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Investigation of immune-modulatory properties of non-digestible oligosaccharides and lactic acid bacteria

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I. Abstract

Background: Over the past decades the incidence of chronic inflammatory, non-communicable diseases (NCD), such as allergies or inflammatory bowel diseases is increasing worldwide. NCD are characterized by chronic, underlying low-grade inflammation and can be associated with dysbiosis, loss of mucosal homeostasis and tolerance involving dysbalanced intestinal and systemic immune responses. Infant formulas containing non-digestible oligosaccharides (NDO) similar to the composition of oligosaccharides in human breast milk, lactic acid bacteria (LAB) or a combination of LAB and NDO have been shown to harbor anti-inflammatory properties and preventive effects towards immune-regulatory disorders. Food-based strategies to achieve or maintain gut homeostasis, a well-balanced microbiota and a properly functioning immune system have therefore gained enormous interest, suggesting for example the dietary use of LAB like Lactobacilli or NDO.

Aim: The aim of this work is to investigate the immune-modulatory potential of non-digestible short chain galacto- and long chain fructo-oligosaccharides (scGOS/lcFOS) mimicking the natural distribution of oligosaccharides in human breast milk in presence or absence of certain LAB strains in human monocyte-derived dendritic cells (MoDC) and the ensuing T cell response. Furthermore the objective is to investigate a potential immune-modulatory effect of certain LAB in an *in vitro* co-culture model of human intestinal epithelial cells (IEC) and peripheral blood mononuclear cells (PBMC).

Methods: Immature human MoDC prepared from peripheral blood of healthy non-atopic volunteers were screened *in vitro* after stimulation with a specific scGOS/lcFOS mixture in presence or absence of LAB. IL-10 and IL-12p70 release was analyzed after 24 hours in cell-free supernatants by ELISA, as well as the phenotype of MoDC by flow cytometry. A luminex-based assay was conducted to assess further cytokine and chemokine release by MoDC. To investigate the resulting T cell response, stimulated MoDC were co-incubated with naïve T cells in allogeneic stimulation assays, intracellular Foxp3 expression was determined as well as the functionality of the induced T cells to suppress responder T cells. For the second part of *in vitro* experiments, human intestinal epithelial cells (IEC) were grown on transwell filters and stimulated apically with LAB or medium. Basolateral underlying CD3/CD28-activated human PBMC were screened for TNFα, IFNγ, IL-10, IL-13 after 24 hours and IEC for galectin-9 release after 48 hours in cell-free supernatant by ELISA. To investigate the induced T cell type, intracellular expression of IL-10, IFNγ and $CD25^+Foxp3^+$ cells was determined among $CD4^+$ T cells.

Results: Oligosaccharides *per se* induced no relevant amounts of pro-inflammatory IL-12p70 and upregulated maturation markers only slightly in comparison to untreated and LPS-stimulated MoDC. However, NDO promoted anti-inflammatory/immune-regulatory IL-10 release by MoDC. Furthermore, scGOS/lcFOS mixtures exerted a significant enhancing effect on LAB induced IL-10 secretion by MoDC while, again, no increase in IL-12p70 production was observed. Blocking toll-like receptor 4 (TLR4) abrogated the increase in IL-10 in both the stimulation with NDO only and stimulation of MoDC with LAB and NDO in combination, suggesting that scGOS/lcFOS act partly via the pattern recognition receptor TLR4. Finally, scGOS/lcFOS-treated MoDC were shown to upregulate the number of Foxp3 positive, potential suppressive regulatory T cells following allogeneic stimulation. In the used co-culture system of IEC and PBMC the tested LAB showed different properties to induce cytokine release by PBMC. TNFα release by PBMC was significantly enhanced concerning exposure of IEC to *L.rhamnosus* and *L.paracasei 2*. The tested LAB differ furthermore in their capacity to induce enhanced IL-10 release by PBMC compared to medium control in presence of IEC. *L.rhamnosus* and the two tested *L.paracasei* strains significantly enhanced IL-10 cytokine production of PBMC in the presence of IEC. In absence of IEC these effects of LAB disappeared. Enhanced galectin-9 release by IEC could be determined after stimulation with *L.paracasei 2.* Intracellular T cell staining for IL-10 was conducted to determine if the IL-10 detected in supernatants was derived from $CD4^+$ T cells. No significant differences were observed between control and LAB stimulated IEC on intracellular $CD4^+$ cytokine production. Foxp3 analysis showed no significant difference in induction by LAB, in absence of IEC however there was a higher percentage of Foxp3 positive $CD4^+$ T cells observed by comparing medium controls in absence and presence of IEC.

Conclusion: Results of this study indicate anti-inflammatory and direct, microbiota independent, immune-modulatory properties of scGOS/lcFOS mixtures on human MoDC. These results provide a possible explanation for clinical observed anti-inflammatory, immune-modulatory effects of these NDO involving a mechanism at least partly via TLR4. The tested combinations of LAB and scGOS/lcFOS might represent a useful dietary ingredient for the maintenance of intestinal homeostasis via the induction of potential suppressive $Foxp3^+$ regulatory T cells. Furthermore results of the human *in vitro* co-culture model of IEC and PBMC indicate an important role of intestinal epithelial cells in maintaining gut homeostasis and modulating immune responses beyond their function as simple physical barrier. The hereby tested LAB induce enhanced IEC dependent immune-regulatory and anti-inflammatory IL-10 secretion of PBMC. Exposure of IEC to LAB led to enhanced galectin-9 release of IEC and caused enhanced TNFα

secretion by PBMC. Therefore LAB might be considered as potential tool to influence dysbalanced immune responses by dietary supplementation. Nonetheless, conclusions have to be drawn carefully from *in vitro* cell systems as the numerous interactions *in vivo* and the complexity of the intestinal barrier and gut associated lymphoid tissue confer obvious limitations to *in vitro* experiments on cellular level. A combination of *in vitro* cell test systems, *in vivo* animal research as well as clinical studies in humans are necessary to provide profound evidence about the mechanism of action, spectrum of effects and possible scope of application for food supplements including LAB and NDO and their impact on health or disease of the host.

Zusammenfassung

Hintergrund: In den vergangenen Jahrzehnten treten nicht übertragbare Erkrankungen, die mit chronischer Entzündung einhergehen, wie zum Beispiel Allergien oder chronisch entzündliche Darmerkrankungen, weltweit gehäuft auf. Ein Zusammenhang dieser Erkrankungen mit einer veränderten Zusammensetzung der Bakterienbesiedlung im Darm sowie Verlust der Darmhomöostase und deregulierten Immunreaktionen wird diskutiert. Deshalb werden zunehmend nahrungsbasierte Konzepte wie beispielsweise der Einsatz von Milchsäurebakterien oder Oligosaccharid-Mischungen mit einer Muttermilch-ähnlichen Kombination von Galacto- und Fructooligosacchariden erforscht, um dem deregulierten Immunsystem und Störungen des Gleichgewichts der Mikrobiota im Darm entgegenzuwirken.

Ziel: Ziel dieser Arbeit ist es, eine mögliche immun-modulatorische Wirkung von unverdaulichen Oligosaccharid-Mischungen (in einem Mischungsverhältnis, wie sie in menschlicher Muttermilch vorkommen) allein oder in Kombination mit speziellen Milchsäurebakterien auf zellulärer Ebene in humanen Immunzellen zu untersuchen. Darüber hinaus sollen in einem humanen Kokultur-System von Darmepithelzellen und Immunzellen mögliche Effekte von vier verschiedenen Laktobazillen-Stämmen untersucht werden.

Methoden: Für den ersten Teil dieser Arbeit wurden Monozyten aus peripherem Blut von gesunden, nicht-atopischen Spendern isoliert und in Gegenwart von IL-4 und GM-CSF zu dendritischen Zellen (DCs) kultiviert. Diese Zellen wurden anschließend mit Oligosaccharid-Mischungen und den unterschiedlichen Bakterienstämmen in verschiedenen Konzentrationen inkubiert. Nach 24 Stunden wurde die Zytokinsekretion mittels ELISA oder luminex-basiertem Testverfahren, sowie der Phänotyp der DCs mittels Durchflusszytometrie gemessen. Um die durch diese DCs induzierte T-Zellantwort zu untersuchen, wurden die DCs nach 24-stündiger Stimulation mit naiven T-Zellen in einem allogenen Stimulationstestverfahren ko-inkubiert und nach sieben Tagen die induzierten T-Zellen phänotypisch und funktionell mittels Durchflusszytometrie analysiert. Im zweiten *in vitro* Teil dieser Arbeit wurde mit einem humanen Kokultur-System aus einer intestinalen Epithelzelllinie und Immunzellen aus dem peripheren Blut von gesunden Erwachsenen gearbeitet. Nach apikaler Stimulation der Epithelzellen mit vier verschiedenen Laktobazillus Stämmen oder Zellkulturmedium wurde im basolateral generierten Überstand der darunter liegenden CD3/CD28-aktivierten humanen Immunzellen TNFα, IFNγ, IL-10, IL-13 nach 24 Stunden und die Galektin-9 Sekretion der Epithelzellen nach 48 Stunden mittels ELISA gemessen und

der Phänotyp der induzierten T-Zellen mittels intrazellulärer Färbung von IL-10, IFNγ und Foxp3 und Durchflusszytometrie bestimmt.

Ergebnisse: Die Oligosaccharid-Mischungen selbst induzierten in humanen DCs keine Sekretion des pro-inflammatorischen IL-12p70, sowie eine nur geringe Hochregulierung von Reifungsmarkern, allerdings bedingten sie die Produktion von anti-inflammatorischem/ immun-regulatorischem IL-10 seitens der stimulierten DCs. Darüber hinaus induzierten die Oligosaccharid-mischungen eine deutliche Erhöhung der durch Milchsäurebakterien hervorgerufenen IL-10 Sekretion, was sich in Bezug auf IL-12p70 nicht zeigte. Durch Blockierung des Toll-ähnlichen Rezeptors 4 (TLR4) konnte die durch die Oligosaccharide induzierte hohe IL-10 Produktion der DCs unterbunden werden. Dies könnte darauf hinweisen, dass Oligosaccharide zumindest zum Teil ihre direkte Wirkung auf DCs über den Mustererkennungsrezeptor TLR4 ausüben. Außerdem zeigte sich eine erhöhte Prozentzahl an Foxp3 positiven T-Zellen in der allogenen Kokultur von Oligosaccharid-stimulierten DCs mit naiven T-Zellen. Dies könnte auf eine Induktion von regulatorischen T-Zellen hinweisen, was sich im Suppressions-Testsystem bestätigen ließ. Im humanen Kokultur-System von intestinalen Epithelzellen und Immunzellen (PBMC, mono-nukleäre Zellen des peripheren Blutes) zeigten sich Unterschiede zwischen den verschiedenen Laktobazillus Stämmen hinsichtlich deren Kapazität Zytokinausschüttung der Immunzellen zu induzieren. *L.rhamnosus* und *L.paracasei 2* erhöhten signifikant die TNFα Sekretion der PBMC. *L.rhamnosus* und die zwei getesteten *L.paracasei* Stämme induzierten zudem erhöhte IL-10 Ausschüttung der PBMC, wenn Epithelzellen in der Kokultur vorhanden waren. Ohne Epithelzellen im Experimentaufbau war der IL-10-erhöhende Effekt der Laktobazillen nicht nachzuweisen und es bestand eine höhere Sekretion von IL-10 und eine höhere Prozentzahl an $CD4^+CD25^+Foxp3^+$ Zellen was auf eine wichtige modulierende Wirkung von Epithelzellen auf Immunzellen hinweist. Nach Stimulation mit *L.paracasei 2* wurden im Überstand der Epithelzellen nach 48 Stunden eine erhöhte Galektin-9 Konzentration im Vergleich zur Mediumkontrolle gemessen.

Fazit: Diese Studie zeigt, dass die getesteten Oligosaccharid-Mischungen neben ihrem bekannten Wirkmechanismus auf die Mikrobiota im Darm direkte, anti-inflammatorische, immunregulatorische Effekte auf menschliche Immunzellen, zum Teil über den Mustererkennungs-Rezeptor TLR4, ausüben. Die untersuchten Kombinationen von Milchsäurebakterien und Oligosacchariden könnten als potentiell gesundheitsfördernder Nahrungszusatz zum Erhalt des Gleichgewichts im darmassoziierten und systemischen Immunsystem durch eine mögliche Induktion von regulatorischen T-Zellen beitragen. Daten dieser Studie könnten eine mögliche Grundlage zur Entwicklung von präventiven Ansätzen gegen Krankheiten, die mit einem deregulierten Immunsystem einhergehen,

wie zum Beispiel Allergien, liefern. Die vier im humanen Kokultur System getesteten Laktobazillus Stämme zeigten teilweise immun-modulatorische Effekte durch die Induktion einer erhöhten IL-10 und TNFα Sekretion der Immunzellen und einer erhöhten Galektin-9 Ausschüttung der Epithelzellen. Diese Ergebnisse liefern erste Hinweise, dass ausgewählte Laktobazillen nach eingehender Untersuchung in verschiedenen Testsystemen deregulierte Immunantworten durch Nahrung positiv beeinflussen könnten. Allerdings sollten Schlussfolgerungen, die allein auf *in vitro* Ergebnissen auf zellulärer Ebene aufbauen vorsichtig interpretiert werden, da hierbei die Komplexität der Situation *in vivo* nicht in Betracht gezogen wird. Deshalb ist eine Kombination von *in vitro* und *in vivo* Studien bis hin zu klinischen Studien im Menschen nötig um nahrungsbasierte Konzepte zur Beeinflussung von Krankheit oder Gesundheit des Menschen zu entwickeln, ihre exakte Anwendung zu definieren und ihre Wirkung fundiert zu belegen.

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„Human monocyte-derived dendritic cells stimulated with specific non-digestible oligosaccharides mimicking the functionality of breast milk oligosaccharides induce $CD4^{+}Foxp3^{high}$ T cells“

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”Human monocyte-derived dendritic cells stimulated with specific non-digestible oligosaccharides in presence or absence of lactic acid bacteria induce $CD4^{+}Foxp3^{high}$ T cells”

Lehmann, S., Hiller, J., van Bergenhenegouwen, J., Knippels, L., Garssen, J., Traidl-Hoffmann, C.

Innate Immune Memory, March 18th-20th 2015, Wellcome Trust Genome Campus, Hinxton, Cambridge, UK.

List of abbreviations

˚C	degree celsius
AP-1	activation protein 1
APC	antigen presenting cell
ASA	allogeneic stimulation assays
B.	Bifidobacterium
CCR	CC-chemokine receptor
CD	cluster of differentiation
cfu	colony forming units
CLR	C-type lectin receptor
CTLA	cytotoxic T lymphocyte-associated antigen
DC	dendritic cells
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DSS	dextran sodium sulfate
ELISA	enzyme linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FAE	follicle associated epithelium
FBS	fetal bovine serum
FMO	fluorescence minus one
FOS	fructo-oligosaccharides
Foxp3	forkhead-box-protein P3
FSC	forward scatter
GALT	gut associated lymphoid tissue
GOS	galacto-oligosaccharides
GM-CSF	granulocyte macrophage colony-stimulating factor
HLA	human leukocyte antigen
HMO	human milk oligosaccharides
IEC	intestinal epithelial cells
Ig	immunoglobulin
IFN	interferon
IL	interleukin
IP-10	interferon gamma-induced protein 10
IRF	interferon response factor
L.	Lactobacillus

LAB	lactic acid bacteria
LAG	lymphocyte activation gene
LAL	limulus amebocyte lysate
LPB	lipopolysaccharide-binding protein
lc	long chain
LPS	lipopolysaccharide
LTA	lipoteichoic acid
m	milli
µ	micro
MACS	magnetic activated cell sorting
MAMP	microbe associated molecular pattern
MAPK	mitogen-activated protein kinase
MDC	macrophage-derived chemokine
MHC	major histocompatibility complex
MIP	macrophage-inflammatory protein
MLN	mesenteric lymph node
MoDC	monocyte-derived dendritic cells
MRS	deMan, Rogosa Sharpe (medium)
NDO	non-digestible oligosaccharides
NFkB	nuclear factor k B
NOD	nuclear oligomerization domain
OD	optical density
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
pg	picogram
PI	propidium iodide
PI3K	phosphatidylinositiol 3-kinase
PGN	peptidoglycan
PRR	pattern recognition receptor
PP	peyer patches
rh	recombinant human
RIG	retinoic acid inducible gene
rpm	revolutions per minute
RPMI	roswell park memorial institute (medium)

RT	room temperature
SEM	standard error of the mean
sc	short chain
SCFA	short chain fatty acids
SSC	side scattered light
TA	teichoic acid
TARC	thymus and activation regulated chemokine
TGF	transforming growth factor
Th	helper T cell
TLR	toll-like receptor
TNF	tumor necrosis factor alpha
Treg	regulatory T cells
TRIF	toll/IL-1R domain-containing adaptor inducing IFN
U	enzyme unit

II. Introduction

Our body is continuously exposed to microbes. The mucosal surfaces of the intestine, skin, airways and reproductive tract are in direct contact with the external environment and e.g. its bacteria, fungi, viruses, parasites and allergens. These interfaces between the environment and the host are lined by continuous layers of specialized epithelial cells that prevent the entry of microbes by providing protective physical barriers (Gallo 2012). On the one hand it is crucial for the host to exhibit appropriate immunity against pathogens that overcome these barriers, on the other hand there is a need to maintain tolerance against harmless commensals, food or self-antigens to maintain health. If loss of tolerance occurs, allergy is a possible consequence, defined as inappropriate overreaction of the immune system towards usually harmless substances called allergens (Ring 2014). Allergy has, next to non-IgE mediated allergy types, been described as overactive Th2 type effector immune response, leading to maturation of B cells resulting in immunoglobulin E (IgE) production by plasma cells. IgE antibodies bind to Fc receptors on mast cells, evoking after another allergen exposure mast cell degranulation, causing acute allergic symptoms like eczema, urticaria, rhinitis, diarrhea, up to anaphylactic reactions (Annunziato 2015, Ring 2014). The causes leading to allergy and how sensitization occurs in humans remain largely unknown, as allergies are complex diseases mediated by genetic, life-style associated and environmental factors (Gilles 2012, 2014, Traidl-Hoffmann 2009).

