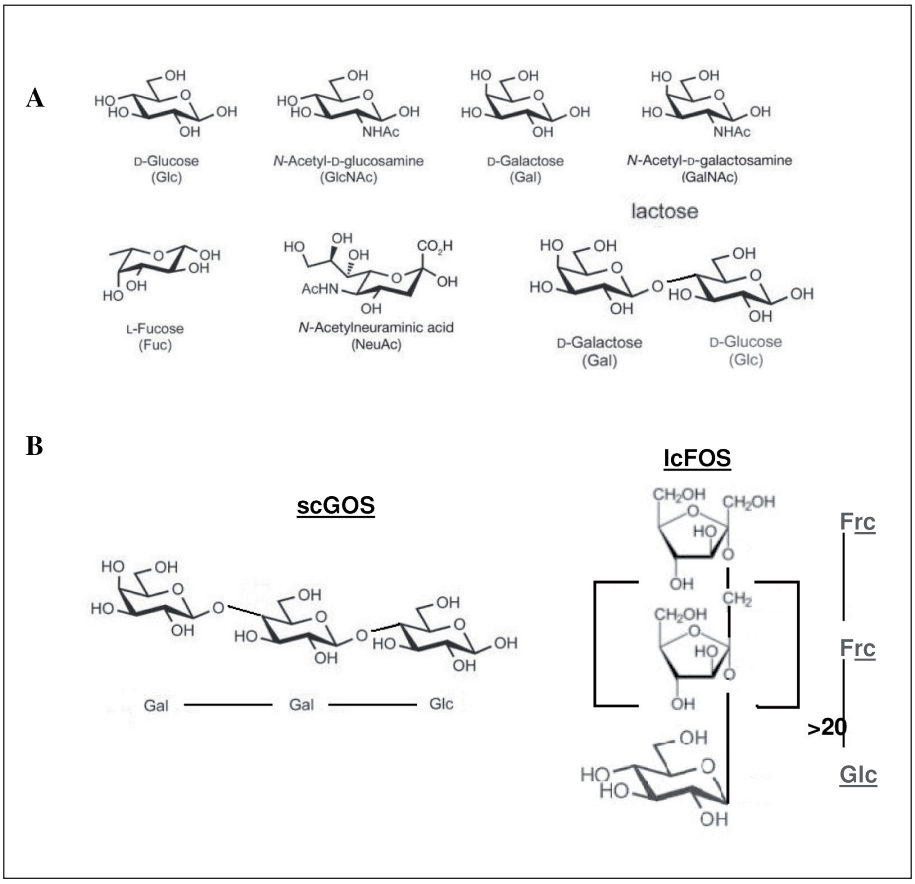

Figure 1 Carbohydrate structures of milk oligosaccharides and scGOS/ lcFOS. *(A) Carbohydrates in human breast milk contain monomeric carbohydrates, including D-glucose, D-galactose, N-acetylglucosamine, N-acetylgalactosamine, L-fucose and sialic acid, and non-digestible oligosaccharides containing a core molecule characterized by repeats of galactose (Gal) and N-acetylglucosamine via β-glycosidic linkages bound to lactose. (B) Based on the structures of non-digestible oligosaccharides present in human breast milk a specific 9:1 mixtuse of scGOS ([Gal-β(1,4)]3-8-Glc; Glc, glucose) and lcFOS ([Frc-β(2,1)]>20-Glc; Frc, fructose) was formulated.*

DC-SIGN is one of the most extensively studied C-type lectins expressed by dendritic cells. Activation of DC-SIGN modulates Toll-like receptors signaling of dendritic cells in a Raf-1 dependent way to induce an IL-10-mediated immune response (Gringhuis et al., 2007). Likewise, it has been shown that probiotics are able to instruct dendritic cells to induce regulatory T cells in a DC-SIGN-dependent manner, suggesting C-type lectins as interesting targets for treatment of inflammatory diseases (Smits et al., 2005). In addition, *Lactobacillus casei* induced the expression of the mannose receptor on gut immune cells (Galdeano and Perdigon, 2006). Interestingly, activation of the mannose receptor on immature monocyte-derived dendritic cells results in high IL-10 production but no IL-12, which could contribute to tolerogenic immune responses (Chieppa et al., 2003). In contrast, *L. casei* induces significant levels of interferon (IFN)-γ possibly via activation of Toll-like receptors (Dogi et al., 2008), suggesting the collaboration of C-type lectins and Toll-like receptors in suppression of allergic immune responses.

Galectins and intestinal epithelial cells

Upon dietary intervention, prebiotics like scGOS/lcFOS reach the lumen of the intestine intact, where they can interact with cells in the intestinal wall. Intestinal epithelial cells express and secrete various galectins, which may recognize non-digestible oligosaccharides. To date, intestinal epithelial cells were found to express galectin-2, -3, - 4, -6, -7 and a specific long isoform of galectin-9 (Lahm et al., 2001; Nio-Kobayashi et al., 2008). Although it has not been shown, milk oligosaccharides or prebiotics like scGOS/lcFOS may bind galectins, which results in modulation of the immune response in the intestine. Galectins have been described to be involved in many physiological processes, including cell signaling, cell adhesion, chemotaxis and cell apoptosis. Although many lymphoid cells also express galectins, we mainly focus on galectins expressed by intestinal epithelial cells, since the intestinal epithelium covers a large surface at the interface between the intestinal lumen and the mucosal immune system. **Table 2** summarizes the mechanisms by which galectins modulate immune response.

Galectins can induce glycoprotein lattice formation on the cell surface as a result of their dimeric or multimeric structure, which has been extensively reviewed (Garner and Baum, 2008; Grigorian et al., 2009; Rabinovich and Toscano, 2009; Sacchettini et al., 2001). Interestingly, galectin multimerization and lattice formation was recently found to be regulated by the structure of the linker region in tandem-repeat type galectins (Earl et al., 2011). Galectins are also involved in the formation of lipid rafts, plasma membrane microdomains facilitating signal transduction, and polarization of enterocytes through organization of membrane domains. To date, only galectin-4 has been shown to be a stabilizer of lipid rafts in the microvillar membrane of enterocytes (Braccia et al., 2003). In addition to facilitating signal

transduction by lipid raft stabilization, galectins regulate the signaling threshold of the T cell receptor on T cells, which depends on the formation of galectin-glycoprotein lattices. Galectin-1 has been reported to antagonize T cell receptor signaling by inducing partial phosphorylation of the T cell receptor δ-chain. Although galectin-1 antagonizes T cell proliferation by suppression of IL-2 production, IFN-γ production and expression of the activation marker CD69 by T cells is not altered upon partial δ-chain phosphorylation. Moreover, chemical disruption of lipid rafts abrogated T cell receptor phosphorylation, indicating the importance of lipid raft integrity (Chung et al., 2000).

Table 2 - Immune modulation by galectins

Glycan-binding receptor	Receptor/Ligand	Signal Transduction	Biological Effect	Refs
Galectin-1	Glycoproteins on $CD4^+CD25^+$ T_{reg}	CD45 clustering on effector T cell via cell-contact with T_{reg}	T_{reg} function ↑ Pro-inflammatory cytokines ↓ IL-10 secretion ↑; T_{reg} expansion	Van der Leij *et al*, 2007 Garin et al., 2007
	?	iNOS activity ↓	NO production mφ↓	Correa et al., 2003
	?	L-arginase activity ↑	Alternative activation mφ ; Pro-inflammatory cytokines ↓	Correa et al., 2003
	TCR	Partial ζ–chain phosphorylation	IL-2↓ ; IFN-γ, CD69 expression unaltered	Chung et al., 2000
	CD2 (LFA-2)	-	Modulation APC-T cell interaction	Walzel et al., 2000
	ECM proteins	-	Cell adhesion/migration	Taylor and Drickamer, 2007 Liu, 2005
Galectin-3	CD98	PI3 kinase ↑	Alternative activation mφ ↑	MacKinnon et al., 2008
	?	-	Chemotactic for monocytes, neutrophils and mast cells	Sano et al., 2000 Nieminen et al., 2005 Chen et al., 2006
	TCR	TCR signaling ↓	T cell inactivation	Demetriou et al., 2001
		NF-κB↑ AP-1↑	IL-8 ↑ by lamina propria fibroblasts	Lippert et al., 2007
Galectin-4	-	-	Lipid raft stabilizer on intestinal epithelial cells	Braccia et al., 2003
	CD3	?	IL-10 ↑ TNF-α, IL-17 ↓	Paclik et al., 2008
	?	PKCθ ↑	IL-6 secretion $CD4^+$ T cell ↑	Hokama et al., 2004

Table 2 - continued

Glycan-binding receptor	Receptor/Ligand	Signal Transduction	Biological Effect	Refs
Galectin-9	?	p38 ↑, ERK1/2 ↑	Increased dendritic cell maturation CD40/CD54/CD80/ CD83/CD86/ HLA-DR ↑	Dai et al., 2005
	-	-	IL-12 ↑, T_h1 cytokines↑	Dai et al., 2005 Su et al., 2010
	TIM-3	-	T_h1 / T_h17 cell apoptosis	Sziksz et al., 2010 Yamamoto et al., 2007 Zhu et al., 2005 Seki et al., 2008
	-	-	T_{reg} induction	Seki et al., 2008
	IgE	-	Suppression of mast cell degranulation	Niki et al., 2009
	CD44	-	Prevention CD44-hyaluronic acid interaction; inflammation ↓	Katoh et al., 2007
	-	-	Inflammatory cytokines ↓	Seki et al., 2008

(APC, antigen presenting cell; DC, dendritic cell; ECM, extracellular matrix; IEC, intestinal epithelial cell; iNOS, inducible nitric oxide synthase; mφ, macrophage; NO, nitric oxide; TCR, T cell receptor; Treg, regulatory T cell)

Likewise, galectin-3 has also been shown to associate with the T cell receptor resulting in the suppression of T cell receptor activation. This was dependent on Mgat5 (β1,6 N-acetylglucosaminyltransferase) activity, an enzyme responsible for β1,6GlcNAc branching of cell surface glycoproteins creating binding sites for galectins. Mgat-/- mice showed disrupted association of galectin-3 to the T cell receptor, and enhanced clustering and signal transduction of the T cell receptor (Demetriou et al., 2001). Interestingly, binding of galectin-9, possibly through T cell immunoglobulin mucin (TIM)-3 receptor on dendritic cells in the immunological synapse, enhanced maturation of dendritic cells in a p38-dependent manner, inducing maturation of dendritic cells and IL-12 production, enhancing Th1 mediated immune responses (Dai et al., 2005).

Galectins may not only be involved in the formation of glycoprotein lattices on individual cells, but also in mediating cell-cell interaction via glycoproteins present on the surface of different cells or interactions between cells and the extracellular matrix. Hence, galectins may play a role in the formation of a close contact zone, for instance, between an antigen presenting cell and T cells. Galectin-1 is known to bind CD2 (also known as LFA-2) on T cells, an adhesion molecule of the immunological synapse contributing to small close-contact zones between T cells and antigen presenting cells (van der Merwe et al., 2000; Walzel et al., 2000). Hence, destabilization of T cell – antigen presenting cell interactions by galectin-1 may suppress T cell activation. Furthermore, galectins can bind extracellular matrix proteins and integrins, modulating cell adhesion in immune and inflammatory processes (Liu, 2005; Taylor and Drickamer, 2007). Therefore, galectins may play a role in migration of inflammatory cells.

Therapeutic potentials

Galectins are extensively studied *in vitro* and in animal models for inflammatory bowel disease, which includes Crohn's disease and ulcerative colitis, a chronic and relapsing inflammatory disease of the intestine resulting from dysregulated interactions with the microflora. Inflammatory bowel disease is a chronic and relapsing inflammatory disease of the intestine resulting from dysregulated interactions with the microflora. However, dietary intervention with scGOS/lcFOS is mainly known for its ability to suppress food allergy and its effects in inflammatory bowel disease remain to be examined. Although inflammatory bowel disease can be Th1, Th2 or Th17 mediated, food allergy is a Th2 driven pathology, in which tolerance towards harmless antigens like food-derived components is lost. Loss of oral tolerance to either the microflora or food-derived antigens results in inflammation and production of pro-inflammatory cytokines, including IFN-γ (Th1), IL-4, IL-5, IL-13 (Th2), IL-17 and IL-23 (Th17) and IL-6, IL-8 and tumor necrosis factor (TNF)-α, and recent evidence point out that galectins can influence the cytokines produced by immune cells and may therefore serve as interesting targets for pharmacological intervention.

Allergy

Although scGOS/lcFOS is extensively studied for its potential to inhibit allergic disease, less is known what the underlying mechanisms are. We recently found that galectin-9 is involved in the suppression of food allergy upon dietary intervention with a diet containing a probiotic bacterial strain and scGOS/lcFOS (de Kivit et al., 2012). Interestingly, it was shown that TLR9 signaling was essential for the suppression of allergic symptoms induced by scGOS/lcFOS (de Kivit et al., submitted). The exact role of galectin-9 in allergic disease is however not clear. Ligation of TIM-3 on

dendritic cells by galectin-9 results in a Th1 polarized immune response as naïve CD4+ T cells produce IFN-γ, but not IL-4 and IL-5, when co-cultured with galectin-9-treated dendritic cells (Dai et al., 2005). Recently it has also been described that in low concentration, galectin-9 directly induces production of IFN-γ by Th1 cells (Su et al., 2010). Furthermore, galectin-9 can induce the development of regulatory T cells, while suppressing Th17 differentiation in a mouse model for autoimmune arthritis (Seki et al., 2008). In addition, galectin-9 also directly suppresses mast cell degranulation by binding to IgE and preventing IgE-antigen complex formation and this may explain why treatment with scGOS/lcFOS and/or probiotics suppresses food allergy despite high levels of IgE found in serum (Abrahamsson et al., 2007; Grüber et al., 2010 ; Kalliomäki et al., 2007; Lam et al., 2008; Niki et al., 2009). In this perspective, also regulatory T cells have been described to inhibit mast cell degranulation (Gri et al., 2008), hence galectin-9 can directly inhibit the acute allergic response by targeting the mast cell, but also induces the development of Th1 and regulatory T cells which contribute to suppression of allergic disease. However, it has also been described that galectin-9 is involved in the development of allergic disease, by stimulating Th2 driven immune responses or inducing apoptosis of Th1 cells through binding of the TIM-3 receptor on activated Th1 cells (Sziksz et al., 2010 ; Yamamoto et al., 2007; Zhu et al., 2005).

Besides galectin-9, other galectins have been studied in relation with allergic inflammation as well. Galectin-1 is known to have anti-inflammatory capacities in by enhancing the expansion and IL-10 production of regulatory T cells, resulting in suppression of pro-inflammatory cytokines, including IFN-γ and TNF-α by effector T cells (Garin et al., 2007; van der Leij et al., 2007). Furthermore, galectin-1 suppresses pro-inflammatory nitric oxide production by macrophages via alternative activation of macrophages (Correa et al., 2003). Alternative activation of macrophages involves development of macrophages with anti-inflammatory properties that produce anti-inflammatory molecules including IL-10 (van Ginderachter et al., 2006). Galectin-3 is also associated with alternative macrophage activation through binding of CD98 on macrophages (MacKinnon et al., 2008). However, galectin-3 is mainly known as a pro-inflammatory mediator by attracting monocytes and macrophages and inducing cytokine production by neutrophils and mast cells (Chen et al., 2006; Nieminen et al., 2005; Sano et al., 2000).

Inflammatory bowel disease

Although treatment of inflammatory bowel disease with scGOS/lcFOS has not yet been studied, galectins are known to be involved in the pathophysiology of inflammatory bowel disease. In trinitrobenzene sulfonic acid (TNBS)-induced colitis, galectin-1 was shown to suppress the development of intestinal inflammation. Administration of galectin-1 resulted in decreased plasma TNF-α, IL-12 and IFN-γ and abrogated the ability of lamina propria CD4+ T cells to secrete IFN-γ (Santucci et al., 2003). Furthermore, expression of endogenous galectin-1, detected on intestinal epithelial cells, was reduced after TNBS treatment. Galectin-1 is known to induce IL-10 production by monocytes and to lesser extent by T cells, thereby possibly restoring homeostasis in the intestine (van der Leij et al., 2007). Interestingly, both galectin-1 administration immediately following TNBS administration and administration two weeks after induction of colitis resulted in alleviation of clinical symptoms, demonstrating both prophylactic and therapeutic effects of galectin-1 (Santucci et al., 2003). Interestingly, using a dextran sulfate sodium (DSS)-colitis model, it was shown that expression of galectin-2 by intestinal epithelial cells, but not galectin-1, was reduced after administration of DSS in drinking water. This might indicate that altered expression of specific galectins may contribute to the pathophysiology of inflammatory bowel disease. Indeed, exogenous galectin-2 treatment resulted in improvement of clinical scores of acute colitis by suppressing inflammatory IL-6 secretion and enhancing the production of IL-10 by lamina propria mononuclear cells (Paclik et al., 2008a). Likewise, galectin-4 produced by intestinal epithelial cells also enhances IL-10 production, while reducing TNF-α and IL-17 secretion (Paclik et al., 2008b). However, another study reported that galectin-4 exacerbated intestinal inflammation by inducing IL-6 production by CD4+ T cells, although galectin-4 expression by intestinal epithelial cells was not increased (Hokama et al., 2004). Hence, there is still some controversy on the involvement of galectins in inflammatory bowel disease.

Besides the role in animal models for inflammatory bowel disease, intestinal biopsies of healthy and inflammatory bowel disease patients also revealed a role for galectin-3 in humans. Epithelial-derived galectin-3 was found to induce activation of lamina propria fibroblasts, resulting in the production of CXCL-8 in a NF-κB and AP-1 dependent way (Lippert et al., 2007). This is possibly responsible for infiltration of neutrophils that are attracted by CXCL-8 into the intestinal wall, which is characteristic in inflammatory bowel disease. Surprisingly, expression of galectin-3 was reduced in inflammatory bowel disease patients (Lippert et al., 2008), possibly as a result of high TNF-α production as it was demonstrated that TNF-α reduces galectin-3 expression *in vitro* (Jensen-Jarolim et al., 2002; Müller et al., 2006). In addition, in Crohn's disease patients, autoantibodies against galectin-3 were found to be increased, which correlates with disease activity (Jensen- Jarolim et al., 2002). However, disrupted galectin-3 – T cell receptor interactions on

T cells as a result of suppressed galectin-3 secretion by intestinal epithelial cells may also result in enhanced T cell activation (Demetriou et al., 2001). Food allergy and inflammatory boewel disease both involve an inflammatory immune response, which causes tissue damage, resulting in disease symptoms. Galectins have not only been described to be involved in disease pathogenesis, but play a major role in the inflammatory response as well. It has been shown in allergic lesions that galectin-9 prevents binding of CD44 on lymphocytes to hyaluronic acid, thereby inhibiting airway inflammation and hyperresponsiveness by suppressing interaction of lymphocytes to the extracellular matrix (Katoh et al., 2007). In addition, galectin-9 suppresses the production of inflammatory cytokines (IL-1β, IL-6, MCP-1 and TNF-α), which results in the suppression of inflammation in collagen-induced arthritis (Seki et al., 2008).

Concluding remarks

It is known that non-digestible carbohydrates present in human breast milk have an effect on the development of the immune system and the composition of the gut microflora. Functionally resembling the non-digestible carbohydrate composition of breast milk, a 9:1 mixture of scGOS/lcFOS has been formulated. Promising studies have been performed using this oligosaccharide mixture. Supplementation of these oligosaccharides was found to suppress Th2 and enhance Th1 and regulatory T cell immune responses *in vivo*. However, the mechanisms by which non-digestible oligosaccharides may induce health beneficial effects when orally supplemented via the diet remains to be elucidated. Recently, preliminary experiments performed within in our group suggest an important role for scGOS/lcFOS in combination with Toll-like receptor ligation of intestinal epithelial cells by specifically enhancing a Th1 and regulatory T cell type effector immune response. Future research may focus on regulation and effects of the expression of glycan-binding molecules by intestinal epithelial cell via Toll-like receptor activation on innate and adaptive immune responses. Furthermore, by simulating inflammatory conditions in the intestine, as is the case in allergic inflammation or inflammatory bowel disease, possible therapeutic potentials of glycan-binding receptors may be studied. In conclusion, intestinal epithelial cells may be crucial in immune modulation mediated by non-digestible oligosaccharides, in which a possible crosstalk between lectins and Toll-like receptors may be involved. Therefore, intestinal epithelial cells may serve as interesting targets for modulation of innate and adaptive immune responses in the intestine, and may open new avenues in the treatment of inflammatory disorders of the intestine using non-digestible oligosaccharides that can be applied via the diet.

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3

Apical TLR ligation of intestinal epithelial cells drives a Th1-polarized regulatory or inflammatory type effector response *in vitro*

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Abstract

Intestinal epithelial cells (IEC) separate the mucosal immune system from the external milieu. Under inflammatory conditions, Toll-like receptor (TLR) expression by IEC is increased. In a transwell co-culture model immune modulation by IEC upon TLR ligation was studied. Human IEC (HT-29 and T84) grown on filters were apically or basolaterally exposed to TLR4 or TLR9 ligands and co-cultured with CD3/CD28-activated healthy donor PBMC in the basolateral compartment. TLR4 ligation of IEC (HT-29) enhanced the production of TNF-α and IEC-derived MDC and decreased numbers of Foxp3+ regulatory T cells. Neutralization of TSLP abrogated TLR4-induced TNF-α secretion. In contrast, apical TLR9 ligation of IEC (HT-29 and T84) enhanced IFN-γ and IL-10 secretion and increased the number of activated Th1 cells. The increase in IFN-γ secretion depended on the presence of IEC. Furthermore, CD14 expression on monocytes was reduced coinciding with enhanced intracellular IL-10 and decreased TNF-α production. However, basolateral TLR9 ligand exposure of HT-29 cells resulted in enhanced IFN-γ, IL-6 and TNF-α, while IL-10 secretion remained unaltered. TLR4 and TLR9 ligands reduced IL-13 secretion in presence and absence of apically exposed IEC and enhanced IL-12 secretion in presence of IEC. These data suggest that TLR4 ligation of IEC drives an inflammatory, while apical TLR9 ligation drives a regulatory Th1 effector immune response in vitro in a polarized manner. IEC may be important modulators of the mucosal effector immune response.

Introduction

The gastrointestinal mucosa is constantly discriminating between harmless and potentially dangerous antigens and micro-organisms. The mucosal immune system contributes to oral tolerance induction, while producing a protective inflammatory response in case of injury or infection. The gut-associated lymphoid tissue is covered by a single layer of epithelial cells. Besides acting as a barrier, intestinal epithelial cells (IEC) are crucial in maintaining homeostasis. However, in diseases like inflammatory bowel disease (IBD) and food allergy, tolerance towards the intestinal flora or dietary antigens is broken, resulting in local pathological inflammation (Cardoso et al, 2008; Sartor 2008). Emerging evidence points out the importance of IEC in orchestrating the mucosal immune response providing cross-talk with cells of the innate and adaptive immune system (Artis, 2008).

Enteric bacteria in the lumen of the gut are recognized among others by Toll-like receptors (TLR) expressed by different cells in the gastrointestinal tract. IEC express a broad spectrum of TLR on the apical membrane, including TLR2, TLR4 and TLR9 (Cario et al, 2002; Uehara et al, 2007). Under basal conditions, IEC are unresponsive to TLR2 and TLR4 (Abreu et al, 2001; Melmed et al, 2003), while apical TLR9 signaling contributes to colonic homeostasis (Lee et al, 2006). Epithelial surface expression of these TLR was found to be increased under inflammatory conditions (Abreu et al, 2002; Singh et al, 2005; Ewaschuk et al, 2007). Interestingly, administration of TLR9 agonists has been used successfully to dampen exacerbated immune activation in animal models of allergic sensitization (Zhu et al, 2007) and IBD (Abe et al, 2007; Rachmilewitz et al, 2004; Rachmilewitz et al, 2002). TLR ligands in the gut lumen come in direct contact with IEC. Furthermore, recent evidence suggests that the functional properties of intestinal dendritic cells are conditioned by IEC (Butler et al, 2006). This suggests that IEC may also contribute to intestinal homeostasis (Artis, 2008). Therefore, targeting specific TLR on IEC may open new avenues for therapeutic strategies to modulate mucosal immune responses. To address the contribution of different TLR ligands to the modulation of the effector response by IEC, a transwell co-culture model was used. IEC were cultured on insert filters with activated healthy donor PBMC added to the basolateral compartment. In this co-culture model it is shown that TLR4 and TLR9 differentially modulate the immune response in the presence of IEC.

Materials and Methods

Culture of intestinal epithelial cells

Human colon adenocarcinoma HT-29 cells (ATCC, HTB-38; passages 136-150), used as IEC, were cultured in 25 cm2 culture flasks (Greiner, Frickenhausen, Germany) in McCoy's 5A medium (Gibco, Invitrogen, Carlsbad,

CA, USA) supplemented with 10% heat-inactivated FCS (Gibco), penicillin (100U/mL)/streptomycin (100µg/mL) (Sigma, UK). Caco-2 cells (ATCC, HTB-37; passage 30-35) were cultured in 25cm2 culture flasks (Greiner) in minimum essential medium (MEM; Invitrogen) supplemented with 10% heat-inactivated FCS (Gibco), penicillin (100U/mL)/streptomycin (100µg/mL) (Sigma), 1mM pyruvate (Sigma) and 0.1 mM non-essential amino acids (Gibco). Human colon carcinoma T84 cells (ATCC, CCL-248; passage 145) were cultured in 25cm2 culture flasks (Greiner) in Dulbecco's modified Eagle medium/F-12 (DMEM/F-12, 1:1) with GlutaMAX (Gibco) supplemented with 10% heat-inactivated FCS (Gibco) and penicillin (100U/mL)/streptomycin (100µg/mL) (Sigma). Cells were kept in an incubator at 37°C and 5% CO2. Medium was refreshed every 2 to 3 days and cells were passaged once a week.

Purification of PBMC

Human peripheral blood mononuclear cells (PBMC) from healthy donors were isolated from buffy coats (Sanquin, Amsterdam, The Netherlands). PBMC were purified using Ficoll-Paque Plus (GE Healthcare Life Sciences, Uppsala, Sweden) gradient centrifugation (1000 x g, 20min). PBMC were collected and washed in PBS/2% FCS. Remaining erythrocytes were removed by adding 5mL 4°C lysis buffer (4.14g NH4Cl, 0.5g KHCO3, 18.6mg Na2EDTA in 500 mL demi water, pH adjusted to 7.4 and filter sterilized) for 5 minutes on ice. PBMC were resuspended in RPMI 1640 (Lonza, Verviers, Belgium) supplemented with 2.5% FCS, penicillin (100U/mL)/streptomycin (100µg/mL) (Sigma) and sodium pyruvate (1mM; Sigma) (PBMC medium).

Transwell co-culture model

One week prior to the experiments HT-29 cells were seeded 5 times diluted on 0.4µm, 1cm^2 transwell filters (Costar, Corning Incorporated, NY, USA) and grown to confluence. Confluence of unpolarized HT-29 cells was determined by examination of the filters using light microscopy as these cells do not create transepithelial electrical resistance (TER; approximately 150 Ωxcm^2). The TER of filters alone was 125 Ωxcm^2. Transwell cultures (12 well) with confluent HT-29 monolayers were used for co-culture with 1.5mL PBMC (2x10^6 cells/mL) (van Hoffen et al, 2010) using PBMC medium and kept in an incubator at 37°C and 5% CO2. Immune cells were stimulated with anti-CD3 (clone CLB-T3/2) and anti-CD28 antibodies (clone CLB-CD28; both 1:10,000, Sanquin). TLR agonists (TLR2: Pam3CysSK4, 1 µg/mL, EMC microcollections GmbH, Tubingen, Germany; TLR4: purified *E. coli* 0111:B4 LPS, 1 µg/mL, Invivogen, San Diego, USA; TLR9: M362 CpG oligodinucleotide type C, 5 µM, Invivogen) were added apically to HT-29 monolayer in 0.5 mL. In additional cultures TLR ligands were added directly to 2x10^6 cells/mL PBMC in 12 well plates to determine direct PBMC responses (TLR2 and TLR4 0.25µg/mL; TLR9 1.25µM assuming equal distribution in upper and lower compartment). After 24h, culture supernatants from the basolateral

compartment were stored at -20°C until cytokine measurement and immune cells were collected. Furthermore, after 24h of co-culture IEC were placed on fresh medium for 24h to determine basolateral mediator release by IEC. For transwell experiments using polarized T84 and Caco-2 cells, 4 weeks prior to the experiment cells were seeded 5 times diluted on 0.4µm, 1cm^2 transwell filters. Confluency was determined by measuring the TER. Upon confluence the resistance was approximately 2000 Ωxcm^2 (T84) and 1200 Ωxcm^2 (Caco-2).

Epithelial mediator function

Neutralization of TSLP (2.5µg/mL, sheep-anti-human TSLP IgG, R&D Systems, Abingdon, UK) was performed during 24h co-culture. Sheep IgG (R&D Systems) was used as isotype control. In addition, anti-CD3/CD28 stimulated PBMC were incubated with increasing concentrations of recombinant human TSLP (0.1–10ng/mL, R&D Systems).

ELISA

Production of cytokines and chemokines in the collected PBMC or IEC supernatants was measured by means of ELISA. Concentrations of IL-6 (PeliPair reagent set, Sanquin), IL-10, IL-12p40/p70, IL-13, IFN-γ, TNF-α (Biosource CytoSetsTM, Nivelles, Belgium), TSLP, IP-10 and MDC (R&D Systems) were measured according to the manufacturer's instruction.

TLR expression by HT-29 cells

HT-29 cells were cultured to confluence in 6 well transwell chambers (Corning) as described and co-cultured with 2.5 mL anti-CD3/CD28 stimulated PBMC (2x10^6 cells/mL). After 24h IEC were trypsinized (trypsin/EDTA (2mM) for 5min) and resuspended in PBS to 2x10^6 cells/mL. Cells (4x10^5) were blocked for 10 min on ice with 6% goat serum and stained with anti-TLR2 (Imgenex, San Diego, USA), anti-TLR4 (Imgenex), anti-TLR9 (Imgenex) or isotype control (IgG1 and IgG2b; BD Biosciences, San Jose, CA, USA) for 45 min on ice, followed by incubation with polyclonal FITC-conjugated goat anti-mouse Ig (BD Biosciences) for 30 min on ice and fixation with 1% paraformaldehyde (Sigma). Flow cytometric analysis was performed using a FACSCalibur (BD Biosciences).

Phenotype analysis of CD4+ T cells and monocytes

After co-culture with IEC, lymphocytes were collected. Th1 cells were triple stained with CD4-PerCP-Cy5, CD69-PE and CD183-Alexa Fluor® 488 (CXCR3), Th2 cells were stained with CD4-PerCP-Cy5, CD69-PE and CRTH2-Alexa Fluor® 647 (all from BD Biosciences). Cells were then fixed with 0.5% paraformaldehyde. To determine Treg cells, surface staining with CD4-PerCP-Cy5 and CD25-FITC (BD Biosciences) was followed by permeabilization with BD CytoFix/CytoPermTM for 30min on ice. Cells were washed with permeabilization buffer (BD Biosciences) and blocked with 1% normal rat serum for 15min on ice. Cells were then stained with Foxp3-PE (eBioscience, San Diego, Ca, USA). To characterize the monocytes GolgiPlugTM (1 µL/10^6 cells; BD Biosciences) was added in the basolateral compartment after co-culture for 18h with IEC.

Monocytes were collected 6h later by incubating the cells with ice cold PBS for 5min on ice. Surface expression of CD14 was analyzed using CD14-PerCP-Cy5 (BD Biosciences). Monocytes were fixed and permeabilized as described previously. Intracellular IL-10 and TNF-α staining was performed using mouse-anti-human IL-10-PE and mouse-anti-human TNF-α-FITC (BD Biosciences). For intracellular cytokine staining in T cells, PBMC were restimulated using PMA (50ng/mL, Sigma) and ionomycin (750ng/mL, Invitrogen) for 1h before incubation with GolgiPlugTM (BD Biosciences). Cells were stained using CD4-PerCP-Cy5 (BD Biosciences). Intracellular IL-10 and IFN-γ staining was performed using mouse-anti-human IL-10-PE and mouse-anti-human IFN-γ-APC (BD Biosciences).

Statistics

Results are presented as means (±SEM) of at least 3 independent experiments. Cytokine production by PBMC or lymphocytes and effects on immune cell phenotype were compared using one-way ANOVA, followed by Dunnett's post hoc test. Correlation was tested using the Pearson's correlation coefficient. Cytokine production by PBMC in response to neutralization of TSLP or isotype controls was compared to non-treated groups using the paired Student's t-test. Analyses were performed using Graphpad Prism 4.0 and SPSS, version 16.0. $P < 0.05$ was considered statistically significant.

Results

IEC-dependent differential cytokine release upon apical TLR ligation of IEC HT-29 cells were grown on transwell filters and co-cultured with PBMC in the basolateral compartment. In this system it was evaluated whether apical TLR ligation of IEC modulates the cytokine response by unstimulated PBMC and CD3/CD28-activated PBMC. In addition, as diffusion of ligands to the basolateral compartment may have occurred, direct effects of TLR ligands on PBMC were also determined. After 24h of epithelial exposure to TLR ligands, IL-12 (Th1) secretion by activated PBMC was increased in the basolateral compartment, while only TLR4 and TLR9 ligation of IEC reduced the secretion of IL-13 (Th2) (**Figure 1A** and **1B**, $P<0.05$). The increase in IL-12 secretion upon TLR ligation was found to depend on the presence of IEC since in absence of IEC TLR agonists did not increase IL-12 production (**Figure 1A**). However, in absence of IEC ligation of TLR4 or TLR9 was found to reduce Th2 type IL-13 secretion (**Figure 1B**, $P<0.05$). In addition to IL-12 and IL-13, the secretion of Th1-type IFN-γ and pro-inflammatory TNF-α by PBMC was measured as well. TLR9 ligation of HT-29 cells enhanced IFN-γ secretion, whereas TLR4 ligation of IEC enhanced TNF-α (**Figure 1C** and **1D**, $P<0.05$). Both responses depended on the presence of IEC since direct stimulation of activated PBMC with TLR4 or TLR9 ligand did not result in enhanced production of IFN-γ or TNF-α (**Figure 1C** and **1D**).

IL-10 is an important regulatory cytokine, while IL-6 is known to break immunological tolerance. IL-10 secretion by activated PBMC was enhanced upon TLR9-stimulation of IEC (**Figure 1E**, P<0.05). In absence of IEC similar effects were found (**Figure 1E**, P<0.05). In contrast, IL-6 secretion was increased when IEC were exposed to TLR4 ligand (**Figure 1F**, P<0.01), while TLR9 ligation of IEC had no effect. TLR4 stimulation also enhanced IL-6 secretion by activated PBMC in absence of IEC (**Figure 1F**, P<0.01). TLR2 only enhanced IL-6 secretion in the absence (106±15.4 pg/mL for controls vs. 1457±107.4 pg/mL for TLR2, P<0.01) and presence of IEC (344±142.1 pg/mL for controls vs. 6055±1591 pg/mL for TLR2, P<0.01). TLR2 ligation of IEC did not modulate the secretion of other cytokines (data not shown).

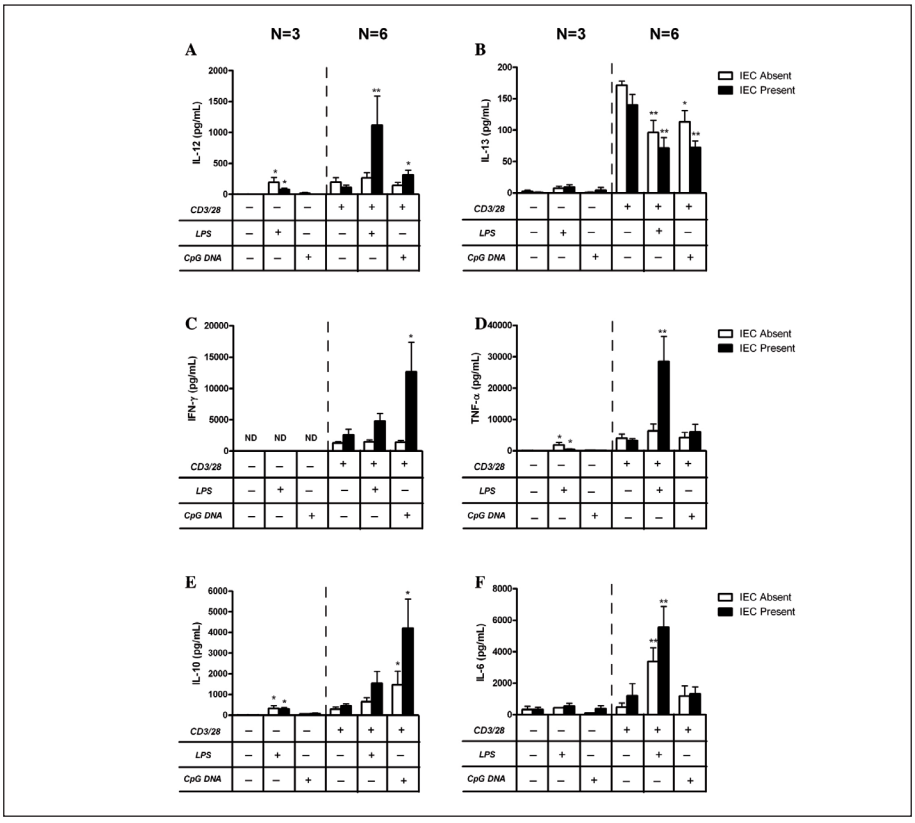

Figure 1 Apical epithelial TLR ligation differentially modulates the effector immune response*. IEC were incubated apically with medium (control), TLR4 (LPS; 1 µg/mL) or TLR9 agonist (CpG DNA; 5 µM) and co-cultured with non-stimulated or CD3/CD28-activated PBMC. PBMC were exposed to TLR4 (0.25µg/mL) or TLR9 agonist (1.25µM). After 24h of culture IL-12 (A), IL-13 (B), IFN-γ (C), TNF-α (D), IL-10 (E), and IL-6 (F) were measured in the (basolateral) supernatant. Data represent n=3-6 experiments with independent PBMC donors, mean ± SEM, *P<0.05, **P<0.01 by ANOVA compared to control. (ND, not detected).*

In unstimulated PBMC cultures, no differential cytokine responses were observed, although TLR4 ligation was able to enhance IL-10, IL-12 and TNF-α secretion by PBMC in the absence and presence of IEC (**Figure 1**, $P<0.05$). In an *in vivo* situation both TLR4 and TLR9 ligand are present in the intestinal lumen and may interact with IEC simultaneously. Therefore, IEC were apically exposed to a combination of TLR4 and TLR9 ligands and co-cultured with anti-CD3/28 activated PBMC for 24h. As shown in **Table 1**, upon simultaneous apical exposure of IEC to TLR4 and TLR9 ligands IFN-γ secretion was further increased ($P<0.05$), while the secretion of regulatory IL-10 remained enhanced. Interestingly, TLR4-induced IL-12 and TNF-α secretion was partly prevented by simultaneous apical stimulation with TLR9 ligand ($P<0.05$). A similar trend was observed for IL-6 (**Table 1**).

Polarized IEC also drive a regulatory Th1 response upon apical TLR9 ligation

HT-29 cells used in co-culture are not polarized. However, Lee et al. (2006) described differential effects of TLR9 ligation on polarized versus unpolarized HCA-7 cells. Therefore, the outcome of apical TLR9 ligation of the unpolarized HT-29 cells on the underlying immune response was compared with the effects of apical TLR9 ligation of polarized T84 cells. As shown in **Figure 2**, apical stimulation of T84 cells with TLR9 ligand resulted in enhanced IFN-γ and IL-10 secretion by activated PBMC, similar to HT-29 cells ($P<0.01$). Apical exposure to TLR ligands did not affect the effector immune response when activated PBMC were co-cultured with Caco-2 cells (data not shown).

Table 1 - Effects of combined apical TLR4 and TLR9 ligation of IEC on cytokine secretion by CD3/CD28-activated PBMC in the presence of HT-29 cells.

	Control	TLR4	TLR9	TLR4 +TLR9
IFN-γ	4393 ± 663	6624 ± 1158	8055 ± 1183**	10338 ± 1553**, #
IL-10	695 ± 70	990 ± 219	2385 ± 151*	1814 ± 162
IL-12	249 ± 150	937 ± 169**	517 ± 252	637 ± 135*,#
TNF-α	5752 ± 147	10367 ± 1313*	4640 ± 356	7519 ± 979#
IL-6	3984 ± 1670	43523 ± 10472*	4993 ± 545	21422 ± 4696*
IL-13	351 ± 118	242 ± 88	106 ± 25*	116 ± 26*

Cytokine levels are shown as pg/mL, mean±SEM of three experiments using independent PBMC donors.

**P<0.05 by ANOVA compared to control*

***P<0.01 by ANOVA compared to control.*

#P<0.05 compared to TLR4 stimulated IEC.

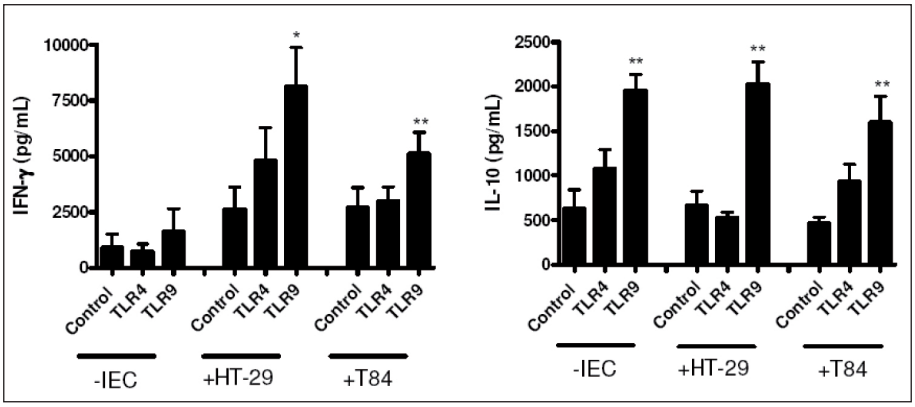

Figure 2 Apical TLR9 ligation of unpolarized HT-29 cells and polarized T84 cells results in a similar cytokine response*. Unpolarized HT-29 and polarized T84 cells were incubated apically with medium (control), TLR4 or TLR9 ligand and co-cultured for 24h with CD3/C28-activated PBMC. Production of IFN-γ and IL-10 was determined in the basolateral supernatant. Data represent n=5 experiments using independent PBMC donors, mean ± SEM,*P<0.05, **P<0.01 by ANOVA compared to medium stimulated IEC.*

Basolateral TLR9 ligation of IEC adds to the inflammatory response

To confirm whether IEC respond in a polarized manner, HT-29 cells were also co-cultured with activated PBMC and basolaterally stimulated with TLR9 ligand for 24h. In contrast to apical TLR9 ligation or direct incubation of CD3/CD28-activated PBMC with TLR9 ligand, basolateral stimulation did not result in enhanced IL-10 secretion by PBMC. Instead, secretion of IL-6 and TNF-α were increased upon basolateral TLR9 ligation (P<0.05), while IL-13 secretion was not suppressed (**Figure 3**).

TLR-activated IEC selectively modulate immune cell phenotype

To assess whether TLR-activated HT-29 cells could alter the phenotype of immune cells, lymphocytes present in the basolateral compartment were analyzed for expression of the chemokine receptors CXCR3 and CRTH2 to characterize Th1 and Th2 cells, respectively (Purwar et al, 2006). Regulatory T cells were characterized by expression of CD25 and Foxp3. In association with an increase in IFN-γ secretion, TLR9 ligation of IEC was found to increase the percentage of activated Th1 cells in the co-culture model (**Figure 4A**, P<0.01). In contrast, TLR4 ligation of IEC reduced the percentage of Treg cells (**Figure 4B**, P<0.05). The expression of CRTH2 remained unaffected (data not shown). Only in the presence of IEC intracellular IFN-γ expression by CD4+ T cells was enhanced upon TLR9 ligation of IEC (**Figure 4C**, P<0.05). Both in presence and absence of IEC the TLR9 agonist enhanced IL-10 expression (**Figure 4C**, P<0.05).