II. 1. Microbiome, host health and disease

The human microbiome and its influence on health or disease of the host has been extensively studied and recent research revealed an important role of microbes in shaping the hosts immune, metabolic, physiologic and even behavioral programming (Purchiaroni 2013, Schwiertz 2010, Trompette 2014, deThije 2014, Cryan 2012). Environmental factors having an influence on the host microbiome can be diet, antibiotic use, hygiene and for infants the maternal microbiota, linked to mode of delivery (Cox 2015, Dominguez-Bello 2010, Collado 2015, Doré 2015, Scott 2013, Voreades 2014). Every part of the body's surface can be characterized by its distinct microbiota, not only intestinal and skin microbiome have been described, but recently also the microbiome of the airways (Karczewski 2013, Grice 2011, Marsland 2014). Next to the bacterial microbiota also viral and fungal colonization of the body's surfaces is recently taken into account (Underhill 2014, Abeles 2014). The composition of the viral, fungal and microbial microbiota differs between body sites like nose, mouth, stomach, lung, skin, intestine and vagina (Figure 1).

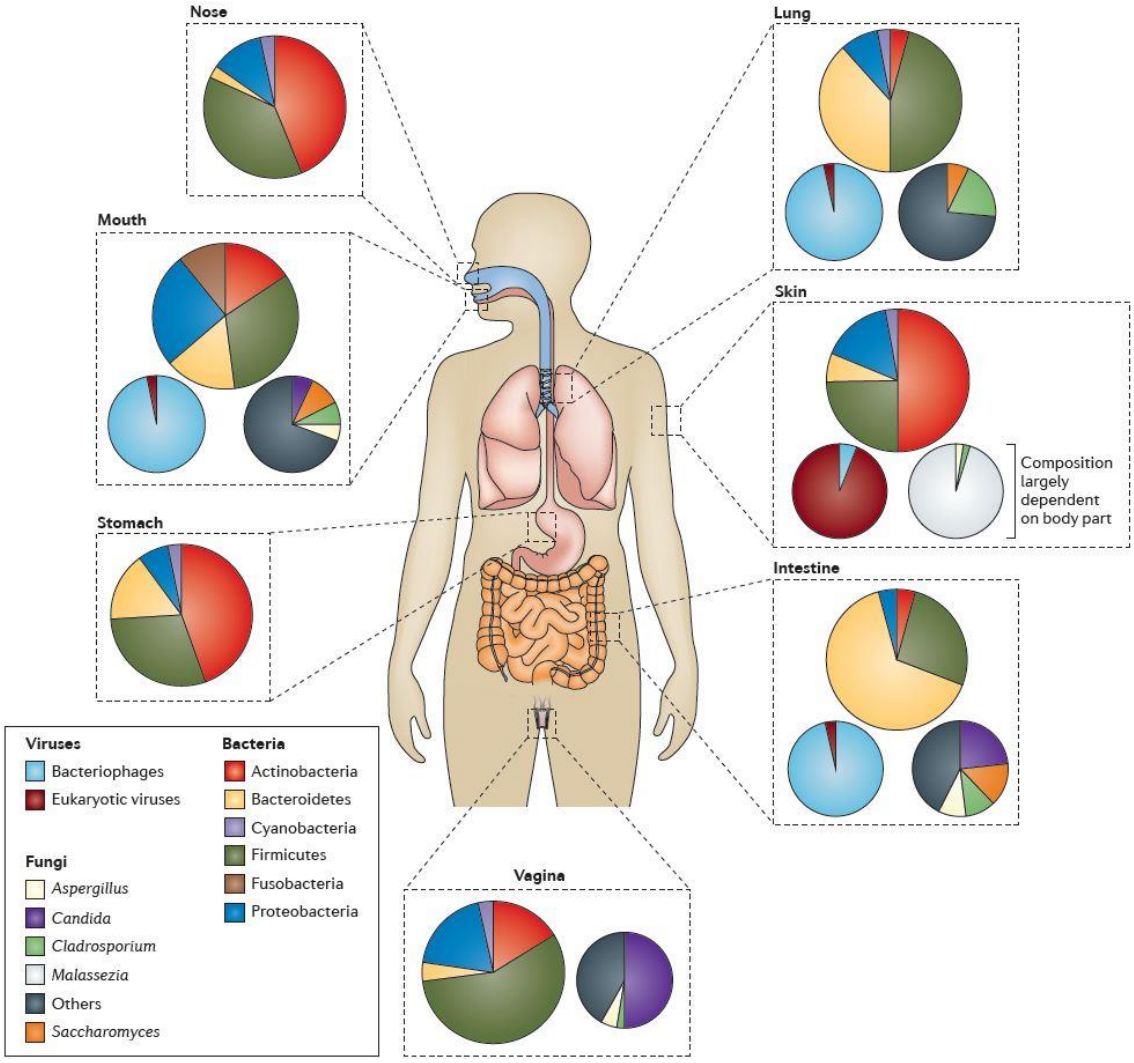


Figure 1. Composition of the bacterial, fungal and viral microbiota at distinct body sites. For bacteria the six most common phyla are displayed (Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Fusobacteria and Proteobacteria).Viruses are grouped into either bacteriophages or eukaryotic viruses and for fungi common genera like Aspergillus, Candida, Cladrosporium, Malassezia and Saccharomyces are shown (Marsland 2014).

In the nose and stomach Actinobacteria and Firmicutes are the predominant phyla, while in the lung Bacteroidetes represent the second most common phylum next to Firmicutes. The skin microbiome is characterized by a high content of Actinobacteria, predominant eukaryotic viruses in contrast to lung, intestine and mouth where Bacteriophages predominate and a composition of fungi varying depending on the body part. The microbiota of the vagina is dominated by Firmicutes, including Lactobacilli and the predominant fungus candida (Marsland 2014, Grice 2011, Frank 2010, Lazarevic 2009, Kim 2009). The four predominate phyla in the intestinal microbiota are Actinobacteria (with genera like Bifidobacterium), Bacteroidetes (e.g. Bacteroides), Firmicutes

(including Lactobacillus and Clostridium) and Proteobacteria (e.g. Enterobacter spp.) with Bacteroidetes being the most prominent phylum (Turnbaugh 2007). The composition of the microbiota itself and of different body surface parts is diverse between individuals also depending on health status. An analysis of gut microbial communities however proposed some years ago three predominant variants or “enterotypes”, dominated by Bacteroides, Prevotella and Ruminococcus. The basis for enterotype clustering is unknown but appears independent of nationality, sex, age, or body mass index (Wu 2011, Arumugam 2011).

It has been shown that the composition of the host microbiota can be associated with potential disease development or to maintenance of health (Prakash 2011, Pfefferle 2014, Power 2014, Clavel 2014, Hoen 2015, Canani 2015). Over the past decades the incidence of chronic inflammatory, non-communicable diseases (NCD), such as allergies or inflammatory bowel diseases is increasing worldwide. These regulatory disorders have often been associated with a dysbalanced immune response and an altered gut microbiota (Kalliomäki 2010, Kau 2011, McLoughlin 2011, Haller 2015, West 2015). Therefore an adequate establishment of the intestinal microbiota and gut colonization in early stages of life is crucial for maintenance of health throughout life (Martin 2010, Penders 2007, Hoen 2015, Roduit 2014). It has been shown that the infant’s immune system matures by exposure to the intestinal microbiota, initiated by microbial colonization of the gut through exposure to bacteria during birth and additionally nutrition and environment in early life (Thavagnanam 2008, Sjogren 2009, Vael 2009, Calder 2006, Penders 2006, Lathrop 2011, Doré 2015). Figure 2 shows the simultaneous development of the intestinal immune system, mucosal barrier, gut colonization, diversification from birth until adulthood. Early life factors like genotype, maternal factors, birth, diet, weaning, environment or microbes are having an impact on development of immune system components and bacterial colonization of the child (Wopereis 2014).

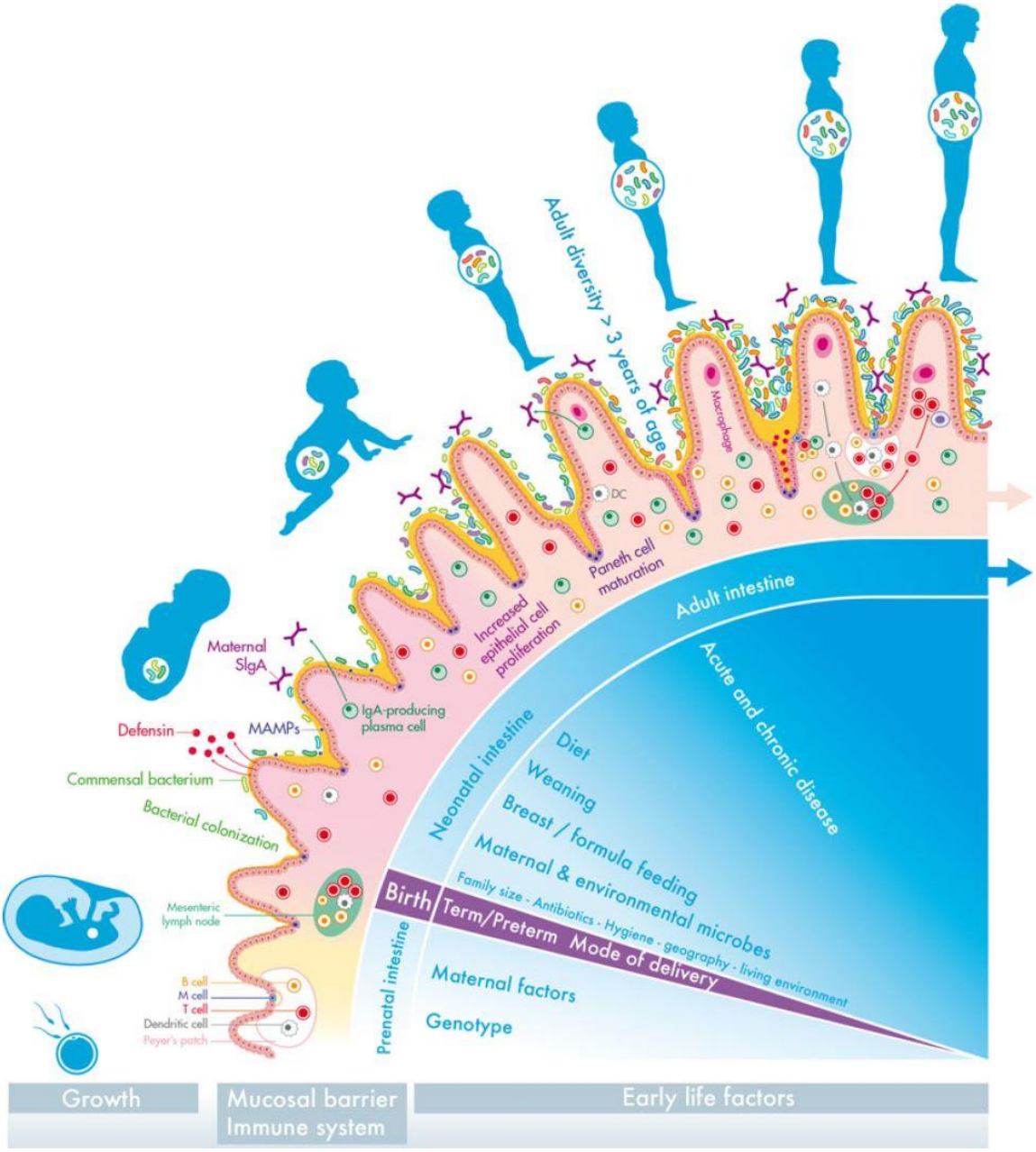


Figure 2. The first thousand days: early life, gut microbiota and immune development – establishing a symbiosis. The infant gut microbiota undergoes dynamic changes during development including microbial diversification, resulting in an adult-like microbiome at about three years of age. Components of the immune system develop simultaneously, establishing adequate functioning immune responses and a tight mucosal barrier. MAMP= microbe associated molecular pattern, Ig= immunoglobulin (Wopereis 2014).

Interestingly, exposure of children to a farm environment in early life is associated with a strong protective effect against development of asthma, hay fever and atopic sensitization (Debarry 2007, von Mutius 2002, Lluis 2014, Loss 2015). The epidemic rise in allergic diseases over the last decades has coincided with progressive westernization, a progress that is accompanied by smaller family size, increased hygiene and excessive antibiotic use (Strachan 1989, von Mutius 2007, West 2014). It has been shown that infants suffering from allergies exhibit a different composed microbiota compared to healthy children (McLoughlin 2011, Penders 2007). Furthermore breastfed neonates exhibit a different intestinal microbial pattern, a more bifidogenic composition of their gut microbiota than bottle-fed infants (Wang 2015). Figure 3 shows the difference in microbial abundance in stool of three months old breast-fed or formula-fed infants (Donovan 2012).

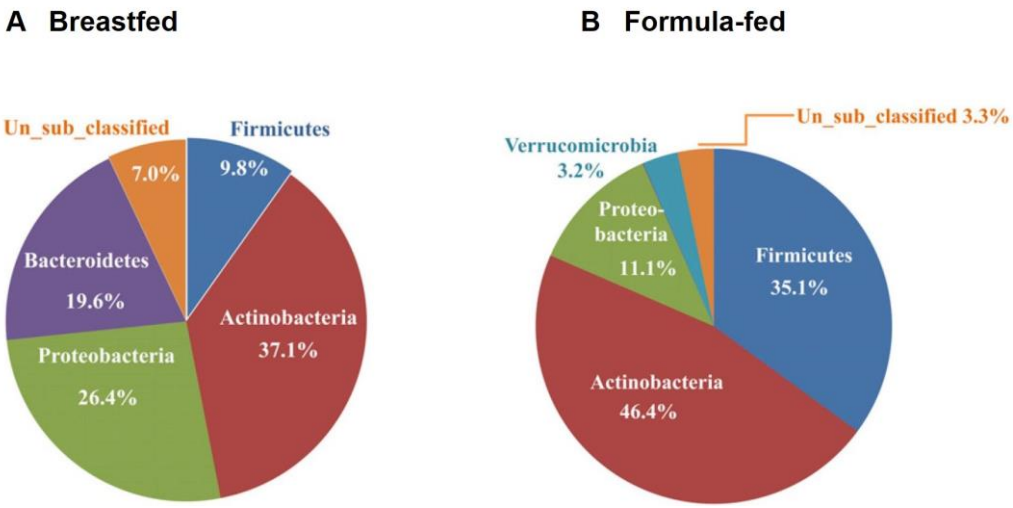


Figure 3. Bacterial phyla in stool of 3 month old breast-fed or formula-fed infants (Donovan 2012).

There is evidence that breastfeeding decreases the incidence of infections (Duijts 2009) and can be protective towards allergic diseases (van Odijk 2003). Human milk has an important role in the establishment of the microbiota and the maturation of the child's immune system in early life (Field 2005, Jeurink 2013). This could be because of its unique composition involving antimicrobial properties and transfer of immunological and tolerogenic stimuli and its high content of non-digestible oligosaccharides (Hoppu 2001, Bode 2012, Hennet 2014).

II. 2. Non-digestible oligosaccharides

Free oligosaccharides are natural occurring in the milk of all mammals. Human milk contains 20–23 g/l oligosaccharides in colostrum and 12–14 g/l in mature milk, making the human milk oligosaccharides fraction the third largest component in human milk after lactose and lipids (Boehm 2007). It has been considered that breast-milk oligosaccharides are growth-promoting factors especially for Bifidobacteria (Gibson 1995, Moro 2002). This fact explains the Bifidobacteria dominated intestinal microbiota of breast-fed infants (Wang 2015). Non-human milk oligosaccharides, such as short chain galacto-oligosaccharides (scGOS) and long chain fructo-oligosaccharides (lcFOS) are used to substitute the described functions of human breast milk in infant nutrition formula (Fanaro 2005, Rinne 2005, Barile 2013). It is already current practice to apply oligosaccharide mixtures that resemble in chemical and functional aspects human milk oligosaccharides as a supplement in infant formula (Figure 4, adapted from Vos 2006).

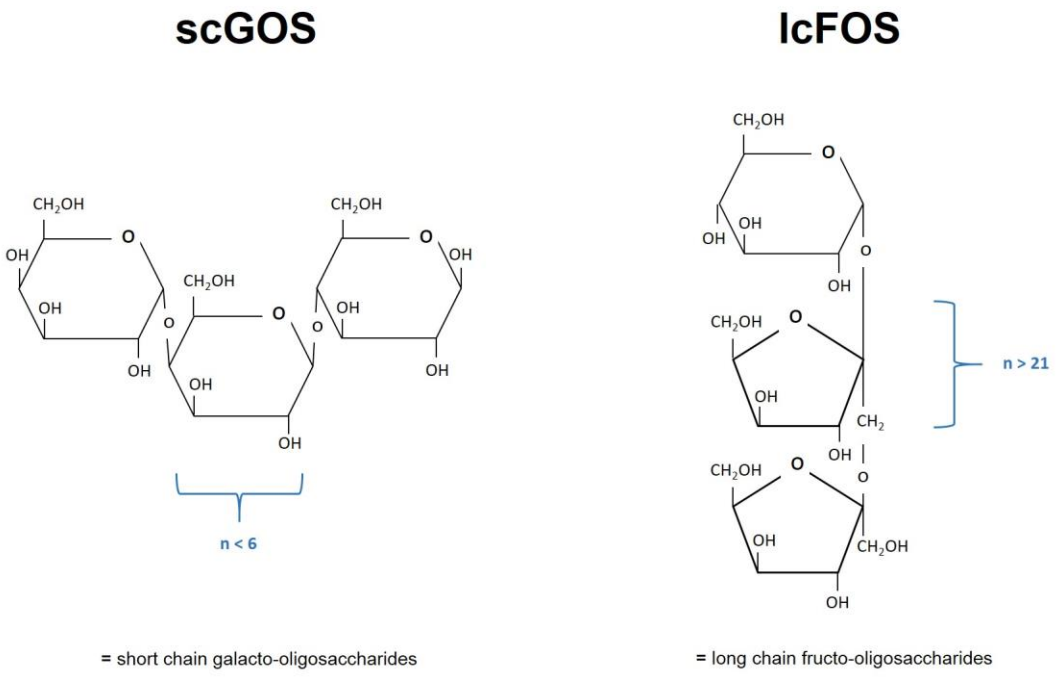


Figure 4. Chemical structure of scGOS/lcFOS used in this study. scGOS=short chain galacto-oligosaccharides, lcFOS=long chain fructo-oligosaccharides (adapted from Vos 2006).

This is a concept in infant nutrition to modify the composition of the gut microbiota and induce a more bifidogenic flora like the one of breastfed children (Boehm 2002, Piemontese 2011, Veereman-Wauters 2011, Oozeer 2013). A study of Moro et al. (2006) indicates that supplementation with short chain GOS and long chain FOS (scGOS/lcFOS) reduces the cumulative incidence of atopic dermatitis during the first six month of life in children at a high risk for allergy, even after five years a protective effect of this oligosaccharide mixture could be determined (Arslanoglu 2012). Also a reduced incidence of infections and less allergic manifestations at the age of two years have been reported (Arslanoglu 2008, Westerbeek 2008). Grüber et al (2010) confirmed that supplementation with scGOS/lcFOS leads to a reduced occurrence of early atopic dermatitis among low-atopy-risk infants and a beneficial effect on mucosal immunity was revealed by Scholtens et al. (2008) showing a higher concentration of fecal secretory IgA. Further human clinical trials exhibited beneficial effects of these non-digestible oligosaccharides like reduced immunoglobulin free light chain levels in infants (Schouten 2011) or a beneficial immunoglobulin profile in infants at high risk of allergy (van Hoffen 2009). Gori et al. (2011) could demonstrate that non-digestible oligosaccharides can induce a modulation of the gut microbiota in adults by decreasing the fecal bacterial load and improve immune activation in HAART (highly active anti-retroviral therapy)-naïve HIV-infected individuals.

Besides this, animal models showed the induction of regulatory T cells by non-digestible oligosaccharides mimicking the functionality of human breast milk oligosaccharides and subsequently the suppression of cow's milk allergy, lower severity of asthma, less respiratory infections, lower IgE and increased viral clearance in mice (Schouten 2010, 2012, Schijf 2012). This specific oligosaccharide mixture has also been tested in a murine vaccination model and an asthma mouse model revealing properties to stimulate delayed-type hypersensitivity, to enhance Th1-dependent vaccination responses and to modulate the early phase of a murine vaccination response (Vos 2007, 2010).

II. 3. Lactic acid bacteria

In the gastrointestinal tract, oligosaccharides selectively stimulate the growth of Bifidobacteria and Lactobacilli (Moro 2002, Knol 2005, Fanaro 2005, Rao 2009, Oozer 2013, Sierra 2015). Lactobacilli belong to the lactic acid bacteria as their end product of carbohydrate metabolism is lactic acid. The genus Lactobacillus comprises a large heterogeneous group of low-G+C Gram-positive, nonsporulating and anaerobic bacteria. Taxonomically, the genus Lactobacillus belongs to the phylum Firmicutes, class Bacilli, order Lactobacillales, family Lactobacillaceae (Lebeer 2008). Bifidobacteria are non-motile, non-spore-forming strictly anaerobic Gram-positive organisms from the phylum Actinobacteria. They are one of the predominant bacterial groups of the human intestinal microbiota (Lee 2010). The dietary use of Lactobacilli as potential immune-modulatory products could represent another promising strategy to prevent or counteract diseases associated with deregulated immune responses or dysbiosis.

Ongoing research and clinical trials indicate that Lactobacilli can have the potential to prevent and reduce the duration of viral or antibiotic associated diarrhea, to alleviate discomfort in connection with diseases of the gastrointestinal tract like to normalize stool frequency as well as consistency in subjects suffering from constipation or lactose intolerance. Furthermore LAB have been shown to have the potential to be effective in certain diseases involving inflammation like pouchitis, irritable colon syndrome or maintenance of remission in ulcerative colitis (Goktepe 2006, Parvez 2010, Hörmannsperger 2010, 2012, Haller 2010, Didari 2015, Lv 2015). Prevention or abatement of allergies and atopic diseases in infants have been described, as well as beneficial effects on microbial aberrancies (de Vrese 2008, Lee 2008, von Schillde 2012, Sanders 2013).

However, potential benefits resulting from LAB supplementation have been shown to be highly strain specific and dependent on several variables differing between different designed studies (Taylor 2007, Kopp 2008, Kalliomäki 2007, 2010, King 2014, Robinson 2014, Kong 2014). The population tested (adults, infants, high/low-risk), symptoms, symptom score used, or clinical endpoints and the disease investigated, as well as the time point and duration of application, e.g. for prevention or intervention, the composition or dose of the LAB applied can have an influence on beneficial or no effects revealed in clinical studies. Heterogeneous results and inconsistent findings are the consequence of a lack of large, well-designed, well-controlled direct comparable clinical studies.

Table 1 shows a selective overview of clinical studies, reviews and meta-analyses covering effects of LAB on various diseases and clinical outcomes, including several populations and compositions of LAB for prevention or treatment, ordered by year.

Author (et al.)	**Year**	**Type**	**LAB**	**Population**	**Disease**	**Effect**
Vijanen	2005	Clinical study	LAB (*Lactobacillus GG*)	infants with atopic eczema–dermatitis syndrome and cow's milk allergy	immunologic effects	↑C-reactive protein levels ↑plasma IL-10 ↑IL-6 ↑E-selectin
Kalliomaki	2007	Clinical trial	LAB (*L.rhamnosus*)	healthy children under seven years	Eczema	risk reduction
Taylor	2007	Clinical trial	LAB (*Lactobacillus acidophilus*)	high risk children	atopic dermatitis	no effect, increased allergen sensitization
Kopp	2008	Clinical trial	LAB (different Lactobacilli)	children	Allergy	no effect
Lee	2008	Meta-analysis	LAB (multiple strains)	prenatal and postnatal	pediatric atopic dermatitis	prevention but not treatment
Haller	2010	Review	LAB (multiple strains and mixtures)	adults	CD, UC, pouchitis, IBS (irritable bowel syndrome)	remission in ulcerative colitis, no effect on Crohn's disease, maintenance of remission in pouchitis, reduction of symptoms in IBS
Hörmanns-perger	2010	Review	LAB (multiple strains and mixtures)	adults	pouchtitis, UC, CD	induction and maintenance of remission
Kalliomäki	2010	Review	LAB (multiple strains)	infants, children, adults	Allergy	heterogenous results, primary prevention of eczema in infants, not adults, no effect by treatment
King	2014	Meta-analysis	LAB (multiple strains)	healthy children and adults	respiratory illness	average duration of respiratory illness episodes, number of days of illness per person↓
Kong	2014	Meta-analysis	LAB (multiple strains)	infants, prenatal, postnatal	food allergy	no preventive effect of prenatal and postnatal probiotic supplementation
Robinson	2014	Meta-analysis	LAB (multiple strains)	preterm infants	necrotizing enterocolitis	prevention of severe NEC and all-cause mortality
Didari	2015	Meta-analysis	LAB (multiple strains)	irritable bowel syndrome patients	irritable bowel syndrome	reduced pain and symptom severity scores
Lv	2015	Meta-analysis	LAB (multiple strains)	adults, children	heliobacter pylori infection	improved eradication rate, side effects of therapy ↓

Table 1. Selective overview of clinical studies, reviews and meta-analyses covering effects of LAB on various diseases and clinical outcomes, including several populations.

II. 4. Lactic acid bacteria in combination with oligosaccharides

A further concept that might be used to inhibit the onset and severity of allergic disorders is the food-based strategy to combine the described non-digestible oligosaccharides mimicking the functionality of oligosaccharides in human milk with breast milk-induced flora, such as Bifidobacteria or Lactobacilli. Dietary intervention with a specific mixture of scGOS/lcFOS in combination with a *Bifidobacterium breve* reduced the severity and incidence of human IgE associated atopic eczema, prevented asthma like symptoms in infants with atopic dermatitis (van der Aa 2010, 2011), restored the bifidogenic milieu of children born through caesarian delivery and has been shown to be able to alter the gut microbiota of formula-fed infants towards a composition of the gut microbiota more like breastfed infants with an increased Bifidobacteria content (Arslanoglu 2007, 2008, Vulevic 2008, Knol 2005). A study of de Kivit et al. (2012) demonstrated in mice and humans that dietary supplementation with scGOS/lcFOS and *Bifidobacterium breve* suppresses allergic symptoms involving a potential role of galectin-9, a soluble type lectin. Also reduced allergen induced T Helper (Th) 2 response and an improved peak expiratory flow in allergic asthmatics have been described after treatment with a combination of scGOS/lcFOS and *B.breve* (van den Pol 2011).

As mentioned for clinical studies investigating beneficial effects of LAB, it can be difficult to draw conclusions from different, heterogeneous clinical studies addressing potential beneficial effects of NDO and combinations of NDO and LAB as results may vary depending on the population, dosage, composition, clinical outcomes and disease analyzed. Table 2 shows a selective overview of clinical studies and reviews covering effects of NDO on various diseases and clinical outcomes, including several populations and compositions of NDO for prevention or treatment, ordered by year.

Author (et al.)	**Year**	**Type**	**NDO**	**Population**	**Disease**	**Effect**
Macfarlane	2006	Systematic review	NDO (various)	adults	bowel habit, calcium absorption, gastrointestinal disorders	bifidobacteria↑, no effect on bowel habit, calcium absorption↑, anti-inflammatory effects, pouchitis improvement
Moro	2006	Clinical study	NDO (scGOS/lcFOS)	infants under 6 month	atopic dermatitis	reduced incidence
Arslanoglu	2007	Clinical study	NDO (scGOS/lcFOS)	infants healthy under 6 months	infection	prevention
Arslanoglu	2008	Clinical study	NDO (scGOS/lcFOS)	infants under 2 years	allergy & infection	reduced incidence
Scholtens	2008	Clinical trial	NDO (scGOS/lcFOS)	healthy infants	mucosal immunity	fecal sIgA↑ bifidobacteria in stool↑

Vulevic	2008	Clinical Study	NDO (trans-galacto-oligosaccharide mixture)	healthy elderly	immune function, microbiota	bifidobacterium counts↑, ↑phagocytosis, NK cell activity, IL-10 ↓IL-6, IL-1β, TNFα, no effects on total or HDL cholesterol
Westerbeek	2008	Clinical trial	NDO (scGOS/lcFOS)	preterm infants	infections	no significant reduction in risk of serious infectious morbidity
Lomax	2009	Review	NDO (various)	children, adults	immune function, infection	benefits regarding inflammatory bowel disease (adults) & atopic dermatitis (children), inconsistent findings in irritable bowel syndrome
Van Hoffen	2009	Clinical trial	NDO (scGOS/lcFOS)	infants at high risk for allergy	allergy	beneficial Ig profile
Grüber	2010	Clinical study	NDO (scGOS/lcFOS and pectin-derived acidic OS)	low atopy risk children	atopic dermatitis	reduced occurrence
Gori	2011	Clinical trial	NDO (scGOS/lcFOS and pectin-derived acidic OS)	HIV infected adults	modulation of gut microbiota and immune activation	bifidobacteria↑, Clostridium↓, reduction of soluble CD14 (sCD14), activated $CD4^+/CD25^+$Tcells natural killer cell activity↑
Hughes	2011	Clinical trial	NDO (galactooligo-saccharides)	healthy students	stress-induced gastrointestinal dysfunction and days of cold or flu	symptoms of gastrointestinal dysfunction & reduced number of days with cold or flu ↓
Schouten	2011	Clinical trial	NDO (scGOS/lcFOS)	infants at risk for allergy	allergy	immunoglobulin free light-chain concentration↓
Arslanoglu	2012	Clinical study	NDO (scGOS/lcFOS)	infants under 5 years	allergy	reduced incidence
Osborn	2013	Review	NDO (various)	infants up to two years	allergy	no effect on asthma, reduction in eczema
Sierra	2015	Clinical study	NDO (galactooligo-saccharides)	healthy, under 1 year old	effect on microbiota, stool, infection, allergy	fecal pH↓, bifidobacterium counts↑, stool frequency↑, softer consistency, no effect on incidence of infections or allergic manifestations

Table 2. Selective overview of clinical studies and reviews covering effects of NDO on various diseases and clinical outcomes, including several populations.

Table 3 shows a selective overview of clinical studies, reviews and meta-analyses covering effects of combinations of LAB and NDO on various diseases and clinical outcomes, including several populations and compositions of LAB/NDO for prevention or treatment, ordered by year.

Author (et al.)	**Year**	**Type**	**LAB/NDO**	**Population**	**Disease**	**Effect**
Jain	2004	Clinical trial	*Lactobacillus acidophilus, Bifidobacterium lactis, Streptococcus thermophilus* and *Lactobacillus bulgaricus* with oligofructose	intensive care unit patients	gut barrier function, sepsis	pathogenic bacteria in nasogastric aspirates↓, no effect on mortality, septic complications or intestinal permeability
Picaud	2010	Observational study	*Bifidobacterium longum, Streptococcus thermophiles* &fructo-OS	infants	infectious diseases	incidence↓
Van der Aa	2010	Clinical trial	scGOS/lcFOS and *Bifidobacterium breve*	infants	atopic dermatitis	severity of eczema↓, Bifidobacteria↑
Van der Aa	2011	Clinical trial	scGOS/lcFOS and *Bifidobacterium breve*	infants with atopic dermatitis	asthma-like symptoms	wheezing↓, less used asthma medication, no effect on total IgE
Van den Pol	2011	Clinical study	scGOS/lcFOS and *Bifidobacterium breve*	adult allergic asthmatics	allergic responses	no effect on sputum eosino-phils, bronchial inflammation, ↓T helper 2 response &improved expiratory flow
De Kivit	2012	Clinical trial	scGOS/lcFOS and *Bifidobacterium breve*	infants with atopic dermatitis	allergy	galectin-9 in serum enhanced
Mugambi	2012	Systematic review	various	full term infant	growth, gastrointestinal outcomes, infections	no effect on growth, LAB+NDO→ increased stool frequency, no impact on stool consistency, colic or vomiting, no effect of NDO on infections
Dang	2013	Meta-analysis	various	infants under 2 years, mothers	eczema	incidence ↓
Pfefferle	2013	Review	various	infants, mothers, prenatal, postnatal	allergy/eczema	↓incidence of eczema, no consistent effects on other allergy outcomes

Table 3. Selective overview of clinical studies, reviews and meta-analyses covering effects of LAB and NDO in combination on various diseases and clinical outcomes, including several populations.

In the context of the previously described evidence in human clinical trials and animal studies for beneficial effects of non-human milk oligosaccharides and Lactobacilli, underlying mechanisms on cellular level are highly interesting. Besides a mode of action involving indirect effects on the immune system via manipulation of the intestinal microbiota, direct effects on immune cells such as dendritic cells and the ensuing T cell response, as well as on epithelial cells could be possible. De Kivit et al. (2013) revealed direct effects of scGOS/lcFOS on intestinal epithelial cells and a Th1/Treg skewing polarization effect including a role of lectins. Others also reported a potential mode of action involving direct immunomodulation of oligosaccharides via a potential effect on immune cells (Eiwegger 2004, 2010, Naarding 2005, Jeurink 2013).

II. 5. Lactic acid bacteria, oligosaccharides and the immune system

The efficacy of potential health beneficial bacteria and NDO mainly depends on interactions with epithelial and immune cells as well as the microbiota of the intestinal mucosa of the host.

II. 5.1. Intestinal epithelial cells

Intestinal epithelial cells (IEC) play an important role in the complex environment of the gut, displaying not only a physical barrier between the gut lumen with external factors and the mucosal immune system. IEC also interfere at the borderline between gut lumen and gut associated lymphoid tissue (GALT) including sampling of the intestinal environment and subsequently influencing and governing the following immune response by modulating immune cells and cytokine responses (Artis 2008, Haller 2008, Hörmannsperger 2012). Intestinal epithelial cells as well as dendritic cells, as luminal front line cells in the human gut express pattern recognition receptors (PRR) to recognize bacteria and their derived effector molecules (de Kivit 2014, Reis e Sousa 2003).

Evidence exists that IEC have immunosuppressive capacities and can create a tolerogenic type of DC (Butler 2006, Iliev 2009, Rescigno 2014). Furthermore it has been shown that IEC are able to discriminate between pathogenic and nonpathogenic bacteria, for example between *E.coli* and lactobacilli (Haller 2000, 2004). Zoumpopoulou et al. (2009) revealed in a murine IEC and DC co-culture model that IEC respond poorly to Gram-positive bacteria, in contrast to *Escherichia coli*, which strongly activated them. Campeau et al. (2012) confirmed that murine intestinal epithelial cells have the capacity to modulate subsequent antigen-presenting and T cell responses to bacterial DNA (bifidobacteria and salmonella) with pathogenic but not commensal bacterial DNA inducing effector CD4 T lymphocytes.