Interestingly, TLR9 ligation of IEC increased the number of IL-10+IFN-γ+ double positive CD4+ T cells (0.77±0.22 % for controls vs. 1.53±0.32 % for TLR9 exposed IEC, P<0.05). TLR2 ligation of HT-29 cells had no effect (data not shown).The secretion of IFN-γ was found to correlate positively with IL-10 only in the presence of TLR-exposed HT-29 cells (**Figure 4C**, r^2=0.4942, P<0.05; in the absence of IEC r^2 = 0.0001,

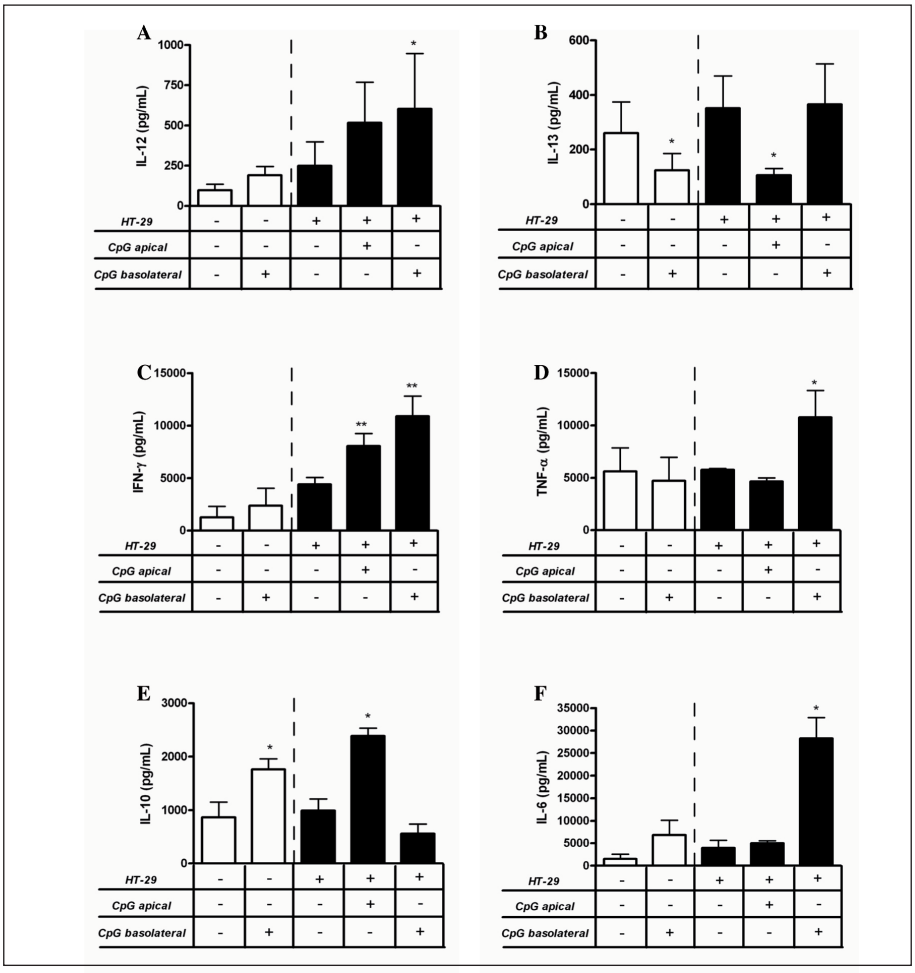

Figure 3 Basolateral TLR9 ligand exposure of IEC abrogates the induction of IL-10, while enhancing TNF-α secretion. *CD3/CD28-activated PBMC were cultured for 24h in the absence or presence of HT-29 cells. Co-cultures were apically or basolaterally exposed to TLR9 ligand or activated PBMC was directly stimulated with TLR9 ligand in the absence of IEC. After 24h of culture IL-12 (A), IL-13 (B), IFN-γ (C), TNF-α (D), IL-10 (E), and IL-6 (F) were measured in the (basolateral) supernatant. Data represent n=3 independent PBMC donors, mean ± SEM, *P<0.05, **P<0.01 by paired Student's t-test (in the absence of IEC) or ANOVA (in the presence of IEC) compared to controls.*

P=0.9787, data not shown). Hence, although TLR9 induced IL-10 secretion by activated PBMC does not depend on the presence of IEC, the combined secretion of IFN-γ and IL-10 is likely to be controlled by IEC. No correlation was found between IL-10 and TNF-α secretion (data not shown).

In addition, the effects of apical TLR-exposure of HT-29 cells on the response of monocytes were studied. TLR9 ligation of IEC resulted in a down-regulation of CD14 expression (**Figure 5A**, P<0.01) and enhanced intracellular IL-10 expression by monocytes (MFI 31.7±11.8 for controls vs. 38.8±14.3 for TLR9, **Figure 5B,** P<0.05), while TNF-α expression was decreased (MFI 26.8 ± 8.8 for controls vs. 18.1±5.8 for TLR9, **Figure 5C**, P<0.01). TLR2 or TLR4 ligation of HT-29 cells had no effect.

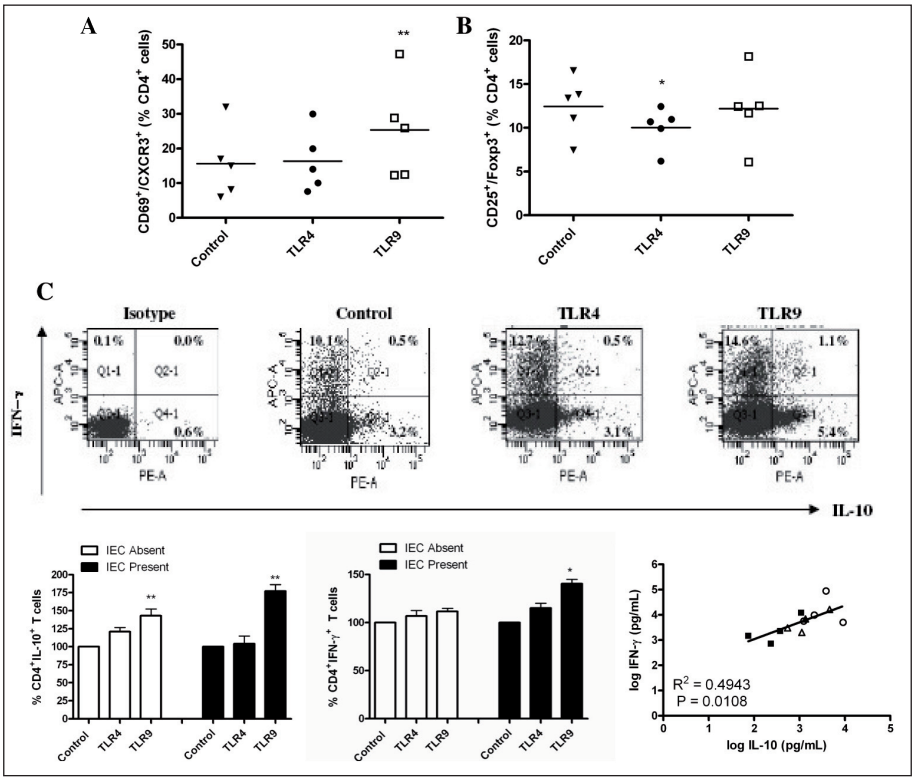

Figure 4 Modulation of T cell phenotype by apical TLR-exposed IEC. *HT-29 cells were co-cultured with CD3/CD28-activated PBMC and exposed to medium (control) or TLR agonists. After 24h lymphocytes were collected and analyzed for the expression of CD4/CD69/CXCR3 (A) and CD4/CD25/Foxp3 (B). (C) Intracellular cytokine expression in CD4+ T cells determined after 24h co-culture with IEC, controls were set at 100%. Correlation between IL-10 and IFN-γ secretion after 24 h by CD3/CD28-activated PBMC in the presence of TLR-exposed HT-29 cells. Data represent n=3-5 independent PBMC donors, mean ± SEM, *P<0.05, **P<0.01 by ANOVA compared to control.*

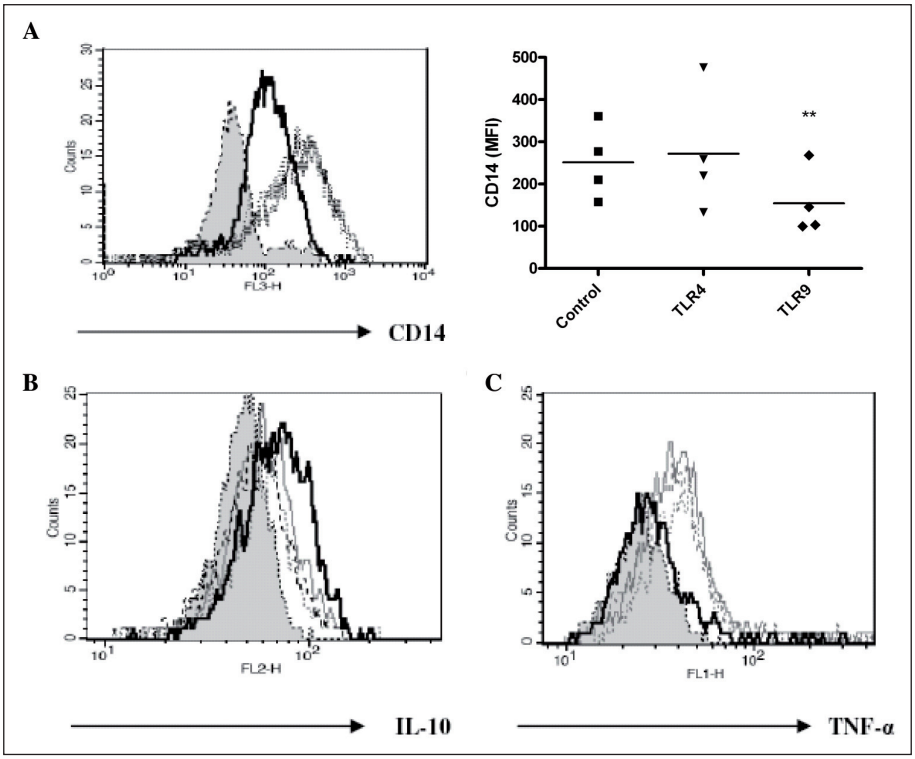

Figure 5 Monocytes adopt an anti-inflammatory phenotype after apical epithelial TLR9 ligation. *HT-29 cells were co-cultured with CD3/CD28-activated PBMC and exposed to medium (control) or TLR agonists. After 24h monocytes were collected and analyzed for CD14 (A), IL-10 (B) and TNF-α (C) expression. Representative plots are shown. Filled histogram: isotype control, black line: TLR9 exposed IEC, grey lines: control and TLR4 exposed IEC. Data represent n=3-5 independent PBMC donors, mean ± SEM, **P<0.01 by ANOVA compared to control.*

Surface TLR expression on IEC is enhanced in co-cultures with activated PBMC

To confirm whether activated PBMC affect TLR expression by IEC, which may have contributed to the immune modulatory function of IEC upon TLR ligation, the surface expression of TLR4 and TLR9 by HT-29 cells upon co-culture with activated PBMC was determined. Under basal conditions, low surface expression of TLR was observed. Upon co-culture with activated PBMC, surface expression of TLR2 (data not shown), -4 and -9 was increased (**Figure 6**, P<0.05).

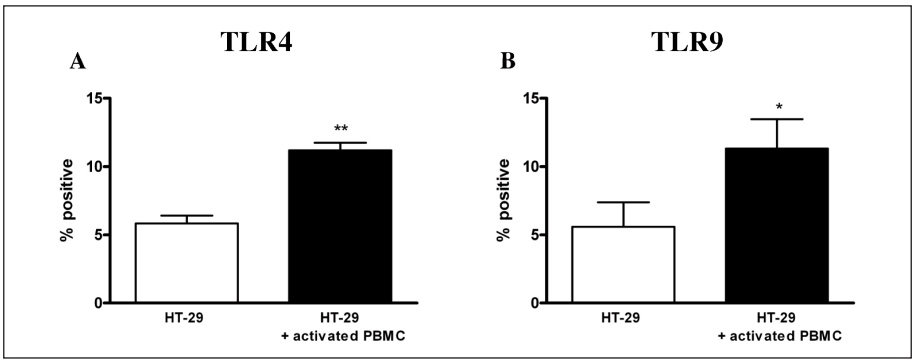

Figure 6 Enhanced TLR4 and TLR9 expression on IEC exposed to activated PBMC. *HT-29 cells were co-cultured for 24h to activated PBMC and surface expression of TLR4 and TLR9 was analyzed by means of flow cytometry. Expression of these TLR was enhanced when compared to control cultures in absence of PBMC. Data represent n=5 independent experiments, mean ± SEM, * P<0.05, **P<0.01 compared to control.*

TLR differentially regulate IEC-derived mediator release

To determine the release of epithelial-derived mediators, HT-29 cells were incubated for 24h on fresh medium after 24h of co-culture with stimulated PBMC. Secretion of CCL22 (MDC),CXCL8 (IL-8), CXCL10 (IP-10) and thymic stromal lymphopoietin (TSLP) were measured. Epithelial exposure to TLR4 ligand enhanced basolateral secretion of MDC (**Figure 7A**, P<0.05). In contrast, IEC exposed to TLR9 ligand showed decreased production of IL-8 (**Figure 7B,** P<0.01) and IP-10 (**Figure 7C**, P<0.05). TLR2 ligation of IEC did not modulate MDC, IL-8 or IP-10 secretion (data not shown).

TSLP is a known immune regulatory mediator selectively released by epithelial cells in low amounts and has been shown to induce an inflammatory Th1 response characterized by high TNF-α secretion (Ito et al, 2005). Therefore, the possible contribution of TSLP in immune modulation mediated by IEC in the transwell model was analyzed. TSLP concentrations were below detection limit. However, using a neutralizing antibody, TSLP neutralization was found to abrogate the additional TNF-α release induced upon epithelial exposure to TLR4 ligand (**Figure 7D**, P<0.05). To confirm the involvement of TSLP in TNF-α secretion by activated PBMC, these cells were exposed to recombinant human TSLP. Recombinant TSLP was found to enhance TNF-α secretion dose-dependently (**Figure 7E**, 1 ng/mL, P<0.05; 10ng/mL, P<0.01). No effects were observed on IFN-γ or IL-10 secretion (data not shown). Matched isotype controls did not affect TLR4-induced TNF-α secretion (data not shown).

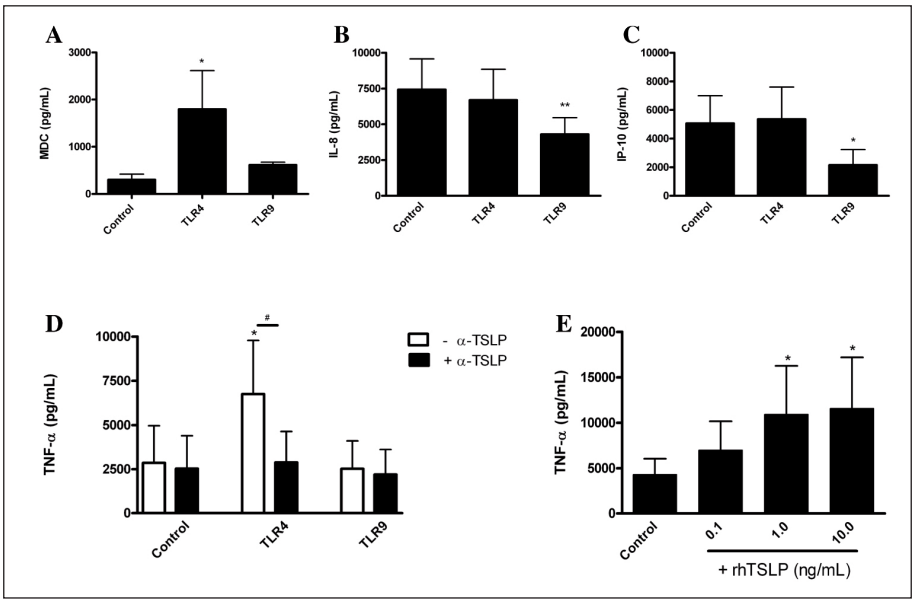

Figure 7 TLR ligation of IEC modulates IEC-derived mediator release. *HT-29 were co-cultured with CD3/CD28-activated PBMC and exposed to medium (control) or TLR ligands for 24h. IEC were placed on fresh medium and after 24h of culture, basolateral MDC (A), IL-8 (B), and IP-10 (C) were measured. (D) HT-29 cells co-cultured with activated PBMC exposed to TLR agonists in the presence or absence of neutralizing antibodies against TSLP. (E) TNF-α production was measured in the supernatant from activated PBMC incubated with increasing concentrations recombinant human TSLP (rhTSLP). Data represent n=5 independent PBMC donors, mean ± SEM, *P<0.05, **P<0.01 by ANOVA compared to control, #P<0.05 by paired Student's t-test compared to TLR4 exposed co-cultures without α-TSLP.*

Discussion

In the current study an *in vitro* co-culture model was used to study effects of TLR ligands on the cross-talk between IEC and the effector immune response. Previously, immune modulatory capacities of IEC were shown using an IEC/PBMC co-culture model when IEC were apically exposed to either pathogenic or non-pathogenic bacteria (Haller et al, 2000, 2002; Parlesak et al, 2004). Different from these studies, in this study a co-culture model was used in which IEC (HT-29 cells) were co-cultured with CD3/CD28-activated PBMC to simulate an effector immune response in the intestinal mucosa (van Hoffen et al, 2010). As bacteria may signal via TLR expressed at the surface of IEC during inflammation, the contribution of specific TLR in modulation of the immune response was investigated.

In the co-culture model, it was shown that using activated PBMC, but not when using non-stimulated PBMC, HT-29 cells differentially alter the effector immune response depending on the type of TLR ligation. TLR4 and -9 ligation of IEC were able to enhance the secretion of IL-12 by activated PBMC, which depended on the presence of IEC. However, apical TLR4 ligation of IEC was found to further enhance TNF-α secretion, whereas apical TLR9 ligation of IEC mainly enhanced IFN-γ combined with IL-10 secretion. In the absence of IEC, TLR4 and TLR9 ligands were not able to enhance TNF-α and IFN-γ secretion, respectively, hence in particular these effects were found to depend on the presence of IEC. A similar co-culture model was used to study immune regulatory effects of probiotic bacteria (van Hoffen et al, 2010). In this study it was shown that epithelial cells exposed to *Lactobacillus GG*, but not *Bifidobacterium breve*, enhanced a Th1-type response, indicating a possible role for IEC in modulation of immune responses upon different apical stimuli. These results may imply that IEC play an active role in fine-tuning of the local gut-associated immune response, in particularly under inflammatory conditions.

Villus tip intestinal epithelial cells are highly polarized, while crypt enterocytes are less well differentiated. Epithelial polarity is, under normal conditions, maintained by tight junction complexes and has been reported to be lost under inflammatory conditions (Katz et al., 1989). Polarized IEC are known to generate a tolerogenic response when apically stimulated with TLR9 ligand (Lee et al., 2006). Co-cultures using the polarized T84 cells showed similar responses to apical TLR9 ligation as unpolarized HT-29 cells. Furthermore, despite the absence of polarization in the HT-29 co-cultures, apical and basolateral exposure to TLR9 ligand elicited differential responses. Only apical exposure resulted in a Th1 response that coincided with regulatory IL-10 secretion. This was lost upon basolateral TLR9 ligand exposure, which resulted in enhanced secretion of pro-inflammatory cytokines including TNF-α. This suggests that during inflammatory conditions apical TLR9 ligation of IEC results in a regulatory Th1 response, which may support intestinal homeostasis. In contrast, basolateral TLR9-stimulation of IEC may boost the inflammatory response. Interestingly, the response to apical TLR9-stimulation was dominant over apical TLR4 ligation by partially inhibiting the production of pro-inflammatory cytokines, further indicating the tolerogenic role of apical TLR9 ligation of IEC.

Recently it has been shown that the expression of TLR2 and TLR4 by IEC is up-regulated in patients suffering from inflammatory bowel disease (Frolova et al, 2008). Our co-culture model reveals a role for TLR4 ligation of HT-29 cells in driving inflammatory responses, characterized by enhanced TNF-α production coinciding with decreased numbers of regulatory T cells. A phenotypically similar response has been observed in Crohn's disease (Ricciardelli et al, 2008; Abreu et al, 2003). Furthermore, during episodes of infection or inflammation the production of IL-6 has been shown to suppress the development

and activity of CD4+CD25+Foxp3+ regulatory T cells (Bettelli et al, 2006). Blocking of IL-6 signaling prevented TNF-α expression in the colon and inhibited development of colitis (Noguchi et al, 2007). TLR4, but also TLR2 ligation of IEC enhanced IL-6 secretion in the presence or absence of IEC. However, TLR2-induced IL-6 has also been shown to provide protection during inflammatory injury since it enhances epithelial regeneration (Cario et al, 2004). Subtle changes in signalling cascades initiated in IEC upon TLR ligation may underlie the differential responses observed in the co-culture model. In addition, MDC secretion by HT-29 cells was found to be enhanced after exposure to especially the TLR4 ligand. MDC acts through CCR4 and CCR4 knockout mice were found to display diminished inflammatory responses after intraperitoneal injection with LPS (Chvatchko et al, 2000). TSLP is produced by IEC also and known to condition DC in the intestinal mucosa towards a tolerogenic phenotype (Zeuthen et al, 2008; Rimoldi et al, 2005). However, TSLP has been shown to induce an inflammatory Th1 response as well characterized by high TNF-α secretion (Ito et al, 2005). Neutralizing antibodies abrogated the additional TNF-α release induced upon epithelial exposure to TLR4 ligand in the co-culture model. Additionally, recombinant TSLP enhanced TNF-α secretion by activated PBMC. Hence, IEC may be involved in initiation and perpetuation of the pro-inflammatory immune responses caused by apical TLR4 stimulation.

IEC can actively contribute to the generation of an immune suppressive microenvironment (Christ et al, 1997) and it has been reported that TLR9 ligand enhances the expansion of regulatory T cells. However, the number of regulatory T cells was not found to be increased upon epithelial TLR9 ligation, but the secretion of IFN-γ was enhanced in association with an increase in regulatory IL-10 secretion. Furthermore, the percentage of double positive regulatory type Th1 cells was increased, which suggests the induction of regulatory Th1-type cells (Haringer et al, 2009)Apical exposure of HT-29 cells to TLR9 ligand also induced monocytes to produce IL-10, while down-regulating the production of TNF-α. In parallel, under pressure of TLR9 ligand the monocytes reduced the surface expression of CD14. In line with our results, a change from CD14high monocytes to an IL-10 producing CD14(low) phenotype has been reported upon co-culture with HT-29 cells (Haller et al, 2002). As the intestinal mucosa contains a large number of antigen presenting cells, a shift towards a more tolerogenic phenotype of these cells upon apical TLR9 ligation of IEC may have beneficial effects in intestinal inflammation.

TLR9 expression on IEC has been shown to be up-regulated in the intestine during inflammation (Ewaschuk et al, 2007), a phenomenon which was observed in the co-culture model as well. In addition, apical TLR9 exposure of HT-29 cells reduced the secretion of IP-10 and IL-8 by IEC, chemokines which are involved in attraction of Th1 cells and neutrophils, respectively (Dwinell et al, 2001). In the current study HT-29 as well as T84

cells were found to affect the effector immune response upon apical TLR9 ligation, whereas Caco-2 cells did not. Compared to HT-29 cells, Caco-2 cells show markedly less TLR expression, especially TLR9 (Uehara et al, 2007; Pedersen et al, 2005). Hence, this may reflect the importance of TLR9 in the orchestration of a self- limiting localized regulatory Th1 response. In vivo, oral administration of TLR9 agonists has been shown to provide protective effects in an IL-10-dependent manner (Bleich et al, 2009) in animal models for DSS or TNBS-induced colitis (Rachmilewitz et al, 2002, 2004) and may also prevent or treat Th2 type diseases such as food allergy (Zhu et al, 2007) and asthma (Kitagaki et al, 2002). Together with the results obtained from the current *in vitro* model, showing strengthened Th1 polarization upon TLR9 ligation in the context of high regulatory IL-10 secretion which is supported by IEC, this may suggest the involvement of IEC in mucosal immune regulation via TLR9 ligation. In summary, the co-culture model shows a contribution of IEC to the generation of an inflammatory or a Th1-polarized regulatory type of immune response depending on the type of TLR ligation on IEC (**Figure 8**). This model may help to identify potential targets to modulate inflammatory responses within the intestinal mucosa.

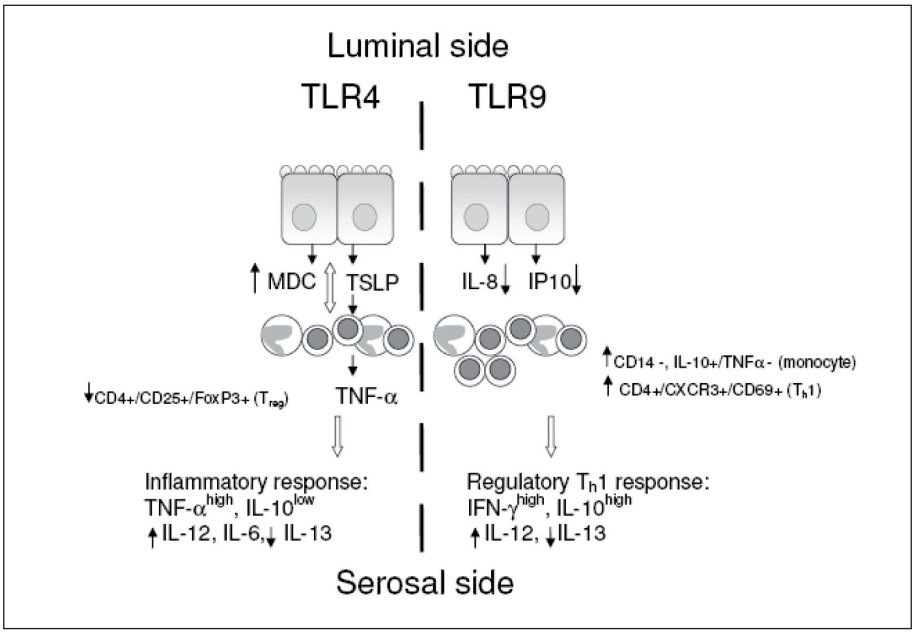

Figure 8 Schematic illustration on the modulation of the effector immune response upon apical TLR4 and TLR9 ligation of IEC.

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4

Intestinal epithelium-derived galectin-9 is involved in the immunomodulating effects of non-digestible oligosaccharides

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Abstract

Dietary intervention using specific non-digestible oligosaccharides (scGOS/lcFOS) in combination with *Bifidobacterium breve* M-16V prevent allergic disease involving galectin-9. In addition, apical TLR9 signalling contributes to intestinal homeostasis. We studied the contribution of galectin-9 secreted by intestinal epithelial cells (IEC; HT-29 and T84) in Th1 and Treg polarization *in vitro*. IEC were grown in transwells, co-cultured with CD3/28-activated human PBMC and apically exposed to genomic DNA derived from *Bifidobacterium breve* M-16V or synthetic TLR9 ligand in absence or presence of scGOS/lcFOS. Cytokine production and T cell phenotype were determined and galectin expression by IEC was assessed. Galectin-9 was neutralized using lactose or a TIM-3-Fc fusion protein. IEC exposed to DNA from *Bifidobacterium breve* M-16V or TLR9 ligand in presence of scGOS/lcFOS enhanced IFN-γ secretion by PBMC and increased the percentage of Th1 and Treg cells. Expression and secretion of galectin-9 by IEC was increased and neutralization of galectin-9 prevented the induction of IFN-γ secretion, while suppressing the production of IL-10 by PBMC as well. Our findings show that galectin-9 secreted by IEC apically exposed to TLR9 ligand in presence of scGOS/lcFOS is involved in Th1 and Treg polarization and may be a promising target to prevent or treat allergic disease.

Introduction

The gastrointestinal immune system is the largest immunological compartment present in the human body, which constantly faces both harmful and harmless antigens present in the intestinal lumen. The intestinal mucosa has therefore the task to discriminate between inducing an immune response in case of pathogen invasion and maintaining tolerance to harmless food antigens or the commensal microbiota. Loss of tolerance towards food antigens may result in the development of food allergy (Cardoso et al., 2008). A monolayer of intestinal epithelial cells (IEC) provides an important barrier between the intestinal lumen and the lamina propria, but is known to be involved in the modulation of innate and adaptive immune responses as well (Artis, 2008; Iliev et al., 2009b).

As IEC are in close contact with the intestinal lumen, dietary components including non-digestible oligosaccharides may affect the homeostatic microenvironment maintained by IEC. Dietary supplementation with a specific prebiotic 9:1 mixture of short-chain galacto-oligosaccharides (scGOS; [Galβ1-4]3-8Glc; Gal, galactose; Glc, glucose) and long-chain fructo-oligosaccharides (lcFOS, ([Frcβ2-1]>20Frcβ2-1Glc; Frc, fructose) (scGOS/lcFOS) has already been proven to protect against the development of allergic symptoms in a murine model for cow's milk allergy as well as in infants at risk for allergy (Schouten et al., 2009; Moro et al., 2010). Supplementation of scGOS/lcFOS with a probiotic strain *Bifidobacterium breve* M-16V is even more effective in suppressing allergic symptoms in mice (Schouten et al., 2009). Human milk contains non-digestible oligosaccharides that are able to shape the intestinal microflora and may induce mucosal tolerance (Field, 2005; Newburg, 2000). In addition, non-digestible oligosaccharides may have direct immune modulatory effects as well.

IEC are known to contribute to effector immune responses and have the capacity to express pathogen recognition receptors including Toll-like receptors (TLR). TLR recognize fragments of among others bacteria. Human IEC were found to express TLR at the surface, which is increased under inflammatory conditions, whereas under homeostasis IEC are unresponsive towards TLR ligands (Cario et al. 2002; Uehara et al., 2007; Singh et al., 2005; Abreu et al., 2001, 2002; Ewaschuk et al., 2007; Melmed et al., 2003). Epithelial TLR9 ligation by unmethylated bacterial CpG DNA was found to induce tolerance to subsequent TLR stimuli, illustrating its involvement in maintenance of intestinal homeostasis (Lee et al., 2006). *In vitro*, IEC modulate effector immune responses in a polarized fashion as apical, but not basolateral TLR9 ligand exposure enhances IFN-γ and IL-10 secretion, while suppressing IL-13 production by PBMC (de Kivit et al., 2011b). In a

murine model for peanut allergy, oral administration of TLR9 agonists prevented allergy, linking TLR9 activation to reduction of allergic disease (Zhu et al., 2007). Upon activation, IEC secrete mediators that shape the phenotype of dendritic cells to induce regulatory T cell (Treg) differentiation and to suppress the development of both Th1 and Th17 cells (Iliev et al., 2009a, 2009b).

The underlying mechanisms by which scGOS/lcFOS exert their immune modulatory effects are unknown. Receptors involved in recognition of carbohydrate structures, lectins, may be involved. One family of soluble type lectins expressed by IEC that contain carbohydrate recognition domains are galectins, which exhibit binding specificity for β-galactosides (Hirabayashi et al., 2002; de Kivit et al., 2011a). IEC were found to express galectin-2, -3, -4 and -9 (Nio-Kobayashi et al., 2009; Wada et al., 1997). Galectins are localized in the cytoplasm, but can be secreted through yet unknown mechanisms as well. Upon secretion, galectins can bind to glycosylated proteins thereby forming galectin-glycoprotein lattices on cell surfaces to regulate immune responses and potentially inducing immunological tolerance (van Kooyk et al., 2008; Rabinovich et al., 2009). We have previously shown that dietary intervention using scGOS/lcFOS in combination with *Bifidobacterium breve* M-16V enhances serum galectin-9 levels, which was associated with suppression of mast cell degranulation (de Kivit et al., 2012). In addition, non-digestible oligosaccharides have been described to support Th1 and Treg cell differentiation *in vivo* as well (Schouten et al., 2010; de Kivit et al., 2012; Vos et al., 2006). To this end, we studied the effects of IEC exposure to scGOS/lcFOS and TLR9 ligand on human PBMC in an *in vitro* co-culture system (de Kivit et al., 2011b). We demonstrate that galectin-9 is expressed and secreted by IEC upon apical exposure to TLR9 ligand and scGOS/lcFOS, which drives a Th1/Treg response.

Materials & Methods

Transwell co-cultures

Culture of human intestinal epithelial cell lines (HT-29 and T84 cells), isolation of human peripheral blood mononuclear cells (PBMC) and transwell co-cultures were performed as previously described (de Kivit et al., 2011b). In short, HT-29 or T84 cells were grown till confluence on transwell insert filters (Corning, NY, USA). Confluence was examined by light microscopy or transepithelial electrical resistance (TER; 125 Ωxcm^2 for HT-29 cells and 1500 Ωxcm^2 for polarized T84 cell monolayers). IEC were co-cultured with $3x10^6$ CD3/28-activated PBMC for 24h. IEC were apically exposed to either TLR9 ligand (M362 type C, 5.0 μM, Invivogen, San Diego, CA, USA) alone or in combination with 0.5% w/v of a 9:1 mixture of scGOS (Vivinal GOS, Borculo Domo) and lcFOS (Raftiline HP, Orafti) (scGOS/lcFOS, Immunofortis™). For determination of galectin secretion by IEC, HT-29 cells were placed on fresh

medium for another 24h after co-culture with CD3/28-activated PBMC. To study the involvement of galectins in immune modulation, lactose (100mM; Sigma, Zwijndrecht, The Netherlands) was added to the basolateral compartment during co-culture. Sucrose (100mM; Sigma) was used as negative control. To specifically block galectin-9, a TIM-3-Fc fusion protein (1.0µg/mL; R&D Systems, Minneapolis, MN, USA) was added to IEC/PBMC co-cultures.

Culture and isolation of genomic DNA of *Bifidobacterium breve* M-16V

Bifidobacterium breve M-16V (Morinaga Milk Industry) was cultivated in Mann Rogosa Sharp (MRS) broth (Oxoid) for 48 hrs at 37°C under anaerobic conditions. Genomic DNA was extracted and purified by CTAB extraction as described (Wilson, 2001) with additional lysozyme treatment of the cell suspension; 2mg/ml, 5 min at 20°C.

ELISA

Concentrations of IL-6, IL-10, IL-12, IFN-γ, TNF-α (Biosource CytoSets™, Nivelles, Belgium) and IL-17A (Arcus Biologicals, Modena, Italy) were measured according to manufacturer's protocol. For galectin-4 and galectin-9 ELISA, high binding EIA/RIA 96-well plates (Costar, Corning Inc, NY, USA) were coated with 0.75µg/mL primary antibodies (R&D Systems) dissolved in PBS overnight at 4°C. Plates were blocked for 1h with 1% BSA in PBS, samples were then added for 2h, followed by incubation with 0.75µg/mL biotinylated secondary antibody (R&D Systems) in 1% BSA in PBS for 1h. Plates were incubated with streptavidin-HRP (R&D Systems) for 1h followed by development with tetramethylbenzidine (TMB, Thermo Scientific, Rockford, IL, USA) for 10 min. The reaction was stopped with 2M H2SO4 and optical density was measured at 450nm (de Kivit et al., 2012).

Flow cytometry

Th1 cells were stained with CD4-PE-Cy5 (eBioscience, San Diego, CA, USA), CD69-PE (eBioscience) and CXCR3-AlexaFluor 488 (BD Biosciences) and fixed with 0.5% paraformaldehyde. Treg cells were stained with CD4-PE-Cy5 and CD25-AlexaFluor488 (eBioscience), followed by intracellular staining with Foxp3-PE using the FoxP3 Staining Set according to manufacturer's protocol (eBioscience). For intracellular cytokine staining, PBMC were re-stimulated with phorbol-12-myristate-13-acetate (50ng/mL; Sigma) and ionomycin (750ng/mL; Sigma) for 6h in presence of a Golgi-transport inhibitor according to manufacturer's instructions (GolgiPlug, BD Biosciences, San Jose, CA, USA), followed by intracellular staining using IFN-γ-APC (BD Biosciences) as described above. Flow cytometric analysis was performed using a FACSCantoII (BD Biosciences).

cDNA synthesis and real-time PCR

HT-29 cells were washed once in PBS 2h after co-culture with CD3/28-activated PBMC and taken up in 200µL RNAlater™ (Qiagen GmbH, Hilden, Germany). Samples were stored at -20°C until cDNA synthesis. mRNA was isolated using the mRNA capture kit (Roche, Mannheim, Germany) and real-time PCR reactions were performed as previously described (Garcia-Vallejo et al., 2004). GAPDH was used as reference gene. Relative target mRNA abundance was calculated by applying the formula: relative mRNA abundance = 100 x $2^{Ct[GAPDH] - Ct[target\ mRNA]}$. Primers for all galectins were designed using the computer software Primer Express 2.0 (Applied Biosystems, Carlsbad, CA, USA).

Fluorescence microscopy staining of galectin-9 in IEC

IEC were fixed using 4% formalin in PBS for 10min, permeabilized in 0.1% Triton X-100 (Sigma) and 1% BSA in PBS for 15min and incubated with anti-human galectin-4 or -9 antibodies or normal goat IgG as isotype control (all 0.75µg/ml; R&D Systems) in 0.1% Triton X-100 and 1% BSA in PBS for 1h. IEC were incubated with secondary Alexa Fluor546 donkey anti-goat IgG (Invitrogen, Carlsbad, CA, USA) in 0.1% Triton X-100 and 1% BSA for 30min and embedded in Hoechst. Object glasses were stored at 4°C until microscopic examination.

Statistics

Statistical analyses were performed using paired Student's t-test or one-way ANOVA for repeated measurements followed by Bonferroni's post hoc test. Analyses were performed using GraphPad Prism 5.0. $P < 0.05$ was considered statistically significant.

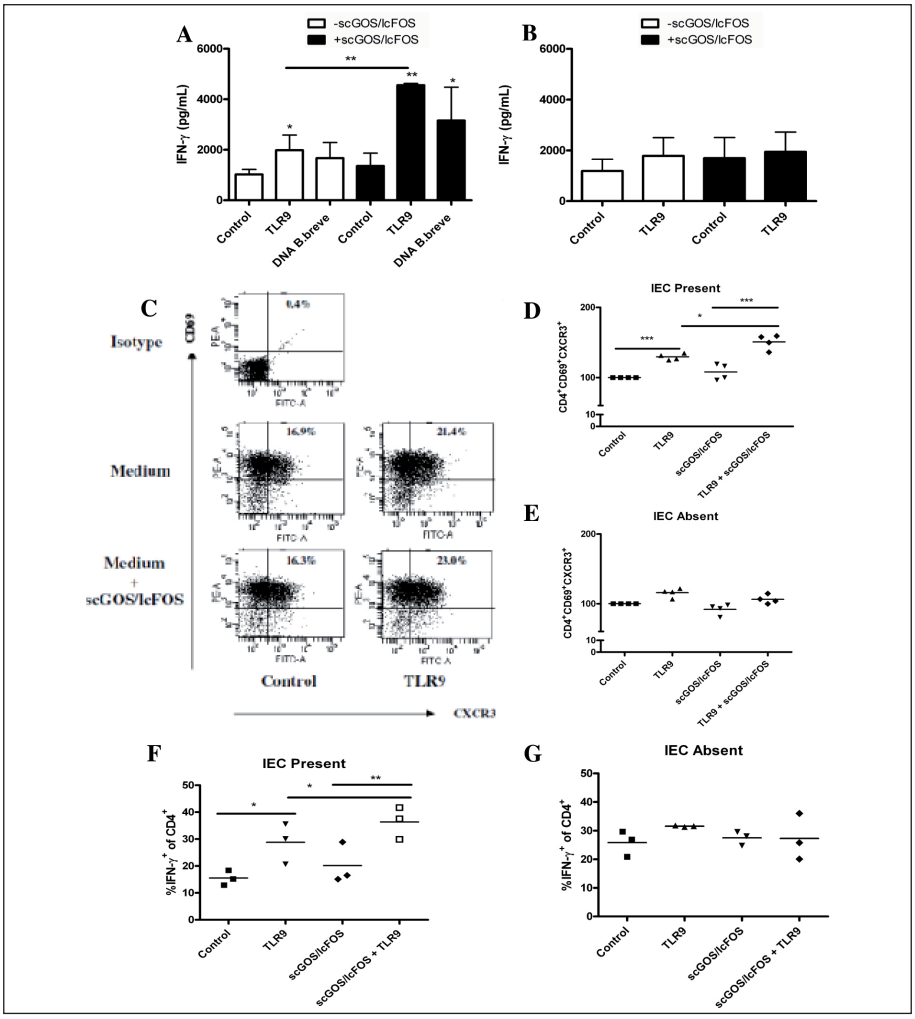

Figure 1 TLR9 ligation of IEC in presence of scGOS/lcFOS results in an enhanced Th1 type effector response. *HT-29 cells were apically exposed to TLR9 ligand or DNA from Bifidobacterium breve M-16V in presence or absence of scGOS/lcFOS and co-cultured for 24h with CD3/28-activated PBMC. In addition, PBMC were directly stimulated with TLR9 ligand in presence of scGOS/lcFOS. Exposure of IEC to TLR9 ligand or DNA from Bifidobacterium breve M-16V increased IFN-γ secretion by PBMC, which was further enhanced in presence of scGOS/lcFOS (A). This was not observed in the absence of IEC (B). Likewise, the percentages of activated Th1 cells (CD4+CD69+CXCR3+) and CD4+IFN-γ+ cells were only increased by apical TLR9 ligation of IEC, which was further enhanced by the presence of scGOS/lcFOS (C, D, F). Direct stimulation of PBMC with TLR9 ligand in absence or presence of scGOS/lcFOS did not alter the number of Th1 cells (E, G). Data represent mean ± SEM of 3-4 independent PBMC donors. *P<0.05, **P<0.01, ***P<0.001.*

Results

The scGOS/lcFOS mixture enhances a TLR9-induced Th1 response via IEC
To investigate the molecular mechanisms by which scGOS/lcFOS exerts its immunomodulatory effects, we used an *in vitro* co-culture model with human IEC and PBMC to study IEC-immune cell cross talk. It was hypothesized that DNA of *Bifidobacterium breve* may activate TLR9 on IEC as apical TLR9 ligation of IEC can induce a regulatory type Th1 response (de Kivit et al., 2011b). IEC apically exposed to purified DNA from *Bifidobacterium breve* M-16V or a synthetic TLR9 ligand enhanced IFN-γ secretion by activated PBMC, which was potentiated by scGOS/lcFOS (**Figure 1A**). Direct stimulation of CD3/CD28-activated PBMC with CpG DNA in the presence of scGOS/lcFOS did not enhance IFN-γ secretion (**Figure 1B**). Furthermore, it was observed that scGOS/lcFOS did not increase IFN-γ production on their own, indicating that the presence of both scGOS/lcFOS and the bacterial DNA is essential for the observed additional effect. In addition, an increased percentage of activated Th1 cells, characterized as CD4+CD69+CXCR3+ cells, were observed when IEC were apically exposed to TLR9 ligand, which was further increased by scGOS/lcFOS (**Figure 1C** and **1D**). In the absence of IEC, the percentage of activated Th1 cells remained unaltered (**Figure 1E**). To confirm the induction of Th1 cells, intracellular IFN-γ staining was performed. Similarly, only in presence of IEC, increased percentage of IFN-γ producing CD4+ T cells were observed upon apical exposure of IEC to TLR9 ligand, which was boosted by scGOS/lcFOS (**Figure 1F** and **1G**).

Treg cells are increased by IEC exposed to TLR9 ligand in presence of scGOS/lcFOS

In addition to the induction of a Th1 polarized effector response, we studied whether IEC apically exposed to TLR9 ligand in presence of scGOS/lcFOS enhances IL-10 production and Treg cell responses as well. TLR9 ligation on IEC, as well as exposure of IEC to DNA from *Bifidobacterium breve* M-16V, resulted in increased IL-10 secretion. TLR9-induced IL-10 secretion by CD3/CD28-activated PBMC was not affected by scGOS/lcFOS (**Figure 2A**). In the absence of IEC, the TLR9 ligand enhanced IL-10 secretion as well, and a similar tendency was observed in presence of scGOS/lcFOS (**Figure 2B**). However, the percentage of Treg cells were only increased when IEC were apically exposed to TLR9 ligand in the presence of scGOS/lcFOS (**Figure 2C-E**). Likewise, intracellular IL-10 expression by CD4+ T cells was increased upon TLR9 ligation in presence and absence of IEC, however, scGOS/lcFOS did not enhance IL-10 production by CD4+ T cells (**Figure 2F** and **2G**).