Under inflammatory conditions, toll-like receptor (TLR) expression is upregulated in IEC (Viljanen 2005, Marschan 2008). It has been shown that intestinal epithelial cells express TLR and therefore can recognize LAB (Wells 2011). Several studies confirmed the capacity of intestinal epithelial cells to recognize LAB in human and murine cell studies via TLR (Vinderola 2005, Zeuthen 2008). Leucocyte-epithelial crosstalk in modulating cytokine responses in the gut mucosa has been described as well as the fact that IEC transmit information about apical stimuli like microbes to underlying immune cells, which orchestrate critical immune responses (Hörmannsperger 2009, Ratajczak 2007).

II. 5.2. Dendritic cells

One aim of this study is to evaluate direct immune modulatory effects of non-digestible oligosaccharides and lactic acid bacteria on human monocyte-derived dendritic cells. These innate immune cells have a pivotal role by managing pro-inflammatory reactions as first line of defense against pathogens but also sending out anti-inflammatory, tolerance inducing signals involving adequate adaptive immune responses including T cell plasticity (Manickasingham 2003). As dendritic cells are located directly under the epithelial cell line in the gastrointestinal tract, peyer's patch dendritic cells are able to sample antigens by extending their dendrites into the gut lumen (Lelouard 2012). Therefore ingested infant formula supplements could play a role in maturation and activation of dendritic cells and subsequently the modulation and skewing of T cell responses, which play an important role for the balance between tolerance and immunity. The complex ecosystem of the gut, intestinal barrier and gut associated lymphoid tissue as well as commensals, LAB, NDO and food antigens present in the gut lumen are schematically displayed in Figure 5.

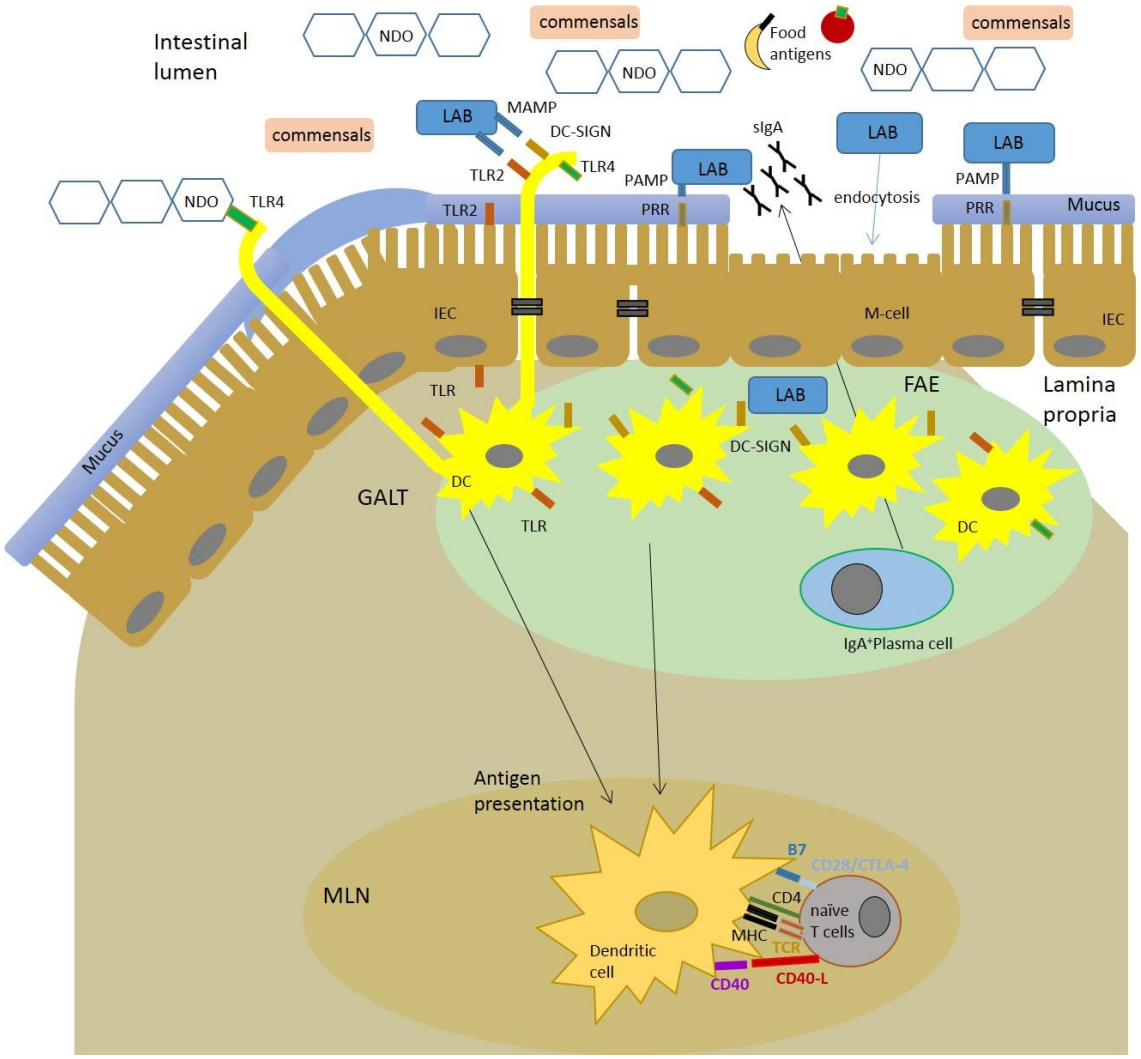


Figure 5. Dendritic cells (DC) in the gut. The gut mucosa is rich in antigen presenting cells (APC) which reside underneath the epithelial cell layer in an immature state sensing constantly the microenvironment for foreign antigens or invading pathogens. APC are specialized in capturing antigen, internalizing and processing it, consequently playing a crucial role in the orchestration of the adaptive immune response. DC can receive conditioning signals by intestinal epithelial cells and are able to reach their dendrites through the tight junctions of epithelial cells. NDO=non-digestible oligosaccharides, LAB=lactic acid bacteria, IEC= intestinal epithelial cell, TLR=toll-like receptor, MLN=mesenteric lymph node, GALT=gut associated lymphoid tissue, FAE= follicle associated epithelium, PAMP=pathogen associated molecular pattern, PRR=pattern recognition receptor, MAMP=microbe associated molecular pattern, TCR= T cell receptor. Figure based on Lebeer 2008.

DC exhibit multiple receptor systems called pattern recognition receptors (PRRs) including intra- and extracellular molecules such as scavenger receptors, c-type lectin-receptors (CLRs), toll-like receptors (TLRs) and nod-like receptors (NLR, nucleotide oligomerization domain-containing protein). These receptors recognize a wide variety of different types of structural components of microbes, foreign antigens or commensals, inducing either immunogenic or tolerogenic DC (Jeong 2011, Hubo 2013).

Microbial substances that stimulate innate immunity are called pathogen-associated molecular patterns (PAMPs) or microbe associated molecular pattern (MAMPs). Different classes of microbes (e.g. viruses, Gram-negative bacteria, Gram-positive bacteria, fungi) express different PAMPs/MAMPs unique to these microbes. Some pattern recognition molecules are expressed on the cell surface of DC, where they may bind extracellular PAMPs. For example lectin-like receptors like C-type lectin family receptors like dectins are found on the cell surface and recognize fungal glycan. Another example on the cell surface are mannose receptors or DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin). Some PRRs are expressed on endosomal membranes and recognize nucleic acids of microbes that have been phagocytized. NOD receptors and RIG-like receptors are cytosolic sensors that recognize bacterial peptidoglycans or viral RNA (Lebeer 2010).

There are different functional TLRs in humans. TLRs are integral membrane glycoproteins that contain leucine-rich repeats flanked by characteristic cysteine-rich motifs in their extracellular regions, which are involved in ligand binding and a Toll/IL-1 receptor (TIR) homology domain in their cytoplasmic tails, which is essential for signaling. Some TLRs are expressed in endosomes and some on the cell surface. Ligands recognized by the different TLRs are structurally diverse and include products of all classes of microorganisms. Bacterial lipopolysaccharide (LPS), the major bacterial cell component of Gram-negative bacteria, is recognized by TLR4 on the cell surface. TLR2 recognizes bacterial peptidoglycans, lipoproteins and lipoteichoic acid from Gram-positive bacteria. In contrast, TLRs 3, 7, 8, and 9 are mainly expressed inside cells on endosomal membranes, where they detect several different nucleic acid ligands expressed by microbes, such as double-stranded RNA and unmethylated CpG motifs common in prokaryotic DNA. Ligand binding to the leucine-rich domains causes physical interactions between TLR molecules and the formation of TLR dimers (McClure 2014).

Activation of DC via PRRs leads to the activation of several intracellular signal transduction pathways and ultimately transcription factors regulating the expression of cytokine genes, inducing cytokine production by DC (Edwards 2002). Signaling pathways initiated by ligand binding to TLRs include for example adaptor proteins like MyD88 or TRIF (Toll/IL-1R domain-containing adaptor inducing IFN), leading finally to the activation of transcription factors like NFkB (nuclear factor k B), AP-1 (activation protein 1) or IRF (interferon response factor) (Ahmed 2013). DC maturation is associated with functional and phenotypic changes such as up-regulation of cell surface and costimulatory molecules CD80, CD83, CD86 and HLA-DR (Joffre 2009). It has been postulated that partially or semi-mature intestinal DC producing little IL-12 are involved in induction of tolerance favoring the induction of a type 3 response (oral tolerance),

whereas active immunity is induced by stimulated fully mature DC with either low production of IL-12, leading to a type 2 response or high-level IL-12 production, favoring a type 1 response (Smit 2011, Novak 2004). Mature DC present internalized and processed antigens to naïve T cells. This bridging function is an important part of the initiation of adaptive immune responses (Rescigno 2010). The cytokine production profile and maturation status of DC depend on the nature of the stimulus and direct the polarization of naïve T cells towards the T cell phenotype T helper (Th) 1, Th2, Th17, Th22, Th9 or regulatory T cell differentiation (Saurer 2009, Wolk 2010).

II. 5.3. T cells

Each subset of T cells obtains defined repertoires of effector functions. Helper T cells (Th) are $CD4^+$ and activate both humoral and cellular immune responses, whereas $CD8^+$ T cells, also known as cytotoxic T cells show cytotoxic activity against tumor cells as well as against cells infected with intracellular microbes. Cytokines released by DC exert largely opposite immune-regulatory effects and the different T cells subsets mediate their corresponding actions via various cytokines produced (Eyerich 2014, Jutel 2011; Figure 6).

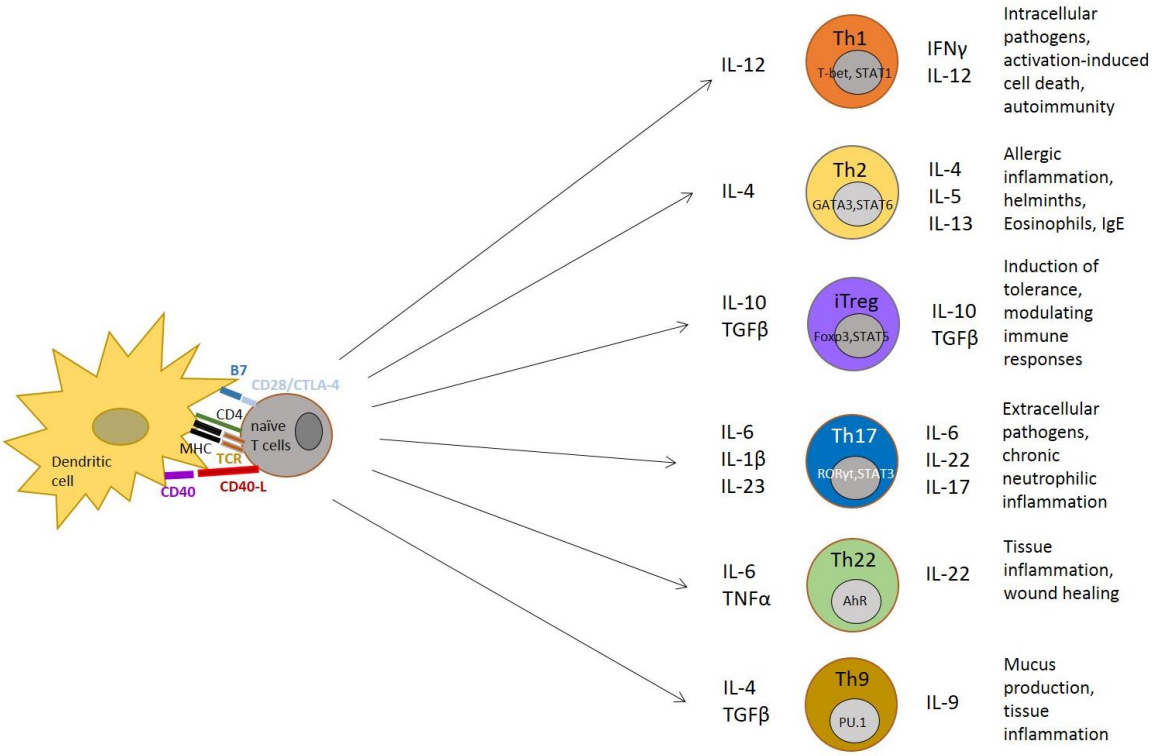


Figure 6. T cell subsets induced by dendritic cells. IL=interleukin, IFN=interferon, TCR=T cell receptor, Th= T helper cell (adapted from Jutel 2011).

IL-12 skews T cell differentiation towards Th1 responses, which are usually associated with inflammatory reactions as Th1 cells participate in cell mediated immunity involving defense against viruses and bacteria. Th2 development relies on IL-4. Th2 cells are known to be a part of antibody-mediated immune responses against parasites, a shift towards Th2 cells has been associated with IgE mediated allergy (Deo 2010). Secretion of IL-1β and IL-23 by DC leads to the appearance of Th17 cells (Jutel 2011). It has been shown that Th17 cells play an important role in protective immunity against mucosal infections (Mills 2008). Another Th cell subset that has been described are Th22 cells, present in inflamed skin secreting IL-22 and TNFα (Eyerich 2009). IL-22 can exert either pathogenic or protective effects, it increases innate immunity and a certain role in allergy, inflammatory diseases and infection defense has been reported (Witte 2010, Akdis 2012).

Anti-inflammatory IL-10 release favors regulatory T cell (Treg) differentiation. T cell balance is thought to be maintained by specialized subsets of regulatory T cells that produce suppressive cytokines such as IL-10 and transforming growth factor β (TGF-β). Treg cells are essential in modulating immune responses, maintaining peripheral tolerance and preventing overreaction since they are able to suppress the activation of naïve T cells (Smith 2011). At least three different populations of regulatory T cells have been identified (Liston 2014). Natural $CD4^+$, $CD25^+$, $Foxp3^+$ Treg cells, which originate from the thymus, are characterized by the constitutive expression of the transcription factor Foxp3. Natural Treg cells exert their suppressive effect via contact dependent mechanisms, probably involving the interaction between cytotoxic T lymphocyte-associated antigen (CTLA)-4 and membrane mTGFβ or LAG-3 expressed on their surface and MHC molecules on APC (Skrindo 2011, Freeman 2012). Tr1 cells have been demonstrated to mediate bystander suppressor function by secreting IL-10, likely contributing to the establishment of gut peripheral tolerance, whereas Th3 cells produce TGF-β and are also believed to play a role in oral tolerance (Cavani 2001, Battaglia 2006).

II. 5.4. Overview

The immune-suppressive microenvironment of the gut is controlled by a complex network of IEC and immunological interactions between APC and T cells, allowing the induction of tolerance to food antigens or commensals. Immunity against invading pathogens involving an intact mucosal barrier and appropriate immune responses is crucial for host health (Hörmannsperger 2012). The composition of the microbiota since birth, as well as luminal content including LAB and NDO can have an impact on the monolayer of IEC expressing PRR like TLR and subsequently interacting with the gut associated lymphoid tissue. DC possess dendrites that can reach into the gut lumen, therefore these cells can sense luminal content in the complex environment of the gut and consequently provide a bridging function to the adaptive immune system by influencing the polarization of T cells into subsequent different subtypes according to the particular stimulus (Figure 7).

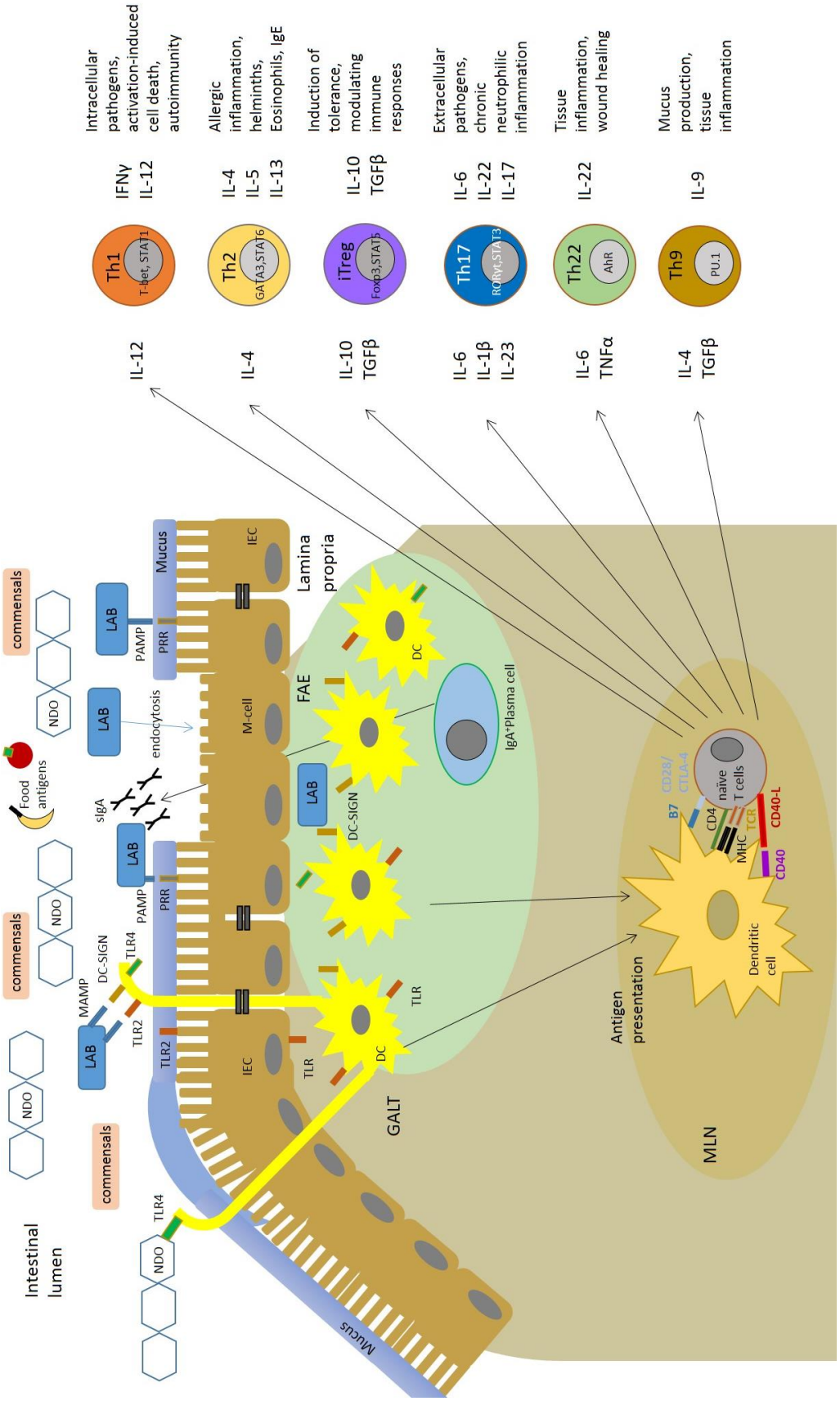


Figure 7. Overview: complex environment of the gut. A monolayer of IEC and mucus provide an intact mucosal barrier. IEC transmit conditioning signals to APC, such as dendritic cells (DC) that constitute an interface between the innate and adaptive immune system, orchestrating polarization of T cells into different subtypes according to luminal stimuli.

Aim of the study

Accumulating evidence exists that an altered composition of the microbiota (dysbiosis) and dysbalanced local and systemic immune responses, including loss of mucosal homeostasis and tolerance, contribute to chronic inflammatory non-communicable diseases such as allergies or inflammatory bowel diseases. Infant formulas containing non-digestible oligosaccharides (NDO) similar to the composition of oligosaccharides in human breast milk, lactic acid bacteria (LAB) or a combination of LAB and NDO have been shown in animal studies and human clinical trials to harbor anti-inflammatory properties and preventive effects towards immune-regulatory disorders. Food-based strategies involving these ingredients to achieve or maintain gut homeostasis, a well-balanced microbiota and a properly functioning immune system have therefore gained enormous interest.

However, it is largely unknown if NDO may have a direct effect on immune cells and the exact underlying mechanisms of potential interactions of NDO and human antigen presenting cells are still unclear. The aim of this work is therefore to investigate the immune-modulatory potential of non-digestible short chain galacto- and long chain fructo-oligosaccharides (scGOS/lcFOS) mimicking the natural distribution of oligosaccharides in human breast milk in presence or absence of certain LAB strains on human monocyte-derived dendritic cells (MoDC) and the ensuing T cell response. *In vitro* cultured human monocyte-derived DC will be used to investigate cytokine secretion profiles and phenotypical alterations of MoDC after stimulation with LAB, NDO or both. Many studies have focused on strain specific effects of different bacterial strains on T cell polarization towards different effector T cell subtypes. Especially the induction of regulatory T cells as well as anti-inflammatory cytokine profiles play an important role in the prevention of allergies and related disorders. However, only little is known about the immune-regulatory potential and effects on T cell responses elicited by NDO in humans. Therefore the potential to induce a regulatory T cell type using phenotypical and functional analysis of T cell responses after allogeneic stimulation will be tested. Ongoing research indicates an important role of intestinal epithelial cells in maintaining gut homeostasis and modulating immune responses. IEC regulate mucosal integrity in the homeostatic IL-10/TGF-β dominated tolerogenic environment in the gut, they express toll-like receptors (TLR) and thereby can recognize LAB. Therefore a further objective of this study is to investigate possible effects and a potential immune-modulatory impact of certain LAB in an *in vitro* co-culture model of human intestinal epithelial cells (IEC) and peripheral blood mononuclear cells (PBMC).

Results from this project will provide an insight into a possible mode of action of specific NDO and effects of certain LAB in human cell systems, discussed in the context of potential food-based strategies involving LAB and NDO and their impact on health or disease of the host.

III. Methods

III. 1. Analysis of the effect of non-digestible oligosaccharides and lactic acid bacteria using a human *in vitro* MoDC - T cell system

III. 1.1. Subjects and ethic statement

For experiments described in this part of the work peripheral blood of healthy, non-atopic donors was used. Volunteers were screened for total serum immunoglobulin E (IgE) levels and for specific IgE against common allergens as described before (Traidl-Hoffmann 2002) and non-atopic individuals were defined by total IgE lower 100 kU. The ethical committee of Technische Universität München approved the study and all volunteers enrolled in the study had given their written informed consent.

III. 1.2. Bacteria preparation

The *Bifidobacterium breve* strain NutRes200 (Morinaga Milk Industry) as well as the *Lactobacillus rhamnosus* strain NutRes1 were kindly provided by Prof. Johan Garssen, Department of Pharmacology and Pathophysiology, Utrecht University. In the course of experiments for this study several Lactobacilli and Bifidobacteria strains have been investigated with regard to their immune-modulatory capacities. The results however will focus on certain tested LAB, one *L.rhamnosus* and one *B.breve* strain. *L.rhamnosus* was available as 0.5 ml cultures of 5.9×10^9 cell counts stored at -80°C. Beforehand it was grown at 37°C in deMan, Rogosa Sharpe (MRS) broth (Oxoid) under anaerobic conditions. Bacteria were harvested in the early stationary phase, washed and aliquoted for storage. Cell counts were determined by plating serial dilutions (cfu). Lyophilized *B.breve* was diluted in D-PBS (Gibco/Invitrogen) and a 25×10^9 cfu/ml stock solution was prepared and stored at -80°C. Before lyophilization, *B.breve* was cultured in MRS broth (Oxoid) for 48 hours at 37°C under anaerobic conditions. To prepare bacterial strains for stimulation experiments, stocks were thawed, diluted appropriately in D-PBS (Gibco/Invitrogen) and concentrations of 1×10^5-1×10^7cfu/ml were generated by serial dilution in cell culture medium.

III. 1.3. Preparation of NDO mixtures

GOS liquid (scGOS; Friesland Campina, 45% scGOS), containing 25% moisture, 15% lactose and 15% other carbohydrates (mainly glucose), FOS powder (inulin, high performance, 96.5%, lcFOS, Orafti) containing 3.5% moisture were kindly provided by Prof. Johan Garssen, Department of Pharmacology and Pathophysiology, Utrecht University, in separate 50 ml tubes, stored at 4°C. To prepare a scGOS/lcFOS (9:1) mixture (final experimental concentration 5 mg/ml) 10 mg FOS powder were dissolved in 9.7 ml warm cell culture medium and 5 ml of dissolved FOS was added to 100 mg GOS liquid. After 0.45 and 0.22 µm filter sterilization the GOS/FOS mixture was ready to be used in the stimulation experiments. Oligosaccharide mixtures were freshly prepared for every experiment. Potential endotoxin contamination of the NDO mixtures was analyzed using a PyroGene Recombinant Factor Endotoxin Detection System (Lonza) conducted by Elke Bartusel from the Environmental Toxicology Group of ZAUM (Center of Allergy&Environment).

III. 1.4. Generation of monocyte-derived dendritic cells

For the generation of monocyte-derived dendritic cells (MoDC) PBMC were isolated from peripheral blood of healthy volunteers by density gradient centrifugation (LymphoprepTM, Axis-Shield). Heparinized blood was diluted 1:1 with D-PBS and layered carefully on the lymphoprep fluid and centrifuged for 15 minutes at 2200 rpm without brake. After centrifugation the mononuclear cells were collected from the distinct band at the plasma/lymphoprep interface and washed several times with D-PBS containing 5 mM EDTA (Gibco/Invitrogen). $CD14^+$ monocytes were purified by MACS (magnetic activated cell sorting, $CD14^+$ beads, Miltenyi Biotec). Bead-coupled antibodies against CD14 were incubated for 15 minutes together with freshly isolated PBMC at 4°C according to the manufacturer's instructions. Afterwards monocytes were separated by positive selection through a magnetic column and cultured in RPMI 1640 supplemented with 10% FBS (HyClone), 2 mmol/l L-glutamine, 20 mg/ml gentamycin (all from Invitrogen Life Technologies) and 50 µmol/l 2-mercaptoethanol (Fluka) at 37°C, 5% CO_2 in the presence of 50 U/ml rhGM-CSF and 50 U/ml rhIL-4 (both Promocell). Medium refreshment was conducted every two days.

III. 1.5. Stimulation of MoDC with scGOS/lcFOS and LAB

Immature MoDC were seeded into 96-well flat bottom plates (Nunc) at a density of $1x10^5$ cells/ml in complete DC medium and treated with the bacterial strains at concentrations of $1x10^5$-$1x10^7$ cfu/ml. Due to the used bacteria and DC concentrations in the experimental setting the bacteria-DC ratio for $1x10^7$ cfu/ml bacteria was 100:1, for $1x10^6$ cfu/ml it was 10:1 and for $1x10^5$ cfu/ml 1:1. Bacterial stimulation was performed either in the absence or presence of 5 mg/ml scGOS/lcFOS mixture (9:1). To block TLR4 activity, a TLR4 antagonist (5 µg/ml, LPS-RS Ultrapure, InvivoGen) was used according to the manufacturer's instructions. LPS (100 ng/ml, Sigma) served as positive control for MoDC stimulation indicating functional MoDC stimulation. Untreated MoDC were used as negative control. After 24 hours of incubation at 37°C and 5% CO_2 supernatants were collected and further analyzed. The phenotype of MoDC was determined and MoDC subsequently further co-cultured in allogeneic stimulation assays.

III. 1.6. Allogeneic stimulation assay of naïve T cells

The capacity of oligosaccharide-matured MoDC to instruct the polarization of T lymphocytes was examined performing allogeneic stimulation assays (ASA). After 24 hours and taking off the cell-free supernatants, stimulated MoDC were washed, counted and co-incubated with naïve $CD4^+CD45RA^+$ T cells at a DC:T cell ratio of 1:10 in T cell medium containing RPMI supplemented with 1% L-glutamine, 1.12% nonessential amino acids, 1.12% sodium pyruvate, 1% pen-strep (all from Gibco), 1% 5 mM mercaptoethanol (Fluka) and human serum (5%, Lonza) for 7 days. Allogeneic $CD4^+CD45RA^+$ T cells were isolated from PBMC of non-atopic donors using the $CD4^+CD45RA^+$ naïve T Cell Isolation Kit II (Miltenyi Biotech), using negative selection to obtain untouched $CD4^+CD45RA^+$ naïve T cells. A positive control for Treg polarization was generated by priming naïve T cells with 5 ng/ml rhTGF-β (Promokine), 200 U/ml rhIL-2 (Novartis), 5 µg/ml anti-IL-12 and 1 µg/ml aIFN-γ (both eBioscience) and co-culturing them with MoDC that have been incubated with medium only. 20 U/ml rhIL-2 was added to the cells on the third day of the ASA.

In Figure 8 the experimental setup of the allogeneic stimulation assay method is schematically displayed.

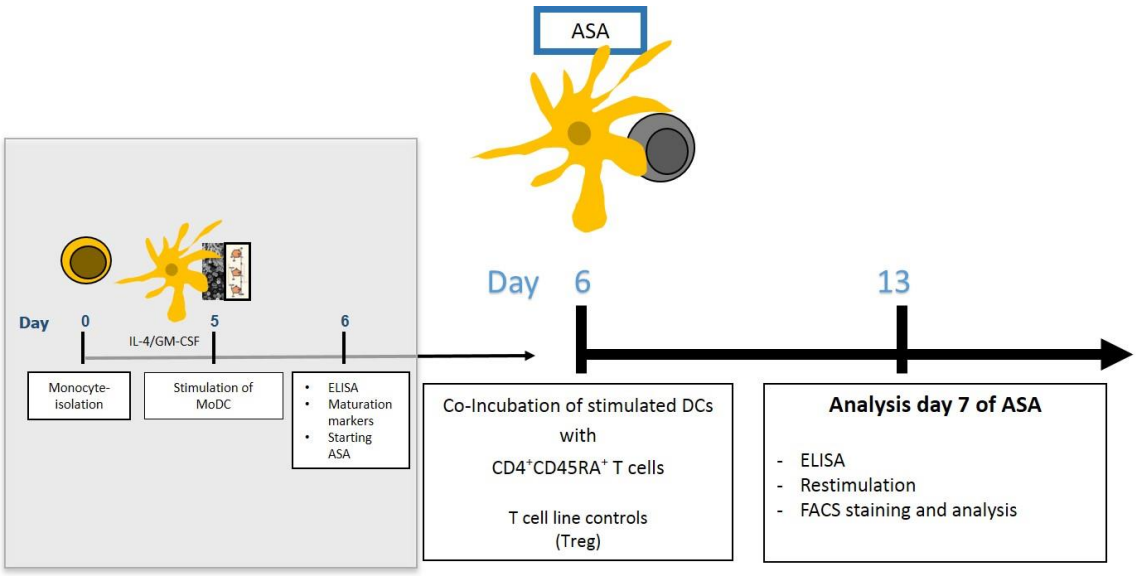


Figure 8. Allogeneic stimulation assay method

III. 1.7. Suppression assay

To determine the potential functionality of regulatory effector T cells induced in the ASA, these cells were harvested on day seven of the ASA, after collecting supernatants and co-incubated with allogeneic freshly isolated responder T cells ($CD4^+$ T cell isolation Kit II, Miltenyi) at a ratio of 1:1 for five days in 96-well plates (BD) coated with anti-CD3 (1 µg/ml, BD). Soluble anti-CD28 (1 µg/ml, BD) and rhIL-2 (100 U/ml) were added. Responder T cells were stained with a CellTrace Violet Cell Proliferation Kit (Life Technologies) according to the manufacturers' instructions at a final concentration of 6 µM. The mix of ASA cells and violet stained responder $CD4^+$ T cells was then stained (after five days of co-incubation) with CD4 APC-Cy7 (BD Biosciences) and violet-positive cells were acquired on a FACS Fortessa (Becton Dickinson). Suppressive capacity was determined by setting gates on proliferated and non-proliferated cells and analyzing the ratio of these cell populations.

In Figure 9 the experimental setting of the suppression assay method is schematically shown.

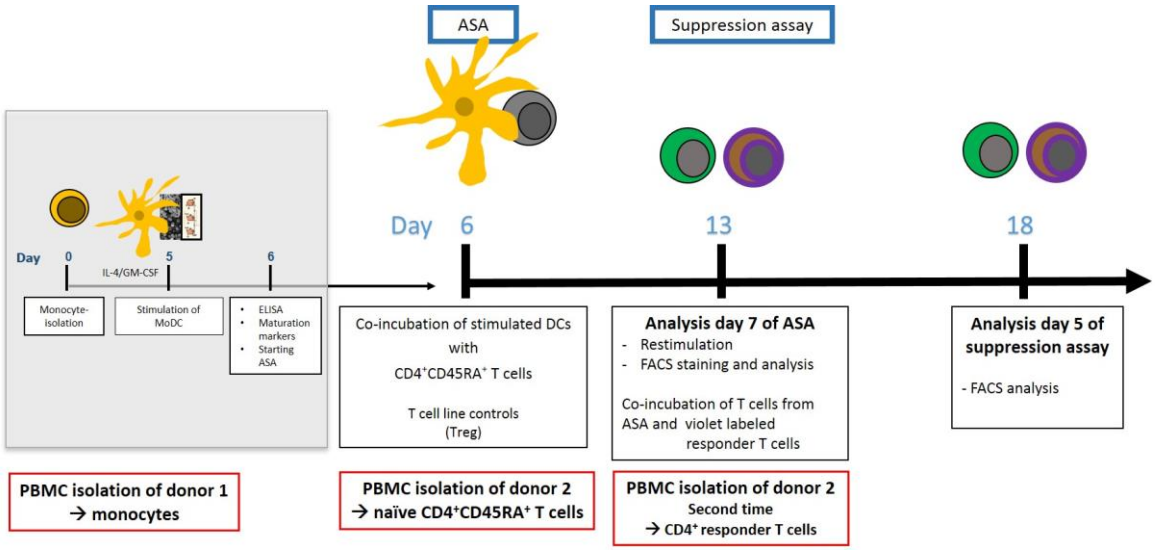


Figure 9. Suppression assay method

III. 1.8. ELISA and luminex-based assay

After 24 hours of incubation of MoDC and LAB, NDO or LPS at 37°C and 5% CO_2, the cytokines IL-10 (BD Biosciences) and IL-12p70 (eBioscience) were analyzed in cell-free supernatant by ELISA according to the manufacturer's protocol. Furthermore, a broader selection of mediators released by MoDC was analyzed with a luminex-based assay conducted by the Multiplex Core Facility Laboratory for Translational Immunology, Department of Pediatric Immunology, University Medical Centre Utrecht.