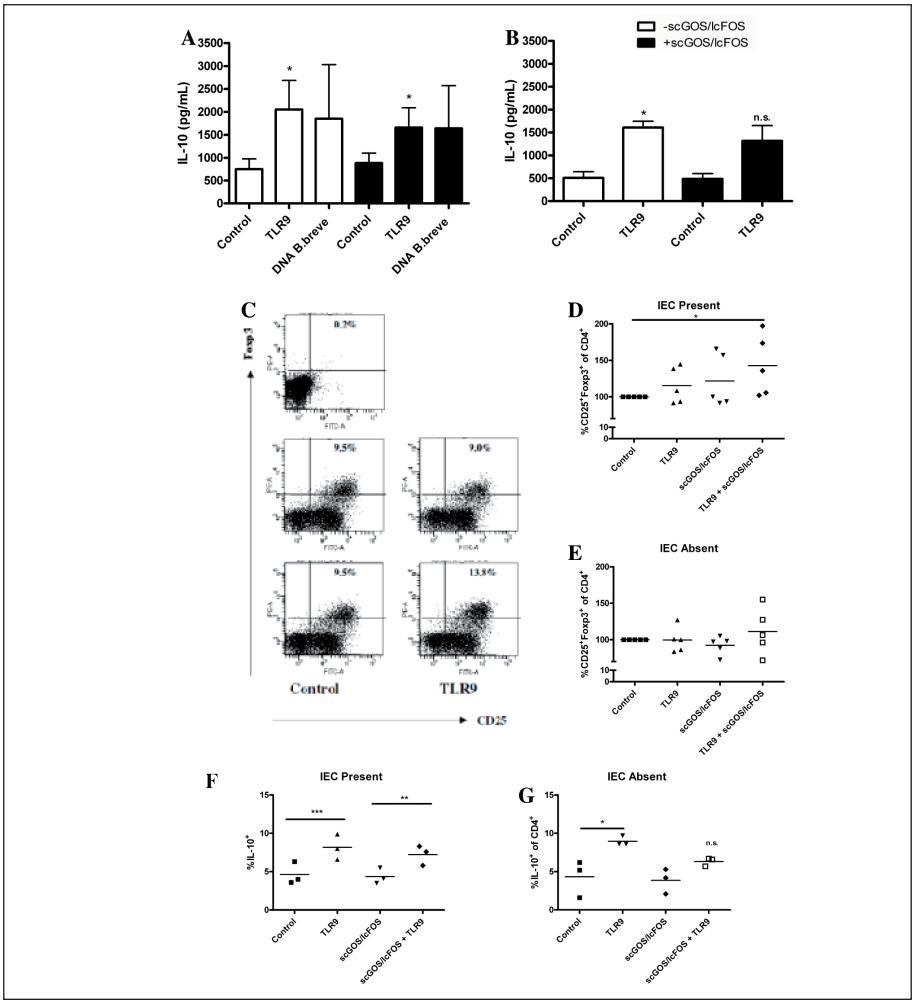

Figure 2 TLR9 ligation of IEC in presence of scGOS/lcFOS results in an enhanced Treg type effector response. *HT-29 cells were apically exposed to TLR9 ligand or DNA from Bifidobacterium breve M-16V in presence or absence of scGOS/lcFOS and co-cultured for 24h with CD3/28-activated PBMC. In addition, PBMC were directly stimulated with TLR9 ligand in presence of scGOS/lcFOS. Exposure of IEC to TLR9 ligand or DNA from Bifidobacterium breve M-16V increased IL-10 secretion by PBMC, which was not affected in presence of scGOS/lcFOS (A). Similar results were observed in absence of IEC (B). However, the frequency of Treg cells (CD4+CD25+Foxp3+) was only increased by apical TLR9 ligation of IEC in presence of scGOS/lcFOS (C, D). Direct stimulation of PBMC with TLR9 ligand in absence or presence of scGOS/lcFOS did not modulate the number of Treg cells (E). Intracellular IL-10 expression by CD4+ T cells was increased by TLR9 ligand, and remained unaltered in the presence of scGOS/lcFOS (F). Similar results were observed in the absence of IEC (G). Data represent mean ± SEM of 3-5 independent PBMC donors, *P<0.05.*

Galectin-9 expression by IEC is increased upon exposure to TLR9 ligand and scGOS/lcFOS

Galectins expressed by IEC may be involved in the induction of Th1 and Treg cell development. To address this possibility, we evaluated whether apical exposure of HT-29 cells to TLR9 ligand and scGOS/lcFOS modulates epithelial galectin expression. First, it was assessed which galectins are expressed by HT-29 cells when co-cultured with CD3/CD28-activated PBMC (**Figure 3A**). Galectin-4 and the long isoform of galectin-9 were highly expressed by HT-29 cells. Apical TLR9 ligation of IEC increased galectin-9 mRNA expression by HT-29, but not galectin-4, while in presence of scGOS/lcFOS galectin-9 expression was further enhanced (**Figure 3B** and **3C**). The galectin-9 up-regulation was confirmed at the protein level by immunofluorescence microscopic analysis of the HT-29 cell monolayer (**Figure 3D**). Furthermore, enhanced basolateral secretion of galectin-9 was observed upon epithelial exposure to TLR9 ligand, which was significantly potentiated by the combination with scGOS/lcFOS (**Figure 3E**). Galectin-4 expression by HT-29 cells was not modulated upon apical exposure of HT-29 cells to TLR9 ligand or scGOS/lcFOS (**Figure 3C, 3D** and **3F**). Hence, TLR9 ligand and scGOS/lcFOS specifically enhanced galectin-9 expression and secretion by IEC.

IEC-derived galectin-9 secretion is involved in induction of an IFN-γ and IL-10 polarized type effector response

In order to study the involvement of galectins in the induction of Th1 and Treg differentiation, galectin function was inhibited by the addition of lactose to the basolateral compartment of IEC/PBMC co-cultures. Lactose binds galectins through their carbohydrate recognition domain and thereby prevents binding of galectins to glycan structures expressed on the cell surface. To determine the specificity of the intervention, sucrose was used as a negative control. Neutralization of galectins by lactose in the basolateral compartment in IEC/PBMC co-cultures exposed to TLR9 ligand and scGOS/lcFOS resulted in reduction of IFN-γ and IL-10 secretion (**Figure 4A** and **4B**), while the production of IL-17 was increased (**Figure 4C**). In addition, IL-6, IL-12 and TNF-α secretion were increased by adding lactose to the basolateral compartment during co-culture (data not shown). Thus, inhibition of galectins induced by IEC exposed to TLR9 ligand and scGOS/lcFOS resulted in a switch from an IFN-γ+IL10+ towards a pro-inflammatory IL-17 immune response.

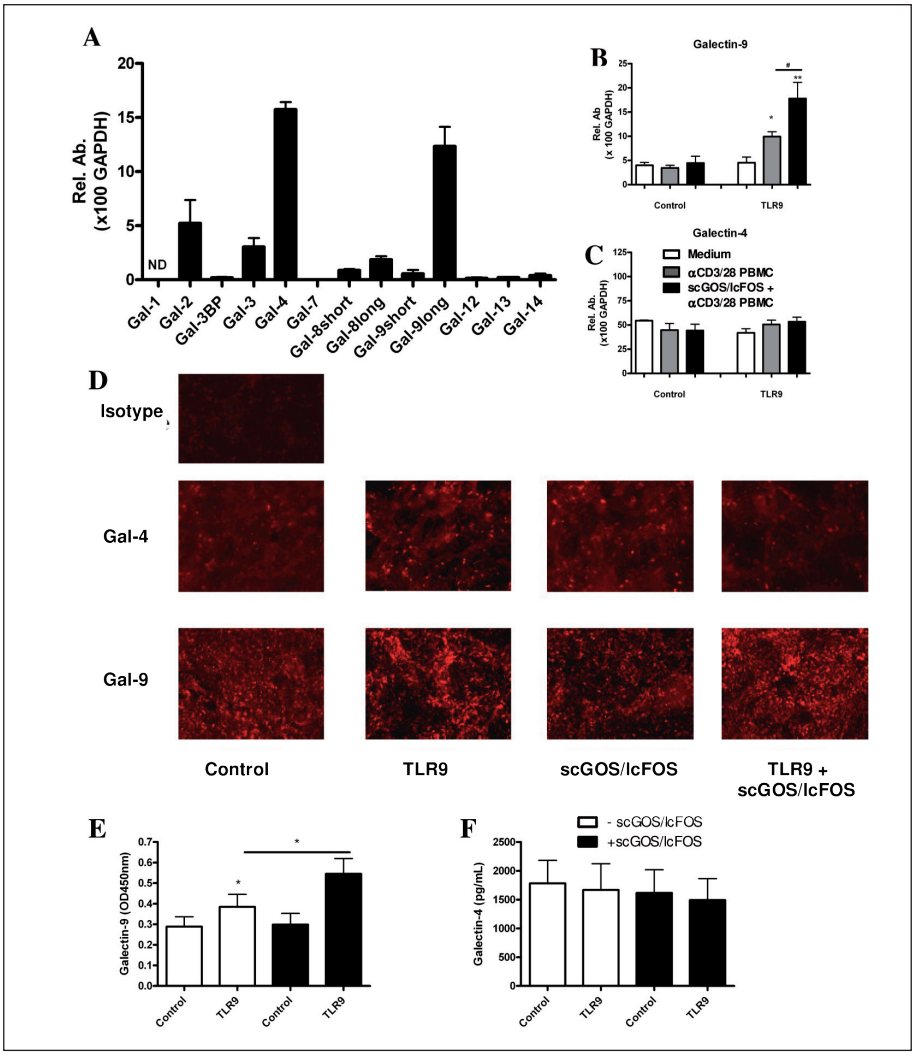

Figure 3 Expression and secretion of galectin-9 in epithelial cells in IEC/PBMC co-cultures is regulated by TLR9 ligation and scGOS/lcFOS. *(A-C) Analysis of galectin mRNA expression by HT-29 cells by quantitative PCR analysis. Evaluation of the expression profile of galectins by IEC (A). Apical TLR9 ligation of IEC, in the absence or presence of scGOS/lcFOS, specifically increases galectin-9 expression (B, C). Protein expression of galectin-4 and -9 and its modulation upon TLR9 ligation and scGOS/lcFOS was confirmed by immunofluorescence microscopic staining of IEC monolayers (D). ELISA was performed in the basolateral supernatant of IEC monolayers (E). TLR9 ligation of IEC in presence of scGOS/lcFOS enhanced galectin-9, but not galectin-4 secretion. Data represent n=3 (A-D) or n=6 (E) independent PBMC donors, mean ± SEM, *,#P<0.05, **P<0.01.*

To prove that galectin-9 is involved in inducing an IFN-γ and IL-10 mediated effector response, a TIM-3-Fc fusion protein was used to specifically neutralize secreted galectin-9. TIM-3-Fc prevented increased IFN-γ and IL-10 secretion by activated PBMC in HT-29/PBMC co-cultures (**Figure 5A-C**). Similar results were obtained using the polarized T84 cell line (**Figure 5D-F**). These data collectively suggest that IEC enhance an IFN-γ and IL-10 type effector response upon apical exposure to TLR9 ligand and scGOS/lcFOS through secretion of galectin-9.

Discussion

The intestinal mucosa is constantly facing a high load of antigens amongst others derived from the diet and micro-organisms. The intestinal epithelium serves as the first line defense against foreign antigens. A single monolayer of IEC forms a tight barrier between the intestinal lumen and the lamina propria. The intestinal epithelium express tight junction proteins to provide a physical barrier, contains goblet cells producing mucus and Paneth cells that actively secrete antimicrobial peptides effectively shaping the intestinal microbiota (Linden et al., 2008; Salzman et al., 2010). In addition, IEC have been shown to condition dendritic cells to induce Treg cells (Iliev et al., 2009a), indicating that besides providing a barrier, IEC actively take part in the innate immune response and shaping adaptive immunity. As IEC are at the interface between the intestinal lumen and the lamina propria, IEC may serve as an interesting target for intervention strategies to modulate immune responses in the gut.

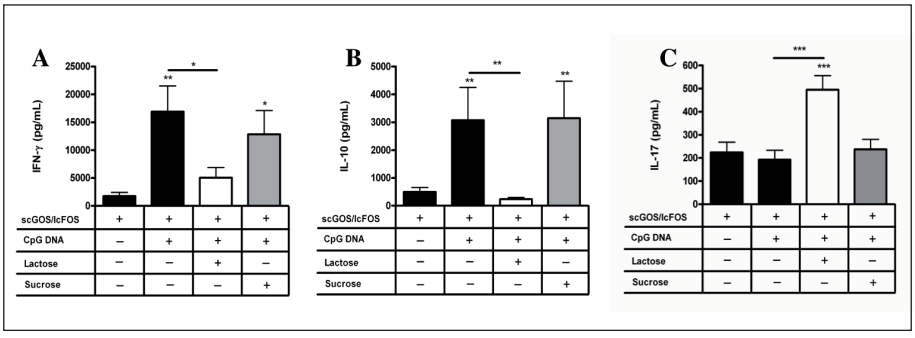

Figure 4 Neutralization of galectins by lactose redirects the TLR9 and scGOS/lcFOS-induced Th1/Treg response to an inflammatory Th17 response. *IEC/PBMC co-cultures were performed in the presence of lactose or sucrose in the basolateral compartment. Sucrose served as negative control. Secretion of IFN-γ (A), IL-10 (B) and IL-17 (C) were measured after 24h of culture. Presence of lactose, but not sucrose, suppressed IFN-γ and IL-10 secretion, while the production of TNF-α, IL-17, IL-12 and IL-6 were increased (C-F). Data represent n=6 independent PBMC donors, mean ± SEM, *P<0.05, **P<0.01, ***P<0.001*

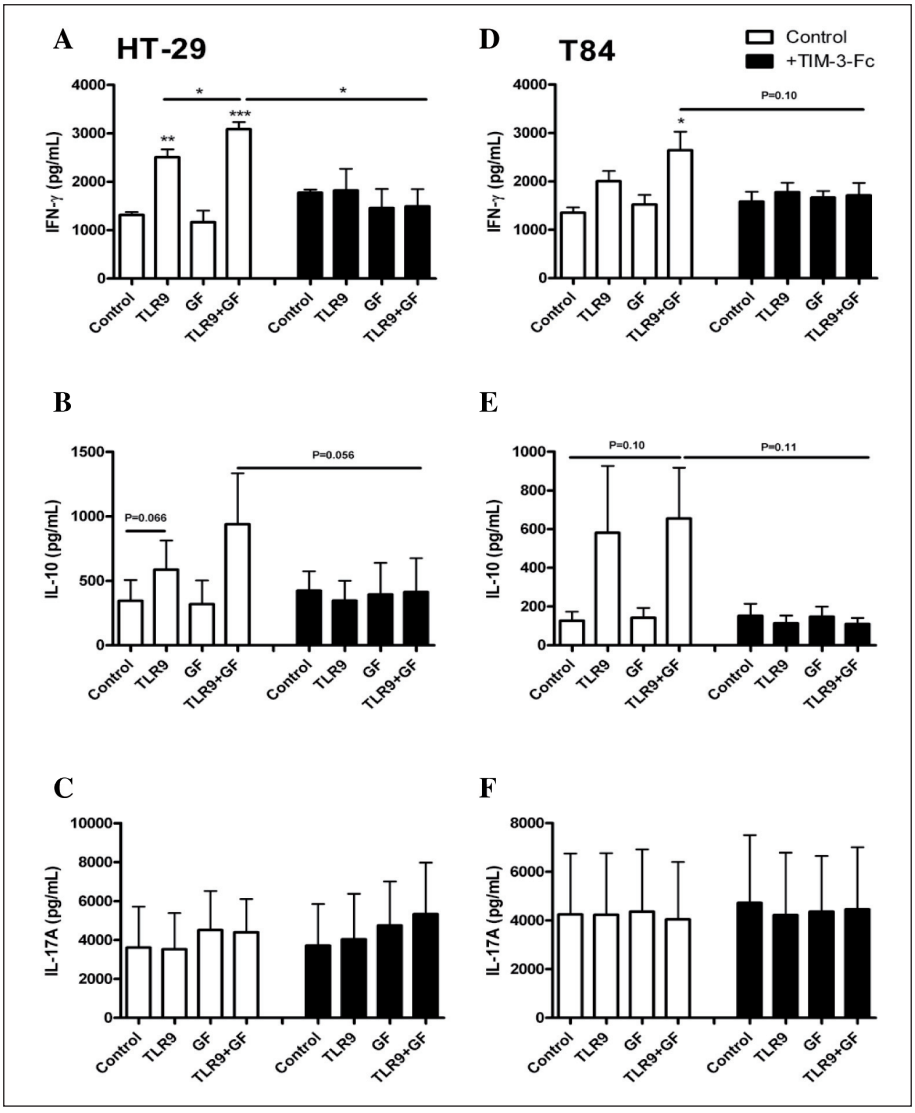

Figure 5 Galectin-9 neutralization in IEC/PBMC co-cultures abrogates IFN-γ and IL-10 secretion by PBMC. *HT-29 (unpolarized) and T84 (polarized) IEC were co-cultured with CD3/28-activated PBMC for 24h and apically exposed to TLR9 ligand in presence or absence of scGOS/lcFOS. Basolateral galectin-9 was neutralized using TIM-3-Fc fusion protein. Galectin-9 neutralization abrogated the induction of IFN-γ(A, D) and IL-10(B, E) by PBMC, but did not modulate IL-17A secretion by PBMC(C, F). Data are represented as mean ± SEM of 3 independent PBMC donors. *P<0.05, **P<0.01, ***P<0.001*

IEC are unresponsive to TLR stimuli under non-inflammatory conditions and it has been shown that modulation of immune responses by TLR ligands can only be observed under inflammatory condition, which is mimicked when IEC are co-cultured with CD3/28-activated PBMC (Abreau et al., 2001; Melmed et al., 2003; de Kivit et al., 2011b). Upon co-culture of IEC with CD3/28-activated PBMC, we observed that exposure of IEC to scGOS/lcFOS combined with TLR9 ligand most effectively enhanced IFN-γ secretion and the number of Th1 cells and Treg cells in an epithelial cell-dependent manner. This is in line with *in vivo* studies showing that scGOS/lcFOS most effectively suppress food allergic symptoms when combined with *Bifidobacterium breve* M-16V (Schouten et al., 2009). Similar to a synthetic TLR9 ligand, genomic DNA from *Bifidobacterium breve* M-16V enhanced IFN-γ production by PBMC, indicating an additive effect of scGOS/lcFOS on TLR9-induced Th1 polarization via IEC. Dietary supplementation of scGOS/lcFOS is known to support a Th1 type effector response as shown in a murine vaccination model and prevents the allergic response in cow's milk allergic mice in association with increased Th1 and Treg polarization in mesenteric lymph nodes (Schouten et al., 2009; Vos et al., 2006; van 't Land et al., 2010). Although a relative small increase in CXCR3+ Th1 cells was found, CD4+ T cells markedly increased the production of IFN-γ. In addition, splenocytes of whey-sensitized mice fed a scGOS/lcFOS diet protected recipient mice from developing whey-induced allergic symptoms. Depletion of CD25+ Treg cells from splenocytes before transfer into recipients did not the development of acute allergic symptoms, indicating a possible induction of allergen-specific Treg (Schouten et al., 2010). Treg cells suppress effector immune responses through several mechanisms, including production of IL-10. However, our data show that although apical exposure of IEC to TLR9 ligand in presence of scGOS/lcFOS further enhances CD4+Foxp3+ T cells, IL-10 production by CD4+ cells was not further enhanced. We have previously shown that TLR9 exposed IEC enhances IL-10 production by antigen presenting cells (de Kivit et al., 2011b). Since IEC are described to condition dendritic cells, further studies are needed to reveal whether IEC-derived galectin-9 is involved in conditioning dendritic cells and concomitantly T cell activation and polarization. Our results, together with *in vivo* and human studies have shown that dietary intervention using scGOS/lcFOS can induce Th1 and Treg polarization, indicating that the IEC/PBMC co-culture model represents the *in vivo*.

Although *in vivo* studies point out a potential role for scGOS/lcFOS as an effective dietary intervention strategy to modulate intestinal immune responses, the underlying mechanisms are poorly understood. Only recently, we have observed that IEC may secrete galectin-9 in sensitized mice fed scGOS/lcFOS and Bifidobacterium breve M-16V upon oral

allergen challenge, thereby potentially shifting the balance towards a Th1 and Treg driven effector response. Furthermore, it was shown that serum galectin-9 levels were most effectively increased in mice fed scGOS/lcFOS in combination with *Bifidobacterium breve* M-16V, stressing the potential additive effect between non-digestible oligosaccharides and TLR ligation (de Kivit et al., 2012). Our *in vitro* observations suggest that apical TLR9 ligation is involved in galectin-9 expression and secretion by IEC. Regulation of galectin expression is largely unknown. However, in endothelial cells, galectin-9 expression is regulated by IFN-γ in a PI3K/IRF3-signaling pathway, involving HDAC3 (Alam et al., 2011; Imaizumi et al., 2007). Although more research is necessary to identify how galectin expression in IEC is regulated possibly involving TLR, secretion of galectin-9 by IEC may induce IFN-γ and IL-10 production by PBMC, indicating that galectin-9 is involved in regulating adaptive immune responses.

Galectin-9 is a ligand for the TIM-3 receptor expressed by Th1, Th17 and dendritic cells. Activation of TIM-3 has been described to induce apoptosis of Th1 and Th17 cells, but also to activate dendritic cells, which instruct naïve CD4+ T cells to produce IFN-γ, but not IL-4 or IL-5 (Zhu et al., 2005; Dai et al., 2005). Therefore, galectin-9 may induce Th1 polarization and via an auto-feedback mechanism suppress exaggerated Th1 responses by inducing Th1 cell apoptosis and inducing Treg cell polarization. However, in contrast to the present observations in this study showing the involvement of galectin-9 in suppression of allergic symptoms, galectin-9 has been shown to sustain Th2 polarized food allergic response as well (Chen et al., 2011). Hence, the mucosal microenvironment may play a crucial role in the function of galectin-9. Galectin-9 is mainly known for its capacity to induce apoptosis of activated Th1 cells (Zhu et al., 2005). Neutralization of galectin-9 in the performed IEC/PBMC co-cultures showed that TLR9-induced IFN-γ boosted by scGOS/lcFOS can be abrogated. This was observed using both unpolarized HT-29 cells and polarized T84 cell lines. Dose-response experiments using recombinant galectin-9 added to CD3/28-activated PBMC indeed showed that galectin-9 can suppress IFN-γ and IL-17 production. However, lower galectin-9 concentrations induced IFN-γ and IL-10 secretion (de Kivit et al., 2012), again indicating that the function of galectin-9 may be dependent on the mediators locally produced in the intestinal mucosal environment. Likewise, *in vivo* studies have shown that galectin-9 is involved in the suppression of Th17-mediated immune responses, through promoting the induction of Treg cells (Seki et al., 2008). Neutralization of galectin-9 tended to suppress IL-10 secretion as well. Although TLR9 ligation on PBMC on its own can induce IL-10 production, apical TLR9 ligation of IEC results in secretion of galectin-9 that has the ability to regulate IL-10 production as well. This indicates that galectin-9 secreted by IEC, as observed in the

co-culture model, may be an important regulator of Th1 and Treg polarization.

In summary, our data show apical exposure of IEC to TLR9 ligand in presence of scGOS/lcFOS specifically induces the expression of galectin-9 by IEC, resulting in Th1 and Treg polarization. Our findings indicate the possible involvement of galectin-9 derived from IEC as a potential mechanism by which scGOS/lcFOS enhance Th1 and Treg development. Hence, the presented data add to the current knowledge by which non-digestible oligosaccharides prevent the development of allergic disease.

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5

Galectin-9 induced by dietary synbiotics is involved in suppression of allergic symptoms in mice and humans

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Editorial:

Galectin-9: a suppressor of food allergy?

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The prevalence of food-induced allergy, which affects approximately 5% of children and 3–4% of adults, is on the rise. It is manifested in a variety of symptoms and disorders involving the gastrointestinal and respiratory tracts as well as skin (Sicherer and Sampson, 2010). The pathogenesis of allergic diseases, such as allergic asthma, allergic dermatitis or food allergy is characterized by an imbalance of T helper type 1 (Th1) and type 2 (Th2) function. There are different strategies to modulate immune responses in allergic diseases and many are aimed at decreasing the Th2 response and/or stimulate Th1 immunity (Nguyen and Casale, 2011). Selected probiotic strains, such as *bifidobacteria* or *lactobacilli* proved to be effective in preventing allergy by enhancement of the Th1 response (Gourbeyre et al., 2011). The modification of the fecal flora with growth of *bifidobacteria* could be achieved in infants by dietary supplementation with a special mixture of galacto- and fructo-oligosaccharides (Moro and Arslanoglu, 2005). In adults with asthma, synbiotics (90% short-chain galacto-oligosaccharides, 10% long-chain fructo-oligosaccharides and *Bifidobacterium breve* M-16V) were shown to decrease the influx of inflammatory cells into the lung and reduce allergen-induced Th2 response (van de Pol et al., 2011). In addition, synbiotics may prevent asthma-like symptoms in infants with atopic dermatitis (van der Aa et al., 2011). Moreover, probiotic bacteria reduced the incidence of eczema in high-risk children (Niers et al., 2009) and *Lactobacillus GG* taken during pregnancy was shown to prevent eczema in a randomized controlled trial (Boyle et al., 2011). The underlying mechanism of the protective effects of synbiotics on allergic inflammation is little known. Recently, a probiotic mixture was demonstrated to be effective in redirecting allergen-specific Th2-polarized immune responses towards Th1-T regulatory responses and in the protection against anaphylactic reactions induced in a murine model of food allergy (Schiavi et al., 2011). In the present issue of *Allergy*, de Kivit et al. reported that treatment of whey sensitized mice with

a mixture of galacto- and fructo-oligosaccharides and *Bifidobacterium breve* M-16 (GF/Bb) diminished allergen-induced inflammatory symptoms while significantly increasing the levels of galectin-9 in the intestinal epithelial cells and serum (de Kivit et al., 2012). The authors raised the possibility that galectin-9 is involved in the beneficial effects of GF/Bb in whey-induced food allergy.

Galectin-9 as an immune regulator

Soluble pattern recognition molecules with carbohydrate binding capabilities, such as ficolins, pentraxins and the collagenous lectins (collectins) (Forbes and Haczku, 2010; Orgeig et al., 2010; Haczku, 2008), have been implied in diverse host defense and immune regulatory activities. Galectins are beta-galactoside binding lectins containing a highly conserved sequence motif in their carbohydrate recognition domain (Barondes et al., 1994). Although all galectins bind galactose, they have different affinity to oligosaccharides (Hirabayashi et al., 2002). Galectins were shown to regulate various cellular functions mainly related to inflammatory processes, including cell growth, apoptosis, cell adhesion, migration and immune responses (Liu and Rabinovich, 2010). Recently, increased levels of galectin-9, a member of tandem-repeat type galectins were reported in models of allergic airway (Sziksz et al., 2010; Yamamoto et al., 2007) and skin inflammation (Igawa et al., 2006) as well as food allergy (Chen et al., 2011). The role of galectin-9 in immunoregulation appears to be complex. Originally galectin-9 was suggested to induce the death of Th1 lymphocytes via the T-cell-immunoglobin-domain and mucin-domain (Tim)-3 (Zhu et al., 2005). Recently, however, Su et al. suggested that galectin-9 regulates T-cell function independently from Tim-3 and can elicit the production of pro-inflammatory cytokines in a dose-dependent manner (Su et al., 2011). Dai et al. found that galectin-9 can stimulate the maturation of dendritic cells and promote Th1 effector responses by triggering the production of IFN-γ and IL-2 (Dai et al., 2005). On the other hand, galectin-9 was shown to induce differentiation of naive T cells into Treg cells, suppress the differentiation of Th17 cells *in vitro* and to decrease the levels of IL-17 dose-dependently in experimental autoimmune arthritis *in vivo* (Seki et al., 2008). In the report by de Kivit et al., these latter observations were well corroborated by showing that elevated galectin-9 expression was associated with increased Th1/Th2 and Treg/Th2 ratio in whey-sensitized mice fed GF/Bb (de Kivit et al., 2012). Galectin-9 directly increased the proportion of CD69/CXCR3 and CD25/FOXP3 positive CD4 positive T- cells as well as IFN-γ and IL-10 production in a dose-dependent manner *in vitro*, indicating induction of Th1 and Treg immunity.

Galectin-9 in food allergy

Given the increased expression of galectin-9 in basolateral epithelial cells and the above effects on T-cells, de Kivit et al. hypothesized that this molecule has a significant immunoregulatory function during food-induced allergic inflammation and that galectin-9 may mediate the effects of GF/Bb supplementation in murine model of cow's milk allergy (**Figure 1**). This hypothesis is in contrast to an earlier proposal by Chen et al. who suggested that enhanced expression of galectin-9 in the intestinal epithelial cells of patients with food allergy may contribute to the maintenance of the allergic status of the intestine (Chen et al., 2011). The work of de Kivit et al. demonstrates however that elevated levels of galectin-9 in the serum and intestinal epithelial cells of whey-sensitized mice after GF/Bb treatment in fact negatively correlated with airway hyper-responsiveness and serum mast cell protease levels (van der Aa et al., 2011). Further, while GF/Bb administration diminished the IgE-mediated mast cell degranulation and therefore alleviated the severity of the

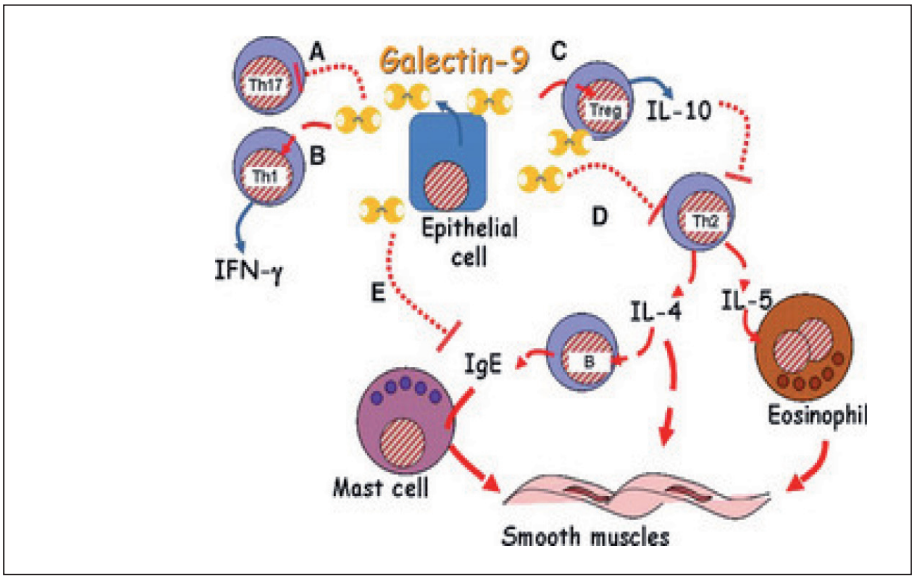

***Figure 1. Galectin-9 plays a protective role during the allergic immune response**. Galectin-9 production and release is upregulated by epithelial cells in response to inflammatory stimuli. Released galectin-9 suppresses Th17 function (A) but enhances Th1 (B) as well as Treg (C) activation. Th2 immunity is suppressed by galectin-9 both indirectly through Treg cells and directly (D), diminishing IL-4 and IL-5 production and the ensuing activation of eosinophilic granulocytes and IgE-producing B-cells. Galectin-9 can additionally, directly bind IgE and prevent antigen-IgE complex formation and mast cell degranulation (E), thereby attenuating the physiological consequences (i.e. smooth muscle activation) of the allergic response.*

disease, these protective effects of synbiotic treatment were partially abolished by the neutralization of serum galectin-9. The GF/Bb-induced suppression of allergic symptoms was also associated with increased galectin-9 levels in the serum of children suffering from IgE-mediated atopic dermatitis. In addition to the potential Th1 polarizing effects and stimulation of Treg differentiation, galectin-9 may have a beneficial role in interfering with IgE-mediated events. Indeed, Niki et al. recently described that galectin-9 binds strongly to IgE, a heavily glycosylated immuno-globulin, preventing the antigen-IgE complex formation and thereby exerting anti-allergic effects (Niki et al., 2009).

In summary, galectin-9, an epithelial product is expressed in mucosal surfaces during inflammatory responses. It is particularly interesting that administration of synbiotics, such as galacto- and fructo-oligo saccharides and *Bifidobacterium breve* M-16 co-treatment, significantly enhances release of galectin-9 in the gastrointestinal tract. The results of de Kivit et al. strongly suggest that the protective effects of synbiotic treatment are, at least partly, mediated by a dual anti-allergic action of galectin-9 via modulation of Th1 and Treg cell polarization and IgE sequestration resulting in attenuated mast cell degranulation (de Kivit et al., 2012). Based on these findings clinical verification of the therapeutic significance of galectin-9 is warranted.

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Abstract

Background: Prebiotic galacto- and fructo-oligosaccharides (scGOS/lcFOS) resembling oligosaccharides in human milk reduce the development of atopic disorders. However, the underlying mechanisms are still unclear. Galectins are soluble type lectins recognizing β-galactoside containing glycans. Galectin-9 has been shown to regulate mast cell degranulation and T cell differentiation. In this study, the involvement of galectin-9 as a mechanism by which scGOS/lcFOS in combination with *Bifidobacterium breve* M-16V protects against acute allergic symptoms was investigated. **Methods**: Mice were sensitized orally to whey, while being fed a diet containing scGOS/lcFOS and *Bifidobacterium breve* M-16V (GF/Bb) or a control diet. Galectin-9 expression was determined by immunohistochemistry in the intestine and measured in the serum by ELISA. T cell differentiation was investigated in the mesenteric lymph nodes as well as in galectin-9-exposed PBMC cultures. Sera of the mice were evaluated for the capacity to suppress mast cell degranulation using a RBL-2H3 degranulation assay. In addition, in a double-blind, placebo-controlled multicentre trial, galectin-9 levels were measured in sera of 90 infants with atopic dermatitis that received hydrolyzed formulae with or without GF/Bb. **Results**: Galectin-9 expression by intestinal epithelial cells and serum galectin-9 levels were increased in mice and humans following dietary intervention with GF/Bb and correlated with reduced acute allergic skin reaction and mast cell degranulation. In addition, GF/Bb enhanced Th1 and Treg cell differentiation in mesenteric lymph nodes and in PBMC cultures exposed to galectin-9. **Conclusions**: Dietary supplementation with GF/Bb enhances serum galectin-9 levels, which associates with the prevention of allergic symptoms.

Introduction

The gastrointestinal immune system is the largest and most complex immunological organ of the human body. It effectively maintains homeostasis to harmless food antigens. Loss of tolerance towards food antigens results in food allergy (FA) (Cardoso et al., 2008), which is in majority characterized by induction of a Th2 polarized immune response during the sensitization phase resulting in production of allergen-specific IgE. Subsequent exposure to the allergen causes diarrhea and ultimately anaphylactic shock through IgE-mediated mast cell degranulation. Intestinal epithelial cells (IEC) provide a first line barrier between luminal contents and immune cells. However, emerging evidence points out that IEC are important regulators involved in modulating immune responses (Artis, 2008; Iliev et al., 2009).

In a murine model for cow's milk allergy (CMA), dietary supplementation of a specific prebiotic 9:1 mixture of short-chain galacto-oligosaccharides (scGOS; [Galβ1-4]3-8Glc; Gal, galactose; Glc, glucose) and long-chain fructo-oligosaccharides (lcFOS, ([Frcβ2-1]>20Frcβ2-1Glc; Frc, fructose) (scGOS/lcFOS) reduces the acute hypersensitivity response (AHR) to whey –a major allergen in CMA (Schouten et al., 2010). scGOS/lcFOS structurally and functionally resemble non-digestible oligosaccharides present in human milk, which are involved in maturation of immune responses of young infants and oral tolerance induction (Field, 2005; Newburg, 2000). A synbiotic diet containing scGOS/lcFOS and *Bifidobacterium breve* M-16V (GF/Bb) reduced AHR in the CMA model even more pronounced (Schouten et al., 2009) and reduced the atopic dermatitis score in infants suffering from IgE-mediated eczema after 12 weeks treatment. Asthma-like symptoms and the prevalence of asthma medication one year later were declined as well (van der Aa et al., 2010; van der Aa et al., 2011). The underlying mechanisms by which scGOS/lcFOS exerts its protective effect on AHR are unknown, however scGOS/lcFOS may interfere with mast cell degranulation and may induce Th1 and Treg cell polarization.

Galectins are soluble type lectins expressed by IEC exhibiting binding specificity for β-galactosides (Leffler et al., 2002; Hirabayashi et al., 2002). IEC express galectin-2, -3, -4 and -9 (Nio-Kobayashi et al., 2009; Wada et al., 1997), which are localized in the cytoplasm, but are secreted through yet unknown mechanisms. Galectins are involved in regulation of immune responses and tolerance induction by inducing signaling through formation of galectin-glycoprotein lattices on cell surfaces (Rabinovich and Toscano, 2009; van Kooyk and Rabinovich, 2008). Recently, galectin-9 was shown to neutralize IgE and to induce Treg type immune responses (Niki et al., 2009; Seki et al., 2008). We hypothesized that galectin-9 is involved in the suppression of allergic disease.

Material and Methods

Murine model for CMA

Animal use was performed in accordance with guidelines of the Dutch Committee of Animal Experiments. The CMA model was performed as described (Schouten et al., 2009). Three-week-old specific pathogen-free C3H/HeOuJ mice (Charles River Laboratories, Maastricht, The Netherlands) were fed a control diet, *Bifidobacterium breve* M-16V (2% wt:wt $2x10^9$ CFU/g; probiotic), scGOS/lcFOS (1%wt:wt, 9:1 scGOS:lcFOS; prebiotic) or a combination of both (GF/Bb) as described (Schouten et al., 2010). Prebiotics were exchanged for the same amount of total carbohydrates in the diet. AHR was measured after 1h after i.d. challenge with whey in the ear pinnae (10µg/100µL PBS). Ear thickness was measured in duplicate using a digital micrometer (Mitutoyo, Veenendaal, The Netherlands). Ear swelling was calculated by subtracting basal ear thickness from the ear thickness after 1h. Oral challenge was performed using 100mg whey/0.5mL PBS (**Figure 1A**).

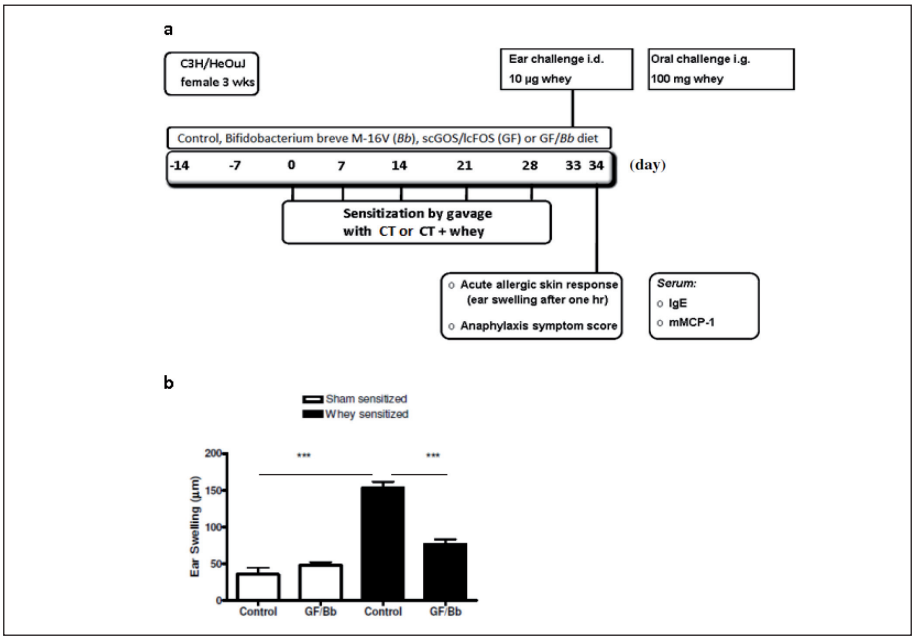

Figure 1 scGOS/lcFOS in combination with Bifidobacterium breve M-16V (GF/Bb) suppress AHR in mice. *(A) Schematic overview of the murine CMA model. Mice were fed a control diet or a diet supplemented with either Bifidobacterium breve M-16V, scGOS/lcFOS or a combination of both starting 2 weeks before and during 5 consecutive weeks of sensitization to whey, using cholera toxin (CT) as adjuvant. (B) One hour after intradermal challenge with whey in the ear, AHR was reduced in whey-sensitized mice treated with GF/Bb compared to whey-sensitized mice. Data represent mean±SEM of 6 mice, ***P<0.001.*

Immunohistochemistry

Mouse swiss rolls from proximal ileum were fixed in neutral 10% formalin for 24h and paraffin embedded. 5µm sections were mounted on coated slides, dewaxed and microwaved in 0.01M sodium citrate for 10min and cooled down for 30min. Aspecific background was blocked with PBS/5% rabbit serum/1%BSA for 30min and incubated overnight at 4°C with polyclonal goat-anti-mouse galectin-9 antibody (R&D Systems, Minneapolis, MN, USA) in PBS/1%BSA. Galectin-9 was detected with biotinylated donkey-anti-goat antibody (Dako, Glostrup, Denmark) in PBS/1%BSA for 1h. Slides were incubated in 3%H2O2/PBS for 30min, followed by ABC-HRP complex (Vector Laboratories, Burlingame, CA, USA) for 1h. Staining was visualised using DAB for 10min and counterstained with Mayer hematoxylin.

ELISA

IL-10, IFN-γ (Biosource CytoSetsTM, Nivelles, Belgium), IL-17A (Arcus Biologicals, Modena, Italy) and mouse mast cell protease (mMCP)-1 (Moredun Scientific, Midlothian, UK) were measured according to manufacturer's protocol. Specific serum immunoglobulins were measured as described (Schouten et al., 2009). For galectin-9, high-binding EIA/RIA 96-well plate (Costar, Lowell, MA, USA) were coated with polyclonal goat-anti-human or anti-mouse galectin-9 antibodies(R&D Systems) in PBS overnight at 4°C. Plates were blocked 1h with PBS/1%BSA. Samples (1:2, 1:5 or 1:10 dilutions) were added for 2h and incubated 1h with biotinylated polyclonal goat-anti-human galectin-9 antibodies (R&D Systems) in PBS/1%BSA. Plates were incubated 1h with streptavidin-HRP (R&D Systems) followed by development with tetramethylbenzidine (TMB, Thermo Scientific, Rockford, IL, USA). The reaction was stopped with 2M H2SO4 and optical density (OD) was measured at 450nm. The 1:5 dilution was most optimal for the mice sera, with OD values ranging between 0.05 – 2.416 after correcting for the OD value of the negative control (0.054). Samples were added in a single plate, allowing direct comparisons of the OD values.

RBL-2H3 assay

The release of β-hexosaminidase by the rat basophil leukemic RBL-2H3 cell line was measured as a model for mast cell degranulation. RBL-2H3 cells were cultured in minimal essential medium (MEM) supplemented with L-glutamine, 10%RPMI1640, 10%FCS and penicillin(100U/mL)/streptomycin(100µg/mL)(Sigma, Zwijndrecht, The Netherlands). RBL-2H3 cells ($1x10^5$) were cultured in 96-well culture plates for 1h at 37°C/5%CO2. RBL-2H3 cells were incubated with 15%v/v anti-DNP-OVA IgE and washed in Tyrode buffer. Cells were stimulated with DNP-HSA (25ng/mL)(Sigma) for 1h. Incubation with Triton X-100 was used as 100% degranulation. Supernatants were collected and incubated 1h with 4-methylumbelliferyl-N-acetyl-β-D-glucsoaminide. The reaction was stopped with glycine

(pH=10.7) and β-hexosaminidase release measured by fluorescence (excitation: 360nm, emission: 460nm). Lactose (100mM, sucrose as negative control) or murine T-cell immunoglobulin and mucin (TIM) domain-3-Ig fusion protein (R&D Systems) were used to neutralize serum galectin-9.

cDNA synthesis and real-time PCR

Mesenteric lymph nodes (MLN) were collected in 200µL RNAlaterTM (Qiagen GmbH, Hilden, Germany), 1cm proximal ileum was snap frozen in liquid nitrogen. Samples were stored at -20oC untill cDNA synthesis. mRNA was isolated using the mRNA capture kit (Roche). Real-time PCR was performed as described (Garcia-Vallejo et al., 2004). GAPDH was used as reference gene. Relative target mRNA abundance was calculated by: relative mRNA abundance=$100x2^{Ct[GAPDH] - Ct[target\ mRNA]}$. Primers were commercially purchased (SABiosciences–Qiagen GmbH, Hilden, Germany).

Purification of PBMC

Human peripheral blood mononuclear cells (PBMC) from healthy donors were isolated from buffy coats (Sanquin, Amsterdam, The Netherlands) and purified using Ficoll-Paque Plus (GE Healthcare Life Sciences, Uppsala, Sweden) gradient centrifugation(1000g, 20min). PBMC were collected and washed in PBS/2%FCS. Remaining erythrocytes were removed using lysis buffer (4.14g NH4Cl, 0.5g KHCO3, 18.6mg Na2EDTA in 500mL water, pH=7.4 and filter sterilized) for 5min on ice. PBMC were resuspended in RPMI1640 (Lonza, Verviers, Belgium) supplemented with 2.5%FCS, penicillin(100U/mL)/streptomycin (100µg/mL) and sodium pyruvate(1mM; Sigma).

PBMC stimulation

PBMC were stimulated with anti-CD3 (CLB-T3/2) and anti-CD28 antibodies (CLB-CD28; both 1:10,000, Sanquin) in the presence of recombinant galectin-9 (0.04mM–1.0mM, kindly provided by Dr. L.G. Baum, UCLA School of Medicine, Los Angeles, USA)(Bi et al., 2008) for 24h.

Flow cytometry

Cells were stained with CD4-PerCP-Cy5.5 (OKT-4), CD69-PE (FN50), CD25-AlexaFluor488 (BC96), Foxp3-PE (236A/E7)(eBioscience, San Diego, CA, USA) and CD183(CXCR3)-AlexaFluor488 (1C6, BD Biosciences, San Jose, CA, USA). Cells were fixed with 0.5% paraformaldehyde or permeabilized using the FoxP3 Staining Set according to manufacturer's protocol (eBioscience). Flow cytometric analysis was performed using a FACSCantoII (BD Biosciences).

Human serum samples

In a double-blind, placebo-controlled multicentre trial, 90 infants with atopic dermatitis received a hydrolyzed formula with or without GF/Bb for 12 weeks as described (van der Aa et al., 2010). The hydrolyzed formula contained 1.3×10^9 CFU/100mL *Bifidobacterium breve* M-16V and 0.8g/100mL scGOS/lcFOS (9:1). At baseline and week 12, a 2-3mL blood sample was collected.

Statistics

Data obtained from the CMA model and clinical study were tested using unpaired Student's t-test or one-way ANOVA. Data obtained from PBMC cultures were analyzed using a one-way ANOVA. Correlation was tested by Pearson's correlation coefficient. Analyses were performed using Graphpad Prism 4.0 (La Jolla, CA, USA). $P<0.05$ was considered statistically significant.

Results

GF/Bb prevents AHR in mice

To study the contribution of GF/Bb in prevention of AHR, a murine model for CMA was used in which mice were orally sensitized and challenged with whey (**Figure 1A**) (Schouten et al., 2009). Whey-sensitized mice showed an increased AHR, and upon dietary supplementation with GF/Bb AHR was reduced, showing a preventive effect of the diet on the development of AHR (**Figure 1B**). However, reduction of allergy was not associated with decreased levels of whey-specific IgE (416±139.7 vs. 633.6±106.2 arbitrary units in allergic mice compared to whey-sensitized mice fed GF/Bb).

Allergic mice fed GF/Bb show specific basolateral galectin-9 expression by IEC

Galectin-9 has been described to neutralize IgE and to induce a Treg mediated immune response (Niki et al.., 2009; Seki et al., 2008). To assess whether galectin-9 is involved in suppression of allergic symptoms induced by GF/Bb *in vivo*, galectin-9 expression in the intestine was investigated. Immunohistochemical staining revealed expression of galectin-9 by IEC (**Figure 2A-E**). Localization of expression of galectin-9 was not changed in whey-sensitized and challenged mice or control mice fed GF/Bb (**Figure 2A-C**). However, whey-sensitized mice fed GF/Bb show specific galectin-9 staining at the basolateral side of IEC (**Figure 2D**), suggesting that GF/Bb induces basolateral epithelial secretion of galectin-9. Galectin-9 expression in ileum and MLN and serum galectin-9 levels were increased in whey-sensitized mice fed GF/Bb (**Figure 2F-H**). The separate components of GF/Bb enhanced serum galectin-9 levels as well, albeit less effectively, suggesting a possible interaction between *Bifidobacterium breve* M-16V-induced TLR signaling and scGOS/lcFOS. Increased serum galectin-9 levels correlated with reduced AHR (**Figure 2I**), which depended on sensitization with whey as sham sensitization did not increase serum galectin-9 levels despite oral challenge to whey. Galectin-4 expression was not modulated by the diet, indicating a galectin-9 specific effect (data not shown).

Galectin-9 enhances Th1 and Treg cell development

To examine whether T cell polarization in whey-sensitized mice fed GF/Bb is modulated, we evaluated the expression of the transcription factors T-bet, GATA-3, RORγT, and Foxp3 as a reflection of respectively Th1, Th2, Th17 and Treg cells in draining MLN as galectin-9 is known for its capacity to induce Th1 cell apoptosis (Zhu et al., 2005). T-bet and Foxp3 expression tended to increase in MLN, while GATA-3 expression was not modulated by GF/Bb (**Figure 3A-C**). Expression of RORγT was expressed at low levels in MLN (**Figure 3D**). Unexpectedly, the Th1/Th2 and Treg/Th2 ratio were significantly increased in whey-sensitized mice fed GF/Bb indicating induction of Th1 and Treg immunity (**Figure 3E** and **3F**). The Treg/Th1 ratio was not modulated (**Figure 3G**). Furthermore, whey-sensitized mice showed a decrease in the Treg/(Th1+Th2) ratio, indicating that Treg cell development over effector T cell induction is suppressed upon whey sensitization compared to controls, while dietary supple mentation with GF/Bb reversed the Treg/(Th1+Th2) ratio in favor of Treg cell development (**Figure 3H**).

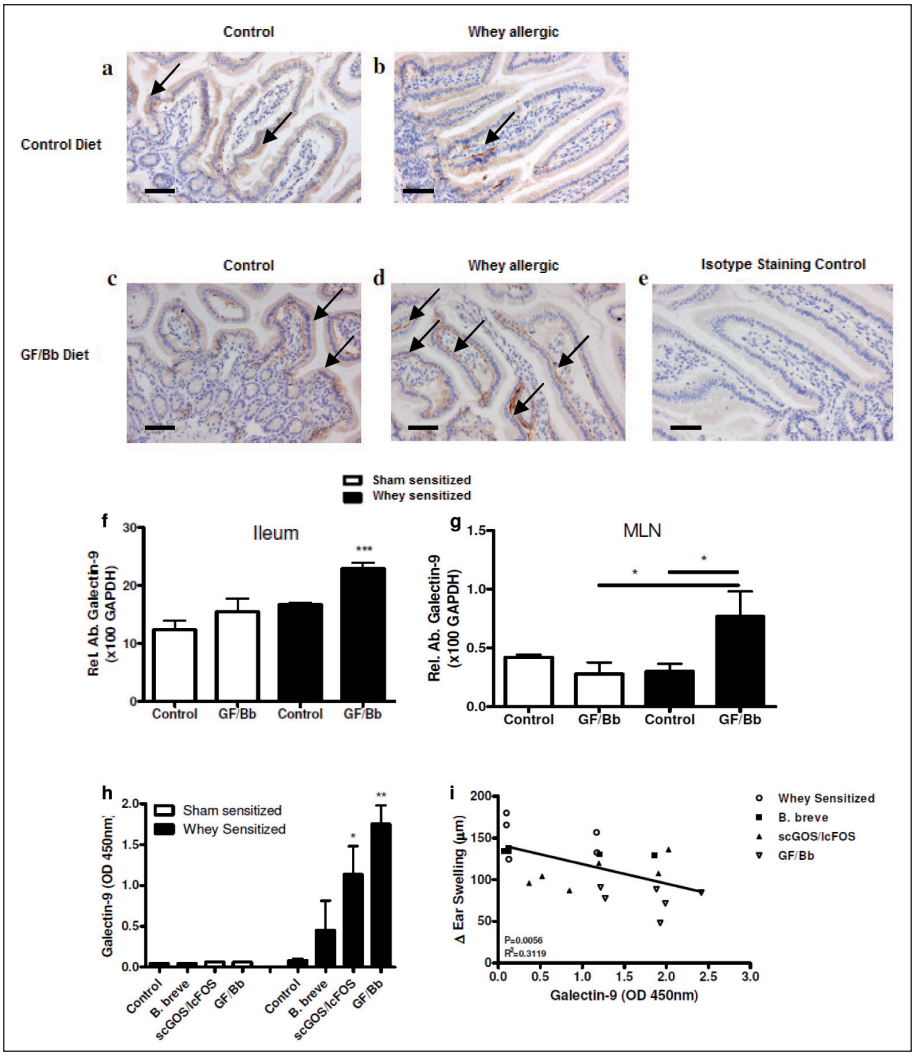

Figure 2 Galectin-9 is basolaterally expressed by IEC and increased in serum in whey allergic mice fed GF/Bb to protect against allergy. *Immunohistochemical analysis of galectin-9 expression in the proximal ileum (A-E). Diffuse galectin-9 staining was observed in the epithelium of sham or whey sensitized mice fed the control diet (A, B) as well as in sham sensitized mice fed GF/Bb (C). In whey-sensitized mice fed GF/Bb, specific basolateral galectin-9 expression was observed (D). Isotype staining control showed no background (E). In whey-sensitized mice – but not sham sensitized mice – fed GF/Bb, galectin-9 expression in ileum(F) and MLN(G) was increased, which was reflected by increased serum galectin-9 levels(H). Increased serum galectin-9 levels correlated with a reduction in AHR in whey-sensitized mice(I). Data represent n=5-6 mice per group, mean±SEM, *P<0.05, **P<0.01.*

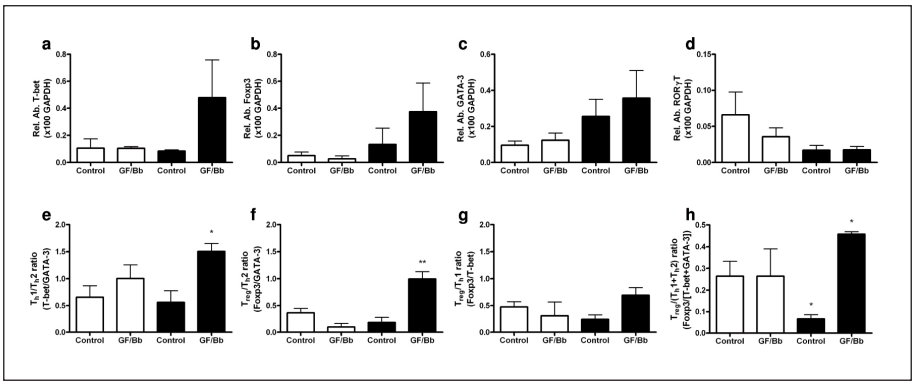

Figure 3 Whey-allergic mice fed GF/Bb show enhanced Th1 and Treg cell development in MLN*. T cell polarization in MLN was evaluated by analyzing the expression of T-bet (Th1, A), Foxp3 (Treg, B), GATA-3 (Th2, C) and RORγT (Th17, D). The expression of T-bet and Foxp3 tended to increase in whey-sensitized mice fed GF/Bb, while GATA-3 expression was not changed. Expression of RORγT was hardly detected. Whey-sensitized mice fed GF/Bb showed an increased Th1/Th2 (E) and Treg/Th2 ratio (F), but the Treg/Th1 ratio remained unaltered (G). The ratio between Treg cells and effector Th1 and Th2 cells (H) was decreased in whey allergic mice and increased in whey-allergic mice fed GF/Bb Data represent n=4 mice per group, mean±SEM, *P<0.05, **P<0.01.*

To study the involvement of galectin-9 in induction of Th1/Treg differentiation of human cells, CD3/CD28-activated PBMC were exposed to increasing concentrations of recombinant galectin-9. Galectin-9 induced the development of Th1 and Treg cells as analyzed within the live CD4+ T cell population (**Figure 4A and 4B**), resulting in increased secretion of IFN-γ and IL-10 and suppressed IL-17 production (**Figure 4C**).

Sera of GF/Bb treated whey-sensitized mice suppress mast cell degranulation

To investigate the involvement of mucosal mast cells as a target of GF/Bb, serum mMCP-1 concentrations were measured. Suppression of AHR was mast cell-associated as serum mMCP-1 concentrations were reduced, indicating that intestinal mast cell degranulation was inhibited (**Figure 5A**). In whey-sensitized mice fed GF/Bb, enhanced serum galectin-9 levels correlated with reduced serum mMCP-1 concentrations (**Figure 5B**). To evaluate whether galectin-9-containing sera from GF/Bb treated whey-sensitized mice were able to reduce mast cell degranulation, a RBL-2H3 degranulation assay was performed. Before IgE cross-linking by the hapten TNP-OVA, RBL-2H3 cells were sensitized with anti-TNP-OVA IgE in presence of sera from GF/Bb-treated animals either sham or whey sensitized. Only serum derived from whey-sensitized GF/Bb treated mice was able to

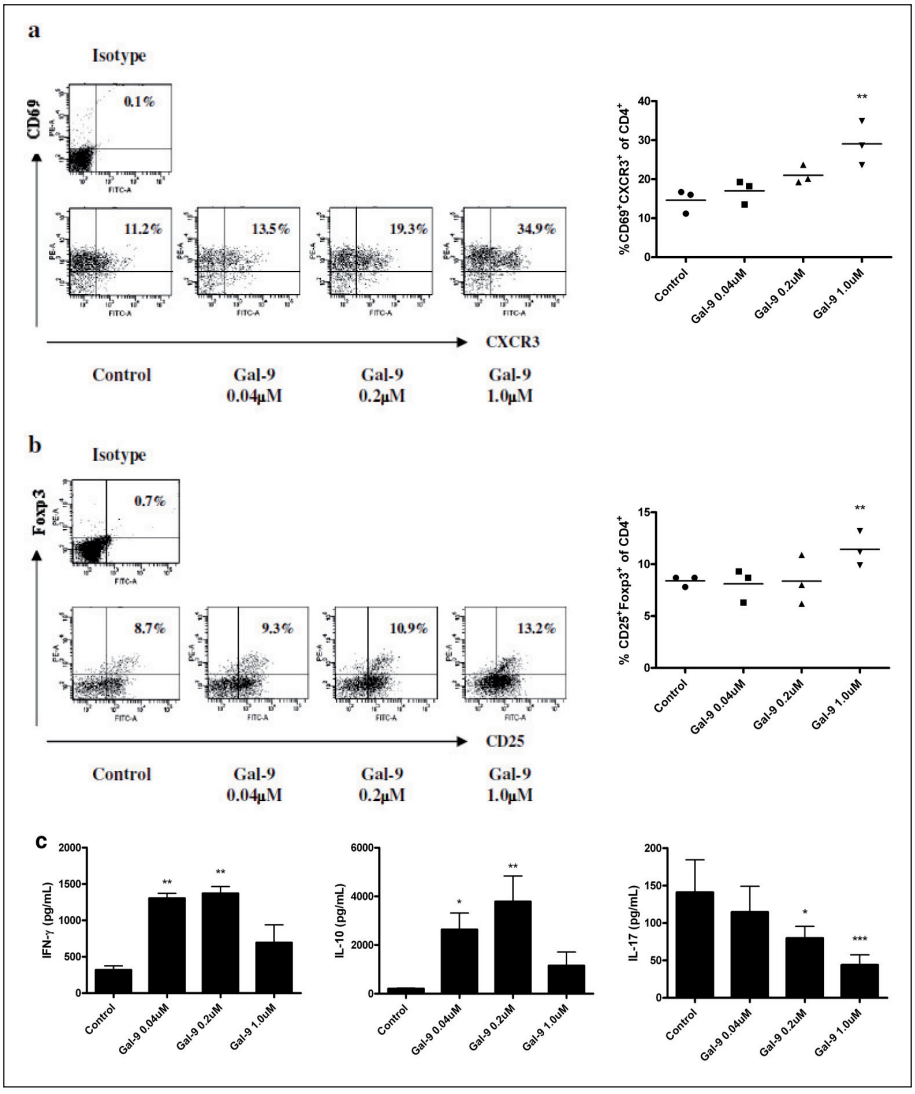

Figure 4 Recombinant galectin-9 induces the development of a Th1 and Treg effector response. *CD3/CD28-activated PBMC were incubated with either medium or increasing concentrations of recombinant galectin-9 for 24h. The number of activated Th1 cells (CD4+CD69+CXCR3+; A) and Treg cells (CD4+CD25+Foxp3+; B) were determined by FACS. Galectin-9 enhanced the percentage of Th1 and Treg cells dose dependently. (C) Cytokines produced by galectin-9 exposed PBMC were measured in the supernatant by ELISA. Data represent n=3 independent PBMC donors, mean±SEM, *P<0.05, **P<0.01, ***P<0.001.*

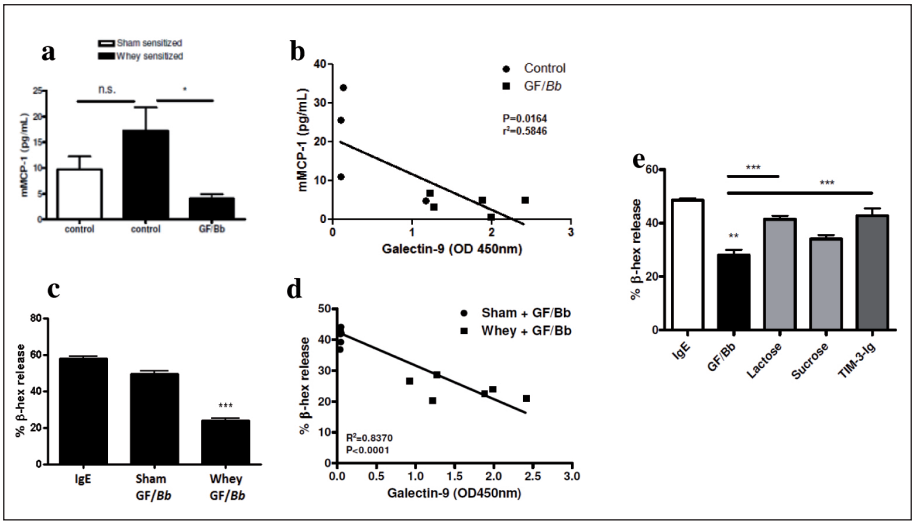

Figure 5 Dietary intervention with GF/Bb suppresses mast cell degranulation. *In vivo mucosal mast cell degranulation was evaluated by serum mMCP-1. Whey-sensitized mice showed a tendency towards increased serum mMCP-1 concentrations after challenge with whey, which was abrogated when mice were fed GF/Bb during sensitization (A). The suppression in serum mMCP-1 concentrations negatively correlated with serum galectin-9 levels (B). In vitro, mast cell degranulation was evaluated by β-hexosaminidase release by RBL-2H3 cells upon IgE cross-linking. RBL-2H3 cells were primed with anti-TNP-OVA IgE in the presence of sera derived from sham or whey-sensitized mice fed GF/Bb. Sera from whey-sensitized mice fed GF/Bb suppressed mast cell degranulation after stimulation with TNP-OVA (C). Anti-TNP-OVA in presence of fetal calf serum was used as control (IgE). The reduced β-hexosaminidase release correlated with increased serum galectin-9 levels (D). Neutralization of galectin-9 by lactose or TIM-3-Ig fusion protein partially abrogated the protective effects of sera from whey-sensitized GF/Bb fed mice (E). Sucrose was used as control for lactose intervention. Data represent n=4-6 mice per group, mean±SEM,*P<0.05, **P<0.01, ***P<0.001, n.s. not significant.*

suppress IgE-mediated degranulation (**Figure 5C**). Indeed, increased serum galectin-9 levels from whey-sensitized mice fed GF/Bb correlated with a reduction of RBL-2H3 cell degranulation (**Figure 5D**). To prove the role of galectin-9 in suppression of RBL-2H3 cell degranulation, we incubated serum of whey-sensitized mice fed GF/Bb with lactose or TIM-3-Ig fusion protein. Neutralization of galectin-9 by lactose or TIM-3-Ig partially abrogated the protective effect of sera derived from whey-sensitized mice fed GF/Bb (**Figure 5E**).

Increased serum galectin-9 levels are associated with GF/Bb-induced suppression allergic symptoms in humans

Recently, it was reported that children suffering from IgE-mediated atopic dermatitis showed reduced allergic symptoms upon treatment with GF/Bb for 12 weeks (van der Aa et al., 2010). To determine the link between serum galectin-9 and reduction of allergic symptoms, we investigated whether galectin-9 levels in serum of these infants were elevated upon treatment with GF/Bb. Serum galectin-9 levels were not different at baseline of intervention. However, after 12 weeks of GF/Bb treatment, serum galectin-9 levels were increased compared to placebo-treated patients (**Figure 6A** and **6B**). IgE levels were not affected upon treatment with GF/Bb (van der Aa et al., 2010).

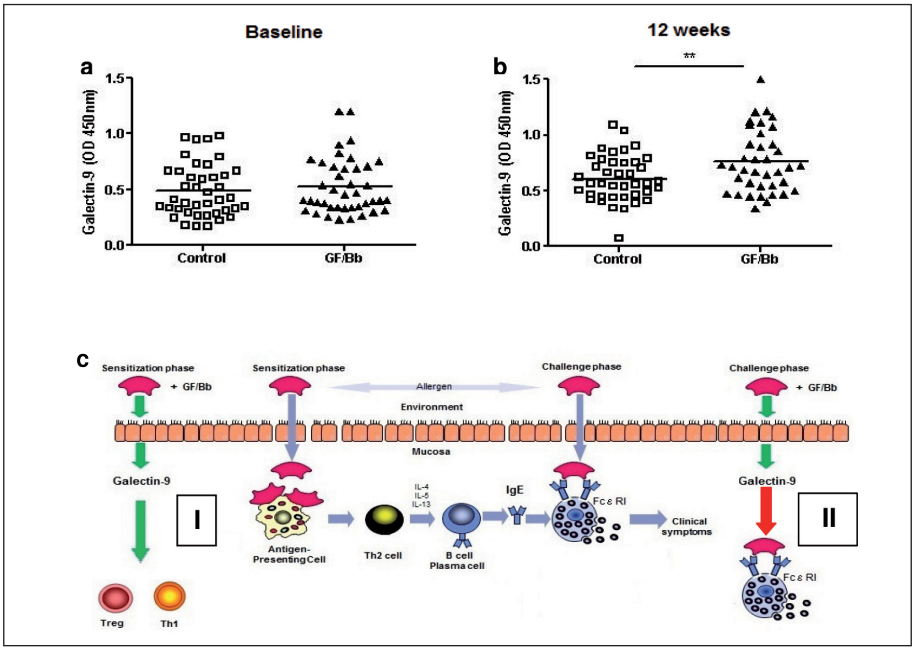

Figure 6 Serum galectin-9 is enhanced in infants that were effectively treated with GF/Bb. *ELISA was performed on serum collected at baseline (A) or at 12 weeks (B) after treatment with the GF/Bb diet of infants suffering from atopic dermatitis. Each patient represent one symbol. Treatment with GF/Bb resulted in enhanced serum galectin-9 levels. The horizontal bar represents mean, **P<0.01. (C) A schematic view of the mechanisms by which GF/Bb suppress IgE-mediated allergic reactions. During allergic sensitization, a Th2 type effector response is raised against an allergen, which results in IgE production by B cells. Upon repeated exposure to the allergen, IgE-primed mast cells degranulate, resulting in clinical symptoms (blue arrows). Supplementation of GF/Bb to the diet two weeks before and during sensitization to whey induces galectin-9 secretion by the intestinal epithelium driving Th1 and Treg cell development (I). During the challenge phase, galectin-9 induced by GF/Bb directly inhibits mast cell degranulation (II). Green arrows: stimulation; red arrows: suppression*

Discussion

scGOS/lcFOS mimic structural and functional properties of non-digestible oligosaccharides present in human milk, which are involved in maturation of immune responses and reduce the risk of developing allergic disease in humans (Field 2005; Newburg 2000; van der Aa et al., 2010; Arslanoglu et al., 2008). scGOS/lcFOS combined with *Bifidobacterium breve* M-16V most effectively reduced AHR in whey-allergic mice (Schouten et al., 2009). Here we demonstrate a novel mechanism by which GF/Bb suppresses allergic symptoms, involving suppression of both mast cell degranulation as well as generating a Th1 and Treg driven immune response through galectin-9.

Classical IgE-mediated allergy is characterized by a Th2 driven immune response against an allergen, resulting allergen-specific IgE-mediated mast cell degranulation. Hence, GF/Bb-induced protection against allergy may affect T cell polarization and mast cell degranulation. Dietary supplementation with scGOS/lcFOS supports a Th1 type effector response and prevents CMA in mice through Treg cells (Schouten et al., 2010; Schouten et al., 2009). We demonstrated that whey-sensitized mice fed GF/Bb showed increased basolateral galectin-9 expression by IEC and increased serum galectin-9 levels. Galectin-9 is a ligand for the TIM-3 receptor expressed by Th1, Th17 and dendritic cells (DC). Activation of TIM-3 induces apoptosis of Th1 and Th17 cells (Zhu et al., 2005). We surprisingly found that galectin-9 was able to drive Th1 and Treg cell development, resulting in increased IFN-γ and IL-10 secretion, but also suppressing IL-17 secretion by activated PBMC. However, a high concentration of galectin-9 abolished the Th1/Treg effector response, possibly by inducing T cell apoptosis. *In vivo* studies, however, have shown that galectin-9 is also involved in suppression of Th17-mediated immune responses, while promoting Treg cell induction (Seki et al., 2008). Furthermore, it has been described that galectin-9 interacting with TIM-3 results in activation of macrophages (Jayaraman et al., 2010). Hence, galectin-9 may induce immunity, while preventing Th1 driven pathology through Th1 cell apoptosis, resulting in a self-regulating immune response.

Galectin-9 has been described to activate DC, which elicit the secretion of IFN-γ, but not IL-4 and IL-5, by CD4+ T cells, promoting a Th1 type immune response (Dai et al., 2005). We observed increased T-bet and Foxp3 expression in MLN, reflecting Th1 and Treg polarization. Upon oral challenge, DC present in the intestinal lamina propria may have been exposed to IEC-derived galectin-9 and migrated towards MLN to induce a Th1 and Treg immune response. Collectively, our data indicate that GF/Bb treatment suppresses AHR through galectin-9-induced Th1 and Treg cell skewing. Although it is uncertain by which mechanisms galectin-9 is involved in

induction of adaptive immunity via DC, future experiments are necessary to investigate whether galectin-9 secreted by IEC directly targets T cells or whether DC are conditioned by galectin-9 to instruct Th1 and Treg cell development.

Despite the observation that GF/Bb largely prevented AHR, IgE levels in serum remained elevated. This was also observed in clinical allergy prevention studies in infants at risk, evaluating the effect of pro-biotics or scGOS/lcFOS on allergy prevention (Abrahamsson et al., 2007; Kalliomäki et al., Lam et al., 2008; Grüber et al., 2010). Using a murine model for CMA, increased serum galectin-9 levels were found to negatively correlate with AHR and serum mMCP-1 levels. Furthermore, sera of whey-sensitized mice fed GF/Bb suppressed IgE-mediated RBL-2H3 cell degranulation, which was partially prevented by neutralizing serum galectin-9 by lactose or TIM-3-Ig. Galectin-9 binds to IgE with high affinity preventing antigen-IgE complex formation to reduce mast cell de-granulation (Niki et al., 2009). Likewise, serum galectin-9 levels were increased in patients affected with atopic dermatitis treated with a hydrolyzed formula containing GF/Bb. Although it was recently shown that galectin-9 expressed by IEC of the duodenum sustained FA via mast cell-derived tryptase in FA patients and in an OVA-induced murine model for FA (Chen et al., 2011), our data implicate a protective role for galectin-9 in prevention of allergic symptoms.

In summary, dietary intervention with GF/Bb induces Th1 and Treg cell polarization through induction of galectin-9 secreted by IEC. In addition, galectin-9 induced by GF/Bb directly suppresses mast cell degranulation (**Figure 6C**). This study shows that GF/Bb may serve as an effective and safe strategy to prevent IgE-mediated allergy.

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6

Dietary non-digestible oligosaccharides condition DC to induce functional Treg responses via intestinal epithelium-derived galectin-9

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Abstract

Non-digestible oligosaccharides (scGOS/lcFOS; GF), which structurally and functionally resemble human milk oligosaccharides, prevent the development of allergic symptoms in mice and humans. In this study, the role of galectin-9 in DC conditioning and regulation of T cell responses under allergic conditions is addressed. We found that intestinal epithelial cells (IEC) apically exposed to TLR9 ligand in the presence of GF suppressed co-stimulatory molecule expression by DC in a galectin-9 dependent manner. Furthermore, galectin-9 conditioned DC supported both the conversion of naïve CD4+ T cells into functional Foxp3+ Treg cells and the downregulation of pro-inflammatory cytokine secretion by DC upon LPS stimulation. *In vivo*, dietary intervention with GF/Bb induced tolerogenic lamina propria DC, expressing low levels of co-stimulatory molecules. In addition, Th2 cytokines were reduced upon *ex vivo* OVA re-stimulation of LP cells from allergic mice when cultured in presence of galectin-9. Thus, GF/Bb induces tolerogenic DC through secretion of galectin-9 by IEC, thereby suppressing oral allergic sensitization.

Introduction

Intestinal dendritic cells (DC) play a crucial role in the induction of tolerance. Many different DC subsets have been identified, which possess specific functions with respect to activation and differentiation of T cells (Dudziak et al., 2007; Merad and Manz, 2009). Antigens given orally can be recognized by DC present in the Peyer's patches (PP) or via dendrites of CX3CR1+ DC, that protrude the intestinal epithelium allowing direct sampling of the intestinal lumen (Kelsall and Strober, 1996; Niess et al., 2005; Rescigno et al., 2001). Upon antigen uptake, CD103+ lamina propria (LP) DC migrate towards mesenteric lymph nodes (MLN) to present antigens to T cells(Schulz et al., 2009). In the MLN, CD103+ LP DC induce the gut homing receptors CCR9 and α4β7 integrin on T cells (Johansson-Lindbom et al., 2005). Furthermore, CD103+ DC have been shown to induce Foxp3+ regulatory T cells through production of retinoic acid and TGF-β, thereby maintaining tolerance towards oral antigens (Coombes et al., 2007; Sun et al., 2007).

Intestinal epithelial cells (IEC) are the first line of defense against luminal antigens. In addition, IEC have been shown to actively regulate immune responses through conditioning DC. In fact, IEC have been described to produce retinoic acid, TGF-β and TSLP, thereby facilitating the development of CD103+ DC and subsequent Treg responses (Iliev et al., 2009a; Iliev et al., 2009b). In addition, IEC suppress co-stimulatory molecule expression by monocyte-derived DC (moDC) (Butler et al., 2006). Furthermore, conditioned medium from Caco2 cells apically exposed to commensal bacteria suppressed the expression of CD40, CD83 and HLA-DR on DC (Zeuthen et al., 2008). In contrast, it was recently shown that ATP released by bacteria facilitates conversion of naïve CD4+ T cells into Th17 cells. It was shown that ATP activates IEC to secrete soluble mediators supporting DC activation (Yao et al., 2012). Thus, IEC play a crucial role in regulating adaptive immune responses by conditioning intestinal DC.

The induction of CD103+ DC and the resulting Treg conversion is protective in murine models of colitis, but less is known about the role of DC in food allergy. A few studies have been conducted showing the importance of DC in food allergy. Transfer of PP CD11c+B220- DC isolated from cow's milk allergic mice into wild type mice induces cow's milk specific IgE responses and these DC produce less IL-10 compared to control DC (Chambers et al., 2004). In addition, it was recently shown that a shift towards decreased CD103+ DC and increased inflammatory CD11b+ DC occurs in a murine model for peanut allergy, whereas pDC expansion using Flt3 ligand *in vivo* induced tolerance (Smit et al., 2011). Some allergens, including the major peanut allergen Ara h 1 and Der p 1, can drive Th2 responses through activation of DC (Royer et al., 2010; Shreffler et al., 2006). Hence, targeting DC may be an effective strategy to prevent food allergic sensitization.

Dietary intervention using a specific 9:1 non-digestible oligosaccharide mixture of short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides (scGOS/lcFOS; GF), which function similarly to human milk oligosaccharides, protects against development of allergic symptoms in mice and humans (Moro et al., 2006; Schouten et al., 2009; Schouten et al., 2010; van der Aa et al., 2010; van der Aa et al., 2011). Upon combination with *Bifidobacterium breve* M-16V (GF/Bb), the suppression of acute allergic skin responses was most pronounced (Schouten et al., 2009). The genomic DNA of *B. breve* M-16V, like a synthetic Toll-like receptor (TLR)-9 ligand, enhanced Treg cell frequency in an *in vitro* IEC/PBMC co-culture model when combined with GF (de Kivit et al., submitted). Recently, it was shown that DNA of commensals, including *Lactobacilli*, and probiotic bacterial strains contain immunosuppressive DNA sequences that suppress DC activation and support Treg conversion in the intestinal lamina propria (Bouladoux et al., 2012). Bacterial DNA stimulates TLR9, and apical TLR9 activation by IEC is known to contribute to intestinal homeostasis (Lee et al., 2006). We have recently shown that GF/Bb can induce galectin-9 expression *in vivo*, which was involved in suppressing mucosal mast cell degranulation(de Kivit et al., 2012). In addition, galectin-9 can promote Treg responses, but also induces Th1 cell apoptosis (Seki et al., 2008; Zhu et al., 2005). Since galectins, including galectin-9, are amongst the most abundantly expressed factors expressed by IEC (Nio-Kobayashi et al., 2009), we hypothesized that IEC-derived galectin-9 plays a role in conditioning DC. Through the use of *in vitro* cell cultures and an ovalbumin (OVA)-induced murine model for food allergy, we show that GF/Bb suppresses the expression of co-stimulatory molecules by DC and induces Treg cell polarization via galectin-9 secretion by IEC.

Materials and methods

Culture of human intestinal epithelial cell lines

Human IEC lines HT-29 (ATCC; HTB-38; MA, USA) and T84 cells (ATCC, CCL-248) were cultured in McCoy's 5A medium (Lonza, Verviers, Belgium) and Dulbecco's modified Eagle medium/F-12 (DMEM/F-12, 1:1) with GlutaMAX (Gibco Invitrogen, Carlsbad, CA, USA) respectively, supplemented with 10% heat-inactivated FCS (Gibco) and penicillin (100U/mL)/streptomycin (100µg/mL) (Sigma, St. Louis, MO, USA). Cells were cultured in 25cm^2 culture flasks (Greiner, Frickenhausen, Germany) and kept in an incubator at 37^{o}C under 5% CO2.

HT-29 cells were seeded one week before experiments in 6-well 0.4µm transwell insert filters, whereas T84 cells were seeded four weeks prior to the experiment. Confluence of IEC monolayers was assessed by transepithelial electrical resistance measurements for T84 cells (1500

Ωxcm^2) or light microscopy for HT-29 cells as HT-29 cells do not build up resistance (125-150 Ωxcm^2). Before stimulation, IEC were basolaterally exposed to recombinant human TNF-α and IFN-γ (both 10ng/mL; Invitrogen) in RPMI1640 (Gibco) supplemented with 10% heat-inactivated FCS, penicillin(100U/mL)/streptomycin (100µg/mL). After 6-12h, the cytokines were washed away and IEC were apically exposed to 5µM TLR9 ligand (M362 CpG oligonucleotide type C; Invivogen, San Diego, USA) in presence or absence of 0.5%w/v of a 9:1 mixture of scGOS (Vivinal GOS, Borculo Domo) and lcFOS (Raftiline HP, Orafti) for 24h. Basolateral conditioned medium was collected and stored at -20°C.

Culture of monocyte-derived dendritic cells

Healthy donor PBMC were isolated from buffy coats (Sanquin, Amsterdam, Netherlands) after gradient centrifugation using Ficoll-Paque Plus (GE Healthcare Life Sciences, Uppsala, Sweden) and resuspended in RPMI1640 medium (Gibco) supplemented with 10% heat-inactivated FCS, penicillin (100U/mL)/streptomycin (100µg/mL). CD14+ cells were isolated by negative selection using magnetic beads (Miltenyi Biotec, Bergish Gladbach, Germany) according to the manufacturer's instructions. Purity was assessed by flow cytometry and was generally 90-95%. Monocyte-derived dendritic cells (moDC) were generated by culturing 10^6 CD14+ cells in RPMI1640 (Gibco) supplemented with 10% heat-inactivated FCS, penicillin (100U/mL)/streptomycin (100µg/mL), recombinant human IL-4 and GM-CSF (30ng/ml and 15ng/ml respectively; Prospec, NY, USA). In some moDC differentiation cultures, recombinant human galectin-9 (1µg/mL; R&D Systems, Minneapolis, MN, USA) was added or culture medium was supplemented with 50% IEC conditioned medium with or without addition of TIM-3-Fc fusion protein (R&D Systems). Medium was refreshed on day 2, 3 and 5 and moDC were collected on day 7.

RALDH activity assay

RALDH activity was assessed using the ALDEFLUOR assay according to manufacturer's protocol (STEMCELL Technologies, Grenoble, France). In short, 10^6/mL moDC were incubated with ALDEFLUOR for 40min at 37°C. Diethylaminobenzaldehyde (DEAB) was added to negative controls. ALDEFLUOR+ cells were analyzed by flow cytometry, using a FACSCantoII and FACSDiva software (BD Biosciences).

Mixed lymphocyte reactions (MLR)

MoDC were collected and 2x10^5 cells were seeded in 96-well U-bottom cell culture plates (Greiner). MoDC were co-cultured with allogeneic CD4+CD45RA+ T cells for 5 days isolated from PBMC by negative selection using magnetic beads (Miltenyi Biotec), according to the manufacturer's protocol. To facilitate the differentiation of CD4+ T cells into Treg cells, exogenous recombinant human TGF-β (1ng/mL; Prospec) was added to the cultures.

T cell suppression assay

CD4+ T cells were isolated using magnetic beads (Miltenyi Biotec) by negative selection according to manufacturer's protocol and $5x10^4$ collected CD4+ T cells were co-cultured for 4 days with $5x10^4$ autologous carboxyfluorescein succimidyl ester (CFSE)-labeled CD4+CD45RA+ naive cells, in presence of human CD3/28 stimulator beads (Dynal, Invitrogen, according to manufacturer's protocol) and recombinant human IL-2 (20ng/mL; Prospec). Naïve CD4+ T cells were CFSE-labelled (eBioscience) by incubation with 1µM CFSE for 10min at 37°C. The staining was stopped by washing the cells twice with PBS/9%FCS. Maximal proliferation was assessed by adding an additional $5x10^4$ unlabelled CD4+CD45RA+ cells to the CFSE-labelled responder cells instead of collected CD4+ T cells to control for cell crowding in the well. Proliferation was assessed using flow cytometry using a FACSCantoII and FACSDiva software (BD Biosciences).

Mice

Three to four weeks old female specific pathogen free Balb/c mice (Charles River Laboratories, Maastricht, The Netherlands) were fed a control diet or a diet supplemented with *Bifidobacterium breve* M-16V (2% wt:wt, $1x10^{11}$ CFU/g) and GF (1% wt:wt, 9:1 GF; GF/Bb) (Schouten et al., 2009) two weeks before and during oral sensitization to ovalbumin (OVA; Sigma). Mice were sensitized by administering 0.5mL sterile PBS containing 20µg/mL cholera toxin (CT) or 0.5mL sterile PBS containing 20µg/mL CT and 40mg/mL OVA per oral gavage once per week for five consecutive weeks. After sensitization, some mice received 10µg OVA/100µL sterile PBS i.d. in the ear pinnae to assess the acute allergic skin response after 1h. Ear thickness was measured in duplicate using a digital micrometer (Mitutoyo, Veenendaal, The Netherlands). Ear swelling was corrected for basal ear thickness before injection. Mice were challenged by administering 100mg OVA/0.5mL sterile PBS per oral gavage. Feces were collected 15-30min. after challenge and water content was assessed as previously described (Schouten et al., 2008). Mice were sacrificed 18h after the oral challenge and blood was collected in Minicollect® Z Serum Sep vials (Greiner). Blood was centrifuged for 30min. at 14,000xg and sera were stored at -20°C. Animal use was in accordance with guidelines of the Dutch Committee of Animal Experiments.

Cell isolation from murine tissue

Lymphocytes were collected from Peyer's patches (PP), mesenteric lymph nodes (MLN) and lamina propria (LP). PP and MLN were crushed using 100µm cell strainers. Cells were taken up in RPMI1640 medium, supplemented with 5%FCS and penicillin(100U/mL)/streptomycin (100µg/mL). LP lymphocytes were collected from 12cm proximal jejunum. In short, PP were excised and intestines were opened longitudinally, minced into 1cm fragments and briefly washed in Hank's Balanced Salt

Solution (HBSS; Invitrogen) supplemented with 15μM HEPES, pH = 7.2. Next, intestinal fragments were incubated 15min in HBSS supplemented with 15μM HEPES, 5μM Na2-EDTA, 10%FCS and penicillin(100U/mL)/ streptomycin (100μg/mL), pH=7.2 for four times. After incubation, fragments were vortexed 10s to remove IEC. The EDTA-containing solution was washed away by a 5min incubation of the fragments in RPMI1640 medium, supplemented with 10%FCS and penicillin(100U/mL) /streptomycin (100μg/mL), followed by two times incubation in RPMI1640 medium, supplemented with 5%FCS and penicillin(100U/mL)/streptomycin(100μg/mL) and 0.25mg/mL collagenase type VIII (Sigma) for 45min. LP cells were collected by vortexing intestinal fragments for 10s after each 45min incubation to completely disrupt the intestinal tissue. Afterwards, FCS to 10% was added to the cell suspension. LP cells were subjected to Percoll gradient centrifugation (850xg for 20min.). LP lymphocytes were collected from the interphase and cells were washed by adding an excess of RPMI1640 medium, supplemented with 5%FCS and penicillin(100U/mL)/streptomycin (100μg/mL).

Culture of bone marrow-derived DC (BMDC)

BMDC were cultured as described by Lutz et al. (Lutz et al.,1999). Bone marrow cells were collected in 5mL RPMI1640 medium, supplemented with 5%FCS, penicillin(100U/mL)/streptomycin (100μg/mL), non-essential amino acids (Gibco) and 50μM β-mercaptoethanol. Cell suspensions were filtered using 100μm sterile filters and 10^6 bone marrow cells were cultured in the presence of 20ng/mL rmGM-CSF (Prospec). On day 3, an equal volume of culture medium containing 20ng/mL rmGM-CSF was added. On day 6, half of the medium containing cells was removed, centrifuged for 5min at 1500rpm and replaced with fresh culture medium containing rmGM-CSF and BMDC were collected on day 7 or 8. In galectin-9 conditioning experiments, 1μg/mL recombinant mouse galectin-9 (R&D Systems) was added from day 3 till day 7 of culture. At day 7, BMDC were stimulated with 1μg/mL LPS, and expression the of co-stimulatory molecules and cytokine production was analyzed after 48h.

ELISA

OVA-specific serum immunoglobulins were measured using similar methods as previously described for whey-specific immunoglobulins (Schouten et al., 2008). Microlon plates (Greiner) were coated with 20μg/mL OVA in carbonate/bicarbonate buffer, 0.05M, pH=9.6. Plates were blocked for 1h with PBS/5%BSA. Next, samples were applied in multiple dilutions and incubated for 2h, followed by incubation with biotinylated rat anti-mouse IgE, IgG1 or IgG2a (1μg/mL; BD Biosciences) for 90min. Plates were subsequently incubated for 1h with streptavidin-horse radish peroxidase (0.5μg/mL; Sanquin) and were developed using o-phenylen diamine. Reactions were stopped using 2M H2SO4 and optical density was measured at 490nm. using a microplate reader (Bio-Rad).