III. 1.9. FACS analysis

Immature MoDC harvested on day five were >95% pure as analyzed by FACS analysis ($CD14^-$ $CD1a^+$ $HLA\text{-}DR^+$ $CD80^{low}$ $CD83^-$ $CD86^{low}$ $CD40^{low}$). Expression of maturation markers CD80 (FITC-conjugated anti-human, BD), CD83 (PE-conjugated anti-human, eBioscience), CD86 (APC-conjugated anti-human, BD), CD40 (FITC-conjugated anti-human, eBioscience) and HLA-DR (APC-conjugated anti-human, eBioscience) were measured after 24 hours of stimulation of MoDC by flow cytometry. Antibodies were incubated at 4°C for 15 minutes, after two washing steps cells were analyzed by flow cytometry. MoDC were routinely checked for viability by flow cytometry (propidium iodide staining). PI staining is commonly used to detect dead cells as it is a red-fluorescent nuclear and chromosome counterstain that is not permanent to living cells.

After seven days of co-culture, supernatants were taken and cells from ASAs were re-stimulated for two hours with phorbol 12-myristate 13-acetate/ionomycin (both Sigma Aldrich) in the presence of monensin (eBioscience) and for another two hours after adding brefeldin A (BD Biosciences). After two washing steps with cold PBS, dead cells were excluded using the LIVE/DEAD Fixable Aqua dead cell stain kit (Life Technologies) according to the manufacturers' instructions, followed by surface staining of CD4 (APC-Cy7-conjugated anti-human, BD Biosciences) for 30 minutes at 4°C. Cells were then fixed, permeabilized and subsequently stained for intracellular Foxp3 (APC-conjugated anti-human, eBioscience) using a Foxp3 staining kit (eBioscience), involving 30 minutes incubation on a shaker and finally analysis by flow cytometry (FACS Fortessa System, BD FACSDiva 7.0).

III. 1.10. Statistical analysis

Data are presented as mean ± SEM and statistically analyzed using the Mann-Whitney-Test or unpaired student's t test, a $p \leq 0.05$ was considered as statistically significant. Statistical analysis was conducted using GraphPad Prism software (version 6.0).

III. 2. Analysis of the effect of lactic acid bacteria using a human *in vitro* co-culture system of intestinal epithelial cells and peripheral blood mononuclear cells

III. 2.1. Bacteria preparation

The four Lactobacillus strains investigated in this part of the study *L.paracasei 1*, *Lactobacillus helveticus*, *L.rhamnosus* and a second *L. paracasei* (kindly provided by Danone dairy) were grown on MRS agar plates (Bio trading) over night. One colony was picked and grown in MRS broth (Oxoid) over night at 37°C and grown again overnight in 50 ml broth under anaerobe conditions. Concentration determination was conducted by OD measurement at 600 nm and after several washing steps bacteria were stored at -80^{0}C in PBS + 5% glycerol. To prepare bacterial strains for stimulation experiments, at the day of the experiment stocks were thawed, washed with D-PBS w/o (Gibco/Invitrogen) and taken up in the appropriate amount of medium to obtain the requested concentration for stimulation experiments.

III. 2.2. Isolation of peripheral blood mononuclear cells

PBMC were isolated from buffy coats (Sanquin) of healthy donors by density gradient centrifugation (Ficoll-Isopaque, GE Healthcare Life Sciences, Amersham Biosciences). Erythrocytes were removed using lysis buffer (4.14 g NH_4Cl, 0.5 g $KHCO_3$, 18.6 mg Na_2EDTA in 500 ml demi water, pH 7.4). After collecting PBMC and several washing steps with PBS + 2% FCS, purified PBMC were cultured in RPMI 1640 (Lonza) supplemented with 2.5% fetal calf serum (Invitrogen), penicillin (100 U/ml)/streptomycin (100 µg/ml, both Sigma UK) and sodium pyruvate (100 mM, Sigma) at 37°C, 5% CO_2.

III. 2.3. Culturing of intestinal epithelial cells (IEC)

The human colon adenocarcinoma cell line HT-29 (HTB-38, American Type Culture Collection, Manassas, Va., USA, passage n. 145-157) was cultured in McCoy's 5A medium (Invitrogen) supplemented with 10% fetal calf serum (heat-inactivated, Gibco), penicillin (100 U/ml)/streptomycin (100 µg/ml) in 25 cm^2 culture flasks (Greiner) at 37°C, 5% CO_2. Cells were passaged after reaching 70-90% confluence, medium was refreshed every two days. For stimulation experiments cells were seeded one week before the experiment five times diluted into the filter insert of a 12-well transwell plate (Costar #3460, 0.4µm pore size, Corning), medium exchange was conducted every two days.

III. 2.4. Transwell co-cultures

On the day of the experiment freshly isolated PBMC ($2x10^6$/ml in the basolateral compartment of the transwell system in presence or absence of aCD3/aCD28 (both 1:10000, Sanquin) were added to the non-polarized HT-29 cells growing on filter inserts (1 cm^2) in the transwell plate inserts. The IEC were subsequently stimulated apically with either *Lactobacillus helveticus*, *Lactobacillus paracasei* (CNCM I-1518) 1, *L.rhamnosus*, *L. paracasei 2* (all $50x10^6$ cfu/ml) or medium only. Transwell inserts without IEC present, as well as a condition with not stimulated PBMC in the basolateral compartment served as controls. After 24 hours cell-free basolateral supernatants were collected and IEC were washed, transferred to a new plate and incubated with fresh medium to determine cytokine release of IEC basolaterally after another 24 hours. In Figure 10 the experimental setting of the human co-culture model of IEC and immune cells to investigate an influence of selected Lactobacilli on epithelial/immune cell crosstalk is shown.

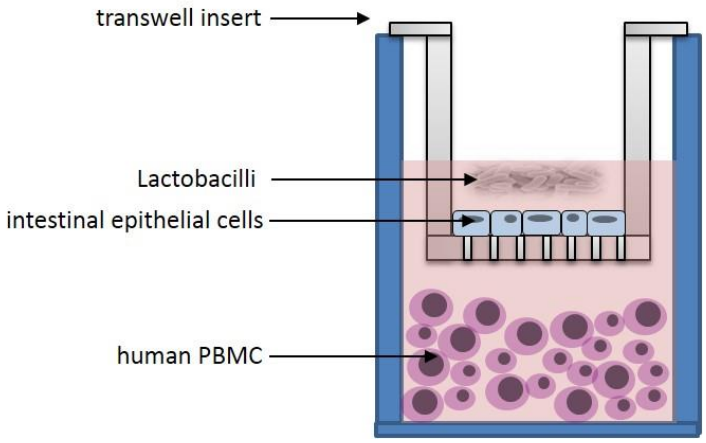


Figure 10. Experimental setting of a human co-culture model of an intestinal epithelial cell line and peripheral blood mononuclear cells. IEC and PBMC are separated by a transwell insert of 0.4 µm pore size. IEC are apically stimulated with lactobacilli.

III. 2.5. ELISA

Cytokines secreted to the basolateral compartment were measured after 24 hours, respectively 48 hours for IEC in cell-free supernatants using the appropriate ELISA kits according to the manufacturer's protocol (IL-10 from U-CyTech biosciences, TNFα and IFNγ both from Biosource, galectin-9 and IL-13 from R&D systems).

III. 2.6. FACS analysis

After taking off the basolateral supernatants, PBMC were restimulated with PMA (phorbol-12-myristate-13-acetate) and ionomycin for four hours at 37°C using Golgiplug (Leucocyte activation cocktail, BD Pharmingen). After four hours of incubation PBMC were harvested with ice-cold PBS. Cells were stained extracellular with CD4-PerCp-Cy5.5 and after fixation and permeabilization intracellular for IL-10-PE and IFNγ-APC or for CD25-AlexaFluor488 extracellular and Foxp3-APC intracellular (regulatory T cells). All antibodies used were purchased from eBioscience. Stained cells were analyzed by fluorescence-activated cell-sorting (FACS Canto).

III. 2.7. Statistical analysis

Data are presented as mean ± SEM and statistically analyzed using one-way ANOVA for repeated measurements with post hoc Dunnett test or paired student's t test. Because of observed high inter-donor variations, repeated measurements were used to analyze the data of 6 donors for FACS analysis and 5-11 donors for cytokines and galectin-9 release of PBMC/IEC. $p \leq 0.05$ was considered as statistically significant. Statistical analysis was conducted using GraphPad Prism software (version 6.0).

IV. Results

IV. 1. Effects of lactic acid bacteria and non-digestible oligosaccharides on human monocyte-derived dendritic cells and the ensuing T cell response

IV. 1.1. Lactic acid bacteria differ in their ability to induce cytokine secretion by MoDC

To investigate the immune-modulatory capacity of different LAB, MoDC of ten healthy volunteers were stimulated with *Bifidobacterium breve* or *Lactobacillus rhamnosus* at a concentration of $1x10^5$, $1x10^6$ or $1x10^7$ cfu/ml with a bacteria-DC ratio of either 100:1, 10:1 or 1:1. Cytokine production (IL-10, IL-12p70) of MoDC was measured by ELISA in cell-free culture supernatant after 24 hours stimulation at 37°C, 5% CO_2. The ability of LAB to induce IL-10 secretion by MoDC was comparable (Fig. 11A), while differences in the induction of IL-12p70 were determined (Fig. 11B). In contrast to *L.rhamnosus, B.breve* induced IL-10 release in the absence of IL-12p70 (Figure 11A, B).

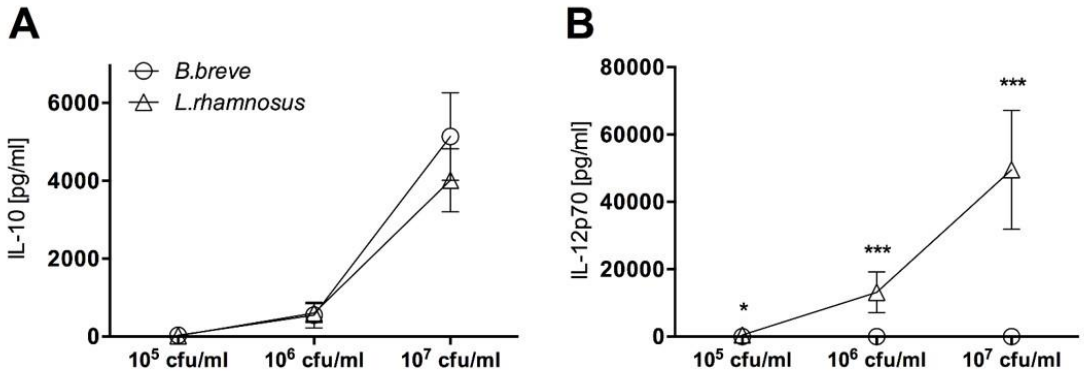


Figure 11. *B.breve* and *L.rhamnosus* differ in their capacity to induce IL-12p70 secretion by human MoDC while IL-10 release is induced in a comparable and dose dependent manner. Immature human MoDC were stimulated with *B.breve* (dot) or *L.rhamnosus* (triangle) in different concentrations ($1x10^5$-$1x10^7$cfu/ml) for 24h. Amounts of IL-10 (A) and IL-12p70 (B) were measured by ELISA in cell-free supernatant. Results are presented as mean ± SEM, ten independent experiments are shown. * $p<0.05$, *** $p<0.001$, Mann Whitney test. LAB=Lactic acid bacteria, MoDC=monocyte-derived dendritic cells.

IV. 1.2. scGOS/lcFOS induce IL-10 secretion of MoDC in the absence of IL-12p70

To test whether scGOS/lcFOS itself can induce cytokine release by MoDC, immature human MoDC were stimulated with scGOS/lcFOS at a concentration of 5 mg/ml. Incubation of MoDC with oligosaccharides induced release of IL-10 (Fig. 12A), while no IL-12p70 release could be detected (Fig. 12B). LPS-stimulated MoDC released both IL-10 and IL-12p70 (Figure 12A, B).

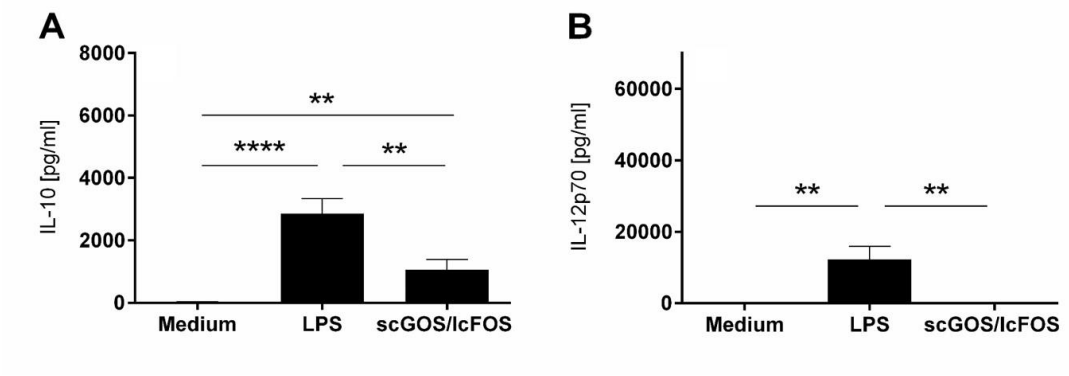


Figure 12. MoDC primed with scGOS/lcFOS are characterized by release of IL-10 while no IL-12p70 secretion is detectable. Immature human MoDC were incubated with scGOS/lcFOS (5 mg/ml) for 24h. Medium-treated MoDC served as negative control, MoDC matured by LPS as positive control for functional MoDC stimulation. Supernatants were analyzed for secretion of IL-10 (A) and IL-12p70 (B) by ELISA. Results are presented as mean ± SEM, ten independent experiments are shown. **** $p<0.0001$, ** $p<0.01$, unpaired student's t test. scGOS/lcFOS=short chain galacto-, long chain fructo-oligosaccharides, MoDC=monocyte-derived dendritic cells.

VI. 1.3. Oligosaccharides induce a lower grade of maturation of MoDC compared to LPS

To investigate if NDO induce a different maturation pattern, MoDC were phenotypically analyzed after incubation with either medium, LPS or oligosaccharides using flow cytometry. Compared to medium, LPS induced maturation as measured by increased mean fluorescence intensity (MFI) specific for maturation markers CD80, CD83 and CD86. In contrast, treatment with oligosaccharides only slightly upregulated maturation markers (Fig. 13A-C). Concerning CD40 and HLA-DR expression no differences between medium, LPS or oligosaccharides-stimulated MoDC could be determined

(Fig. 13D, E). The viability of MoDC analyzed by PI staining was not affected by LPS- or scGOS/lcFOS-stimulation (Fig. 13F).

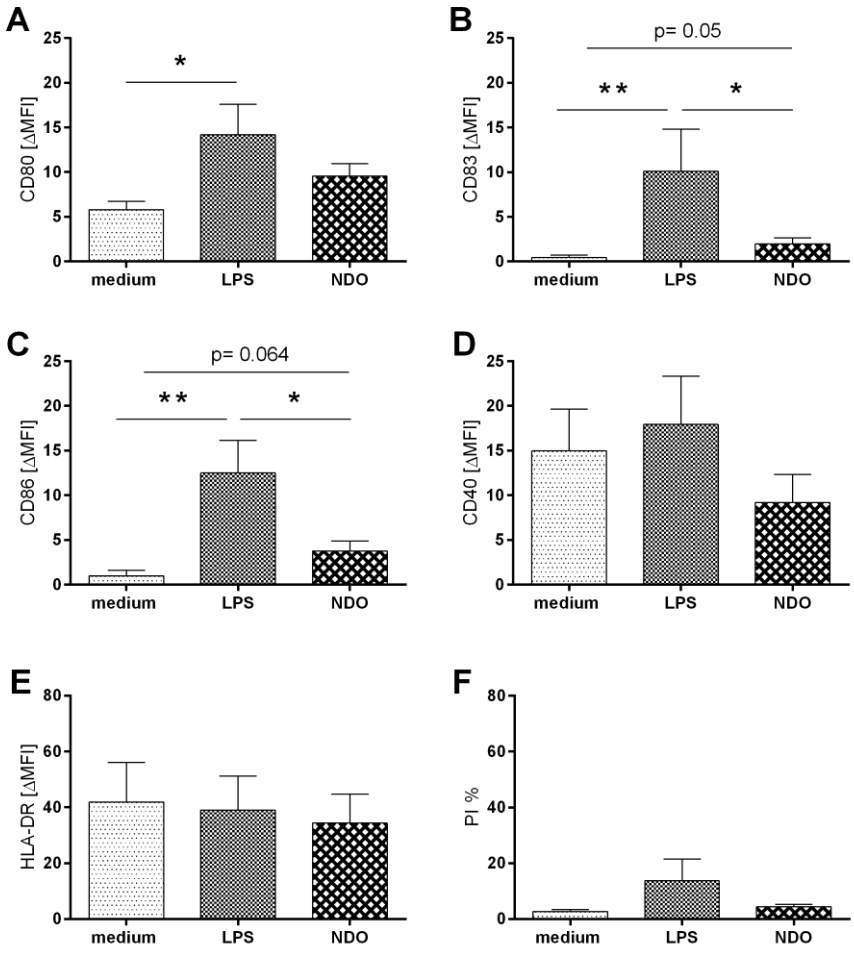


Figure 13. Lower induction of maturation markers CD80, CD83 and CD86 by oligosaccharides compared to LPS. MoDC were stimulated with oligosaccharides (5 mg/ml), LPS or medium for 24h. MoDC matured by LPS served as positive control for functional stimulation. The mean fluorescence intensities (MFI) specific for maturation markers CD80 (A), CD83 (B), CD86 (C), CD40 (D) and HLA-DR (E) as well as cell viability (F; PI=propidium iodide staining) were determined by flow cytometry. Three to five independent experiments are shown. * $p<0.05$, ** $p<0.01$, Mann Whitney test. LPS=lipopolysaccharide, NDO=non-digestible oligosaccharides.