Flow cytometry

Cells were collected and resuspended in PBS/2%FCS. MoDC were characterized using CD16-PE(3G8; BD Biosciences), DC-SIGN-FITC(120507), CX3CR1-FITC (528728; both R&D Systems), CD14-PerCp-Cy5(61D3), CD40-FITC(5C3), CD80-PE(2D10.4), CD83-PE(HB15e), CD86-PE(IT2.2), CD103-APC(B-Ly7), HLA-DR-PE(LN3) or CD103-AlexaFluor®647(B-Ly7)(eBioscience) antibodies. Human CD4+ T cells were extracellularly stained using CD4-PerCP-Cy5.5(OKT-4) and CD25-AlexaFluor488(BC96) antibodies followed by fixation and permeabilization using the Foxp3 staining buffer set (eBioscience) according to manufacturer's protocol. T cells were intracellularly stained using Foxp3-PE (PCH101, eBioscience) antibodies. FITC-labelled mouse IgG2b, PE-labelled mouse IgG2b, PerCP-Cy5.5-labeled mouse IgG1 and eFluor®660-labelled mouse IgG1 antibodies were used as isotype controls(eBioscience). After staining, cells were taken up in PBS/2% FCS, fixed with 2% paraformaldehyde and measured for mean fluorescence intensity.

Murine cells isolated from PP, MLN and LP were collected in PBS/2%FCS, Fcγ-receptors were blocked using 10µg/mL CD16/CD32 antibodies, followed by extracellular staining using CD11c-PerCP-Cy5.5(N418), I-A/I-E-APC-Cy7(M5/114.15.2), CD40-FITC(HM40-3), CD80-APC(16-10A1), CD83-FITC(Michel-17), CD86-APC(GL1), CD103-APC(2E7), CD4-PerCP-Cy5.5(RM4-5) and CD69-FITC(H1.2F3) antibodies (eBioscience). Stained cells were either fixed in 0.5% paraformaldehyde or permeabilized for intracellular staining as described above. Intracellular staining was performed using Foxp3-APC(FJK-16s), T-bet-eFluor660(eBio4B10), GATA-3-PE(TWAJ), IL-4-PE-Cy7(BVD6-2462), IL-10-PE(JES5-16E3) and IFN-γ-APC(XM61.2) (eBioscience). Flow cytometric analysis was performed using a FACSCantoII and FACSDiva software (BD Biosciences).

Cytometric bead assay

Murine IL-1β, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, TNF-α and IFN-γ were measured using a cytometric bead assay according to manufacturer's protocol (BD Biosciences). Flow cytometric analysis was performed using a FACSCantoII and data was analyzed using FCAP software (BD Biosciences).

Statistics

For *in vitro* moDC experiments, data were analyzed using one-way ANOVA for repeated measurements, followed by Bonferroni's post-hoc test. Data derived from murine experiments were analysed by one-way ANOVA, followed by Bonferroni's post-hoc test. $P<0.05$ was considered statistically significant.

Results

IEC apically exposed to TLR9 ligand in presence of GF suppress the expression of co-stimulatory molecules by moDC

Apical TLR9 ligation of IEC contributes to intestinal homeostasis and IEC are described to modulate DC activation (Butler et al., 2006; Lee et al., 2006). Therefore, we assessed whether apical exposure of IEC to TLR9 ligand, in absence and presence of GF, could modulate DC activation. Generation of moDC in presence of conditioned medium derived from IFN-γ/TNF-α-primed HT-29 to simulate inflammatory conditions cells tended to increase the expression of CD80, CD83, CD86 and HLA-DR (**Supplementary Figure 1**). However, differentiation of moDC in presence of conditioned medium derived from HT-29 or T84 (data not shown) cells apically exposed to TLR9 ligand in presence of GF markedly reduced the expression of CD40, CD80, CD83 and HLA-DR compared to control IFN-γ/TNF-α-primed HT-29 cells, whereas CD86 remained unaltered (**Figure 1**). Direct exposure of moDC to TLR9 ligand in the absence or presence of GF did not modulate co-stimulatory molecule expression (**Supplementary Figure 1**). Since IEC-conditioned medium derived from HT-29 cells apically exposed to TLR9 ligand in presence of GF contained increased levels of galectin-9 (**Figure 1B**), we evaluated whether neutralization of galectin-9 abrogated the reduced expression of co-stimulatory molecules. Indeed, the presence of TIM-3-Fc fusion protein – a specific galectin-9 inhibitor – prevented the suppression of co-stimulatory molecules (**Figure 1C-G**).

Galectin-9 conditioned moDC support the differentiation of functional Foxp3+ Treg cells

We next evaluated whether the suppressed expression of co-stimulatory molecules is of functional relevance. Since IEC are known to induce tolerogenic CD103+ DC that induce Treg responses (Iliev et al., 2009b), we assessed the capacity of the IEC conditioned media to generate tolerogenic moDC and subsequently their capacity to induce Foxp3+ Treg cells.

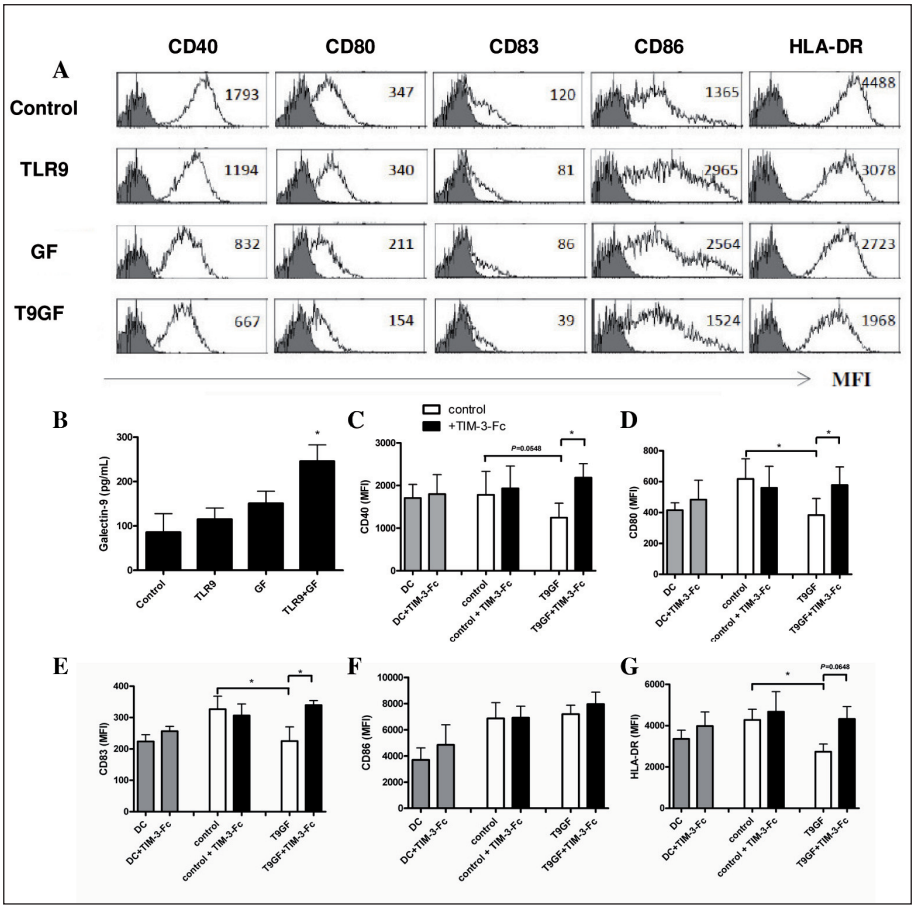

Figure 1 IEC apically exposed to TLR9 ligand in the presence of GF suppress DC activation in a galectin-9-dependent manner. *HT-29 cells were pre-stimulated with TNF-α and IFN-γ, followed by apical exposure to TLR9 ligand in absence or presence of GF. Conditioned media from HT-29 cells were used during moDC differentiation. Expression of co-stimulatory molecules and HLA-DR was measured at day 7 of culture (A). Conditioned medium from HT-29 cells apically exposed to TLR9 ligand suppressed co-stimulatory molecule expression and HLA-DR expression by moDC, which was potentiated by the presence of GF. Apical exposure of HT-29 cells to TLR9 ligand in the presence of GF show increased secretion of galectin-9 (B). Neutralization of galectin-9 in the IEC-conditioned medium from TLR9 ligand exposed IEC in presence of GF abrogated the suppression of co-stimulatory molecule and HLA-DR expression. Direct exposure of moDC (DC) to TIM-3-Fc excludes the involvement of Fcγ receptors (C-G). Mean fluorescence was corrected for isotype background staining. Data represent n=4 independent PBMC donors, mean ± SEM, *P<0.05.*

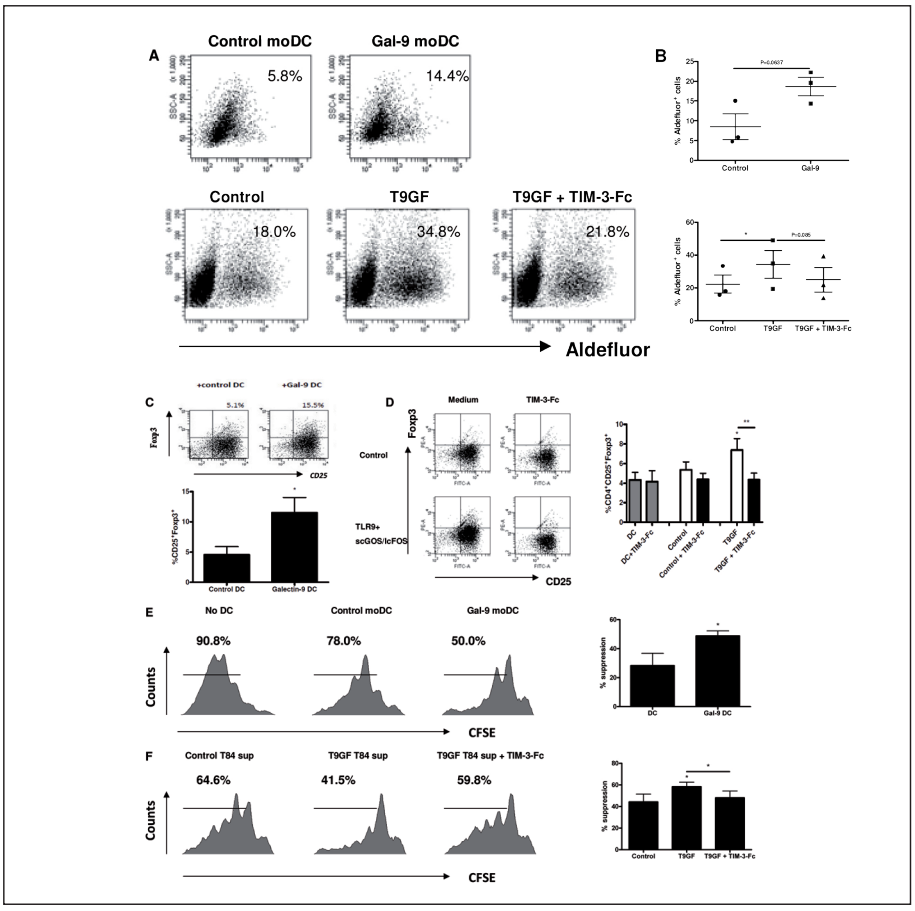

Figure 2 Galectin-9 conditioned moDC support the differentiation of functional Foxp3+ Treg cells. *Monocytes were differentiated into moDC in presence of recombinant human galectin-9 or T84 cell conditioned medium and RALDH activity of moDC was assesed. Galectin-9 conditioned moDC or moDC conditioned by T84 cells apically exposed to TLR9 ligand in presence of GF showed increased RALDH activity by moDC (A, B). MoDC were co-cultured with naive CD4+CD45RA+ T cells for 5 days in presence of exogenous TGF-β. Galectin-9 conditioned moDC had increased capacity to induce Foxp3+ Treg cells (C). Likewise, moDC cultured in presence of conditioned medium from HT-29 cells apically exposed to TLR9 ligand in presence of GF enhanced the conversion of naïve CD4+ T cells into Foxp3+ Treg cells in a galectin-9 dependent manner (D). CD4+ T cells isolated from MLR cultures using galectin-9 conditioned moDC had increased suppressive capacity (E) Likewise, CD4+ T cells derived from MLR cultures using moDC cultured in the presence of supernatant conditioned by T84 cells apically exposed to TLR9 ligand and GF suppressed proliferation of target CD4+ T cells. Addition of TIM-3-Fc to conditioned medium from T84 cells exposed to TLR9 ligand and GF did not generate moDC to induce functional Treg cells (F). Data represent n=4 independent PBMC donors, mean ± SEM, *P<0.05, **P<0.01. T9GF, TLR9 ligand in combination with scGOS/lcFOS.*

Since IEC contribute to the generation of CD103+ tolerogenic DC, we evaluated the capacity of galectin-9 to induce the expression of CD103 on moDC. CD103 expression was not expressed by moDC conditioned with galectin-9 or IEC-conditioned medium from IEC apically exposed to TLR9 ligand in presence of GF (data not shown). However, galectin-9 enhanced the activity of the vitamin A converting enzyme RALDH as measured by increased Aldefluor activity of galectin-9 conditioned moDC compared to control moDC (**Figure 2A** and **2B**). Similarly, medium from HT-29 cells (data not shown) and T84 cells apically exposed to TLR9 ligand in presence of GF enhanced RALDH activity in moDC, which was prevented upon neutralization of galectin-9 in the conditioned medium (**Figure 2A** and **2B**).

As galectin-9 was involved in the suppression of co-stimulatory molecules and the induction of RALDH activity in moDC, moDC were conditioned with recombinant human galectin-9 and co-cultured with naïve allogeneic CD4+ T cells in presence of exogenous TGF-β. Galectin-9 conditioned moDC enhanced Foxp3 expression by CD4+ T cells (**Figure 2C**). Similarly, conditioned medium from HT-29 cells apically exposed to TLR9 ligand in presence of GF generated moDC that have increased capacity to induce Foxp3 expression by CD4+ T cells, which could be blocked using a TIM-3-Fc fusion protein (**Figure 2D**).

Expression of Foxp3 in humans is not restricted to Treg cells (Morgan et al., 2005). Therefore, purified CD4+ cells from mixed lymphocyte cultures were collected and, subsequently, co-cultured with autologous, naïve, CFSE-labeled target CD4+ T cells to assess the suppressive capacity of the generated CD4+Foxp3+ cells. Indeed, galectin-9 conditioned moDC generated CD4+ cells with increased suppressive capacity (**Figure 2E**). Likewise, conditioned medium from HT-29 cells (data not shown) and T84 cells apically exposed to TLR9 ligand in presence of GF generated moDC which induce CD4+ cells that showed a most effective increase in suppressor function (**Figure 2F**).

Galectin-9 conditioned DC secrete lower levels of pro-inflammatory cytokines

To assess the role of galectin-9 in maintaining immunological tolerance, BMDC from Balb/c mice were cultured and conditioned with recombinant galectin-9. BMDC were matured using LPS stimulation and expression of co-stimulatory molecules and cytokine production was assessed after 48h. Galectin-9 conditioned BMDC showed decreased expansion of

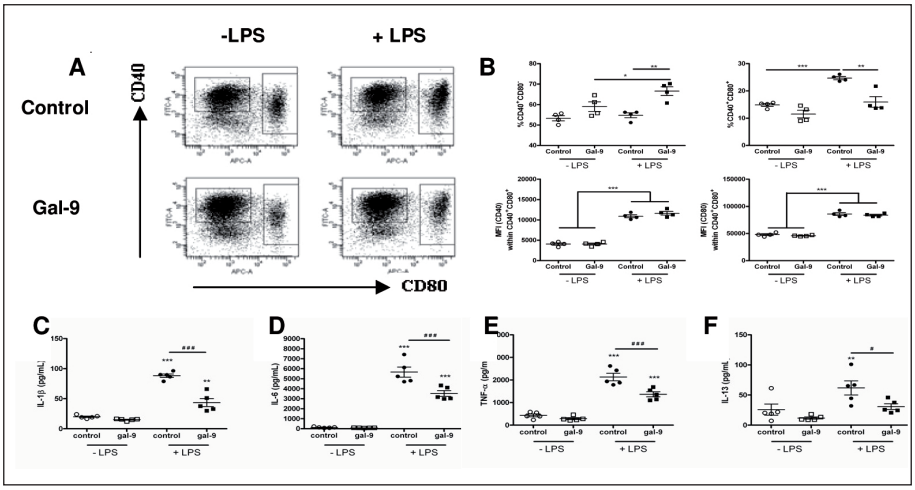

Figure 3 Galectin-9 suppresses pro-inflammatory cytokine production by BMDC upon LPS stimulation. *Wild type Balb/c BMDC were conditioned with recombinant galectin-9 and stimulated with LPS for 48h. Galectin-9 conditioned BMDC were less responsive to LPS stimulation as expansion of CD40+CD80+ BMDC was suppressed compared to control BMDC (A, B). LPS stimulation did not alter the ability of galectin-9 conditioned BMDC to induce CD40 or CD80 expression (B), whereas the production of IL-1β (C), IL-6 (D), TNF-α (E) and IL-13 (F) upon LPS stimulation was reduced. Data represent n=5 BMDC cultures, mean ± SEM, #P<0.05, **P<0.01, ###,***P<0.001.*

CD40+CD80+ BMDC after LPS stimulation (**Figure 3A** and **3B**, upper panel). Galectin-9 did not affect cell viability (**Supplementary figure 2**). Furthermore, LPS stimulation did not affect the induction of co-stimulatory molecule expression by CD40+CD80+ cells, indicating that the BMDC were activated to similar extent as control BMDC (**Figure 3B**, lower panel). LPS stimulation of control BMDC resulted in increased production of the pro-inflammatory cytokines IL-1β, IL-6, TNF-α and the Th2-associated cytokine IL-13. However, galectin-9 conditioned BMDC were less capable of secreting IL-1β, IL-6, TNF-α and IL-13 production (**Figure 3C–F**). However, we could not detect the production of IL-10 by BMDC (data not shown).

Expression of co-stimulatory molecules by LP DC is suppressed by the GF/Bb diet

To evaluate whether non-digestible oligosaccharides can suppress DC activation *in vivo*, we evaluated in an OVA-induced murine food allergy model whether expression of co-stimulatory molecules is reduced in gut-associated lymphoid tissue upon supplementation of GF/Bb to the diet (**Figure 4A**). Serum OVA-specific IgE was suppressed, whereas no effect was observed for OVA-specific IgG1 or IgG2a upon dietary intervention with GF/Bb (**Figure 4B-D**). Sensitization to OVA resulted in a pronounced acute

allergic skin response and increased the water content in the feces. These parameters were suppressed by the GF/Bb diet (**Figure 4E** and **4F**), indicating that the GF/Bb diet can suppress food allergic symptoms. In line with previous observations (de Kivit et al., 2012), serum galectin-9 levels were increased in OVA-allergic mice fed GF/Bb compared to control OVA-allergic mice *(***Figure 4G***).*

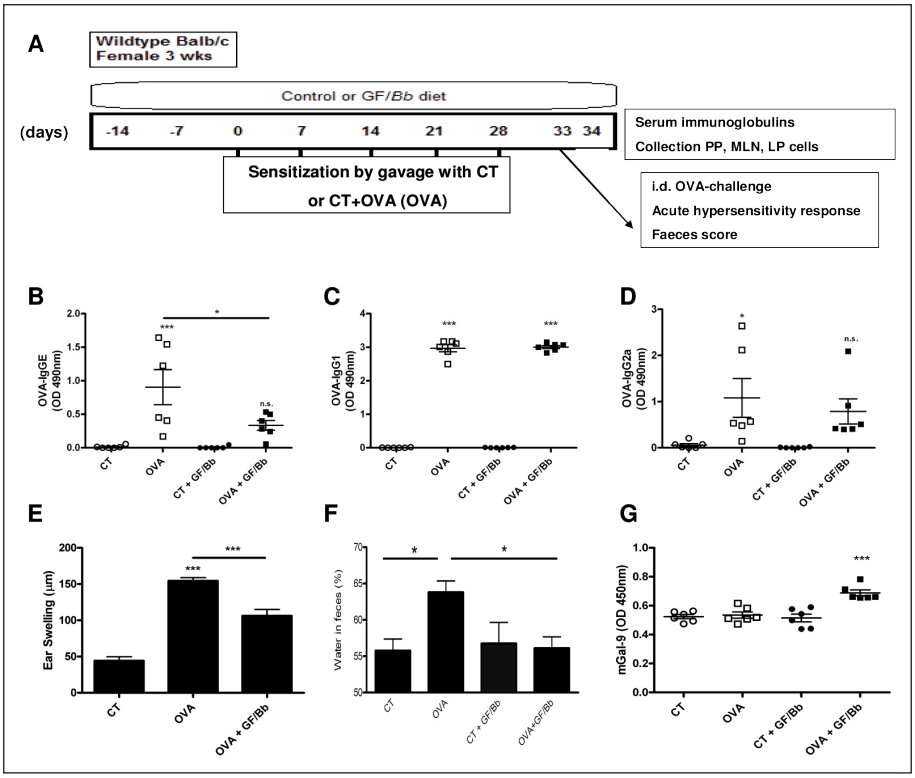

Figure 4 The GF/Bb diet suppresses food allergic symptoms and IgE production in an OVA-induced murine model for food allergy*. Schematic overview of the murine food allergy model (A). OVA-sepcific serum IgE – but not OVA-specific IgG1 and IgG2a – was reduced upon supplementation of GF/Bb to the diet (B-D). OVA-sensitized mice fed GF/Bb showed a reduced acute allergic hypersensitivity response upon i.d. allergen challenge in the ear compared to control OVA-sensitized mice (E). In addition, the GF/Bb diet prevented diarrhea-like symptoms upon oral OVA challenge (F). This was associated with increased serum galectin-9 levels (G). Data are representative for n=2 independent experiments, n=6 mice per group, mean ± SEM, *P<0.05, ***P<0.001.*

Next, lymphocytes were isolated from PP, MLN and small intestinal LP and the expression of CD40, CD80, CD83 and CD86 by CD11c+ cells was analyzed. In the LP, an increased frequency of CD11c+CD40+CD80+ and CD11c+CD83+ cells were observed and supplementation of GF/Bb to the diet suppressed the expression of co-stimulatory molecules by LP CD11c+ cells (**Figure 5A** and **5B**). These effects were not observed in PP and were found to lesser extent in MLN (**Supplementary figure 3**). In addition, in OVA-allergic mice, a increase in the percentage of CD11c+I-A/I-E(mid) cells compared to sham sensitized mice was observed. Interestingly, in OVA-allergic mice, CD11c+I-A/I-E(mid) cells expressed less CD103, whereas in GF/Bb fed mice reduced CD103 expression was partly prevented (**Figure 5C**). Furthermore, LP CD11c+ cells in OVA-sensitized mice produced more IL-4, which was prevented by the GF/Bb diet (**Figure 5D**). These data collectively confirm the ability of non-digestible oligosaccharides to suppress small intestinal LP DC activation after oral administration *in vivo*.

Dietary intervention with GF/Bb enhances LP Treg cell numbers, while suppressing mucosal Th2 responses

We next evaluated whether GF/Bb modulates effector T cell responses *in vivo*. In OVA-allergic mice a reduced percentage of CD4+Foxp3+ Treg cells was observed, whereas the frequency of CD4+CD69+GATA-3+ Th2 cells in the LP was increased compared to non-allergic mice. Supplementation of GF/Bb to the diet partially normalized the percentage of Treg cells back to control levels, whereas the frequency of activated Th2 cells in the LP was reduced (**Figure 5E** and **5F**). These data show that GF/Bb can polarize the effector T cell response towards induction of Treg cells at the expense of the frequency of activated Th2 cells.

Galectin-9 suppresses OVA-specific Th2 responses

To confirm whether galectin-9 can suppress cytokine production *ex vivo*, small intestinal LP cells were isolated from non-allergic or OVA-allergic mice and subsequently re-stimulated with OVA for 5 days in the presence or absence of recombinant mouse galectin-9. An OVA-specific response was observed in mice orally sensitized to OVA, but not in sham sensitized mice, as measured by increased ^{3}H-thymidine incorporation by LP cells. Adding recombinant galectin-9 to the cultures partially prevented the response of LP cells from OVA-sensitizaed mice in response to OVA, whereas in sham sensitized mice, proliferation of LP cells was not affected (**Figure 6A**). Furthermore, OVA re-stimulation of LP cells derived from OVA-allergic mice resulted in increased production of IL-6, TNF-α, IL-4 and IL-5. However, in presence of galectin-9, IL-6, TNF-α, IL-4 and IL-5 secretion was suppressed, whereas production of IFN-γ and IL-13 was not affected (**Figure 6B–G**). Production of IL-10 and IL-12 were not detected (data not shown). These data collectively indicate a role for galectin-9 iwn suppressing mucosal Th2 responses through suppression of DC activation and cytokine production as well as in suppressing an effector Th2 response in the small intestinal LP.

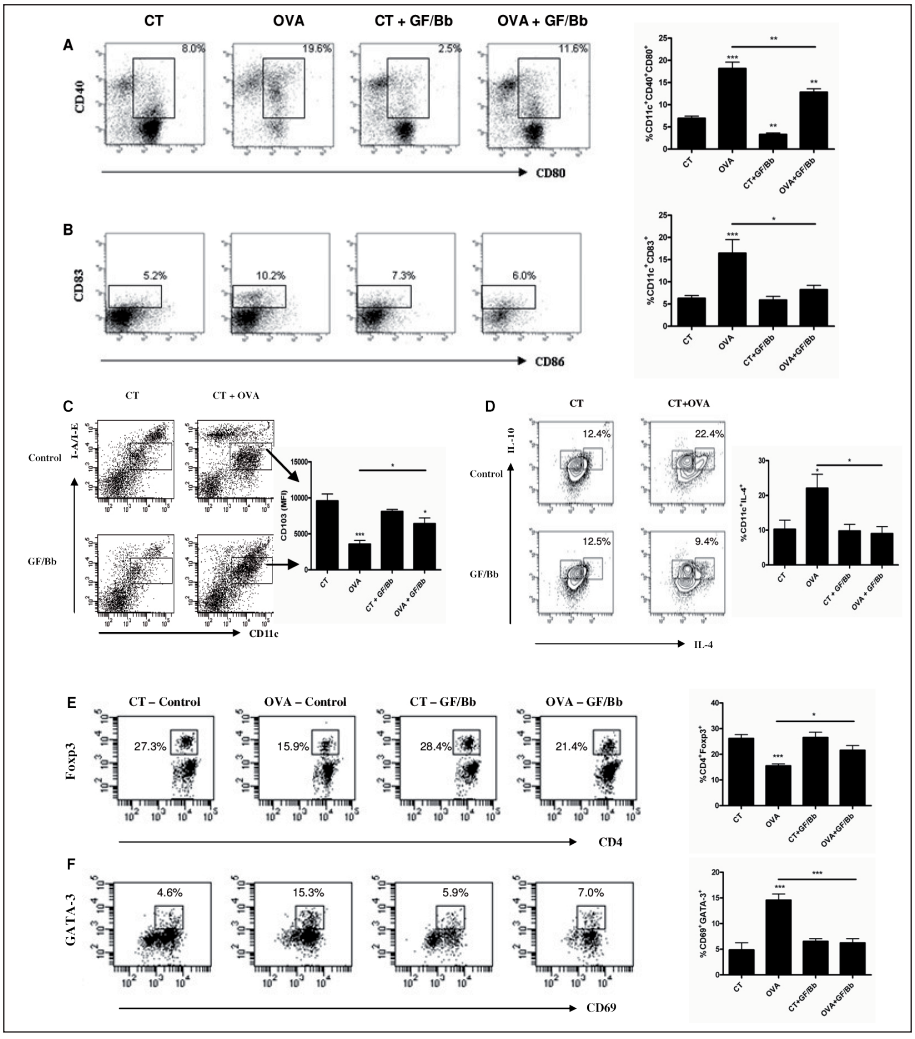

Figure 5 Supplementation of GF/Bb to the diet prevents DC activation and cytokine production in the LP, resulting in a suppressed Th2 response. *Mice were orally sensitized to OVA as shown in figure 3A. CD11c+ cells isolated from the intestinal LP from OVA-sensitized mice expressed increased levels of co-stimulatory molecules, which was prevented upon GF/Bb supplementation to the diet (A, B). Furthermore, OVA-sensitized mice showed an increased percentage of CD11c+MHC-II(mid) cells expressing lower levels of CD103 (C). In OVA-sensitized mice, LP CD11c+ cells showed increased IL-4 production (D). The GF/Bb diet normalized CD103 expression and IL-4 production to the level of non-allergic mice (C, D). Consequently, reduced Foxp3+ Treg cells and increased activated Th2 cells were observed in the LP of OVA-sensitized mice, but not upon dietary intervention with GF/Bb (E, F). Data are representative for n=2 independent experiments, n=6 mice per group, mean ± SEM, *P<0.05, **P<0.01, ***P<0.001.*

Discussion

DC present in the intestinal mucosa play a crucial role in maintaining immunological homeostasis. Despite a high load of antigens present in the intestinal lumen, limited or no inflammatory response is elicited. In fact, tolerance is induced by CD103+ DC present in the LP which effectively induce Foxp3+ Treg cells in the MLN (Coombes et al., 2007; Schulz et al., 2009; Sun et al., 2007). IEC play a role in the induction of CD103+ DC and thereby contribute to induction of immunological tolerance (Iliev et al., 2009a; Iliev et al., 2009b). In the present study, we observed that exposure of IEC to non-digestible oligosaccharides, which structurally and functionally resemble human milk oligosaccharides, results in secretion of galectin-9 and the generation of tolerogenic DC which are characterized by lower expression of co-stimulatory molecules. Furthermore, DC produced less pro-inflammatory cytokines upon stimulation and supported the differentiation of naïve CD4+ T cells into Treg cells.

Under inflammatory conditions, IEC have been shown to increase their surface expression of TLR, including TLR9 Abreu et al., 2002; de Kivit et al., 2011; Ewaschuk et al., 2007; Singh et al., 2005). Apical TLR9 ligation of IEC has been described to contribute to intestinal homeostasis Lee et al., 2006). Exposure of IEC to commensal bacteria has been shown to condition DC to express lower levels of co-stimulatory molecules (Zeuthen et al., 2008). Recently, it was shown that apical exposure of IEC to DNA derived from *Bifidobacterium breve* resulted in an tolerogenic effector response, which is supported by the observation that DNA from commensals as well as probiotic bacterial strains contain immunosuppressive motifs that induce Treg polarization (Bouladoux et al., 2012; Campeau et al., 2012). Furthermore, dietary supplementation of GF increased the presence of *Bifidobacteria*, while decreasing pathogenic *Clostridium* species in the microbiota, thereby preventing the occurrence of infectious and allergic disease (Arslanoglu et al., 2008; Gori et al., 2011).

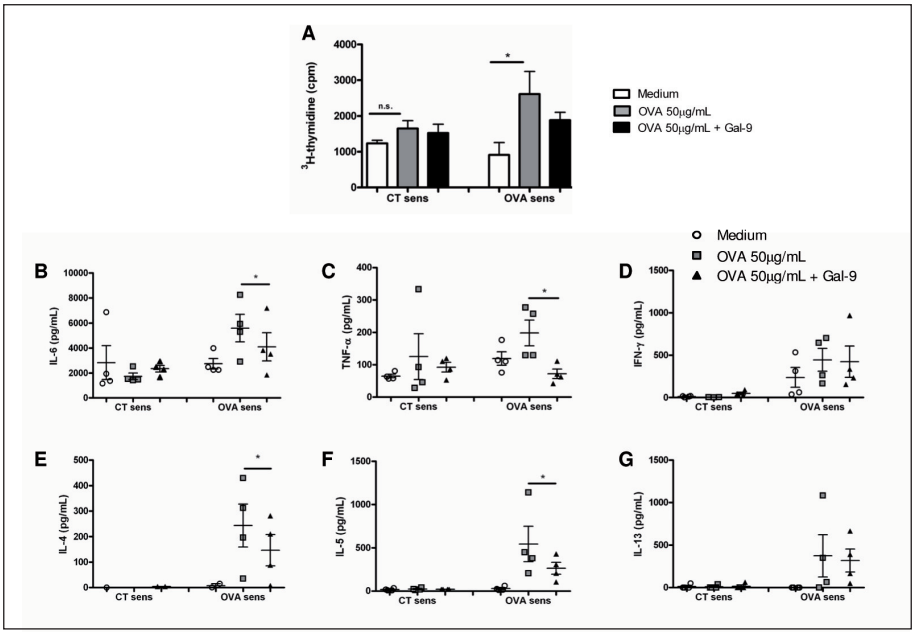

Figure 6 Galectin-9 suppresses OVA-specific pro-inflammatory and Th2-associated cytokine production by LP cells from OVA-sensitized mice*. LP cells from OVA-sensitized mice were isolated and re-stimulated with OVA for 5 days in absence or presence of recombinant galectin-9. Galectin-9 prevented an OVA-specific T cell response as ^{3}H-thymidine incorporation was suppressed (A). Furthermore, galectin-9 inhibited the production of IL-4, IL-5, IL-6 and TNF-α production by LP cells from OVA-sensitized mice upon OVA re-stimulation, whereas the production of IL-13 and IFN-γ were not affected (B-G). Data represent n=4 mice per group, mean ± SEM, *P<0.05.*

In our study, we observed that IEC primed with IFN-γ and TNF-α respond to apical TLR9 stimulation in the presence of GF by secreting soluble mediators that suppress the expression of co-stimulatory molecules by moDC. Moreover, these moDC had increased capacity to induce Foxp3+ Treg cells. This is in line with observations from others that show the capacity of IEC to suppress DC activation (Butler et al., 2006). In addition, we identified galectin-9 as an important mediator secreted by IEC involved in generating DC with tolerogenic capacity. Although galectin-9 conditioned DC effectively induced Treg cells, phenotypical analysis of galectin-9 conditioned DC did not show expression of CD103 despite increased RALDH activity, suggesting that galectin-9 can contribute to immunological tolerance through mechanisms independent of CD103+ DC. Several studies have reported that galectin-9 contributes to induction of tolerogenic antigen presenting cells and Treg induction (Arikawa et al., 2010; Kojima et al., 2011; Seki et al., 2008; Wang

et al., 2009). However, galectin-9 is mainly known for its potential to induce apoptosis of Th1 and Th17 cells (Oomizu et al., 2012; Zhu et al., 2005). Altogether, this suggests, for the first time, a crucial role for galectin-9 in preventing excessive Th1 and Th17 inflammation, while maintaining tolerance through induction of Treg polarization in a Th2 polarized disease.

Dietary intervention with GF/Bb is known to prevent the development of acute allergic symptoms in a murine model for cow's milk allergy model through induction of Treg cells (Schouten et al., 2010). In addition, it was shown that galectin-9 expression and secretion by IEC was increased upon dietary intervention with GF/Bb in whey-sensitized mice (de Kivit et al., 2012). Likewise, in the present study it was observed that dietary intervention with GF/Bb enhanced serum galectin-9 levels in OVA-induced food allergy model. We elaborated on these data by showing reduced expression of CD40, CD80 and CD83 by small intestinal LP DC after oral allergen challenge upon supplementation of GF/Bb to the diet. Moreover, suppressing co-stimulation has been reported to have beneficial effects in allergic disease, since transfer of BMDC, in which CD40 expression was silenced, before and after allergic sensitization to OVA, favored the development of OVA-specific Treg cells and suppressed Th2 cytokine production upon OVA-re-stimulation (Suzuki et al., 2010). However, galectin-9 is reported to induce co-stimulatory molecule expression by DC, thereby sustaining the food allergic response (Chen et al., 2011).

Although *in vitro* galectin-9 did not result in expression of CD103 by DC, OVA-sensitized mice show less CD103 expression by CD11c+ cells and Treg cells in the LP, which was partly prevented upon dietary intervention with GF/Bb. This suggests that other soluble mediators released by IEC, potentially TGF-β or TSLP, may act in concert with galectin-9 to induce oral tolerance. Similar results were observed in a peanut-induced murine allergy model, in which less CD103+ DC were observed in peanut-allergic mice (Smit et al., 2011). Interestingly, in OVA-sensitized mice, infiltration of CD11c+ MHC-II(mid) cells was observed and CD11c+ cells produced more IL-4, which was paralleled by increased percentage of activated Th2 cells in the LP. Although limited studies report on the role of galectin-9 in food allergy, galectin-9 knock-out mice show increased Th2 cytokines and lower amounts of Treg cells in the bronchoalveolar lavage fluid in a model for eosinophilic lung inflammation (Katoh et al., 2012). Hence, galectin-9 may be involved in suppressing food allergy through conditioning DC, resulting in suppression of Th2 polarization and promoting the conversion of naïve T cells to Treg cells.

Cytokines produced by DC are crucial for generating an appropriate effector immune response. Decreased expression of TLR by both IEC and mucosal DC explains unresponsiveness of intestinal DC to TLR ligands, including LPS (Abreu et al., 2001; Cerovic et al., 2009; Melmed et al., 2003). In addition, upon activation of TLR, intestinal DC produce IL-10 instead of IL-12, thereby promoting tolerance (Monteleone et al., 2008). Although it is not known whether galectin-9 regulates TLR expression by DC, BMDC conditioned with galectin-9 were less responsive to TLR4 activation as LPS stimulation resulted in less production of pro-inflammatory cytokines and lower secretion of the Th2-associated cytokine IL-13. Similar to the observations that LP DC produce less IL-12 upon TLR ligation, galectin-9 conditioned DC did not produce IL-12 secretion upon LPS stimulation. However, we could not detect IL-10 production, indicating that galectin-9 by itself does not induce IL-10 secretion by DC. Further studies are necessary that show whether galectin-9 regulates TLR expression or signaling pathways in DC. Innate responses may play an important role in allergic disease as neonates that developed allergy in later life were shown to be more responsive to TLR stimulation compared to healthy control (Prescott et al., 2008). Similarly, OVA re-stimulation of LP cells from OVA-sensitized mice in presence of galectin-9 suppressed Th2-associated cytokine production and pro-inflammatory cytokine secretion. Although galectin-9 is known to induce Th1 apoptosis as well (Oomizu et al., 2012; Zhu et al., 2005), IFN-γ production was not suppressed.

In conclusion, this study provides new insights by which dietary intervention using non-digestible oligosaccharides prevent the development of food allergy. Galectin-9 secreted by IEC suppresses DC activation and responsiveness to pro-inflammatory stimuli, and promote the differentiation of Treg cells, possibly via the induction of tolerogenic DC in the intestinal mucosa.

Acknowledgements

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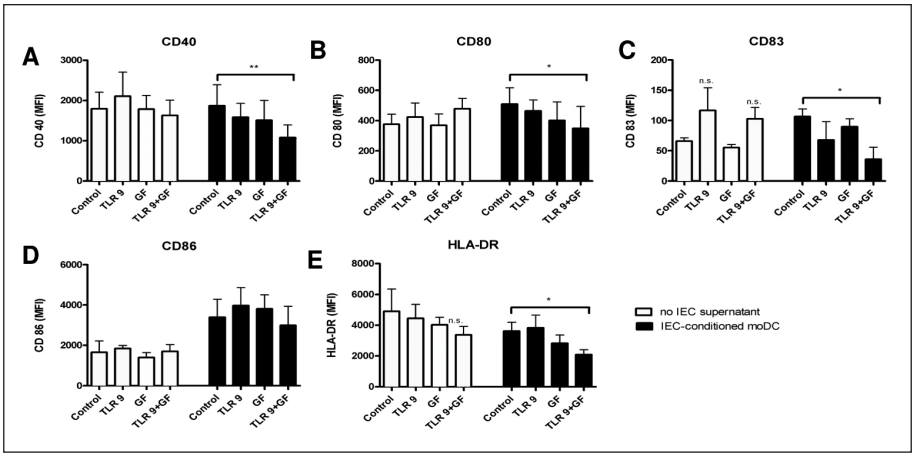
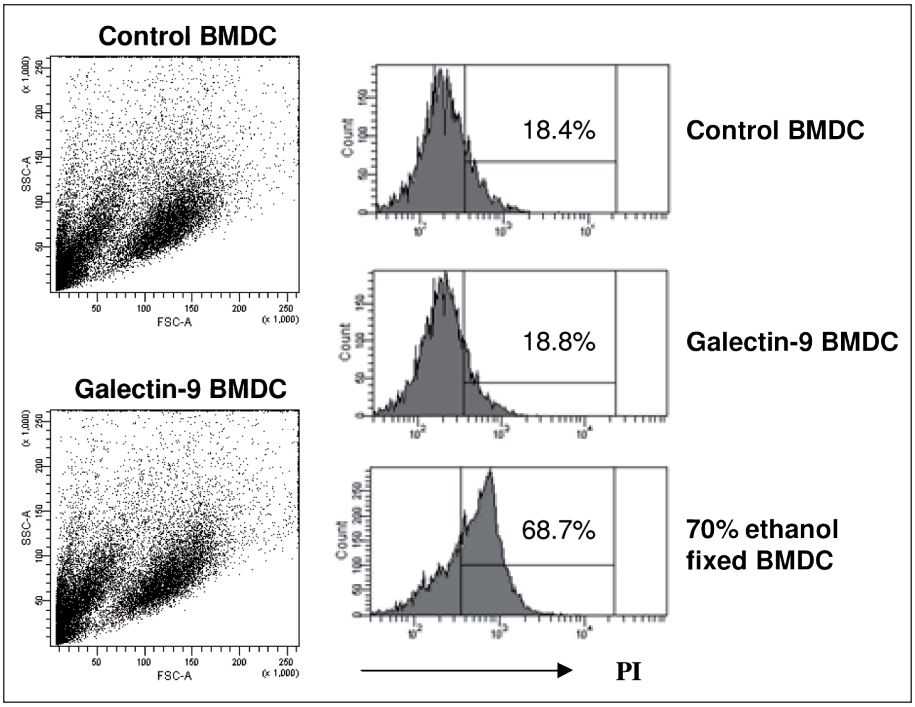
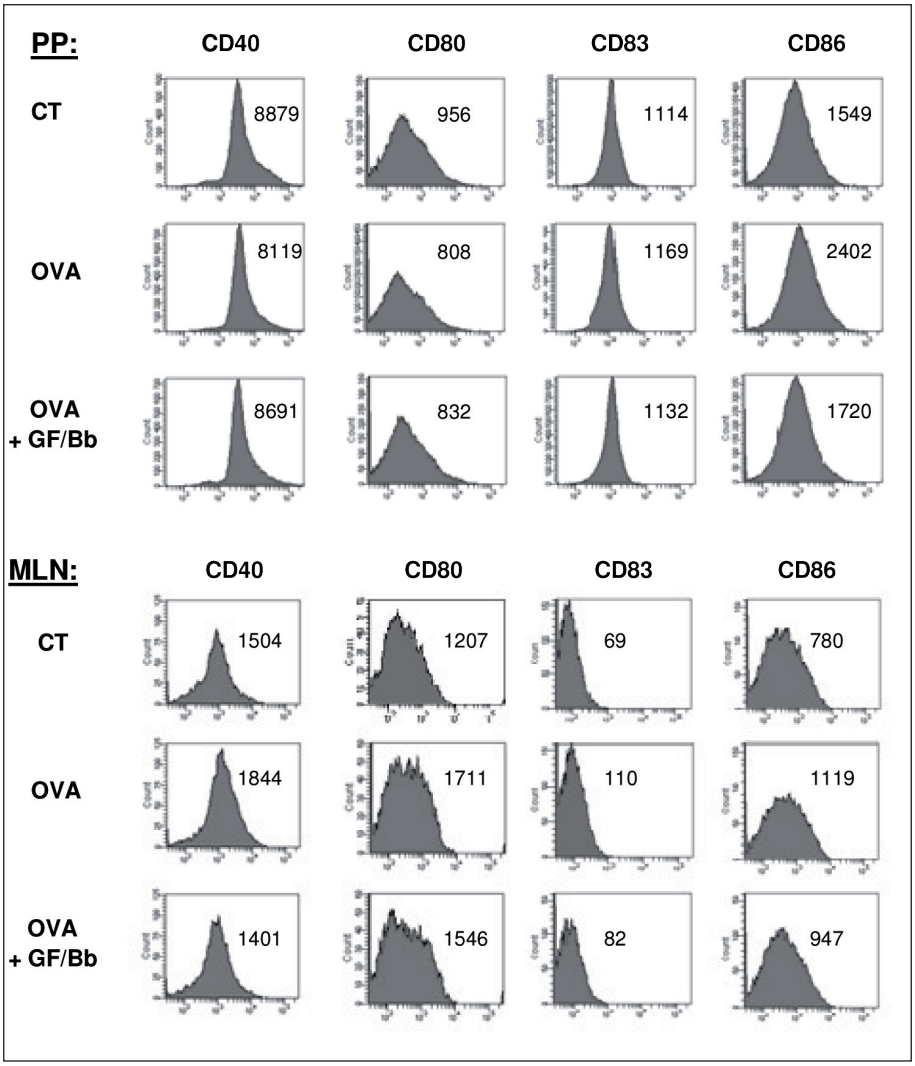
Supplementary figures



Supplementary Figure 1 DC conditioned by IEC apically exposed to TLR9 ligand in presence of GF express lower levels of co-stimulatory molecules and HLA-DR. *CD14+ monocytes were differentiated into moDC in the absence or presence of IEC conditioned medium. TLR9 ligand in absence or presence was directly added to moDC (white bars) or apically added to TNF-α/IFN-γ-primed IEC prior to conditioning of moDC (black bars). After 7 days, surface expression of CD40, CD80, CD83, CD86 and HLA-DR were measured (A-F). Differentiation of monocytes into moDC in presence of IEC-conditioned medium derived from HT-29 cells exposed to TLR9 ligand and GF, but not direct exposure of moDC to TLR9 ligand with or without GF, suppressed the expression of CD40, CD80, CD83 and HLA-DR. Data represent n=4 independent donors, mean±SEM, #,*P<0.05, **P<0.01, n.s. not significant; GF, scGOS/lcFOS.*



Supplementary Figure 2 Galectin-9 does not affect BMDC viability. *Bone marrow cells of Balb/c mice were differentiated into BMDC in the absence or presence of recombinant mouse galectin-9. On day 7, viability was assessed. No difference was observed in the FSC/SSC profile between BMDC generated in the absence or presence of galectin-9. In addition, no difference in PI+ cells was found between BMDC condition with or without galectin-9. Data represent n=5 BMDC cultures.*



Supplementary Figure 3 Expression of co-stimulatory molecules in PP and MLN by LP CD11c+ cells. *Expression of CD40, CD80, CD83 and CD86 by CD11c+ in PP and MLN was assessed. In PP, the expression of CD86 – but not CD40, CD80 or CD83 – by CD11c+ cells was increased in OVA-sensitized mice, which was abrogated upon supplementation of GF/Bb to the diet. In MLN, expression of co-stimulatory molecules by CD11c+ cells was increased in OVA-sensitized mice, which was partially normalized to control levels upon dietary intervention with GF/Bb. Data represent n=6 mice per group, numbers represent mean fluorescence intensity (MFI).*

7

Non-digestible galacto- and fructo-oligosaccharides and *Bifidobacterium breve* M-16V delay the onset of DSS-induced colitis in association with suppressed galectin-4 expression

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Manuscript in preparation

Abstract

Background & Aims: Dietary intervention using non-digestible oligosaccharides (short-chain galacto- and long-chain fructo-oligosaccharides, GF) in combination with *Bifidobacterium breve* M-16V (GF/Bb) is known to support the induction of Treg cells. We addressed whether dextrane sodium sulfate (DSS)-induced colitis can be attenuated by dietary supplementation with GF/Bb and hypothesized a possible involvement for galectins in IBD. **Methods:** A murine DSS colitis model was performed to study the effects of dietary supplementation of Bb, GF and GF/Bb on development of colitis. Galectin-4 and -9 expression in colon as well as dendritic cell (DC) activation and T cell polarization in gut-associated lymphoid tissue (GALT) were analyzed. An *in vitro* intestinal epithelial cell (IEC)/PBMC co-culture model was used to study the mechanism of GF/Bb in preventing an inflammatory response. Galectin-4 and -9 expression were analyzed in human biopsies from UC and CD patients. **Results:** Dietary intervention with GF or GF/Bb delayed the onset of DSS-induced colitis, in association with reduction of galectin-4 expression in the colon. Intestinal inflammation was improved upon supplementation with Bb of GF/Bb. Furthermore, the frequency of CD103+ DC in mesenteric lymph nodes (MLN) was enhanced, which was paralleled by enhanced Treg cell percentage in MLN. In addition, in Peyer's patches, the frequency of Treg cells was increased at the expense of Th17 and Th1 cells. *In vitro*, TLR4 activation enhanced galectin-4 expression by IEC, which was prevented when GF was present. Galectin-4 expression was increased in inflamed intestinal tissue UC and CD patients, whereas galectin-9 expression was reduced. **Conclusions:** GF/Bb attenuates the development of DSS-induced intestinal inflammation, suppresses epithelial galectin-4 expression and supports Treg conversion while suppressing Th1 and Th17 cells in GALT.

Introduction

Inflammatory bowel disease (IBD) is characterized by a chronic inflammation in the intestine. Whereas CD manifests as mainly distinct aphthous transmural lesions throughout the entire gastrointestinal tract, UC lesions remain restricted to the epithelial surface, which only occur in the colon (Baumgart and Sandborn, 2007). Although the etiology of IBD is not exactly known, it is hypothesized that both genetic predisposition and alterations in the intestinal epithelial barrier integrity are involved in the development of IBD (Bouma and Strober, 2003). Altogether, this may result in loss of mucosal tolerance leading to aberrant inflammatory responses towards the microbiota.

In particular, intestinal epithelial cells (IEC) play a crucial role in regulating mucosal immunity and tolerance. IEC not only provide a physical barrier to luminal antigens, but IEC are also important sensors of microbes as they express Toll-like receptors (TLR) (Artis, 2008). TLR4 expression by IEC is low under non-inflammatory condition, however, in CD and UC patients TLR4 expression by IEC is markedly increased (Abreu et al., 2001; Cario and Podolsky, 2000; Erridge et al., 2010). Aberrant TLR4 activation on IEC results in the production of IL-8, which triggers neutrophil infiltration into the lamina propria, leading to tissue damage. Besides recognition of pathogens, IEC play an important role in regulating tolerogenic immune responses in the intestinal mucosa as well to prevent unwanted immune reaction to commensal bacteria. IEC were found to specifically induce generation of tolerogenic CD103+ DC which convert CD4+ T cells into Foxp3+ Treg cells. Indeed, IEC drive the generation of CD103+ DC, thereby attenuating colitis (Iliev et al., 2009; Annacker et al., 2005; Collins et al., 2012). Hence, targeting IEC may be a promising approach to modulate intestinal mucosal immune responses to prevent or treat IBD.

In UC, intervention using *Bifidobacteria* in combination of with non-digestible oligosaccharides has shown to reduce intestinal inflammation (Furrie et al., 2005; Ishikawa et al., 2011). In murine models for IBD the probiotic mixture VSL#3 has been shown to increase epithelial barrier integrity, thereby reducing bacterial translocation into the lamina propria (Mennigen et al., 2009; Corridoni et al., 2012; Bassaganya-Riera et al., 2012). Moreover, *bifidobacterial* strains can induce human IL-10 producing DC expressing lower levels of co-stimulatory molecules, thereby reducing the induction of Th1 cells (Hart et al., 2004). Dietary intervention using a specific mixture of short-chain galacto- and long-chain fructo-oligosaccharides (scGOS/lcFOS, GF) is known to induce Treg polarization in cow's milk allergic mice (Schouten et al., 2010). In particular, GF in combination with *Bifidobacterium breve* M-16V (Bb; GF/Bb) was shown to be most effective in suppressing food allergic symptoms

(Schouten et al., 2009). Non-digestible oligosaccharides support the growth of *Bifidobacteria* and *Lactobacilli* and especially inulin and fructo-oligosaccharides are reported to have protective effects on the development of DSS-induced colitis (Newburg, 2000; Osman et al., 2006). Very recently, we have demonstrated that GF acts directly via TLR9 ligation on IEC, supporting the development of tolerogenic DC and consequently induction of function Treg cells (de Kivit et al., submitted).

IEC have been shown to express an array of galectins, including galectin-2, -3, -4 and -9 (Lahm et al., 2001; Nio-Kobayashi et al., 2009). Galectins specifically bind β-galactoside containing glycans. Galectins are involved in regulation of immune responses and tolerance induction by inducing signaling through formation of galectin-glycoprotein lattices on cell surfaces (Rabinovich and Toscano, 2009; van Kooyk and Rabinovich, 2008). Galectin-4 is expressed in the intestinal tract, specifically by IEC (Gitt et al., 1998; Mathieu et al., 2008). In several murine models for colitis increased galectin-4 expression was found in IEC (Mathieu et al., 2008; Paclik et al., 2008). Therefore, galectin-4 might play an important role in IBD. Though IEC derived galectin-4 has been shown to have immunogenic activity via stimulation of IL-6 production by lamina propria CD4+ T cells, studies in murine colitis models targeting galactin-4 are not clear (Mathieu et al., 2008; Paclik et al., 2008; Hokama et al., 2004). Galectin-9 is an interesting galectin since it has been shown to suppress Th1 and Th17 cell development whilst augmenting Treg cells (Oomizu et al., 2012; Seki et al., 2008). Recently we have shown that dietary intervention with GF/Bb induces the secretion of galectin-9 by IEC (de Kivit et al., 2012). Galectin-9 may have protective capacities in IBD as treatment of mice with a galectin-9 homologous protein isolated from intestinal parasites suppressed the induction of colitis, while enhancing IL-10 and TGF-β production (Kim et al., 2010). We hypothesized that dietary intervention using GF/Bb exerts protective effects on the development of colitis via modulation of galectin-4 and -9 expression. To this end, we performed *in vivo* studies examining the effects of GF/Bb intervention in DSS-induced colitis in mice. Using a IEC/PBMC co-culture system we further addressed the role of galectin-4 in pro-inflammatory response as seen in IBD and the possible modulating effects of GF. Lastly, we investigated the expression of galectins in human colon biopsies obtained from patients suffering from IBD.

Materials and methods

Mice

C57Bl/6 female mice were fed a control a control diet (AIN93G), *Bifidobacterium breve* M-16V (2% wt : wt 1x1011 CFU/g (Morinaga Milk Industries, Japan), GF (1% wt:wt, 9 : 1 scGOS (Friesland-Campina, Amerfoort, The Netherlands) : lcFOS (Orafti, Tienen, Belgium) (Immunofortis™, Nutricia, Zoetermeer, The Netherlands); GF) or a combination of both (GF/Bb) as described (Schouten et al., 2009). Mice were fed the respective diet 2 weeks before and during induction of colitis for 6 days. Colitis was induced by adding 1.5% w/v DSS to the drinking water. Feces consistency (score 0 = normal, 1 = soft but with form, 2 = diarrhea) and development of blood in the feces (score 0 = no blood, 1 = positive test, 2 = visible fresh blood in feces; colo-rectal test kit, Axon Lab AG, Stuttgart, Germany) and body weight was monitored from the time point DSS was added to the drinking water. Animal use was in accordance with guidelines of the Dutch Committee of Animal Experiments.

Flow cytometry

Lymphocytes were collected from Peyer's patches (PP) and mesenteric lymph nodes (MLN) by crushing the tissue on a 100µm cell trainer. Cells isolated from PP and MLN were collected in PBS/2%FCS. Fcγ-receptors were blocked using 10µg/mL CD16/CD32 antibodies, followed by extracellular staining using CD11c-FITC, CD40-FITC, CD80-APC, CD86-APC, CD103-APC, CD4-FITC and CD69-PerCP-Cy5.5 antibodies (all eBioscience). Cells were fixed using 0.5% paraformaldehyde, or fixed and permeabilized using the using the Foxp3 staining buffer set (eBioscience) according to manufacturer's protocol for intracellular staining. Intracellular staining was performed using Foxp3-APC, ROR-γT-PE, T-bet-PE and GATA3-eFluor660 antibodies (all eBioscience). Flow cytometric analysis was performed using a FACSCantoII and FACSDiVa software (BD Biosciences).

Culture of human intestinal epithelial cell lines

The human intestinal epithelial cell (IEC) line HT-29 (ATCC; HTB-38; MA, USA) was cultured in McCoy's 5A medium (Lonza, Verviers, Belgium), supplemented with 10% heat-inactivated FCS (Gibco) and penicillin (100U/mL)/streptomycin (100µg/mL) (Sigma, St. Louis, MO, USA). Cells were cultured in 25cm^2 culture flasks (Greiner, Frickenhausen, Germany) and were kept in an incubator at 37^{o}C under 5% CO2. HT-29 cells were seeded one week before experiments in 12-well 0.4µm transwell insert filters (Corning). Confluence of IEC monolayers was assessed by light microscopy as HT-29 cells do not build up resistance (125-150 Ωxcm^2).

Transwell co-cultures

IEC were co-cultured with $3x10^6$ CD3/28-activated peripheral blood mononuclear cells (PBMC) for 24h as previously described (de Kivit et al., 2011). IEC were apically exposed to either TLR4 ligand (E. coli 0111:B4 LPS, 1.0µg/mL, Invivogen, San Diego, CA, USA) alone or in combination with 0.5% w/v of a 9:1 mixture of scGOS (Vivinal GOS, Borculo Domo) and lcFOS (Raftiline HP, Orafti) (GF). Basolateral supernatants were collected and stored at -20°C till further use.

cDNA synthesis and real-time PCR

HT-29 cells were washed once in PBS 2h after co-culture with CD3/28-activated PBMC and taken up in 200µL RNAlaterTM (Qiagen GmbH, Hilden, Germany). Samples were stored at -20°C until cDNA synthesis. mRNA was isolated using the mRNA capture kit (Roche, Mannheim, Germany) and real-time PCR reactions were performed as previously described (Garcia-Vallejo et al., 2004). GAPDH was used as reference gene. Relative target mRNA abundance was calculated by applying the formula: relative mRNA abundance = $100 \times 2^{Ct[GAPDH] - Ct[target\ mRNA]}$. Primers for all galectin-4 and -9 were designed using the computer software Primer Express 2.0 (Applied Biosystems, Carlsbad, CA, USA).

Human intestinal samples were obtained from surgical resection specimens (according to the guidelines from the Medical Ethics Committee of the Leiden University Medical Center) and include pairs of macroscopically inflamed and normal-appearing (non-inflamed) mucosa from 5 patients with CD (clinically and histologically confirmed), 5 patients with UC (clinically and histologically confirmed), with normal tissue from 5 patients with a colorectal carcinoma, at least 10 cm from the tumor, as controls. From these samples, mRNA was isolated and quality of the RNA was checked by running a 1% agarose gel (detection of 28S, 18S and 5S bands). 200-500 ng total RNA was treated for 30 min at 37°C with DNAse. To inactivate the DNAse, 1 µL EDTA was added and incubated for 10 min at 65°C. Subsequently, the mRNA was directly subjected to cDNA synthesis (Iscript, Bio-Rad) for 60 min at 42°C, followed by 5 min at 85°C. The synthesized cDNA was diluted 10 times in nuclease-free water and stored at -20°C until use.

Histology and Fluorescence microscopy staining

Swiss rolls were made of colons from DSS-treated mice and fixed in 10% neutral formalin for 24h after which they were embedded in paraffin. 5µm Sections were cut and stained with hematoxylin/eosin according to standard methods. Inflammation in the colon was evaluated by scoring the extent of crypt damage and the severity of crypt damage in the colon. Each parameter was scored for severity with a number from 0 (normal) to 4 (severe), and for the amount of affected colon (1, <25%; 2, 25-50%; 3, 50-75%; 4, 75-100%).

The histological score was calculated as a weighted sum of intestinal inflammation and epithelial damage.

5 µm Cryostate sections of human intestinal samples or IEC on insert filters were fixed using 4% formalin in PBS for 10min, permeabilized in 0.1% Triton X-100 (Sigma) and 1% BSA in PBS for 15min. Insert filters or cryostate sections were incubated with anti-human galectin-4 or -9 antibodies or normal goat IgG as isotype control (all 0.75µg/ml; R&D Systems) in 0.1% Triton X-100 and 1% BSA in PBS for 1h. IEC sections were washed with PBS and incubated with secondary AlexaFluor546 donkey anti-goat IgG (Invitrogen, Carlsbad, CA, USA) in 0.1% Triton X-100 and 1% BSA for 30min and embedded in Mowoil containing DAPI. Object glasses were stored at 4°C until microscopic examination using an Olympus BX50 microscope equipped with a Leica DFC 320 digital camera.

ELISA

Concentrations of IL-6, IL-12, TNF-α (Biosource CytoSetsTM, Nivelles, Belgium) and IL-17A (Arcus Biologicals, Modena, Italy) were measured according to manufacturer's protocol.

Statistics

Statistical analyses were performed using two-way ANOVA followed by Bonferroni's post hoc analysis for murine experiments. *In vitro* data were analyzed using one-way ANOVA for repeated measurements, followed by Bonferroni's post hoc test. Analyses were performed using GraphPad Prism 5.0. $P<0.05$ was considered statistically significant.

Results

Dietary intervention with GF/Bb delays the onset of DSS-induced colitis

To test whether dietary intervention using GF/Bb can ameliorate intestinal inflammation, a DSS-induced colitis model was performed. C57Bl6 mice were fed a diet containing Bb, GF or a combination of both (GF/Bb) two weeks before and during DSS treatment. Fecal consistency was improved in DSS-treated mice fed Bb or GF/Bb (**Figure 1A** and **1B**), whereas the development of blood in the stool was attenuated upon dietary intervention with GF (day 3 and 4) or GF/Bb (day 2 and 3) (**Figure 1C**). In addition, histological analysis of the colon indicates that dietary intervention with Bb or GF/Bb tended to attenuate colonic inflammation (**Figure 1D** and **1E**).

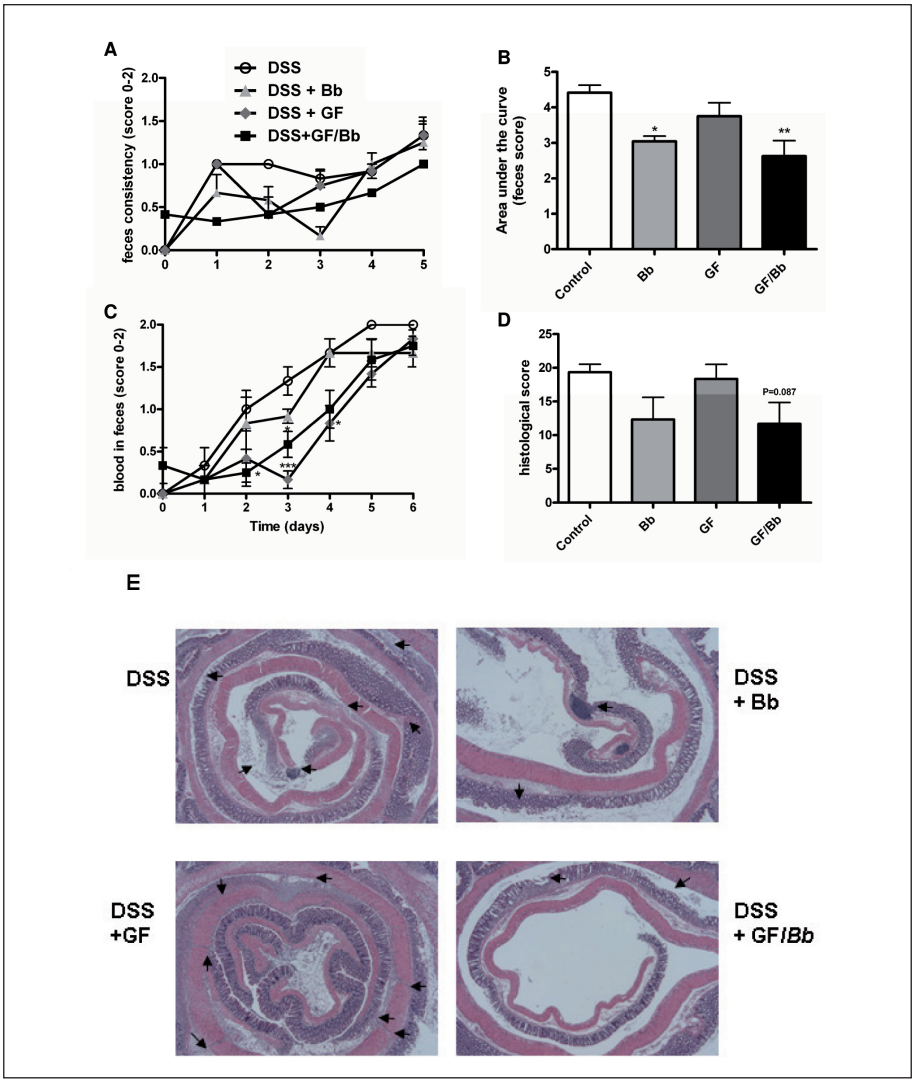

Figure 1 Dietary intervention with Bb, GF and GF/Bb delays the onset of DSS-induced colitis. *Colitis in mice was induced by adding DSS to the drinking water. Mice were monitored for the presence of diarrhea (A, B) and the presence of blood in the feces (C). Dietary intervention with B. breve and GF/Bb reduced the development of diarrhea upon induction of colitis. The development of blood in the stool was suppressed upon dietary intervention with GF or GF/Bb. Furthermore, dietary intervention with Bb and GF/Bb attenuated collonic inflammation in DSS treated mice (D, E). Data are representative for n=6 mice per group, mean ± SEM, *P<0.05, **P<0.01.*

Dietary intervention with GF/Bb modulates DC phenotype in mesenteric lymph nodes (MLN)

Since dietary intervention using Bb, GF or GF/Bb delayed the onset of DSS-induced colitis, we analyzed the expression of co-stimulatory molecules by CD11c+ cells in the MLN. In healthy mice, dietary intervention with *Bifidobacterium breve* M-16V, GF and GF/Bb increased the expression of CD80 and CD86 by CD11c+ cells in MLN. DSS treatment enhanced the expression of CD80 and CD86 ($P<0.05$), but tended to suppress CD40 expression by CD11c+ cells in MLN. Dietary intervention with GF/Bb normalized both CD80 and CD86 expression by MLN CD11c+ cells (**Figure 2A-2C**). In addition, DSS colitis resulted in a lower percentage of CD11c+CD103+ cells in MLN, while dietary intervention Bb, GF or GF/Bb enhanced the frequency of these cells in MLN (**Figure 2D** and **2E**). These data show that dietary intervention with Bb, GF or GF/Bb can contribute to the induction of tolerogenic type DC in the MLN.

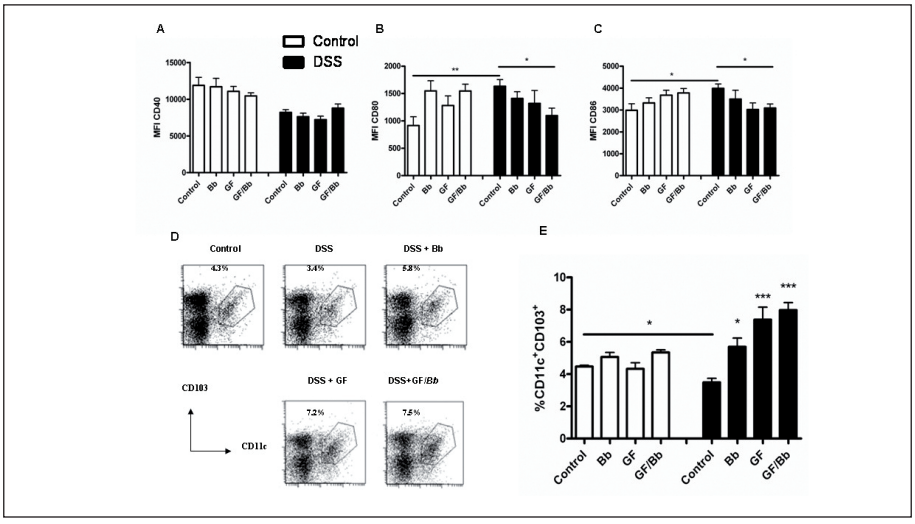

Figure 2 DSS-treated mice fed GF or GF/Bb show reduced expression of co-stimulatory molecules by DC and increased CD103+ tolerogenic DC in MLN. *Expression of CD40 (A), CD80 (B) and CD86 (C) by CD11c+ cells in the MLN was evaluated in control and DSS-treated mice fed Bb, GF or GF/Bb. CD40 expression by MLN CD11c+ cells was reduced in DSS-treated mice while CD80 and CD86 expression were increased. Dietary intervention with Bb, GF and GF/Bb did not modulate CD40 expression, whereas GF/Bb suppressed CD80 and CD86 expression by MLN CD11c+ cells. In addition, the frequency of CD103+ DC in DSS-treated mice was reduced. Dietary intervention with Bb, GF and GF/Bb enhanced the percentage of CD103+ DC in MLN in DSS treated mice (D, E). Data are representative for n=6 mice per group, mean ± SEM, *P<0.05, **P<0.01, ***P<0.001.*

GF/Bb induce Treg cell polarization *in vivo*

As dietary intervention enhances the relative numbers of CD11c+CD103+ cells in the MLN, which can effectively induce Treg cell polarization, we analyzed whether T cell polarization was affected upon dietary intervention with Bb, GF or GF/Bb in MLN (**Figure 3A**). Therefore, CD4+ T cell phenotype was analyzed by FACS and characterized as activated T cells (CD4+CD69+), Th1 cells (CD4+T-bet+GATA-3-), Th2 cells (CD4+ T-bet-GATA-3+), Th17 cells (CD4+Ror-γT+Foxp3-) or Treg cells (CD4+ Ror-γT-Foxp3+). In the MLN, a trend towards an increased percentage of activated T cells was observed in mice undergoing DSS colitis when compared with control mice. Dietary intervention with GF/Bb tended to decrease the percentage of activated T cells in the MLN. In addition, a trend towards decreased Th17 cells in MLN was observed in Bb or GF/Bb treated mice. In line with the increased percentages of CD11c+CD103+ cells in the MLN, in DSS treated mice fed GF/Bb a significant increase in Treg cells was observed in the MLN. A similar trend was observed in mice fed Bb or GF. Surprisingly, these observations were also made in healthy mice as well. No changes were observed for the relative number of Th1 in MLN. The numbers of Th2 cells were too low to be detected (data not shown).

In CD patients it is known that the small intestine is colonized by adherent invasive *E.coli* bacteria that enter the PP (Chassaing et al., 2011). Therefore, T cell subsets were also analyzed in the PP in mice (**Figure 3B**). In the PP, the number of activated CD4+ T cells, as characterized by the expression of CD69, was significantly increased in mice treated with DSS compared to healthy controls. Dietary intervention with Bb, GF or GF/Bb significantly suppresses T cell activation in PP observed in colitic mice. Furthermore, dietary intervention with GF and GF/Bb significantly suppressed the percentage of Th17 cells in PP of colitic mice, whereas the GF/Bb diet also significantly enhanced the frequency of Treg cells in PP of healthy and colitic mice. In contrast to the MLN, all diets significantly suppressed the percentage of Th1 cells in the PP of mice undergoing DSS colitis.

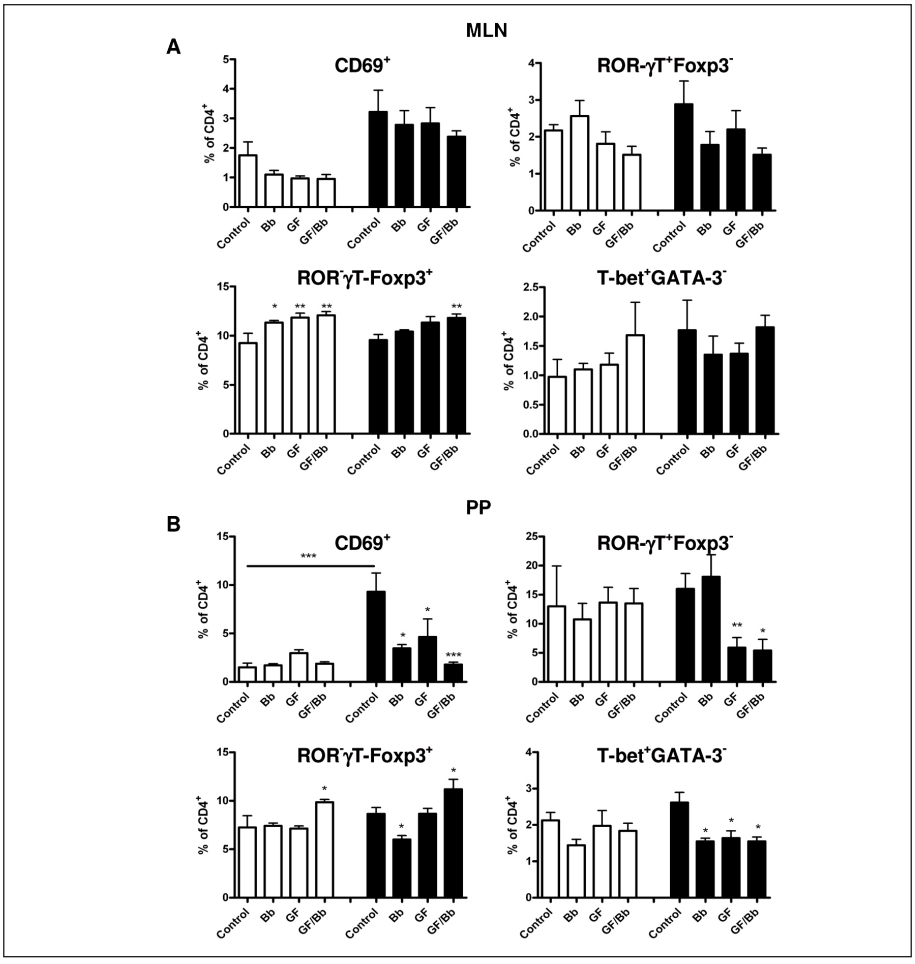

Figure 3 T cell polarization in MLN and PP is modulated upon dietary intervention with Bb, GF or GF/Bb. *In MLN, expression of CD69, ROR-γT, Foxp3 and T-bet by CD4+ T cells are analyzed. Activated CD4+CD69+ T cells tend to be suppressed by supplementation of Bb, GF or GF/Bb to the diet. Similarly, the frequency of CD4+ROR-γT+Foxp3- Th17 cells tends to be reduced, whereas the percentage of CD4+ROR-γT-Foxp3+ Treg cells is increased. No differences are observed for the frequency of CD4+T-bet+GATA-3- Th1 cells (A). In PP, the frequency of activated CD4+CD69+ T cells is increased in DSS-treated mice, while supplementation of Bb, GF or GF/Bb to the diet suppresses the percentage of activated T cells in PP in DSS-treated mice. The frequency of CD4+ROR-γT+Foxp3- Th17 cells is reduced in DSS-treated mice fed GF or GF/Bb, while the percentage of CD4+ ROR-γT-Foxp3+ Treg cells is increased in mice fed GF/Bb. All diets reduce the percentage of CD4+T-bet+GATA-3- Th1 cells in PP of DSS-exposed mice (B). Data are representative for n=4-6 mice per group, mean ± SEM, *P<0.05, **P<0.01, ***P<0.001*

IEC apically exposed to GF prevent TLR4-induced reduction of Treg cells and pro-inflammatory cytokine production

Dietary intervention with GF is known for its capacity to induce Treg cells. We therefore hypothesized that dietary intervention with GF reduces intestinal inflammation through induction of Treg cells. We have previously demonstrated that galectin-9 is involved in the induction of Treg cells (de Kivit et al., submitted). Using an IEC/PBMC co-culture model, it was addressed whether galectin-4 is involved in the pro-inflammatory response seen in IBD. IEC were co-cultured with CD3/28-activated PBMC to simulate an effector immune response. Since TLR4 expression by IEC is increased in IBD patients, IEC were apically exposed to the TLR4 ligand LPS. We have previously shown, using IEC/PBMC co-cultures, that TLR4 expression on IEC is increased upon co-culture of IEC with CD3/28-activated PBMC. Interestingly, we found that ligation of TLR4 by IEC resulted in enhanced expression of galectin-4, but not galectin-9 by IEC (**Figure 4A** and **4B**). Exposure of IEC to TLR4 ligand in combination with GF reversed the increased galectin-4 mRNA expression to control levels (**Figure 4A**). This was observed at the protein level as well (**Figure 4C**). No significant differences were observed on galactin-9 mRNA and protein level, though increase was suppressed TLR4-induced IL-12 and IL-17, but not IL-6 and TNF-α secretion by PBMC in the co-culture system (**Figure 4D**). In addition, GF protected from a TLR4-induced reduction in the percentage of Treg cells in an IEC-dependent manner (**Figure 4E**).

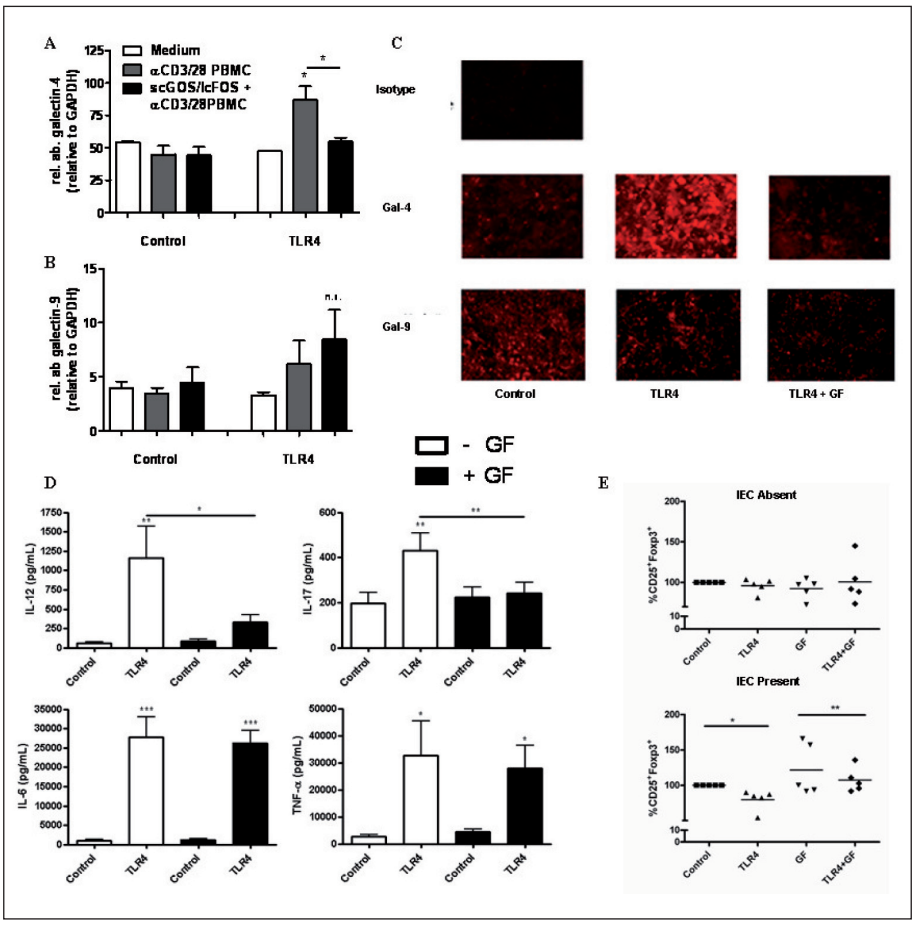

Figure 4 TLR4-induced galectin-4 expression, pro-inflammatory cytokine production and suppression of Treg cells is prevented by GF. *Human intestinal epithelial cells (HT-29) were apically exposed to LPS and co-cultured with CD3/28-activated PBMC. Galectin-4 mRNA expression was measured 4h after co-culture. Galectin-4 protein expression, cytokine production by PBMC and PBMC phenotype were assessed after 24h of culture. Apical exposure of IEC to LPS enhances galectin-4, but not galectin-9 mRNA and protein expression by IEC (A-C). Presence of GF prevents the up-regulation of galectin-4 expression by IEC (A, C). In addition, GF prevents TLR4-induced IL-12 and IL-17, but not IL-6 and TNF-α secretion by activated PBMC (D). In addition, GF prevents TLR4-induced reduction in Foxp3+ T cells only when IEC are present (E). Data are representative for n=5 independent PBMC donors, mean ± SEM, *P<0.05, **P<0.01, n.s not significant.*

Inflamed intestinal tissue shows increased galectin-4 and reduced galectin-9

To address the involvement of galectin-4 and -9 in colitis, mRNA expression of galectin-4 and -9 were analyzed in both colon and ileum. Galectin-4 mRNA expression in the colon was increased in mice undergoing DSS colitis, which was prevented when mice were fed GF or GF/Bb (**Figure 5A**). However, no differences were observed for galectin-9 mRNA expression in the colon of these mice (**Figure 5B**). In the ileum, DSS treatment did not affect galectin-4 expression, whereas the mRNA expression of galectin-9 tended to be reduced. Dietary intervention with Bb, GF and GF/Bb reduced galectin-4 mRNA expression in the ileum (**Figure 5C**). In contrast, galectin-9 mRNA expression in the ileum tended to enhance in DSS treated mice fed GF/Bb (**Figure 5D).**

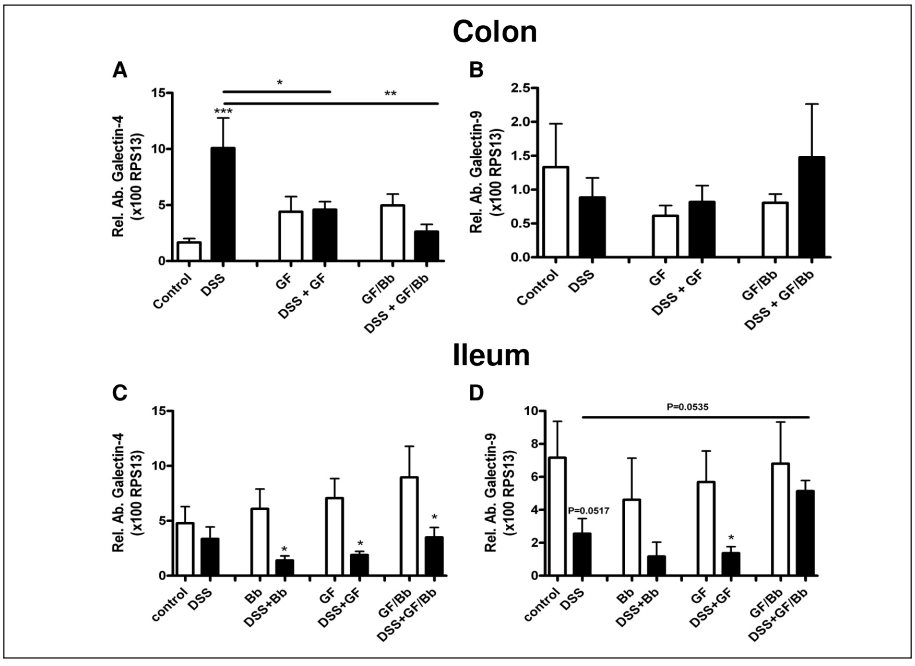

Figure 5 Expression of galectin-4 and -9 mRNA in colon and ileum of mice fed Bb, GF or GF/Bb. *The mRNA expression of galectin-4 and -9 was analyzed control and DSS treated mice fed Bb, GF or GF/Bb. In the colon, galectin-4 mRNA expression was increased upon DSS treatment in mice. Dietary supplementation with GF and GF/Bb normalized galectin-4 expression to the level of healthy control mice (A). In contrast, no significant changes were observed in galectin-9 expression in the colon (B). In the ileum, galectin-4 expression was not affected by DSS treatment. However, in mice uncergoing DSS colitis, dietary intervention with Bb, GF and GF/Bb suppressed galectin-4 expression in the ileum (C). Galectin-9 expression in the ileum tended to be decreased in mice exposed to DSS, whereas dietary intervention with GF/Bb increased the expression of galectin-9 (D). Data are representative for n=6 mice per group, mean ± SEM, *P<0.05, **P<0.01, ***P<0.001*

To show whether galectin-4 and -9 are differentially expressed in humans, the mRNA expression of galectin-4 and -9 as well as of the cytokines TNF-α and IL10 were measured in human intestinal samples from non-inflamed and inflamed tissue derived from UC and CD patients. Colorectal cancer samples served as controls. As expected, comparing inflamed versus non-inflamed tissue within patients, TNF-α expression was increased in inflamed tissue of both UC and CD patients, whereas IL-10 expression was reduced in CD patients (**Figure 6A** and **6B**). Galectin-4 expression was significantly increased in inflamed tissue from UC patients when compared to non-inflamed and control tissue, while in CD patients a similar trend was observed (**Figure 6C**). In contrast, galectin-9 expression was significantly reduced in inflamed tissue in CD patients, whereas a similar trend was found in inflamed UC tissue (**Figure 6D**) when compared to non-inflamed tissue. To confirm the increased galectin-4 and reduced galectin-9 expression during intestinal inflammation in humans, we performed immunohistochemical analysis. Similar to the increased mRNA expression of galectin-4 in inflamed tissue from UC patients, galectin-4 protein expression was increased in IEC in UC patients and to lesser extent in CD patients during inflammation. Furthermore, galectin-9 expression in IEC was decreased in inflamed tissue specimens of both UC and CD patients (**Figure 7**) compared to non-inflamed intestinal tissue.