IV. 1.4. scGOS/lcFOS enhance LAB-induced IL-10 release

To examine whether the presence of scGOS/lcFOS affect LAB-induced MoDC cytokine release, MoDC were stimulated with either *B.breve* or *L.rhamnosus* in the absence or presence of scGOS/lcFOS. The presence of scGOS/lcFOS significantly enhanced IL-10

release for both strains (Fig. 14A, C) while IL-12p70 release was not affected (Fig. 14B, D). For all tested bacterial strains a raise in IL-10 release after addition of scGOS/lcFOS could be observed mainly at the lowest bacteria concentration applied (bacteria-DC ratio 1:1, Fig. 14A, C).

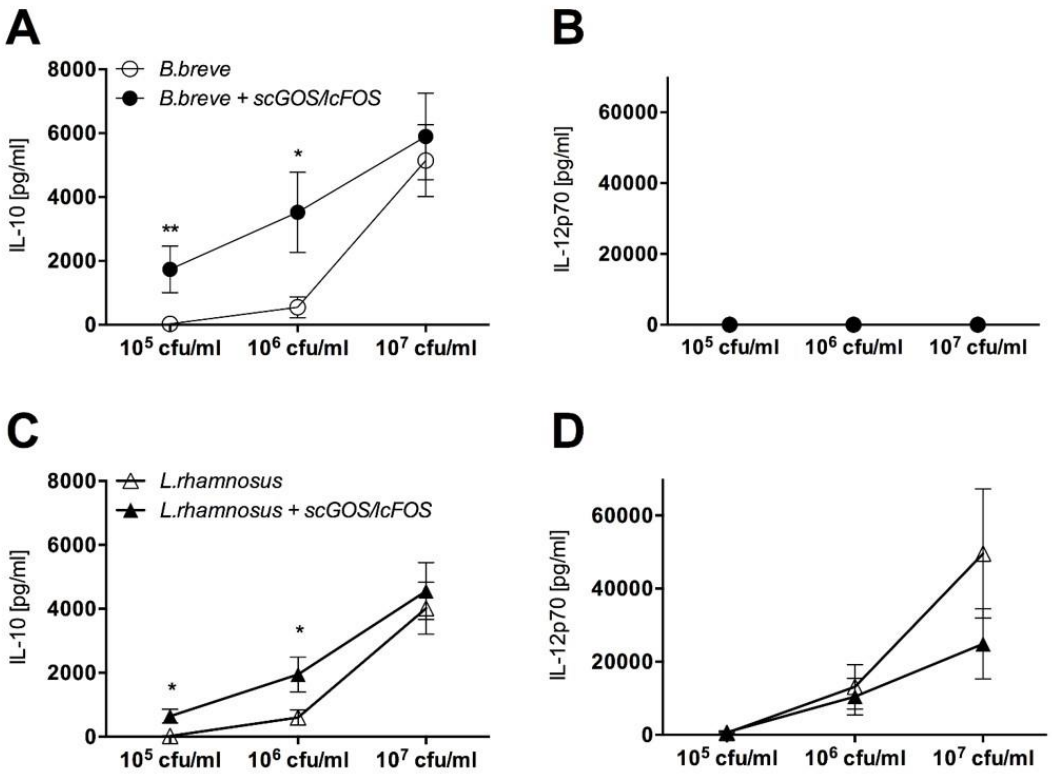


Figure 14. LAB-primed MoDC stimulated with scGOS/lcFOS are characterized by an enhanced release of IL-10 while no enhanced IL-12p70 secretion is detectable. Immature human MoDC were incubated with *B.breve* (clear dots, A, B) or *L.rhamnosus* (clear triangle, C, D) in different concentrations with (black marks) or without (clear marks) scGOS/lcFOS (5 mg/ml) for 24h. Supernatants were analyzed for secretion of IL-10 (A, C) and IL-12p70 (B, D) by ELISA. Results are presented as mean ± SEM, ten independent experiments are shown. * $p<0.05$, ** $p<0.01$, Mann Whitney test. LAB=Lactic acid bacteria, MoDC=monocyte-derived dendritic cells.

To investigate the observed differential effect on cytokine release in more detail, a larger selection of MoDC-derived soluble mediators was analyzed using a luminex-based assay. scGOS/lcFOS was found to enhance mediator release depending both on the analyte measured and the bacterial strain used, suggesting strain-dependent effects (Figure 15A-D). Stimulation of MoDC with bacteria induced cytokine and chemokine release in a dose-dependent manner (Figure 15A, B), addition of scGOS/lcFOS

enhanced the secretion of various mediators by MoDC mainly at low concentrations of LAB applied (Figure 15C, D).

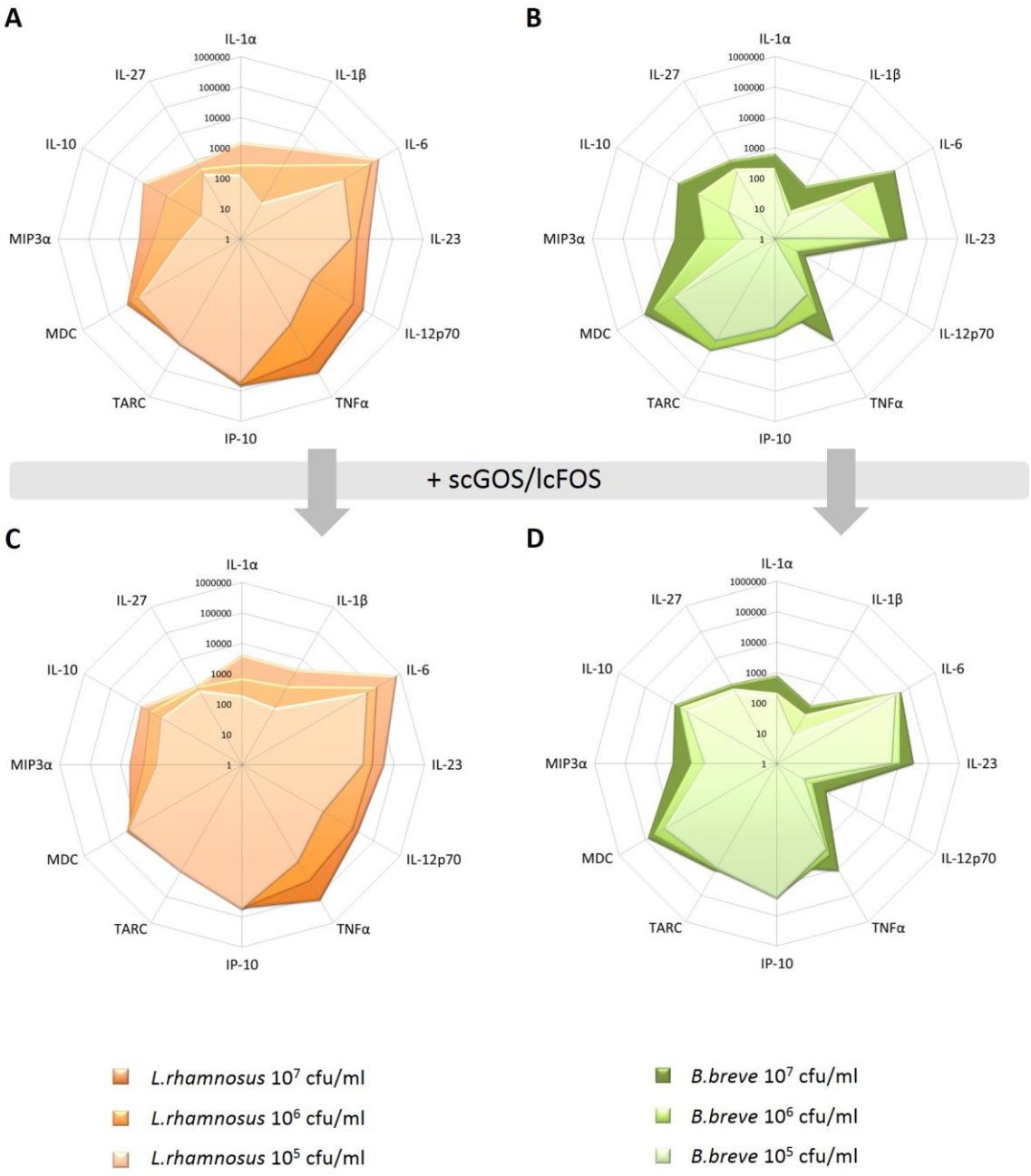


Figure 15. scGOS/lcFOS enhance cytokine and chemokine release by LAB-stimulated MoDC. Immature MoDC were stimulated with *L.rhamnosus* (A, C) or *B.breve* (B, D) in different concentrations ($1x10^5$-$1x10^7$ cfu/ml) in the absence (A, B) or presence (C, D) of scGOS/lcFOS (5 mg/ml) for 24h. The amount of cytokines and chemokines in cell-free supernatants was measured by luminex-based assay (readout: IL-1α, IL-1β, IL-6, IL-23p19, IL-27, TNFα, IP-10 (CXCL10), MDC (CCL22), TARC (CCL17), MIP3α (CCL20) and ELISA (IL-10, IL-12p70)). Results are presented as mean of three independent experiments. Results are shown in pg/ml. LAB=Lactic acid bacteria, MoDC=monocyte-derived dendritic cells.

The observation that, in contrast to LPS, scGOS/lcFOS stimulated MoDC released IL-10 in the absence of IL-12 as analyzed by ELISA, raised the question whether other soluble mediators were also differentially affected. Upon comparing the LPS- with the scGOS/lcFOS-induced MoDC profile of mediator release measured by luminex-based assay, several differences could be observed depending on the secreted mediator. Especially concerning the pro-inflammatory cytokines IL-12 and TNFα a different pattern of cytokine/chemokine secretion was revealed (Fig. 16).

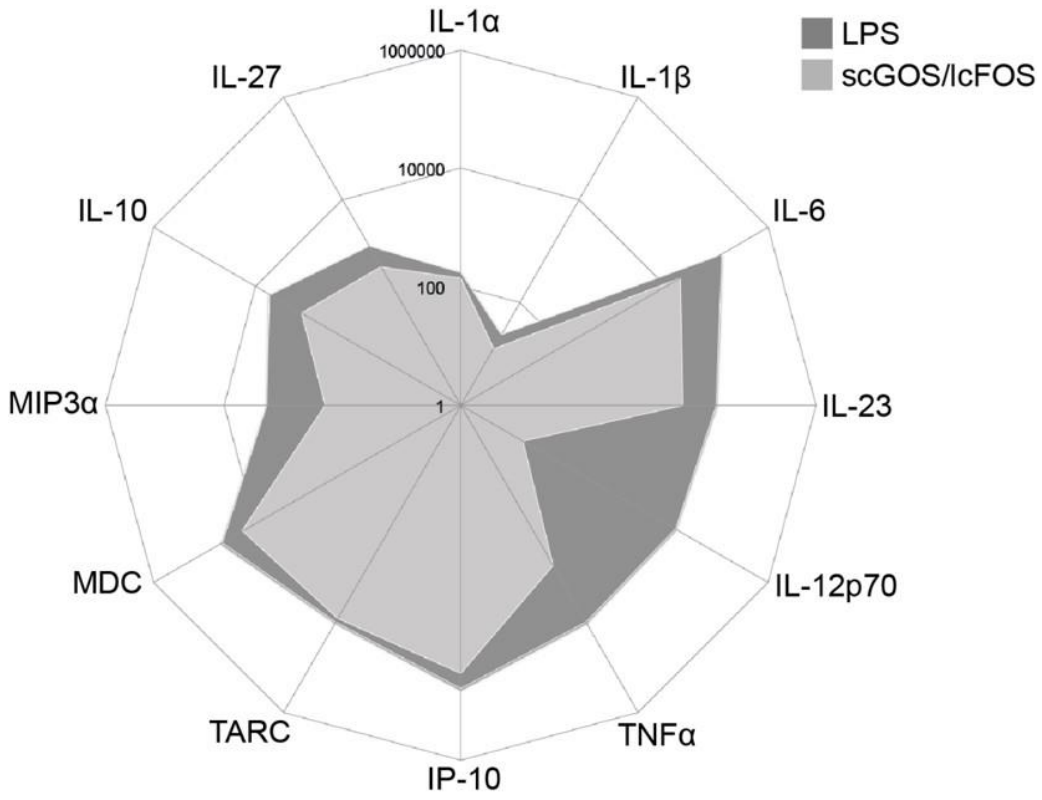


Figure 16. scGOS/lcFOS-stimulated MoDC release cytokines and chemokines in a different manner compared to LPS. MoDC were stimulated with scGOS/lcFOS (5 mg/ml) for 24h. MoDC matured by LPS served as positive control for functional stimulation. The amount of cytokines and chemokines in cell-free supernatants was measured by luminex-based assay (readout: IL-1α, IL-1β, IL-6, IL-23p19, IL-27, TNFα, IP-10 (CXCL10), MDC (CCL22), TARC (CCL17), MIP3α (CCL20) and ELISA (IL-10, IL-12p70)). Results are presented in pg/ml. Three independent experiments are shown.

IV. 1.5. scGOS/lcFOS dose-dependently activate TLR4

NDO were previously shown to stimulate TLR4 (Ortega-Gonzalez 2014). To analyze the potential involvement of TLR4 in the MoDC cytokine release following scGOS/lcFOS stimulation, dose-response experiments in the presence and absence of a TLR4 antagonist were conducted. Stimulating MoDC with scGOS/lcFOS in the presence of a TLR4 antagonist completely abrogated IL-10 secretion by MoDC (Fig. 17).

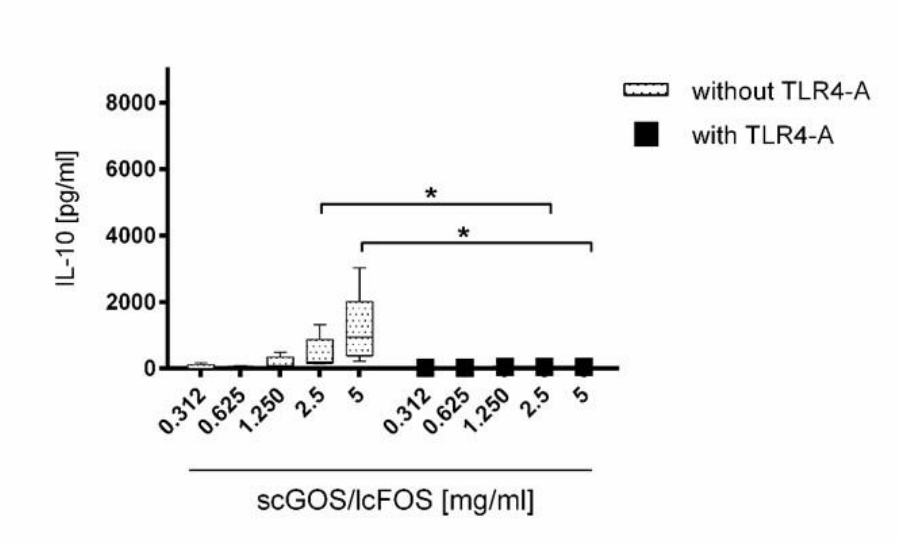


Figure 17. Stimulation with scGOS/lcFOS directly induces a dose dependent IL-10 release by MoDC that is impaired by addition of a TLR4 antagonist. Immature human MoDC were exposed to oligosaccharide mixture scGOS/lcFOS (312 µg/ml - 5 mg/ml) for 24h in the presence (black marks) or absence (grey marks) of a TLR4 antagonist. The amount of IL-10 was measured by ELISA in cell-free supernatant. Results are presented as mean ± SEM, five independent experiments are shown. * $p<0.05$, Mann Whitney test.

Additionally, scGOS/lcFOS dose-dependently increased *B.breve*-induced IL-10 release of MoDC. Blocking of TLR4-activity abrogated this enhancing effect (Fig. 18). Controls for these experiments see Figure 19.

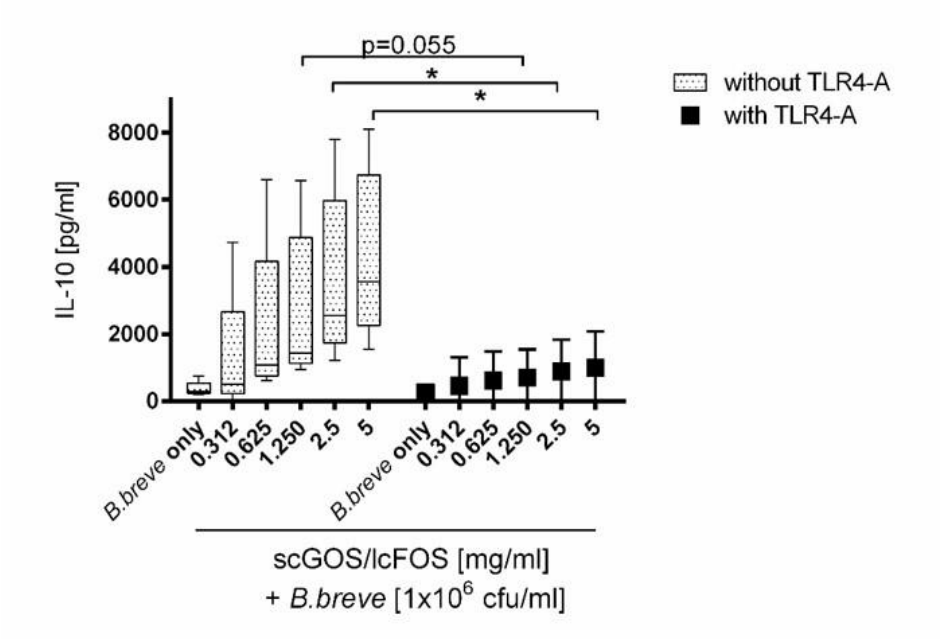


Figure 18. Stimulation with scGOS/lcFOS directly induces a dose dependent enhanced IL-10 release by LAB-primed MoDC that is impaired by addition of a TLR4 antagonist. Immature human MoDC were exposed to oligosaccharide mixture scGOS/lcFOS (312 µg/ml - 5 mg/ml) in the presence of *B.breve* at a concentration of 1x10^{6}cfu/ml in the presence (black marks) or absence (grey marks) of a TLR4 antagonist. The amount of IL-10 was measured by ELISA in cell-free supernatant after 24 hours. Results are presented as mean ± SEM, five independent experiments are shown. * p<0.05, Mann Whitney test.