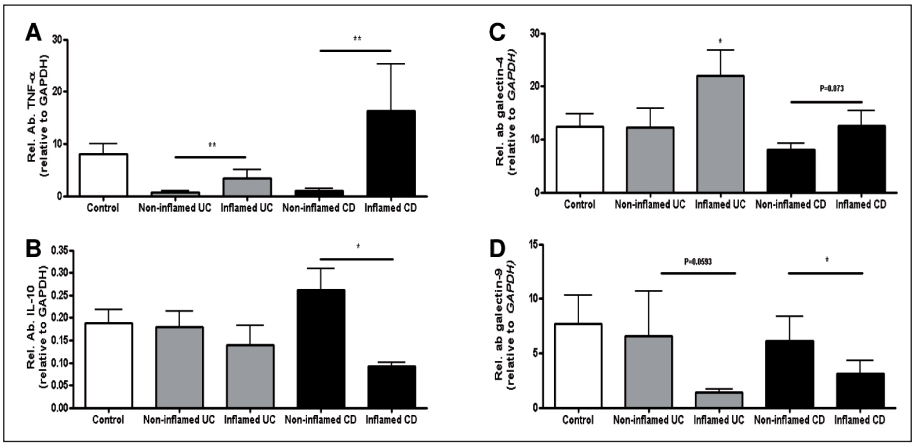

Figure 6 Expression of galectin-4 and -9 mRNA in inflamed colonic tissue from mice and humans. *Expression of TNF-α (A), IL-10 (B), galectin-4 (C) and galectin-9 (D) mRNA was analyzed in human intestinal samples from UC and CD patients. In inflamed tissue from UC and CD patients, increased pro-inflammatory TNF-α mRNA expression is observed, whereas in CD patients a reduction of anti-inflammatory IL-10 expression is observed as well. In UC patients, and to lesser extent in CD patients, increased galectin-4 mRNA expression was found. In contrast, decreased galectin-9 expression is observed in CD patients and to lesser extent in UC patients. Data represent n=10 patients in the control group and n=5 UC and CD patients.*

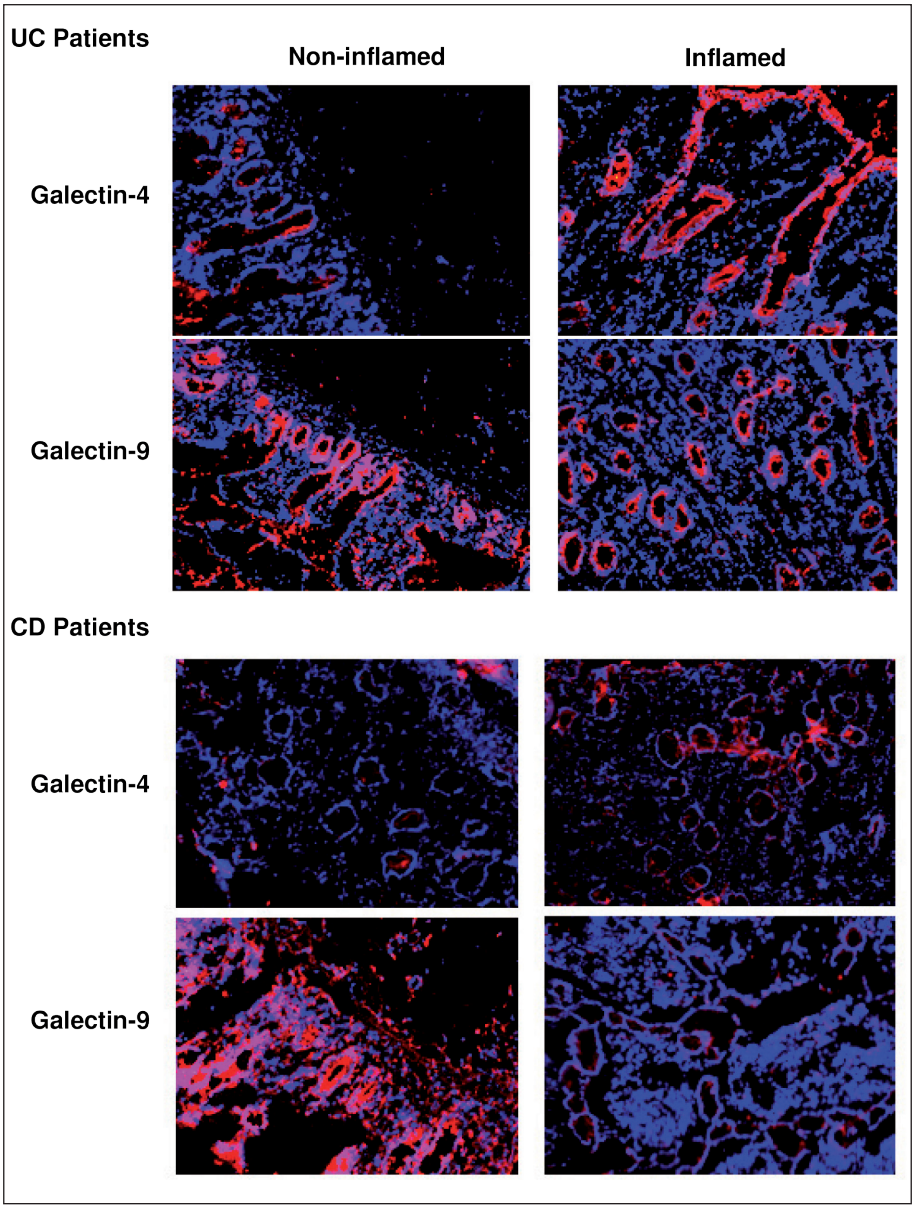

Figure 7 Galectin-4 and -9 protein expression in inflamed and non-inflamed UC and CD tissue. *Galectin-4 and -9 protein expression was analysed by fluorescence microscopic analysis of inflamed and non-inflamed tissue from human UC and CD. In UC patients, increased galectin-4 expression is observed in the intestinal epithelium in inflamed tissue, whereas galectin-9 expression by IEC is slightly reduced. In CD patients, galectin-4 expression is moderately increased, whereas galectin-9 expression by IEC is reduced in inflamed tissue. Data are representative for n=3 patients per group.*

Discussion

In contrast to IEC in non-inflammatory conditions, the intestinal epithelium of IBD patients show a massive increase in the expression of TLR4 (Abreu et al., 2001; Cario and Podolsky, 2000). Furthermore, mice expressing a constitutively active TLR4 are highly susceptible to DSS-induced colitis and DSS-treated mice show increased TLR4 expression by IEC (Fukata et al., 2011; Ortega-Cava et al., 2003). Previously, using an *in vitro* co-culture system, in which IEC were basolaterally co-cultured with human CD3/28-activated PBMC, we observed an inflammatory response upon apical TLR4 exposure of IEC (de Kivit et al., 2011). In the present study, we show that apical exposure of IEC to TLR4 ligand in the presence of GF suppressed IL-12 and IL-17 production by PBMC, indicating anti-inflammatory capacities for non-digestible oligosaccharides via IEC in this system. Surprisingly, TNF-α and IL-6 production by PBMC was not affected. These cytokines are highly expressed by monocytes as well and this may suggest that GF, in presence of LPS, only modulates T cell polarization. CD103+ DC effectively inducing Treg cells are protective for the development of colitis (Iliev et al., 2009; Annacker et al., 2005; Collins et al., 2012). Combined exposure of IEC to TLR4 ligand in the presence of GF prevents TLR4-induced reduction of Foxp3+ Treg cells *in vitro*. Furthermore, we show that TLR4 activation of IEC results in enhanced galectin-4 expression by IEC, which is prevented by the presence of GF. Interestingly, galectin-9 expression tended to enhance upon TLR4 ligation in the presence of GF. These data suggest a possible link between TLR signaling and galectin secretion by the intestinal epithelium during inflammation.

The interaction between the host microbiota and the immune system plays an important role in intestinal homeostasis. With respect to DSS-colitis, neutralization of TLR2 and TLR4 improves disease activity, which was associated with restoration of *Lactobacillus spp.* and *Bifidobacterium spp.* in the gut (Dong et al., 2012). In particular, it was recently shown by us and others that bacterial DNA from *Lactobacilli* and *Bifidobacteria* have suppressive capacities by reducing inflammatory cytokine production and supporting Treg conversion (de Kivit et al., submitted) (Campeau et al., 2012; Bouladoux et al., 2012). Similarly, non-digestible oligosaccharides support the growth of *bifidobacteria* and *lactobacilli* and especially inulin and fructo-oligosaccharides are reported to have protective effects on the development of DSS-induced colitis (Newburg, 2000; Osman et al., 2006). In line with these observations, our study shows a delay in the development of fecal bleeding and diarrhea upon colitis induction by DSS when animals are fed GF or GF/Bb. Colonic inflammation was only improved when animals were fed Bb or combined with GF, suggesting

that TLR activation of IEC is crucial to suppress intestinal inflammation. This is associated with reduced galectin-4 mRNA expression in colon and increased galectin-9 mRNA expression in the ileum, indicating that galectin-4 and -9 are associated with colitis. In addition, CD103+ DC and Treg numbers in MLN were increased by the GF/Bb diet. Depletion of Foxp3+ Treg cells has been shown to aggravate DSS colitis, whereas increased Treg induction protects from colitis (Iliev et al., 2009; Annacker et al., 2005; Collins et al., 2012; Boehm et al., 2012). Hence, dietary intervention using GF or GF/Bb may prevent the development of colitis through suppression of galectin-4 and enhancing Treg cell conversion at the expense of Th17 in the GALT. Furthermore, in the colonic mucosa of IBD patients, DC as well as macrophages are characterized by high expression of co-stimulatory molecules and DC produce large amounts of pro-inflammatory cytokines (te Velde et al., 2003; Hart et al., 2005; Rogler et al., 1999). Interestingly, small aphtoid lesions are most commonly found in the PP from the distal ileum in 70% of CD patients, suggesting that PP are potentially involved in the pathogenesis of CD (Rutgeerts et al., 1984; Olaison et al., 1992). DC present in PP can actively take up adherent invasive *E.coli* bacteria and migrate to MLN to instruct T cell responses (Keita et al., 2008; Salim et al., 2009). Hence, DC conditioned in PP upon dietary intervention with GF/Bb may result in the induction of tolerance in the colon. The GF or GF/Bb-induced suppression of DC and induction of Treg cells in MLN of colitic mice is paralleled by an induction of Treg cells and a suppression of T cell activation as wells as a reduction in Th1 and Th17 polarization in the PP.

In the present study we observe for the first time differential expression of galectin-4 and -9 in human resection specimens from UC and CD patients. Immunohistochemistry shows that the expression of galectin-4 and -9 was highly abundant in IEC. Not much is known about the role of galectins in IBD. Although galectin-4 was shown to exacerbate intestinal inflammation, it is proposed that galectin-4 exerts anti-inflammatory effects by inducing T cell apoptosis in the intestinal mucosa as well (Paclik et al., 2008; Hokama et al., 2004). Especially in inflamed areas of UC patients, galectin-4 expression in the intestinal epithelium was increased. In contrast to galectin-4, the expression of galectin-9 by IEC was reduced in inflamed tissue of IBD patients. Recently, a dysregulation in the TIM-3/galectin-9 axis was shown in CD patients. In this study, in contrast to our observations, galectin-9 expression in the ileum and sigmoid colon did not differ between CD patients and healthy individuals (Morimoto et al., 2011). We recently proposed a possible role for galectin-9 in the induction of Treg cells through conditioning of DC (de Kivit et al., submitted). Hence reduced expression of galectin-9 may be associated with reduced mucosal tolerance in the intestine, whereas increased galectin-4 expression may be associated with increased inflammatory responses.

In summary, dietary intervention with Bb, GF or GF/Bb delays DSS-induced colitis by attenuating the development of diarrhea, fecal bleeding and suppressing colonic inflammation. Dietary intervention with GF/Bb suppresses DC activation, whereas all diets enhance the frequency of CD103+ DC and Treg cell polarization *in vivo*. This is accompanied by reduced galectin-4 expression in the colon. Furthermore, differential expression of galectin-4 and -9 in inflamed areas of the intestine in UC and CD patients is observed, indicating the potential involvement of galectins in chronic inflammatory disease of the intestine. These data indicate the potential use of dietary non-digestible oligosaccharides such as GF/Bb in attenuating the onset of chronic inflammatory disease of the intestine.

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8

Exposure of intestinal epithelial cells to CpG DNA and galacto-/fructo-oligosaccharides protects against epithelial barrier disruption *in vitro*

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Abstract

The intestinal epithelial barrier is maintained by tight junction proteins, including zonula occludens (ZO)-1 and occludin, located at the lateral membranes of intestinal epithelial cells (IEC). Pro-inflammatory cytokines cause disruption of tight junctions by suppressing the expression of or re-localizing tight junction proteins. Intestinal permeability is increased in inflammatory bowel disease and TLR9 ligation of IEC can contribute to maintain intestinal homeostasis. Specific bacterial strains and non-digestible oligosaccharides may be beneficial in remission maintenance. In this study, we assessed whether specific non-digestible oligosaccharides (scGOS/lcFOS) can protect intestinal epithelial barrier integrity under inflammatory conditions. T84 and Caco-2 IEC lines were grown on transwell insert filters and co-cultured with CD3/28-activated PBMC. IEC were apically exposed to scGOS/lcFOS in the absence and presence of TLR9 ligand. Transepithelial resistance (TER) and paracellular permeability were assessed. In addition, the expression and localization of ZO-1 and occludin was analyzed. Apical exposure of T84 cells, but not Caco-2 cells, to TLR9 ligand protected against a decrease in TER and increased paracellular permeability upon co-culture with CD3/28-activated PBMC. This was enhanced by the presence of scGOS/lcFOS. In addition, exposure of T84 cells to TLR9 ligand in the presence of scGOS/lcFOS prevented re-localization of ZO-1 and occludin from the tight junction. In conclusion, these data indicate a potential protective effect of scGOS/lcFOS on intestinal epithelial barrier integrity under inflammatory conditions.

Introduction

Intestinal epithelial cells (IEC) line the intestinal lumen and provide a physical barrier between the intestinal lumen and the immune cells present in the lamina propria. The barrier is maintained by tight junctions located at the lateral membranes of IEC. Several proteins are associated with the tight junctions. Zonula occludens (ZO)-1 and occludin are the best characterized proteins in the junctional complex (Anderson and van Itallie, 1995; Balda and Matter, 1998). ZO-1 is a membrane-associated intracellular protein, whereas occludin is a transmembrane protein (Stevenson et al., 1986; Furuse et al., 1993). ZO-1 is required to retain occludin in the tight junction, but also interacts with the actin cytoskeleton in IEC (Fanning et al., 1998). Thus, the critical interaction between transmembrane tight junction proteins and the cytoskeleton and may influence paracellular transport through the tight junctions. Especially, myosin light chain kinase (MLCK) regulates tight junction permeability through phosphorylation of myosin light chain (MLC). This results in contraction of the peri-junctional actomyosin ring (Cunningham and Turner, 2012). MLCK is activated amongst others by pro-inflammatory cytokines, including IFN-γ (Wang et al., 2005). Activation of MLCK has been described to reduce the transepithelial electrical resistance (TER) and enhance tight junction permeability, associated with redistribution of ZO-1 and occludin (Shen et al., 2006).

Disruption of the intestinal epithelial barrier is characteristic in inflammatory bowel disease (IBD). IBD is characterized by a chronic inflammation in the intestine. Whereas Crohn's disease (CD) manifests as mainly distinct aphthous transmural lesions throughout the entire gastrointestinal tract, ulcerative colitis (UC) lesions remain restricted to the epithelial surface, which only occur in the colon (Baumgart and Sandborn, 2007). In particular, IFN-γ as well as bacteria are known to affect intestinal epithelial permeability and TNF-α can potentiate this effect (Wang et al., 2005). In IBD, the expression of tight junction proteins, including occludin and ZO-1, is dramatically decreased. Down-regulation of tight junction protein expression is associated with increased neutrophil influx into the lamina propria in IBD patients (Kucharzik et al., 2001). In both CD and UC, MLCK expression and MLC phosphorylation are increased (Blair et al., 2006). The disruption in tight junction integrity results in increased baterial translocation from the gut lumen into the lamina propria, reinforcing the inflammation. Hence, improving the barrier integrity of IEC may ameliorate chronic inflammation of the intestine.

It has been shown that treatment of T84 and Caco-2 IEC monolayers with *Lactobacillus plantarum* MB452 and *Lactobacillus casei* protect against disruption of tight junctions through induction of occludin and ZO-1 expression (Karczewski et al., 2010; Eun et al, 2011). Furthermore, fecal derived *Bifidobacteria* grown in presence of human milk oligosaccharides prevented re-localization of occludin out of the tight junction (Chichlowski et al., 2012). These data suggest that specific bacterial strains can exert protective effects on epithelial barrier integrity. Dietary intervention using a specific mixture of short-chain galacto- and long-chain fructo-oligosaccharides (scGOS/lcFOS; GF) in combination with *Bifidobacterium breve* M-16V is known to induce Treg polarization in cow's milk allergic mice (Schouten et al., 2009; de Kivit et al., 2012). In addition, we have recently observed an induction of Treg polarization in DSS-treated mice fed a diet supplemented with GF and *Bifidobacterium breve* M-16V as well (de Kivit et al., submitted). Apical exposure of IEC to DNA of commensal or probiotic bacteria supports an anti-inflammatory response (Bouladoux et al., 2012; Campeau et al., 2012). Bacterial DNA activates TLR9, and it has been observed that DNA from *Lactobacillus rhamnosus* GG prevented TNF-α-induced epithelial barrier disruption (Ghadimi et al., 2010). Furthermore, we have recently indicated that scGOS/lcFOS potentiates apical TLR9 activation in IEC to induce anti-inflammatory Treg responses (de Kivit et al., submitted). It is not known whether apical TLR9 activation influences the expression or activity of MLCK in IEC. However, in IBD pro-inflammatory cytokine production correlates with intestinal epithelial MLCK expression and barrier disruption (Blair et al., 2006). Therefore, we hypothesized that scGOS/lcFOS can exert protective effects on intestinal barrier integrity. We observed that apical exposure of T84 cells to TLR9 ligand in the presence of scGOS/lcFOS had protective effects on TER and transepithelial permeability. This was associated with reduced redistribution of occludin and ZO-1 from the tight junction.

Materials and Methods

Transwell co-cultures

Culture of human IEC lines (Caco-2 and T84 cells), isolation of human peripheral blood mononuclear cells (PBMC) and transwell co-cultures were performed as previously described (de Kivit et al., 2011). In short, Caco-2 or T84 cells were grown till confluence on transwell insert filters (Corning, NY, USA). Confluence was examined by measurement of TER IEC were co-cultured with $3x10^6$ CD3/28-activated PBMC for 24h or 48h. IEC were apically exposed to either TLR9 ligand (M362 type C, 5.0 µM, Invivogen, San Diego, CA, USA) alone or in combination with 0.5% w/v of a 9:1 mixture of scGOS (Vivinal GOS, Borculo Domo) and lcFOS (Raftiline HP, Orafti) (scGOS/lcFOS) (ImmunofortisTM, Nutricia).

Transepithelial permeability

For measurement of transepithelial permeability, IEC were washed with RPMI1640, supplemented with 2.5% FCS, penicillin (100U/mL)/ streptomycin(100µg/mL) (Sigma) and sodium pyruvate (1mM; Sigma) without phenol red 1h prior to theaddition of FITC-dextran. IEC were apically exposed to 1mg/mL 4kDa FITC-dextran (Sigma Zwijndrecht, The Netherlands) for 30-60min. Paracellular transport of 4kDa FITC-dextran was assessed by measuring the concentration of the FITC-Dextran in the basolateral compartment using a Mithras LB 940 fluorometer (Berthold Technologies, Belgium).

Western blot

At 48h after the co-culture experiment, IEC were collected in Laemmli buffer and stored at -20°C until further used. Protein concentration was determined (Bio-Rad DC Assay) and protein was denatured by adding bromophenol blue and 2-mercaptoethanol at 100°C for 5 min. Proteins were separated using a pre-cast 4-20% SDS-PAGE gel (Bio-Rad) or an 8% SDS-PAGE gel for ZO-1 expression. Proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Membranes were blocked with 5% Protifar in TBS-T for 1h. After blocking, membranes were washed and incubated with rabbit anti-ZO-1 IgG (1:1000, Cell Signaling Technology, Boston, USA), rabbit anti-occludin IgG (1:1000, Cell Signaling) and goat anti-actin IgG (1:500, Santa Cruz Biotechnology, Santa Cruz, USA) overnight at 4°C. Membranes were washed and incubated with horseradish peroxidase conjugated secondary antibodies; goat anti-rabbit IgG (1:2000, Cell Signaling) and rabbit anti-goat IgG (1:2000, Santa Cruz), for 1h at room temperature. Western blots were developed using ECL reagents (GE Healthcare, Buckinghamshire, UK) to visualize proteins and the signal was captured with photographic film (Pierce).

Fluorescence microscopy

At 48h after the co-culture experiment, IEC monolayers were fixed for 10 minutes with 4% formalin in PBS. Cells were blocked and permeabilized with 1% BSA and 0.1% Triton X-100 in PBS. Filters were stained using: rabbit anti-human ZO-1, mouse anti-human occludin (Invitrogen, Carlsbad, CA, USA) for 2h at room temperature. Filters were washed three times with PBS, followed by incubation with goat anti-mouse AlexaFluor488 and goat anti-rabbit AlexaFluor568 (Invitrogen). Microscope slides were preserved with Prolong Gold (Invitrogen). Filters were microscopically analyzed using a Olympus BX60 fluorescence microscope equipped with DFC425C color camera (Leica).

Statistics

Statistical analyses were performed using paired Student's t-test or one-way ANOVA for repeated measurements followed by Bonferroni's post hoc test. Analyses were performed using Graphpad Prism 5.0. $P < 0.05$ was considered statistically significant.

Results

TLR9 ligand combined with scGOS/lcFOS protects against barrier disruption in T84 cells co-cultured with CD3/28-activated PBMC

To examine whether TLR9 ligand and scGOS/lcFOS have protective effects on intestinal epithelial barrier integrity, T84 and Caco-2 cells were basolaterally co-cultured with CD3/28-activated PBMC for 24h. IEC were apically exposed to TLR9 ligand in the absence or presence of scGOS/lcFOS and TER was measured. After 24h of co-culture with unstimulated PBMC, TER tended to increase, but was not affected by apical TLR9 stimulation, scGOS/lcFOS or a combination of both (**Figure 1A** and **1B**). As expected, upon co-culture with CD3/28-activated PBMC, TER was significantly decreased in T84 and Caco-2 cells. However, exposure of T84 cells to TLR9 ligand partially protected against a decrease in TER, which was more pronounced upon apical exposure of T84 cells to a combination of TLR9 ligand in the presence of scGOS/lcFOS (**Figure 1C**). Apical exposure of T84 cells to scGOS/lcFOS alone did not protect against a decrease in TER induced by CD3/28-activated PBMC. However, these effects were not observed when using Caco-2 cells (**Figure 1D**).

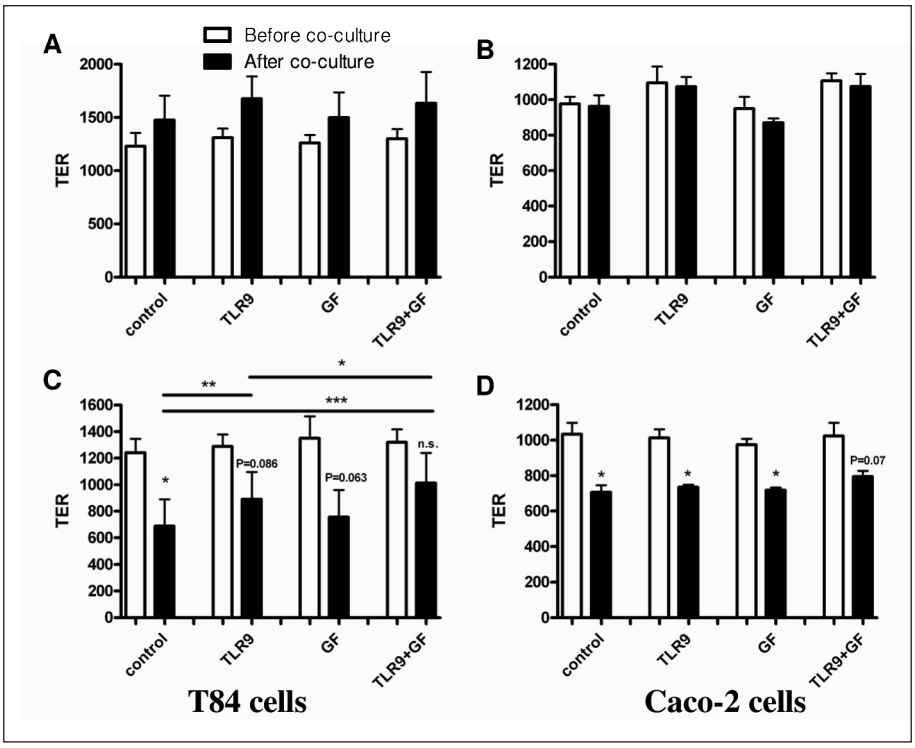

Figure 1 A reduction in epithelial TER under inflammatory conditions is partially prevent upon apical exposure of IEC to TLR9 ligand in the absence or presence of scGOS/lcFOS. *T84 and Caco-2 cells were co-cultured with unstimulated (A, B) or CD3/28-activated PBMC (C, D) and apically exposed to TLR9 ligand, scGOS/lcFOS (GF) or a combination of both. Upon co-culture with unstimulated PBMC, TER was increased in T84 cells, but not Caco-2 cells, and apical exposure to TLR9 ligand or scGOS/lcFOS did not influence TER (A, B). Upon co-culture with CD3/28-activated PBMC, TER was decreased in both T84 and Caco-2 cells. However, only in T84 cells, apical exposure to TLR9 ligand partially prevented a decrease in TER. This was potentiated by the presence of scGOS/lcFOS (C). TER from filters (125Ωxcm^2) was substracted from the TER. Data are representative for n=3 independent PBMC donors, mean ± SEM, *P<0.05, **P<0.01, ***P<0.001.*

An increase in epithelial permeability under inflammatory conditions is prevented upon apical exposure of IEC to TLR9 ligand in presence of scGOS/lcFOS

Next we evaluated whether a protection in the decrease in TER was reflected by a lower permeability of the IEC monolayer. T84 and Caco-2 cells were basolaterally co-cultured with CD3/28-activated PBMC. IEC were apically exposed to TLR9 ligand in the absence or presence of scGOS/lcFOS and TER was measured. Epithelial permeability was assessed at 48h after co-culture by measuring the diffusion of 4kDa FITC-dextran to the basolateral compartment. Co-culture of IEC with CD3/28-activated PBMC resulted in high concentration of 4kDa FITC-dextran in the basolateral compartment. In T84 cells, but not Caco-2 cells, apical exposure to TLR9 ligand, scGOS/lcFOS or especially TLR9 ligand in the presence of scGOS/lcFOS reduced paracellular transport of 4kDa FITC-dextran (**Figure 2**).

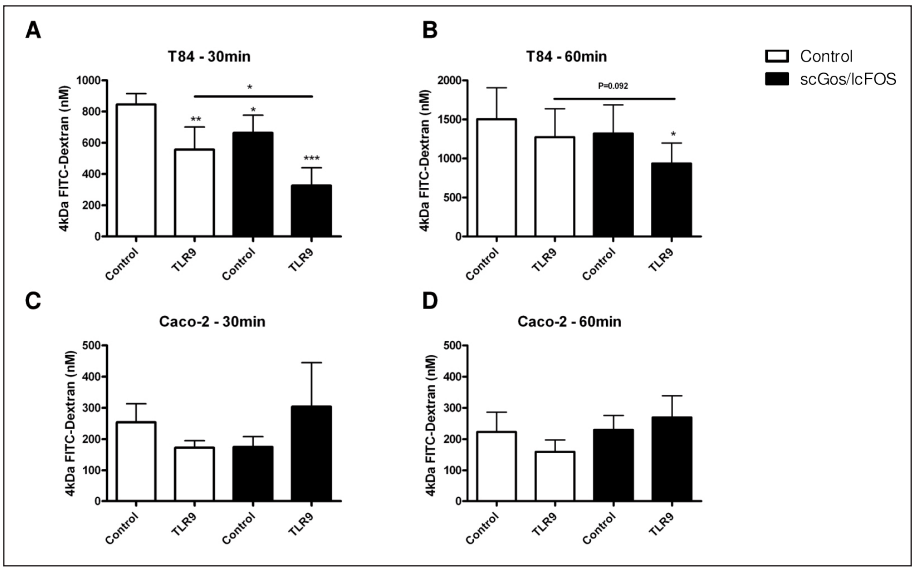

Figure 2 Inflammation induced epithelial permeability is improved upon apical exposure of IEC to TLR9 ligand in the absence and presence of scGOS/lcFOS. *T84 and Caco-2 cells were co-cultured with CD3/28-activated PBMC and apically exposed to TLR9 ligand, scGOS/lcFOS or a combination of both for 48h. Diffusion of 4kDa FITC-dextran into the basolateral compartment was measured for 30 and 60min. In T84 cells apical exposure to TLR9 ligand, scGOS/lcFOS and especially a combination of TLR9 ligand in the presence of scGOS/lcFOS prevented paracellular transport of 4kDa FITC-dextran after 30min and 60min into the basolateral compartment (A, B). These effects were not observed when using Caco-2 cells (C, D). Data are representative for n=3 independent PBMC donors, mean ± SEM, *P<0.05, **P<0.01, ***P<0.001.*

Reduced co-localization of ZO-1 and occludin in IEC upon co-culture with activated PBMC is prevented by apical exposure to TLR9 ligand and scGOS/lcFOS

Since apical exposure of T84 cells to TLR9 ligand in presence of scGOS/lcFOS enhances the TER and barrier function of IEC, the expression of occludin and ZO-1 were analyzed. Western blot analysis showed that the expression of occludin and ZO-1 by T84 cells is reduced upon co-culture with CD3/28-activated PBMC. However, apical exposure of T84 cells to TLR9 ligand in the presence of scGOS/lcFOS did not increase the expression of occludin or ZO-1 (**Figure 3A**). Furthermore, phosphorylation of MLC was partially prevented upon apical exposure of IEC to TLR9 ligand in the presence of scGOS/lcFOS (**Figure 3B**).

Next, fluorescence microscopy was used to analyze the localization of the tight junction proteins in the IEC monolayer 48h after co-culture. Occludin and ZO-1 were re-localized out of the tight junction in T84 cells co-cultured with CD3/28-activated PBMC. However, when T84 cells were apically exposed to TLR9 ligand in the presence of scGOS/lcFOS, localization of occludin and ZO-1 was partially retained in the tight junction (**Figure 3C**). Furthermore, less co-localization of occludin and ZO-1 was observed in T84 cells cultured with CD3/28-activated PBMC, which was improved upon apical exposure to TLR9 and scGOS/lcFOS.

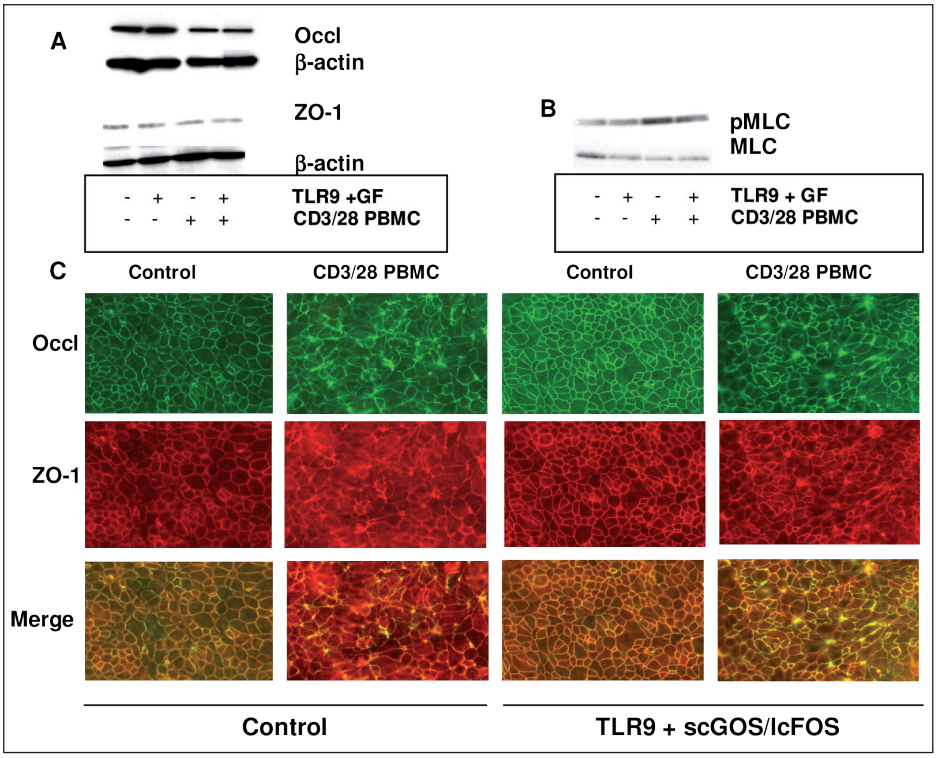

Figure 3 Expression of occludin and ZO-1 in T84 cells. *T84 cells were co-cultured for 48h with CD3/28-activated PBMC and exposed to TLR9 ligand in the presence of scGOS/lcFOS. Western blot analysis showed reduced occludin expression in T84 cells co-cultured with CD3/28-activated PBMC. This was not changed upon apical exposure of T84 cells to TLR9 ligand in the presence of scGOS/lcFOS. Expression of ZO-1 was slightly reduced as well (A). Phosphorylation of MLC was increased upon co-culture of IEC with CD3/28-activated PBMC. However, epithelial exposure of T84 cells to TLR9 ligand in the presence of scGOS/lcFOS prevented MLC phosporylation (B). In T84 cells co-cultured with CD3/28-activated PBMC relocalization of occludin and ZO-1 out of the tight junction occurred. Furthermore, occludin and ZO-1 did not co-localize. However, exposure of T84 cells to TLR9 ligand in the presence of scGOS/lcFOS partially prevented the relocalization of occludin and ZO-1 (C). Preliminary data represent n=1 experiment.*

Discussion

In the present study, preliminary observations show that apical activation of IEC using a TLR9 ligand in the presence of scGOS/lcFOS has protective effects on intestinal epithelial barrier integrity under inflammatory conditions. Apical exposure of IEC to TLR9 ligand in the presence of scGOS/lcFOS partially prevented a reduction in TER and increase in intestinal epithelial permeability. These observations are potentially linked to the prevention ZO-1 redistribution from the tight junction and reduced phosphorylation of MLC, as the expression of occludin and ZO-1 was not affected.

Disruption of the intestinal epithelial barrier is characteristic for example in IBD. In particular, IFN-γ treatment of IEC results in a decreased barrier function through reduction of ZO-1 expression and consequently perturbations in the actin cytoskeleton (Youakim and Ahdieh, 1999). Since ZO-1 binds occludin, re-localization or reduced expression of ZO-1 results in re-localization of occludin, thereby increasing paracellular permeability. Our results indicate that exposure of T84 cells, but not Caco-2 cells, to TLR9 ligand in the presence of scGOS/lcFOS partially prevented epithelial barrier disruption. Co-culture of IEC with CD3/28-activated PBMC disrupted the co-localization of occludin and ZO-1 in T84 cells, which was partially prevented upon apical exposure of IEC to TLR9 ligand in the presence of scGOS/lcFOS. Interestingly, Caco-2 cells express lower levels of TLR9 and may therefore not respond to TLR9 ligand (Uehara et al, 2007; Pedersen et al, 2005). It is known that activation TLR2 on IEC results in an increase in TER and re-localization of ZO-1 involving PKC (Cario et al., 2004). We elaborate on these data by showing that apical TLR9 ligation improved TER of T84 cells as well. Previously, we have shown that TLR9 ligation of IEC in the presence of scGOS/lcFOS enhanced IFN-γ production by PBMC (de Kivit et al., 2011), which would potentially further disrupt the tight junction. However, the anti-inflammatory cytokine IL-10 was enhanced as well and IL-10 is known to maintain epithelial barrier integrity (Madsen et al., 1997). In line with this, we have shown that genomic DNA derived from *Bifidobacterium breve* M-16V has similar immunoregulatory properties comparable to synthetic CpG DNA (de Kivit et al., submitted). These data indicate that, *in vivo*, dietary intervention using *Bifidobacterium breve* and scGOS/lcFOS may enhance the intestinal epithelial barrier integrity, thereby potentially ameliorating chronic inflammation in the intestine.

Although these preliminary data show potential protective effects on epithelial barrier integrity, underlying mechanisms remain to be investigated. Potential involvement of MLC phosphorylation through MLCK may

be an interesting point for future research. Contraction of the peri-junctional actomyosin ring results in re-localization of tight junction proteins from the tight junction complex hereby enhancing permeability. Exposure of IEC to scGOS/lcFOS and TLR9 ligand suppressed MLC phosphorylation, which induces this contraction of the actomyosin ring, which may underlie the mechanism of protection against inflammatory induced barrier disruption. In addition, various claudins are expressed by IEC that contribute to the epithelial barrier function (Prasad et al., 2005). These data provide new insight by which TLR9 ligand, such as bacterial DNA from commensal or probiotic bacterial strains, and scGOS/lcFOS exert protective effects in the intestinal mucosa.

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9

Summary and general discussion

The intestinal mucosa is constantly facing antigens amongst others derived from the diet and the microbiota. The intestinal epithelium serves as the first line defence against foreign antigens. A monolayer of IEC forms a physical barrier between the intestinal lumen and the lymphocytes which reside in the lamina propria (LP). Furthermore, the intestinal epithelium, contains mucus-secreting goblet cells and Paneth cells that actively secrete anti-microbial peptides (Linden et al., 2008; Salzman et al., 2010). In addition, IEC actively take part in the innate immune response through conditioning of dendritic cells (DC). Consequently, IEC shape adaptive immunity and give rise to tolerogenic DC, which induce Treg cells and thereby contribute to oral tolerance. IEC secrete soluble mediators that support the generation of tolerogenic CD103+ DC in the LP (Iliev et al., 2009a and 2009b). Induction of intestinal immune tolerance via modulation of DC function through IEC could be a novel way to prevent or treat inflammatory diseases of the intestine.

Dietary intervention using non-digestible oligosaccharides – in particular a 9:1 mixture of short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides (scGOS/lcFOS) – with or without combination with *Bifidobacterium breve* M-16V prevents the development of acute allergic symptoms, thereby promoting oral tolerance through yet unknown mechanisms (Schouten et al., 2009 and 1010; van der Aa et al., 2010; Arslanoglu et al., 2008). This thesis aims at understanding the mucosal immune system of the intestine and modulation of the gut-associated lymphoid tissue through observations on changes in immune cell subsets and function. Dietary intervention with specific non-digestible oligosaccharides and probiotic bacterial strains was performed in order to induce immunological tolerance in the GALT as a strategy to prevent or treat inflammatory diseases of the gut.

Summary

Directly underneath the intestinal epithelium, DC are present in the LP which have a close interaction with the intestinal epithelium. IEC are able to recognize luminal antigens through Toll-like receptors (TLR) (Cario et al., 2002; Uehara et al., 2007). TLR recognize microbial fragments, including bacterial cell wall fragments, bacterial endotoxins (such as LPS) and bacterial or viral RNA or DNA and hence recognize both antigens derived from the microbiota as well as invading pathogens. Mainly under inflammatory conditions, IEC express TLR at the apical surface, which contribute to both inflammation as well as immune tolerance (Abreu et al., 2002; Singh et al., 2005; Ewaschuk et al., 2007). Increased epithelial surface TLR2 and TLR4 expression is associated with inflammatory bowel disease (IBD) (Frolova et al., 2008). In contrast, apical TLR9 stimulation contributes to intestinal homeostasis (Lee et al., 2006). In **chapter 3** it is

shown, by using an *in vitro* IEC/PBMC co-culture model, that IEC respond differentially and in a polarized fashion to various TLR stimuli. Ligation of IEC with different TLR ligands resulted in differential mediator release from IEC and specific modulation of the effector immune response. It is shown that under inflammatory conditions, surface expression of TLR4 and TLR9 is increased. Stimulation of TLR4 using LPS resulted in a pro-inflammatory Th1-polarized effector response. This response is accompanied by production of TNF-α by monocytes. In contrast, apical TLR9 stimulation instructs a Th1-polarized response in presence of anti-inflammatory cytokine IL-10 secretion, whereas TNF-α production by monocytes was suppressed.

Dietary intervention using scGOS/lcFOS was previously shown to prevent acute allergic symptoms through induction of Treg cells (Schouten et al., 2010). However, the underlying mechanisms are not known yet. Since IEC are at the interface between the intestinal lumen and the LP, the IEC/PBMC co-culture model was used to assess whether scGOS/lcFOS act via IEC or directly on lymphocytes. **Chapter 4** shows, for the first time, that scGOS/lcFOS act via IEC by enhancing the apical epithelial TLR9-induced IFN-γ secretion by PBMC. In addition, scGOS/lcFOS in combination with TLR9 ligand enhanced the development of Treg cells. Since scGOS/lcFOS is a mixture of carbohydrate structures, it was hypothesized that lectins could be involved. **Chapter 2** of this thesis provides an overview of carbohydrate binding receptors and IEC express high amounts of various galectins (Lahm et al., 2001). We have demonstrated the expression of various galectins by IEC under inflammatory conditions as shown in **chapter 4**. Neutralization of galectin function by lactose or specific neutralization of galectin-9 was able to abrogate the regulatory effect of IEC apically exposed to TLR9 ligand in the presence of scGOS/lcFOS.

In **chapter 4** it is shown that apical exposure of IEC to genomic DNA of *Bifidobacterium breve* M-16V in the absence and presence of scGOS/lcFOS results in a similar effector immune response as compared with a synthetic TLR9 ligand. To confirm the involvement of galectin-9 in the protection against food allergy, a well described murine model for whey-induced cow's milk allergy was performed. In this model, it was previously shown that dietary intervention using scGOS/lcFOS, especially upon combination with *Bifidobacterium breve* M-16V (GF/Bb), effectively suppresses the development of acute allergic symptoms (Schouten et al., 2009). Data in **chapter 5** show that upon dietary intervention with GF/Bb the acute allergic hypersensitivity response is negatively correlated with increased serum galectin-9 levels. This depended on immune activation in the intestinal mucosa, since the induction of galectin-9 by GF/Bb was only observed in whey-sensitized animals that received an

oral challenge with whey. Interestingly, serum from whey-sensitized mice fed GF/Bb suppressed *in vitro* mast cell degranulation, which could be partially inhibited by neutralizing galectin-9.

Besides suppressing mast cell degranulation, galectin-9 is mainly known for its capacity to induce Th1 cell apoptosis (Zhu et al., 2005). However, several studies have indicated the capacity of galectin-9 to induce Treg cell polarization (Seki et al., 2008; Wang et al., 2009). In **chapter 5**, increased Th1 and Treg cell polarization was observed in mesenteric lymph nodes (MLN) of whey-allergic mice fed GF/Bb. Intestinal DC play a crucial role in the induction of mucosal tolerance and IEC can condition intestinal DC (Iliev et al., 2009b; Butler et al., 2006). In **chapter 6** it is shown that IEC apically exposed to TLR9 ligand in presence of scGOS/lcFOS suppress the expression of co-stimulatory molecules by monocyte-derived DC (moDC) in a galectin-9 dependent manner. Galectin-9 conditioned moDC had increased capacity to induce Foxp3+ Treg cells with suppressive function *in vitro*. Furthermore, described in **chapter 6** as well, similar observations were made *in vivo* in an ovalbumin (OVA)-induced murine model for food allergy. Dietary intervention with GF/Bb suppresses LP DC activation and cytokine production, while promoting the expansion of CD11c+CD103+ DC. Consequently, the frequency of Treg cells in the small intestinal LP was restored partially to the level of non-allergic mice, whereas the percentage of activated Th2 cells was suppressed compared to allergic mice. *Ex vivo* OVA re-stimulation of LP cells from allergic mice in presence of galectin-9 suppressed the production of Th2, but not Th1 associated cytokine production. Furthermore, it is known that LP DC in the intestinal LP are hyporesponsive towards TLR stimuli (Cerovic et al., 2009). In **chapter 6**, bone marrow-derived DC conditioned with galectin-9 are shown to be less responsive to LPS stimulation.

Since galectin-9 was shown to exert immunosuppressive functions at the level of DC, the use of dietary intervention with GF/Bb in a DSS-induced colitis model was addressed in **chapter 7**. Dietary intervention with GF/Bb did ameliorate disease activity in the DSS model, and disease progression was delayed. This was associated with an increased percentage of CD103+ DC in MLN and concomitantly an increased frequency of Treg cells in the MLN. Interestingly, GF/Bb enhanced the percentage of Treg cells in Peyer's patches (PP) as well and additionally, the percentage of activated T cells as

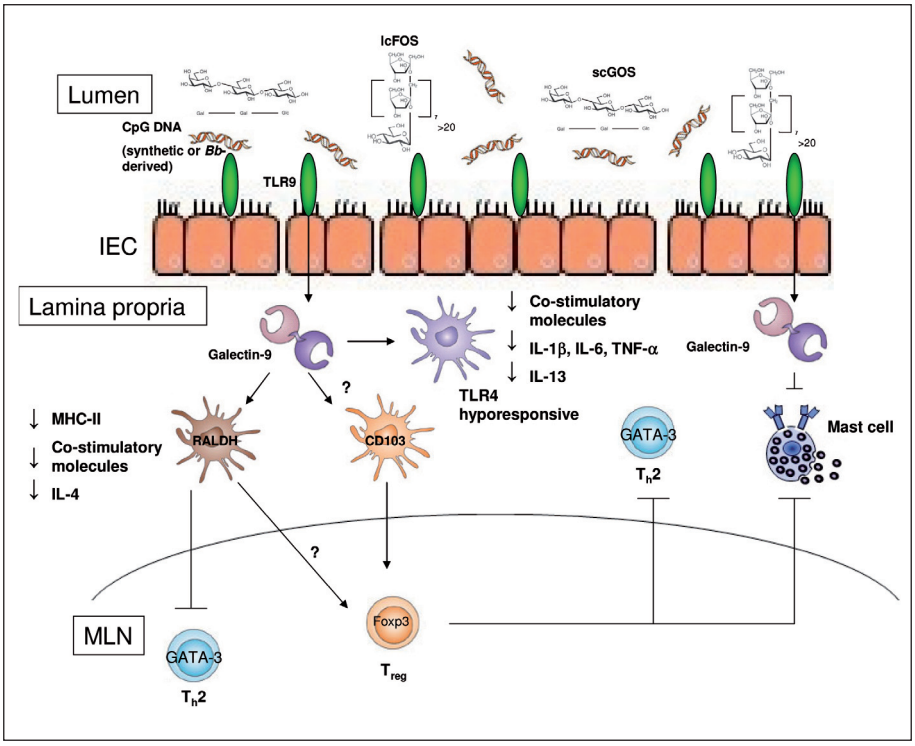

Figure 1 Protective effects of IEC-derived galectin-9 in the suppression of food allergic responses. *Under inflammatory conditions, apical TLR9 expression by IEC is increased (chapter 3). Ligation of TLR9 by synthetic CpG DNA or genomic DNA from Bifidobacterium breve M-16V(Bb) in the presence of scGOS/lcFOS induces galectin-9 secretion by IEC (chapter 4 and 5). Galectin-9 conditions DC to express lower levels of MHC-II and co-stimulatory molecules and induces RALDH activity in DC. Furthermore, galectin-9-conditioned DC produce less pro-inflammatory cytokines upon TLR4 stimulation. In allergic mice fed GF/Bb, an increased frequency of CD103+ DC is observed in the LP. Galectin-9-conditioned DC support Treg conversion, while suppressing Th2 polarization (chapter 5 and 6). In addition, galectin-9 secreted by IEC directly suppresses mast cell degranulation (chapter 5).*

well as the percentage of Th1 and Th17 cells were decreased in PP of mice exposed to DSS while being fed GF/Bb. These observations were associated with *in vitro* observations showing the capacity of scGOS/lcFOS to suppress TLR4-induced epithelial galectin-4 expression. TLR4-induced IL-12 and IL-17 production was suppressed by the presence of scGOS/lcFOS. Furthermore, galectin-4 expression was increased in the colon in DSS treated mice, whereas dietary intervention with *Bifidobacterium breve* M-16V, scGOS/lcFOS and GF/Bb suppressed galectin-4 expression

in both the colon and ileum. In contrast, galectin-9 expression was reduced mainly in the ileum, while GF/Bb enhance ileal galectin-9 expression in mice undergoing DSS colitis. Interestingly, in inflamed tissue of UC and CD patients, increased galectin-4 and reduced galectin-9 expression was observed.

Finally, it is believed that IBD is caused by an aberrant immune activation towards the commensal microbiota and indeed bacterial translocation into the LP is observed in IBD. Preliminary data described in **chapter 8** show that scGOS/lcFOS can have protective effects on the inflammation induced barrier disruption and localization of ZO-1 and occludin in the intestinal epithelial monolayer. This may potentially lower bacterial translocation and consequently the induction of severe inflammation by lymphocytes present under the intestinal epithelial monolayer.

Altogether, the data presented in this thesis indicate a protective role for specific non-digestible oligosaccharides in combination with *Bifidobacterium breve* M-16V in the intestinal mucosa under inflammatory conditions (**Figure 1** and **2**). Non-digestible may contribute to improved barrier integrity of IEC. In addition, IEC apically exposed to scGOS/lcFOS support the induction of tolerogenic DC, which effectively induce Treg cells and thereby suppressing the allergic Th2 type effector response. Strikingly, in a Th1/Th17 polarized murine IBD model, dietary intervention with scGOS/lcFOS resulted in a similar induction of tolerogenic DC and Treg cells. In addition, scGOS/lcFOS reduced both Th1 and Th17 in the gut-associated lymphoid tissue found. These data demonstrate that the immune modulatory effects of specific non-digestible oligosaccharides depend on the inflammatory status of the gut mucosa. Furthermore, galectin-9 secreted by IEC upon exposure to scGOS/lcFOS is involved in suppressing mast cell degranulation during the challenge phase of an allergic response. *In vitro* data indicate an important role for apical TLR9 ligation and scGOS/lcFOS in galectin-9 secretion by IEC. In contrast, scGOS/lcFOS suppressed TLR4-induced galectin-4 expression in IEC. In conclusion, dietary intervention with GF/Bb may contribute to the prevention or treatment of both allergic and chronic inflammatory diseases in the intestine.

IEC as first line of defence – more then ‘ just’ a physical barrier

IEC are well-known for their function as a physical barrier between the intestinal lumen and the lymphocytes present in the intestinal LP. In addition, specialized cells in the intestinal epithelium – Paneth cells and goblet cells – can secrete anti-microbial peptides and mucus contributing to the barrier for micro-organisms to enter the LP. However, at specialized sites – known as Peyer’s patches (PP)– luminal antigens are actively taken up through so-called microfold (M) cells in the follicle associated epithelium. The PP contains DC, T cells and B cells, which upon encountering an antigen become activated to raise an effector immune response. Furthermore, outside the PP, CX3CR1+ DC have been shown to extrude dendrites between the IEC to allow direct sampling of luminal antigens (Rescigno et al., 2001). In addition, the recent hypothesis is that CX3CR1+ DC take up luminal antigens,

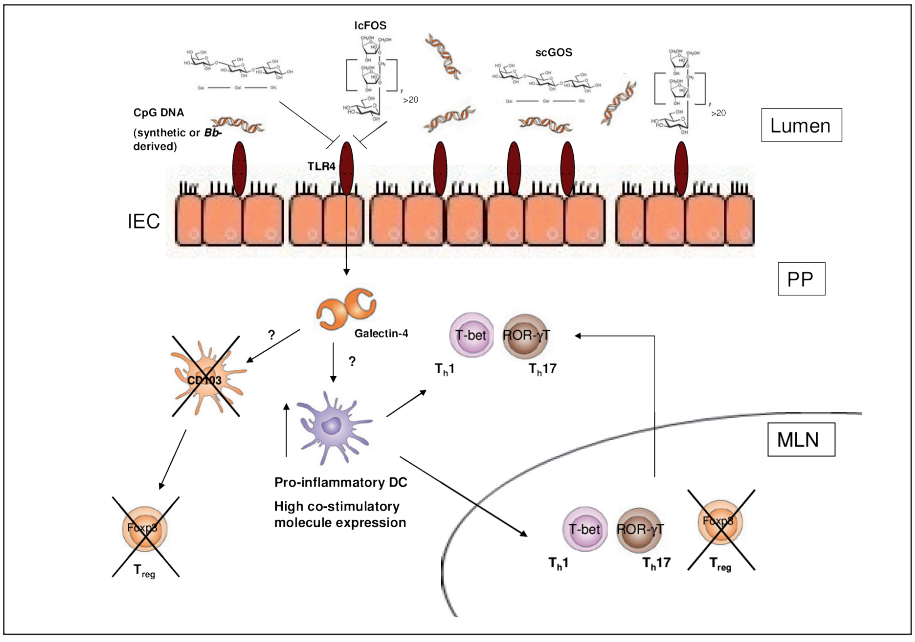

Figure 2 Potential mechanism by which dietary intervention with GF/Bb restores intestinal homeostasis in IBD. *TLR4 activation results in galectin-4 expression by IEC, potentially resulting in a lower frequency of tolerogenic CD103+ DC and an increased frequency of pro-inflammatory DC that express high levels of co-stimulatory molecules. This results in expansion of effector Th1 and Th17 cells and suppression of Treg conversion in PP and MLN. Dietary supplementation with GF/Bb reduces galectin-4 expression by IEC by suppression TLR4 activation on IEC and thereby increases the number of tolerogenic CD103+ DC in MLN. Consequently, Treg cell frequency is increased, whereas the percentage of Th1 and Th17 cells was suppressed in both PP and MLN upon supplementation of GF/Bb to the diet (chapter 7).*

including bacteria, which are then transported through gap junctions to CD103+ DC. Upon activation, CD103+ DC migrate to the draining MLN to instruct Treg conversion via mechanisms dependent on retinoic acid and TGF-β (Coombes et al., 2007; Sun et al., 2007). In addition to creating a barrier, IEC are described to express pathogen recognition receptors, including Toll-like receptors (TLR) and Nod-like receptors (NLR) (Cario et al., 2002; Uehara et al., 2007). Furthermore, in healthy conditions IEC are described to condition intestinal DC, which become tolerogenic and express less co-stimulatory molecules (Iliev et al., 2009b; Butler et al., 2006). Especially during inflammatory conditions, expression of TLR is enhanced by IEC, which is observed in for example inflammatory bowel disease (IBD) (Abreu et al., 2003). Data shown in chapter 4 and chapter 7 indicate that scGOS/lcFOS interact with TLR activation in IEC. Since IEC are generally, under non-inflammatory conditions, hyporesponsive to TLR signals (Abreu et al, 2001; Melmed et al, 2003), this may explain why dietary intervention using *Bifidobacterium breve* M-16V, scGOS/lcFOS or a combination of both (GF/Bb) only has an effect when lymphocytes in the LP are activated. Similar observations were made by van Hoffen et al., showing that adding *Lactobacilli* or *Bifidobacteria* to HT-29/PBMC co-cultures did not result in effector immune response, whereas direct exposure of PBMC to these bacteria resulted in high production of IL-12 and TNF-α (van Hoffen et al., 2010). However, when CD3/28-activated PBMC were used in the co-culture model to mimic inflammatory conditions, *L. rhamnosus* GG was able to enhance IL-12, IFN-γ and TNF-α production by PBMC, indicating the ability of IEC to modulate effector immune responses.

Although scGOS/lcFOS interact with TLR signalling, it is not known by which mechanisms scGOS/lcFOS can enhance TLR9 signaling, while suppressing TLR4 signaling in IEC. Secretion of galectins upon TLR activation on IEC may play an important role in regulating the adaptive immune response in the intestinal mucosa. It is not known what mechanisms and signalling transduction pathways regulate the expression and secretion of galectins. For galectin-9, expression was observed to be regulated through IRF3-signaling (Imaizumi et al., 2007). In nasal polyp fibroblasts, galectin-9 expression was induced in response to IFN-γ, involving mitogen-activated protein kinases (MAPK), phosphatidylinositol 3 phosphate kinase (PI3K), and Janus kinase/signal transducer and activator of transcription (JAK/STAT) signalling pathways (Park et al., 2011). Furthermore, NF-κB signalling has been described to regulate galectin-1 expression in T cells (Toscano et al., 2011). Since TLR activation results in activation of amongst others the NF-κB signalling pathway, further exploration of the involvement of NF-κB signalling and potentially other signalling cascades in IEC regulating expression of galectins may provide further insight into the role of galectins in mucosal immune responses in the intestine.

Restoring mucosal tolerance by specific non-digestible oligosaccharides

In food allergy, a robust Th2 response is generated against a harmless food-derived antigen. Although some allergens can directly activate DC to induce Th2 responses (Shreffler et al., 2011), the question how food proteins can induce excessive Th2 responses leading to food allergy remains a fundamental question. The induction of mucosal tolerance has received a lot of attention, and failure of mucosal tolerance induction may result in food allergy as well as chronic inflammation in the intestine.

TLR9 expression by IEC may play a crucial role in maintaining intestinal homeostasis and administration of TLR9 agonists has been used successfully to dampen effector immune responses in animal models for allergic sensitization (Zhu et al., 2007). Similarly, activation of TLR9 using a synthetic TLR9 ligand or genomic DNA derived from *Bifidobacterium breve* M-16V supported IFN-γ and IL-10 secretion by lymphocytes, and enhanced the frequency of CD4+Foxp3+ Treg cells (Chapter 3 and 4). This is supported by recent findings, showing that genomic DNA from commensals, as well as DNA from *Bifidobacterium breve*, contain immunosuppressive DNA sequences that potentially act through TLR9 that induce tolerogenic Treg responses (Bouladoux et al., 2012; Campeau et al., 2012). Furthermore, IEC can suppress the expression of co-stimulatory molecules via direct cell-cell contact (Butler et al., 2006). In addition, soluble mediators, especially TSLP and TGF-β, present in conditioned medium derived from Caco-2 cells apically exposed to commensal bacteria generate DC expressing lower levels of CD40, CD83 and HLA-DR (Zeuthen et al., 2008). However, TSLP was not detected in the conditioned media used in the presented studies. We have identified galectin-9, released by IEC upon apical exposure to TLR9 agonist in presence of scGOS/lcFOS as the soluble mediator involved in conditioning DC to express lower levels of co-stimulatory molecules (Chapter 6). Interestingly, these observation were only made upon priming IEC with TNF-α and IFN-γ, indicating that IEC only respond to TLR9 agonist and scGOS/lcFOS under inflammatory conditions.

In vivo, tolerance is induced by CD103+ DC present in the LP, which effectively induce Foxp3+ Treg cell in MLN (Coombes et al., 2007; Schulz et al., 2009; Sun et al., 2007). *In vitro* conditioning of DC with either galectin-9 or conditioned medium from IEC apically exposed to TLR9 ligand in the presence of scGOS/lcFOS did not induce CD103 expression by DC, but did enhance RALDH activity by DC. However, dietary intervention with GF/Bb enhanced the percentage of CD103+ DC in both a murine model for food allergy (Chapter 6) and a DSS-induced colitis model (Chapter 7). CD103+ DC have been shown to express indoleamine-2,3-dioxygenase

(IDO), an enzyme involved in tryptophan catabolism, which is essential to drive Treg cell development to prevent colitis (Matteoli et al., 2010). Blockade of the mannose receptor has been shown to prevent induction of Th2 polarization by various allergens as a consequence of IDO up-regulation (Royer et al., 2010). A mixture of 5 probiotic bacterial strains – consisting of *L. acidophilus, L. casei, L. reuteri, B. bifidum* and *S. thermophilus* – has been described to induce IDO expression in DC resulting in suppression of TNBS-induced colitis and atopic dermatitis (Kwon et al., 2010). In addition, tryptophan metabolites produced by IDO during immunotherapy contribute to induction of tolerance in allergic airway inflammation (Taher et al., 2008). In addition to RALDH and IDO, expression of COX-2 has been described to induce oral tolerance through induction of Foxp3+ T cells at the expense of a Th2 effector response (Broere et al., 2009). Hence, unravelling the intracellular signalling cascades in DC upon for instance DC conditioning by IEC is essential to understand how scGOS/lcFOS induce mucosal tolerance.

In the OVA-induced food allergy model, CD103 expression was induced on CD11c+I-A/I-E(mid) cells, which were increased in frequency in allergic mice (chapter 6). Since these cells were only increased in allergen sensitized mice upon oral challenge, these cells may have infiltrated into the tissue. Indeed, TLR activation on IEC results in differential secretion of chemokines – including IL-8, MDC and IP-10 –, indicating a potential role for IEC in attraction of lymphocytes from the circulation into the intestinal mucosa (chapter 3). In the lungs it has been reported that Ly6C(hi) monocytes can repopulate the CD103+ DC population, whereas Ly6C(lo) monocytes give rise to pro-inflammatory CD11b+ DC (Jakubzick et al., 2008). During colitis, monocytes enter the intestinal mucosa and can give rise to CD103+ cells (Rivollier et al., 2012). However, CD103+ DC are thought not to be derived from monocytes, but are derived from a pre-DC precursor cell (Bogunovic et al., 2009). Furthermore, extensive flow cytometric analysis recently showed that infiltrating monocytes become TLR-unresponsive CX3CR1(hi) macrophages, while in inflammation monocytes retain in the CX3CR1(int) stage and produce pro-inflammatory cytokines upon TLR activation (Bain et al., 2012). Interestingly, DC generated when galectin-9 was added to differentiating human monocytes or murine bone marrow cells showed lower expression of co-stimulatory molecules, increased RALDH activity and produced less pro-inflammatory cytokines upon TLR activation. This indicates that galectin-9 may condition uncommitted cells entering the intestinal LP to become tolerogenic. Similarly, monocytes were observed to adopt an anti-inflammatory phenotype *in vitro* as well, producing more IL-10 and less TNF-α (Chapter 3). These monocytes express lower levels of the monocyte marker CD14 indicating that IEC secrete galectin-9, which conditions these monocytes to potentially become moDC with anti-inflammatory capacities. Further

characterization of these cells in the LP is necessary to understand which cells contribute to induction of mucosal tolerance in allergic mice fed GF/Bb.

Induction of Treg responses is essential for the development of mucosal tolerance, since transfer of Foxp3+ Treg cells abrogates the development of food hypersensitivity responses (Yamashita et al., 2012). Furthermore, in DEREG mice, in which Treg cells are depleted upon injection of diphtheria toxin, it was shown that depletion of Treg cells during sensitization exacerbates allergic airway inflammation (Baru et al., 2010). Likewise, transfer of spleen cells from whey-sensitized mice fed specific non-digestible oligosaccharides prevented an acute allergic ear swelling response in recipient mice, which was not observed when Treg cells were depleted (Schouten et al., 2010). Using two murine models for food allergy (Chapter 5 and 6) and a murine DSS-induced colitis model (Chapter 7), it was observed that dietary intervention with scGOS/lcFOS and GF/Bb effectively induces Treg conversion, indicating the capacity of scGOS/lcFOS to induce mucosal tolerance under inflammatory conditions. Interestingly, scGOS/lcFOS on their own did not induce galectin-9 secretion or modulate cytokine production and T cell phenotype *in vitro* (Chapter 4), indicating an important interaction with potentially TLR signalling. This is reflected *in vivo*, since the GF/Bb most effectively prevented the development of acute allergic responses. Furthermore, scGOS/lcFOS alone only modulated DC and T cell phenotype in the DSS-colitis model, whereas GF/Bb also improved the inflammatory response in the intestines. Thus, the observations made in this thesis indicate a role for specific non-digestible oligosaccharides in shaping DC function and supporting Treg conversion. However, TLR activation on IEC, potentially TLR9, is necessary to improve the clinical symptoms in allergic and inflammatory disease in the intestine.

Although dietary supplementation with GF/Bb can prevent the development of acute allergic symptoms, IgE levels generally remain increased (Abrahamsson et al., 2007; Kalliomaki et al., 2007; Gruber et al., 2010). Despite the high IgE levels measured in serum of mice and humans that received GF/Bb, acute hypersensitivity reactions are suppressed. Galectin-9 is known to directly suppress degranulation of mast cells by preventing IgE from binding to the allergen (Niki et al., 2009). In addition, Treg cells induced by the GF/Bb diet interact with mast cells to suppress IgE receptor expression by mast cell degranulation and to directly prevent mast cell degranulation via OX40-OX40L interactions (Kashyap et al., 2008; Gri et al., 2008). Murine studies show that mast cell degranulation within the intestinal mucosa is suppressed upon dietary intervention with GF/Bb, as reflected by reduced serum mMCP-1 concentration (Schouten et al., 2009) (Chapter 5). Surprisingly, serum

mMCP-1 levels were not reduced upon dietary intervention with GF/Bb in the OVA-induced food allergy model in Balb/c mice (data not shown). Hence, the OVA-induced food allergy model may not be suitable for studying mucosal mast cell responses and is more suitable for measuring DC function and T cell polarization. In contrast, C3H/HeOuJ mice used in the murine cow's milk allergy model may be less suitable to study DC and T cell responses. Similar observations have been reported, showing mouse strain-dependent differences with respect to production of immunoglobulins, induction of acute skin reactions and anaphylaxis and the potential to measure allergen-specific re-stimulation responses (Smit et al., 2011).

In addition to allergic disease, in Chapter 7 we show, that dietary intervention attenuated DSS-induced colitis. In IBD, TLR9 ligands have been shown to ameliorate chronic intestinal inflammation (Abe et al., 2007; Rachmilewitz et al., 2002 and 2004). Similar to observations made in the murine models for food allergy (Chapter 5 and 6), dietary intervention with GF or GF/Bb enhanced the frequency of tolerogenic CD103+ DC in MLN. Consequently, the percentage of Treg cells was enhanced, whereas Th1 and Th17 polarization are suppressed. Furthermore, we identified galectin-4 secreted by IEC to be potentially involved in supporting an inflammatory response. However, further experiments are necessary to show the involvement of galectin-4 in intestinal inflammation. Furthermore, galectin-9 expression in the ileum was reduced in mice undergoing DSS colitis, supporting the hypothesis that galectin-9 is involved in maintaining intestinal homeostasis and the induction of Treg cells through DC conditioning. Interestingly, it appeared from the results in Chapter 7 that *Bifidobacterium breve* M-16V was essential to attenuate the inflammation induced by DSS. Since *Bifidobacterium breve* M-16V may signal through TLR9, this again indicates an important interaction between TLR signaling and the protective effects of specific non-digestible oligosaccharides. Although it is known that TLR2 activation on IEC has protective effects on barrier integrity (Cario et al., 2004), intestinal epithelial damage was largely prevented upon dietary intervention with *Bifidobacterium breve* M-16V or GF/Bb (Chapter 7). *In vitro*, we observed potential protective effects of TLR9 signaling on epithelial barrier function, which was improved by the presence of scGOS/lcFOS (Chapter 8). This indicates, that in addition TLR2 activation on IEC, apical ligation of TLR9 on IEC protects against epithelial barrier disruption under inflammatory conditions. In IBD, increased expression of myosin light chain kinase (MLCK) in IEC has been described, resulting in deficits in the epithelial barrier (Blair et al., 2006). Further experiments may focus on evaluating whether apical exposure of IEC to TLR9 ligand or DNA from *Bifidobacterium breve* M-16V in the presence of scGOS/lcFOS regulates MLCK expression and activity in IEC or whether the

improved epithelial barrier is the result of increased anti-inflammatory cytokine production.

Future directions

The results obtained in this thesis demonstrate an important role for IEC in regulating both innate and adaptive immune responses in both allergic and inflammatory disease of the intestine. In particular, TLR activation by luminal components plays an essential role in the activation of the intestinal epithelium and mediator secretion by IEC. In particular, galectin-4 and -9 were studied, since these galectins are regulated by apical TLR4 and TLR9 activation on IEC. Activation of other TLR, including TLR2 and TLR5, may regulate expression of galectins as well. Furthermore, galectin-1 and -3 are abundantly expressed by the intestinal epithelium as well. Especially, galectin-1 is associated with Treg responses (Garin et al., 2007). Hence, the contribution of galectin-1 in the beneficial effects of GF/Bb on allergic and inflammatory disease is worthwhile studying.

As indicated above, IEC may play a crucial role in attracting immune cells from the circulation into the intestinal mucosa. Expression and secretion of various chemokines upon exposure to TLR activation in the absence and presence of scGOS/lcFOS *in vitro* may provide insight in the contribution of chemotaxis of specific lymphocyte subsets in allergic and inflammatory disease in the intestine. In addition, it was recently shown for TSLP that in addition to IEC, murine CD103+ DC produced TSLP as well. TSLP-producing CD103+ DC were found to act directly on T cells by driving Treg differentiation (Spadoni et al., 2012). Since in Chapter 5 increased galectin-9 expression in whey-allergic mice fed GF/Bb was observed in MLN as well, this may implicate that DC conditioned by galectin-9 in the intestinal mucosa may start producing galectin-9 themselves. Hence, upon migration towards the MLN, galectin-9 producing DC instruct Treg polarization. It remains to be evaluated whether IEC apically exposed to TLR9 ligand in the presence of scGOS/lcFOS induce galectin-9 expression by DC.

In addition to the observation that galectin-9 can condition monocytes differentiating in tolerogenic moDC and bone marrow derived cells into BMDC *in vitro*, conditioning experiments using other galectins is worth studying. In particular, conditioning DC with galectin-1 and galectin-4 may be of interest, since galectin-1 is known for its anti-inflammatory function and as galectin-4 was found to be associated with intestinal inflammation (Chapter 7). In addition, conditioned medium from IEC exposed to a Th2 or Th17 polarized pro-inflammatory cytokine mixture may more physiologically reflect the inflammation in the intestinal mucosa characteristic for food allergy and IBD respectively.

Studying the intracellular signalling pathways in DC that support Treg cell conversion, including the involvement of IDO and COX-2, provide more insight in the mode of action of galectin-conditioned DC. In addition, transfer of the conditioned DC into naïve as well as allergic recipient mice can show the potential of these DC to drive T cell responses *in vivo* and their capacity to protect against food allergy and intestinal inflammation. Similar approaches could be used in murine colitis models. Ultimately, using knock-out models for specific galectins, preferentially knock-outs specifically for galectins expressed by the intestinal epithelium, can provide conclusive evidence of the role of galectins induced by scGOS/lcFOS.

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Appendix

Nederlandse samenvatting
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Nederlandse Samenvatting

De darm wordt voortdurend blootgesteld aan antigenen die aanwezig zijn in de darmholte. Het darmepitheel vormt een eerstelijns verdediging tegen lichaamsvreemde antigenen. Een monolaag van darmepitheelcellen vormt een fysische barrière tussen de darminhoud en de witte bloedcellen in het onderliggende bindweefsel. Het darmepitheel bevat zowel absorptieve cellen als gespecialiseerde slijmproducerende slijmbekercellen en Paneth cellen die antimicrobiële eiwitten in de darmholte uitscheiden. Ondanks dat het darmepitheel een belangrijke barrièrefunctie heeft, kan het voorkomen dat antigenen door het darmepitheel heen komen en in het onderliggende bindweefsel terecht komen. Antigenen worden dan herkend door witte bloedcellen die zich in grote getale in het bindweefsel van de darmwand bevinden. Deze witte bloedcellen bevinden zich zowel in gestructureerde weefsels, die bekend staan als Peyerse platen (PP) en mesenteriale lymfeknopen, als willekeurig in het bindweefsel, de lamina propria (LP). Het darmepitheel met de immunologische weefsels vormen tezamen het mucosale immuunsysteem van de darm. Zowel het darmepitheel en het immuunsysteem zorgen ervoor dat schadelijke antigenen effectief worden geweerd of onschadelijk gemaakt.

Het grootste aandeel aan witte bloedcellen in het menselijk lichaam bevindt zich in de LP. Omdat de meeste antigenen in de darmholte niet schadelijk zijn voor het lichaam, staat het immuunsysteem in de darm continu voor de moeilijke taak om schadelijke en onschadelijke antigenen van elkaar te onderscheiden. Op deze manier is het immuunsysteem in de darm in staat om tolerant te zijn voor onschadelijke antigenen, maar een ontstekingsreactie op te wekken in aanwezigheid van pathogenen. Recent is aangetoond dat darmepitheelcellen een actieve rol spelen in aangeboren afweerreacties door dendritische cellen (DC) te conditioneren. Dendritische cellen zijn effectieve antigeen presenterende cellen in de darm en sturen de adaptieve immuunreactie door B- en T-cellen aan. Darmepitheelcellen sturen de ontwikkeling van tolerogene DC aan die op hun beurt de ontwikkeling van regulatoire T (Treg) cellen stimuleren. Treg cellen spelen een essentiële rol in de onderdrukking van adaptieve immuunreacties door helper T cellen. Deze helper T cellen kunnen worden onderverdeeld in type 1 (Th1), type 2 (Th2) en type 17 (Th17) helper T cellen op basis van de mediatoren (cytokinen) die door deze T cellen geproduceerd worden. De ontwikkeling van Treg cellen draagt bij aan de ontwikkeling van tolerantie tegen antigenen. Tolerogene DC in de darm brengen het oppervlakte-eiwit CD103 tot expressie. Mediatoren geproduceerd door darmepitheelcellen blijken de expressie van CD103 te kunnen bevorderen. Op deze manier kunnen darmepitheelcellen dus een aantrekkelijk aangrijpingspunt zijn voor de inductie van immunolgische tolerantie om mogelijk (allergische) ontstekingsziekten van de darm te voorkomen of te behandelen.

Voedselallergie en chronische darmontsteking (IBD) zijn verschillende vormen van ontstekingsziekten in de darm. Voedselallergie is in de meeste gevallen een zogenaamde type I overgevoeligheidsreactie tegen onschadelijke antigenen afkomstig van de voeding die wordt veroorzaakt daar een overmatige Th2 activatie. De ontwikkeling van een Th2 reactie wordt gestuurd door onder andere DC. De inductie van een Th2 reactie tegen een onschadelijk antigeen leidt tot de productie van het antilichaam IgE door B-cellen (plasmacellen). IgE bindt aan de mestcel en herhaaldelijke blootstelling aan specifieke antigeen veroorzaakt clustering van mestcelgebonden IgE. Dit leidt tot vrijzetting van ontstekingsmediatoren door de mestcel (onder andere histamine), wat leidt tot de klinische symptomen karakteristiek voor een voedselallergische reactie. In tegenstelling tot voedselallergie is de oorzaak van IBD nog niet volledig bekend. Men vermoedt dat IBD wordt veroorzaakt door verlies van tolerantie tegen de bacteriën (de microflora). IBD kan worden onderverdeeld in ulceratieve colitis (UC) en de ziekte van Crohn. In zowel UC als de ziekte van Crohn wordt de ontstekingsreactie veroorzaakt door een overmatige Th17 activatie geïnduceerd door DC. In zowel voedselallergie als IBD is er een verstoring in de homeostase in het mucosale immuunsysteem van de darm die in stand wordt gehouden door complexe interacties tussen het darmepitheel, DC en de cellen van het adaptieve immuunsysteem. Inzichten in deze interacties kan mogelijk dus leiden tot nieuwe behandelingsstrategieën voor voedselallergie en IBD

Dieetinterventie met specifieke niet-verteerbare oligosacchariden – in het bijzonder een 9:1 mengsel van korte keten galacto- en lange keten fructo-oligosacchariden (scGOS/lcFOS) – kan de ontwikkeling van een voedselallergische reactie in muizen voorkomen. Dit effect is sterker wanneer scGOS/lcFOS wordt gecombineerd met de bacteriële stam *Bifidobacterium breve* M-16V (GF/Bb). In jonge patiënten kan GF/Bb de ontwikkeling van atopisch eczeem verminderen. De samenstelling van scGOS/lcFOS is gebaseerd op de samenstelling van neutrale niet-verteerbare oligosacchariden in humane melk. Hoewel dieetinterventie effectief is gebleken in het voorkomen van voedselallergie, is het effect van scGOS/lcFOS in IBD nog niet bekend. In muismodellen voor voedselallergie is gebleken dat de inductie van Treg cellen een belangrijk mechanisme is, waardoor scGOS/lcFOS een acute overgevoeligheidsreactie tegen verschillende allergenen kan verminderen. Dit proefschrift richt zich op het verkrijgen van meer inzicht in het mucosale immuunsysteem van de darm en modulatie van subtypen en functie van witte bloedcellen in de darm. Hiertoe zijn zowel *in vitro* als *in vivo* studies uitgevoerd om het effect van GF/Bb op de modulatie van de aangeboren en adaptieve immuunreacties te onderzoeken.

Een *in vitro* co-cultuur model voor het bestuderen van interacties tussen darm-epitheelcellen en witte bloedcellen

Aangeboren immuunreacties worden onder andere op gang gebracht door activatie van Toll-like receptoren (TLR). Deze TLR worden tot expressie gebracht door onder andere DC, maar ook darmepitheelcellen. TLR herkennen onder andere fragmenten van de bacteriële celwand (TLR2), endotoxines (zoals LPS) geproduceerd door Gram-negatieve bacteriën (TLR4) en bacterieel CpG DNA (TLR9). De expressie van TLR op darmepitheelcellen is verhoogd in situaties van ontsteking. Expressie van TLR2 en TLR4 is verhoogd in patiënten met IBD en dragen mogelijk bij aan het veroorzaken van een ontstekingsreactie. In tegenstelling tot activatie van TLR2 en TLR4 draagt activatie van TLR9 op het luminale membraan van darmepitheelcellen bij aan de handhaving van homeostase in de darm. In **hoofdstuk 3** en **4** is een celkweeksysteem beschreven waarin humane darmepitheelcellen samen met witte bloedcellen uit humaan bloed in kweek zijn gebracht. Dit systeem is gebruikt om de bijdrage van darmepitheelcellen in de modulatie van immuunreacties te bestuderen. Darmepitheelcellen blijken verschillend te reageren op luminale blootsteling aan TLR stimulaties. Activatie van TLR4 op darmepitheelcellen leidt tot een Th1 en Th17 gepolariseerde ontstekingsreactie en TNF-α productie door monocyten, terwijl luminaal TLR9 activatie juist een gereguleerde Th1 reactie induceert samen met productie van het anti-inflammatoire cytokine IL-10 door monocyten.

De onderliggende mechanismen waarmee scGOS/lcFOS leidt tot de inductie van Treg cellen zijn onbekend. In **hoofdstuk 4** van dit proefschrift wordt beschreven dat scGOS/lcFOS een TLR9-geïnduceerde Th1 respons en Treg polarizatie in het co-cultuur systeem versterkt. Vergelijkbare resultaten werden waargenomen wanneer het DNA van *Bifidobacterium breve* M-16V werd vergeleken met een synthetisch TLR9 ligand. Aangezien scGOS/lcFOS bestaat uit een mengsel van suikers, bestaat er de mogelijkheid dat suikerbindende receptoren (lectines) een mogelijke rol spelen waarmee scGOS/lcFOS de immuunreactie kan moduleren. **Hoofdstuk 2** in dit proefschrift geeft een overzicht van de effecten van suikerbindende eiwitten (lectines) op een immuunreactie. Met name darmepitheelcellen blijken grote hoeveelheden galectins to expressie te brengen en uit te kunnen scheiden. Neutralisatie van deze galectins, en met name galectin-9, voorkomt het beschermende effect van TLR9 ligand en scGOS/lcFOS blootstelling van darmepitheelcellen (**hoofdstuk 4**). Dit geeft aan dat galectin-9 mogelijk een bijdrage kan leveren in het aansturen van een regulatoire Th1 reactie.

In vivo dieetinterventie met GF/Bb ter preventie van ontsteking in de darm

Om de bevindingen verkregen met het *in vitro* co-cultuur systeem *in vivo* te bevestigen, zijn dieetinterventie studies in een muismodel voor

voedselallergie uitgevoerd. In **hoofdstuk 5** en **6** zijn muismodellen voor respectievelijk koemelkallergie en kippeneiwitallergie gebruikt. Het veel gebruikte koemelkallergie model, geïnduceerd met het koemelkallergeen wei, is gebruikt om het effect van GF/Bb op het voorkomen van mestcel-reacties te bestuderen. In **hoofdstuk 5** van dit proefschrift is voor het eerst aangetoond dat de acute overgevoeligheidsreactie negatief correleert met galectin-9 concentraties in het serum. Dit was mede afhankelijk van activatie van het immuunsysteem in de mucosa van de darm, aangezien galectin-9 productie alleen werd waargenomen wanneer muizen waren gesensitiseerd tegen wei. Alleen wanneer het serum van wei gesensitiseerde muizen die een GF/Bb dieet hebben gehad werd gebruikt in een mestcel degranulatie experiment, bleek de degranulatie van mestcellen galectin-9-afhankelijk geremd te worden. Daarnaast is in de mesenteriale lymfeknopen verhoogde Th1 en Treg polarizatie waargenomen wanneer wei-gesensitiseerde muizen het GF/Bb dieet ontvingen. Ook in kinderen blijkt dieetinterventie met GF/Bb IgE-afhankelijke atopisch eczeem te verminderen, wat gepaard ging met een verhoogde hoeveelheid galectin-9 in het serum.

In **hoofdstuk 6** van dit proefschrift is *in vitro* laten zien dat galectin-9 de DC opgekweekt vanuit humane monocyten conditioneert en daarmee Treg polarisatie stimuleert. Deze Treg waren functioneel in een T cel suppressie experiment. Daarnaast is in een ovalbumine (OVA) geïnduceerd muismodel voor kippeneiwitallergie aangetoond dat DC in de LP verminderd actief zijn en daarnaast meer CD103 tot expressie brengen wanneer muizen het GF/Bb dieet ontvingen gedurende de sensitisatie. Dit ging gepaard met verlaagde Th2 en verhoogde Treg frequentie in de LP. Tenslotte bleek galectin-9 in een *ex vivo* OVA re-stimulatie experiment productie van Th2-geassocieerde cytokinen te onderdukken.

Omdat galectin-9 geïnduceerd door het GF/Bb dieet een essentiële rol speelt in de conditionering van DC en de inductie van Treg cel polarisatie, hebben we in **hoofdstuk 7** van dit proefschrift de effecten van het GF/Bb dieet in een dextraan sodium sulfaat (DSS)-geïnduceerd muismodel voor colitis onderzocht. Dieetinterventie met *Bifidobacterium breve* M-16V of GF/Bb resulteerde in minder ontsteking in de dikke darm, terwijl dieetinterventie met GF of GF/Bb de ontwikkeling van bloederige diarree vertraagde. De vertraging in het ziekteproces ging gepaard met een verhoogde frequentie CD103+ DC en Treg in de mesenteriale lymfeknopen, terwijl het percentage Th17 cellen was verlaagd. Onverwacht vonden we dezelfde trends terug in de PP. *In vitro* studies in het co-cultuur model laten zien dat scGOS/lcFOS de expressie van TLR4-geïnduceerde galectin-4 expressie remt. Dit ging gepaard met een normalisatie in de hoeveelheid Treg cellen en een vermindering van de productie van IL-12 en IL-17. In muizen blootgesteld aan DSS was de expressie van galectin-4 verhoogd

in vergelijking met onbehandelde muizen. De expressie van galectin-4 in zowel de dunne en dikke darm was verlaagd door het GF/Bb dieet. Daarnaast werd een verhoogde galectin-9 expressie in de dunne darm waargenomen in muizen die het GF/Bb dieet ontvingen. Tot slot werd in ontstoken weefsel van zowel patiënten met UC als ziekte van Crohn een verhoogde expressie van galectin-4 en een verlaagde expressie van galectin-9 gevonden. Toekomstig onderzoek moet uitwijzen of modulatie in galectin-4 en -9 expressie als nieuw aangrijpingspunt voor de behandeling van IBD kan dienen.

scGOS/lcFOS: mogelijke versterking van de barrière-integriteit van het darm-epitheel

De ontstekingsreactie in IBD wordt mogelijk veroorzaakt door overmatige activatie van het mucosale immuunsysteem van de darm als gevolg van translocatie van bacteriën van de microflora in de LP. In **hoofdstuk 8** van dit proefschrift worden de eerste resultaten gepresenteerd die de effecten van scGOS/lcFOS op de barrièrefunctie van het darmepitheel beschrijven. Blootstelling van darmepitheelcellen aan een synthetisch TLR9 ligand in de aanwezigheid van scGOS/lcFOS leidde tot een verbetering van de barrière integriteit onder inflammatoire condities in het co-cultuur model. Dit ging gepaard met een verbeterde co-localisatie van de tight junction eiwitten zonula occludens (ZO)-1 en occludin. De verbeterde barrière-integriteit kan mogelijk de verhoogde bacteriele translocatie door het darmepitheel verminderen en zodoende activatie van het mucosale immuunsysteem verminderen.

Conclusies

De onderzoeksresultaten beschreven in dit proefschrift laten zien dat specifieke niet-verteerbare oligosacchariden (scGOS/lcFOS) in combinatie met *Bifidobacterium breve* M-16V bescherming biedt tegen de ontwikkeling van ontstekingen in de mucosa van de darm. Het GF/Bb dieetconcept draagt mogelijk bij aan een verbetering van de barrière-integriteit van het darmepitheel door de localisatie van tight junction eiwitten te reguleren. Daarnaast is aangetoond dat blootstelling van darmepitheelcellen aan een TLR9 ligand in combinatie met scGOS/lcFOS bijdraagt aan de ontwikkeling van tolerogene DC via de secretie van galectin-9. Galectin-9 geconditioneerde DC dragen effectief bij aan T cel polarisatie richting Treg cellen, resulterend in immunologische tolerantie. Het beschermende effect is geverifieerd in muismodellen voor voedselallergie (een Th2 gedreven ontsteking) en colitis (een Th1/Th17 gedreven ontsteking). Dieetinterventie met GF/Bb was het meest effectief in de onderdrukking van ontsteking in de darm, wat gepaard ging met een verhoogde hoeveelheid tolerogene DC en Treg cellen en modulatie van galectin-4 en -9 expressie in het mucosale immuunsysteem van de darm. Regulatie van galectins kan mogelijk een nieuwe strategie zijn om (allergische) ontstekingsziekten in de darm te voorkomen of te behandelen.

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Sander de Kivit was born on the 17th of February 1988 in Rotterdam, The Netherlands. In 2004 he graduated at the Thorbecke Lyceum in Rotterdam, where he received his athenaeum certificate. Thereafter, he started the bachelor program Biomedical Sciences at Utrecht University. After graduating cum laude for his bachelor's degree in 2007, he started the master program Drug Innovation. During his master program, he conducted a research project for nine months under supervision of dr. Linette Willemsen at the department of Pharmacology and dr. Els van Hoffen at the department of Dermatology/Allergology at the University Medical Center Utrecht. During this project, he studied the interactions between intestinal epithelial cells and immune cells using an *in vitro* co-culture model. In his second year of the master program in 2008, he started his PhD research project at the department of Pharmacology under supervision of dr. Linette Willemsen, dr. Aletta Kraneveld and prof. dr. Johan Garssen. As part of the master program, he conducted a six months research project at the department of Molecular Cell Biology and Immunology at the VU University Medical Center in Amsterdam under the supervision of dr. Eirikur Saeland and prof. dr. Yvette van Kooyk. During this project he started to investigate the effects of exposure of intestinal epithelial cells to specific non-digestible oligosaccharides on the immune response. In 2009, he graduated cum laude for the master program Drug Innovation and continued his PhD studies on the effects of specific non-digestible oligosaccharides on the mucosal immune system of the intestine. During his PhD research project, he received the Young Investigator's Award for the ESPGHAN Annual meeting 2011, the Best Oral Presentation Award for the Section Experimental Gastroenterology (SEG) at the Dutch Experimental Gastroenterology and Hepatology (DEGH) meeting in 2011 and the oral abstract award at the annual EAACI Congress in 2012. In parallel, he was involved in teaching undergaduates attending the bachelor program Pharmacy and the College of Pharmaceutical Sciences in Utrecht. The results of his PhD studies are described in this thesis.

List of Publications

From this thesis:

1. De Kivit, S., Saeland, E., Kraneveld, A.D., van de Kant, H.J., Schouten, B., van Esch, B.C., Knol, J., Sprikkelman, A.B., van der Aa, L.B., Knippels, L.M., Garssen, J., van Kooyk, Y., Willemsen, L.E., 2012. Galectin-9 induced by dietary synbiotics is involved in suppression of allergic symptoms in mice and humans. Allergy 67: 343-352

2. De Kivit, S., Kranveld, A.D., Garssen, J., Willemsen, L.E., 2012. Glycan recognition at the interface of the intestinal immune system: Target for immune modulation via dietary components. Eur. J. Pharmacol. 668 Supp 1: S124-132

3. De Kivit, S., van Hoffen, E., Korthagen, N., Garssen, J., Willemsen, L.E.M, 2011. Apical TLR Ligation of Intestinal Epithelial Cells Drives a Th1-polarized Regulatory or Inflammatory Type Effector Response in vitro. Immunobiology 216: 518-527

Other publications:

1. Borre, Y., Sir, V., de Kivit, S., Westphal, K.G., Olivier, B., and Oosting, R.S., 2012. Minocycline restores spatial but not fear memory in olfactory bulbectomized rats. Eur. J. Pharmacol. 697:59-64

2. Van Esch, B.C., Schouten, B., de Kivit, S., Hofman, G.A., Knippels, L.M., Willemsen, L.E., Garssen, J., 2011. Oral tolerance induction by partially hydrolyzed whey protein in mice is associated with enhanced numbers of Foxp3(+) regulatory T-cells in the mesenteric lymph nodes. Pediatr. Allergy Immunol. 22: 820-826

3. Schouten, B., van Esch, B.C., Hofman, G.A., de Kivit, S., Boon, L., Knippels, L.M., Garssen, J., Willemsen, L.E.M., 2011. A potential role for CD25+ regulatory T-cells in the protection against casein allergy by dietary non-digestible carbohydrates. Br. J. Nutr. 107: 96-105

4. Van Hoffen, E., Korthagen, N.M., de Kivit, S., Schouten, B., Bardoel, B., Duivelshof, A., Knol, J., Garssen, J., Willemsen, L.E.M., 2010. Exposure of Intestinal Epithelial Cells to UV-Killed Lactobacillus GG but Not Bifidobacterium breve Enhances the Effector Immune Response in vitro. Int. Arch. Allergy Immunol. 152: 159-68

List of Abbreviations

AHR	- acute hypersensitivity response
APC	- antigen presenting cell
Bb	- *Bifidobacterium breve* M-16V
BMDC	- bone marrow-derived dendritic cell
CD	- Crohn's disease
CFSE	- carboxyfluorescein succimidyl ester
CMA	- cow's milk allergy
CT	- cholera toxin
CRD	- carbohydrate recognition domain
DC	- dendritic cell
DC-SIGN	- dendritic cell-specific intercellular adhesion molecule (ICAM)-3- grabbing non-integrin
DSS	- dextran sulfate sodium
FA	- food allergy
GALT	- gut-associated lymphoid tissue
GF	- scGOS/lcFOS
GF/Bb	- diet containing scGOS/lcFOS and *Bifidobacterium breve* M-16V
IBD	- inflammatory bowel disease
IEC	- intestinal epithelial cells
IFN	- interferon
lcFOS	- long-chain fructo-oligosaccharides
LP	- lamina propria
MFI	- mean fluorescence intensity
MLC	- myosin light chain
MLCK	- myosin light chain kinase
MLN	- mesenteric lymph node
MLR	- mixed lymphocyte reaction
moDC	- monocyte-derived dendritic cell
OVA	- ovalbumin
PBMC	- peripheral blood mononuclear cells
pDC	- plasmacytoid dendritic cell
PP	- Peyer's patch
scGOS	- short-chain galacto-oligosaccharides
SEM	- standard error of the mean
TER	- transepithelial electrical resistance
Th1/Th2/Th17	- T helper type 1, 2 or 17 cell
TIM	- T cell immunoglobulin mucin
TLR	- Toll-like receptor
TNBS	- trinitrobenzene sulfonic acid
Treg	- regulatory T cell
UC	- ulcerative colitis
ZO-1	- zonula occludens-1