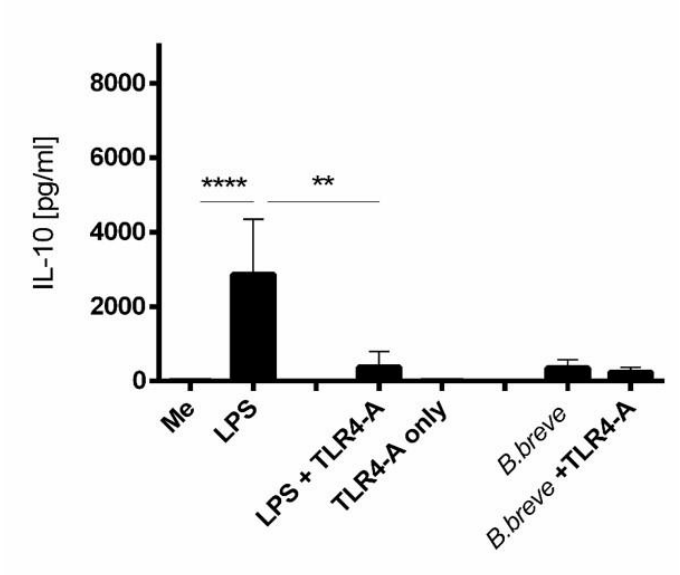


Figure 19. Effect of *B.breve* on MoDC cytokine release is not affected in the presence of a TLR4 antagonist. Immature human MoDC were incubated with *B.breve* (1x10^{6}cfu/ml) or LPS (100 ng/ml) in the presence or absence of a TLR4 antagonist (LPS-RS) for 24h. Medium- and TLR4 only-treated MoDC served as negative control, MoDC matured by LPS as positive control for functional MoDC stimulation. The amount of IL-10 was measured by ELISA in cell-free supernatant after 24 hours. Results are presented as mean ± SEM, five independent experiments are shown. . **** p<0.0001, ** p<0.01, unpaired student's t test.

IV. 1.6. scGOS/lcFOS-stimulated MoDC induce CD4$^+$Foxp3$^+$ T cells

DC release of IL-10 is associated with the induction of Tregs (Boks 2012). Therefore it was investigated whether the IL-10 release following stimulation of MoDC with scGOS/lcFOS would lead to Foxp3 expression which is a phenotypical characteristic of Tregs. MoDC treated with scGOS/lcFOS co-cultured with purified naïve T cells in allogeneic stimulation assays. Compared to control MoDC (medium-treated MoDC Fig. 20A, positive Treg control Fig. 20B), scGOS/lcFOS-stimulated DC induced an upward shift in Foxp3 expressing T cells (Fig. 20C). In Figure 21 the gating strategy by means of FMO (fluorescence minus one) control for these experiments is shown.

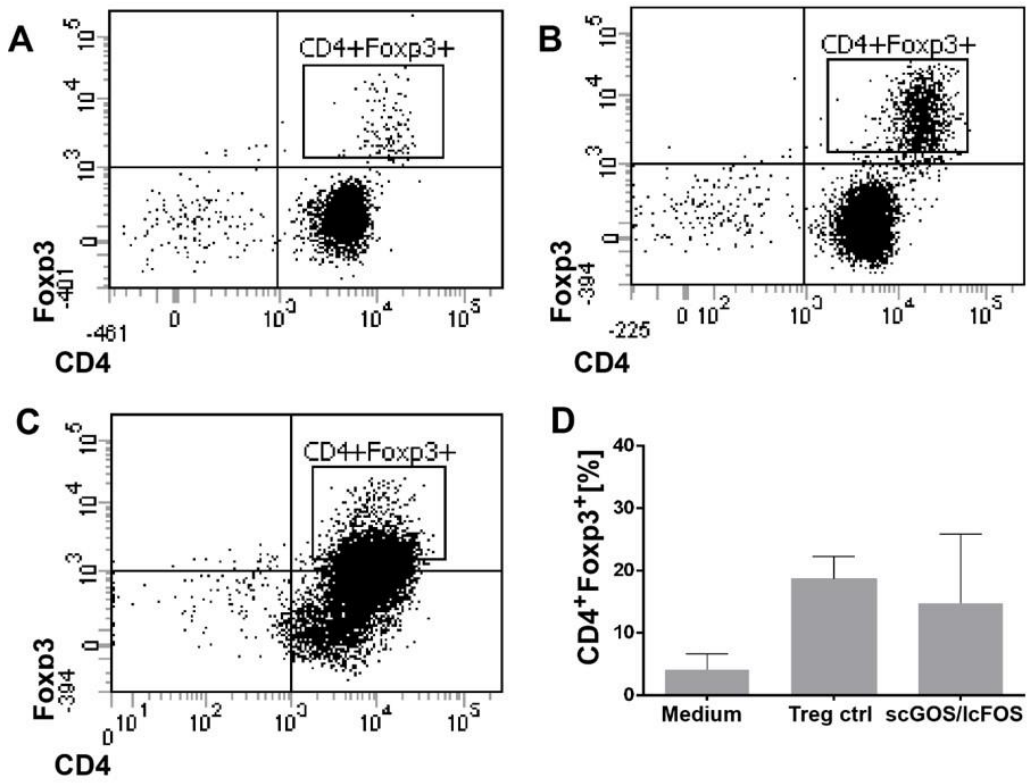


Figure 20. scGOS/lcFOS stimulated MoDC induce CD4$^+$Foxp3$^+$ T cells. MoDC stimulated with scGOS/lcFOS (5 mg/ml) were used to prime naïve CD4$^+$CD45RA$^+$ T cells in an ASA. At day 7, cells were stained for CD4 and intracellular Foxp3 was analyzed by flow cytometry. The dot plots show data from one representative experiment out of three (A-C). T cells that have been co-incubated with medium-treated MoDC served as medium/negative control (A) and a positive control for Treg polarization is shown in (B). CD4$^+$Foxp3$^+$ T cells induced by scGOS/lcFOS stimulated MoDC are shown in (C). Data in (D) are expressed as percentages of CD4$^+$Foxp3$^+$ cells. Results are presented as mean ± SEM, 3 independent experiments are shown (D). ASA=allogeneic stimulation assay, scGOS/lcFOS=short chain galacto-, long chain fructo-oligosaccharides, MoDC=monocyte-derived dendritic cells.

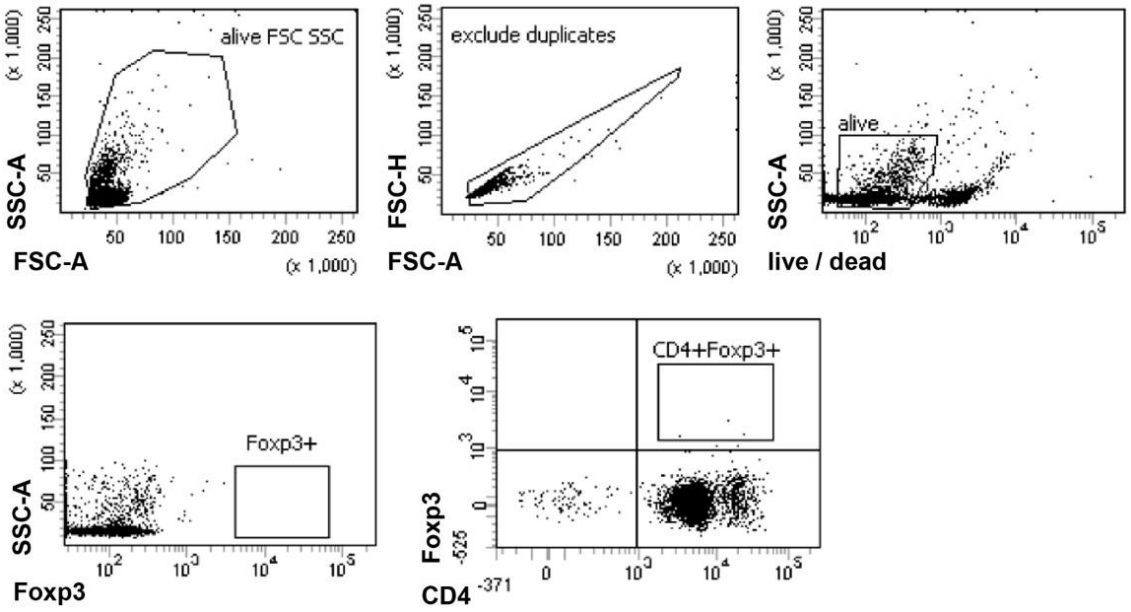


Figure 21. Gating strategy by means of FMO control. MoDC stimulated with scGOS/lcFOS (5 mg/ml) were used to prime naïve $CD4^+CD45RA^+$ T cells in an ASA. At day 7, cells were stained for CD4 but not intracellular Foxp3 and analyzed by flow cytometry. The gating strategy (with FMO control) is shown which was used to obtain percentages of $CD4^+Foxp3^+$ cells shown in Figure 20. $CD4^+Foxp3^+$ cells were derived out the gate of alive cells after excluding duplicates. scGOS/lcFOS=short chain galacto-, long chain fructo-oligosaccharides, ASA=allogeneic stimulation assay, FMO=fluorescence minus one, MoDC=monocyte-derived dendritic cells.

IV. 1.7. T cells induced by scGOS/lcFOS are functional suppressive

To examine whether $Foxp3^+$ T cells differentiated by scGOS/lcFOS-matured MoDC are functional suppressive, these cells were co-incubated with violet-track-labeled $CD4^+$ responder T cells for five days in a suppression assay. The mix of ASA cells and violet stained responder $CD4^+$ T cells was then stained with CD4 APC-Cy7 and violet-positive cells were acquired by FACS. Suppressive capacity was determined by setting gates on proliferated and non-proliferated cells and analyzing the ratio of these cell populations. Analysis of the suppressive function of T cells induced in ASA revealed a suppressive capacity of $CD4^+Foxp3^+$ T cells induced by scGOS/lcFOS-treated MoDC as they inhibited the proliferation of $CD4^+$ responder T cells compared to T cells induced by medium- or LPS-treated MoDC (Figure 22).

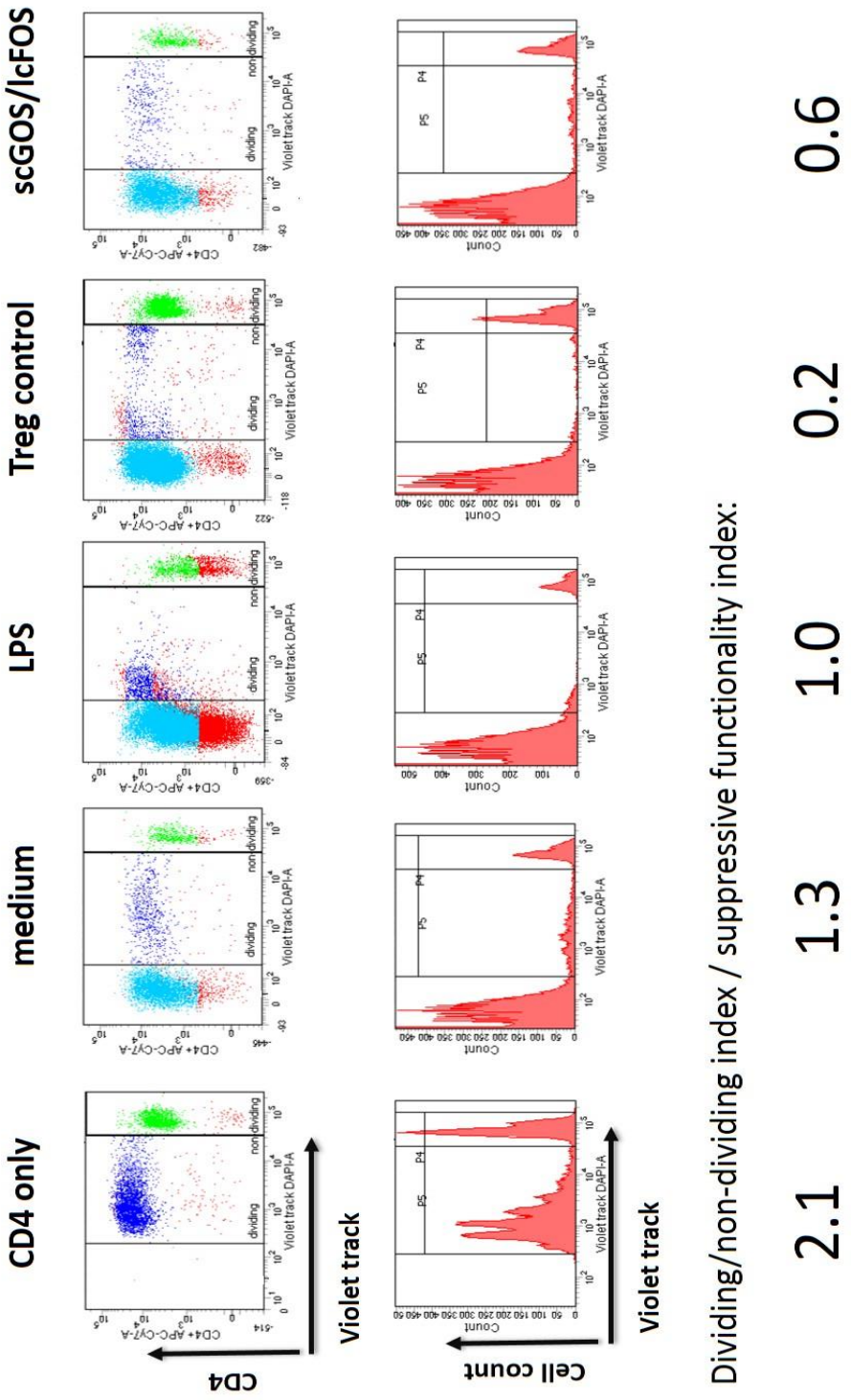


Figure 22. Suppressive capacity of T cells induced by scGOS/lcFOS-treated MoDC. T cells of the allogenic stimulation assays (ASA) were harvested on day seven and co-incubated with freshly isolated, violet-labeled responder $CD4^+$ T cells a ratio of 1:1 for five days in presence of anti-CD3/28 and rhIL-2. The mix of ASA cells and violet stained responder $CD4^+$ T cells was then stained (after five days of co-incubation) with CD4 APC-Cy7. Violet-labeled-$CD4^+$ responder T cells alone served as negative control, T cells from the Treg control of the ASA as positive control for suppression. Proliferation of violet-positive cells was analyzed by flow cytometry and suppressive capacity was determined by dividing dividing cells/ non-dividing cells.

IV. 2. Effects of lactic acid bacteria in a human *in vitro* co-culture system of intestinal epithelial cells and peripheral blood mononuclear cells

Results of this part of the thesis were obtained working at UIPS, Utrecht University as part of a study of the IRT Bioaster consortium.

IV. 2.1. LAB enhance TNFα secretion by PBMC, no evident effect on IFNγ release

To investigate an influence of the tested LAB on IEC and following immune responses, HT-29 cells co-cultured with PBMC in the basolateral part of the previously described human co-culture system were apically stimulated with the four LAB and release of pro-inflammatory TNFα and IFNγ was determined. TNFα secretion by PBMC in the basolateral compartment was significantly enhanced after co-culture with IEC stimulated with *L.rhamnosus* or *L.paracasei 2* (Fig. 23A, B). For *L.paracasei 2* a trend ($p=0.10$) of enhanced cytokine release after stimulation could be observed. Presence (Fig. 23A) or absence (Fig. 23B) of IEC had no influence on TNFα release by PBMC. No effect of LAB on IFNγ release by PBMC after co-culture with IEC could be observed compared to the medium control (Fig. 24A, B).

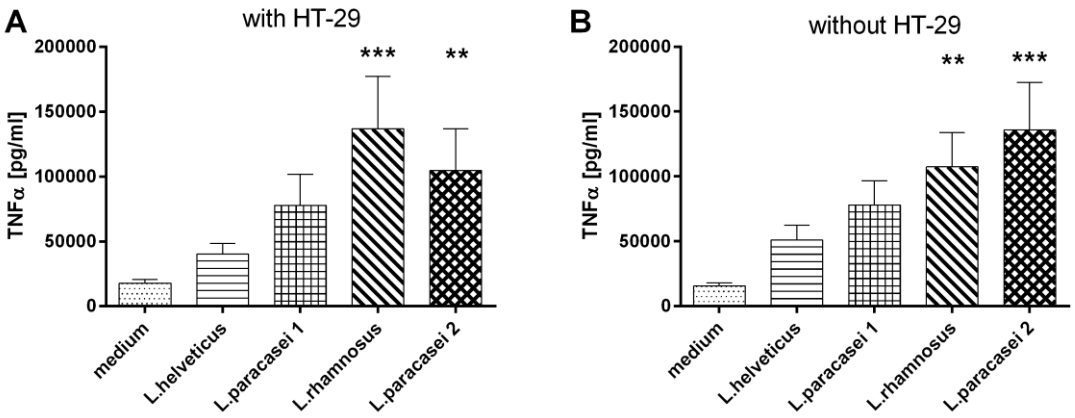


Figure 23. LAB differently induce enhanced TNFα release by PBMC in absence and presence of IEC. Human IEC were seeded into transwell-filter inserts and co-cultured with aCD3/aCD28 stimulated PBMC. Supernatants of PBMC in the basolateral compartment were generated in the presence (A) or absence (B) of apically stimulated IEC (HT-29 cells). IEC were stimulated with four different LAB or medium only (control) for 24 hours. Amount of TNFα was measured by ELISA in cell-free supernatant of the basolateral compartment. Results are presented as mean ± SEM, *** p<0.001, ** p<0.01, one-way ANOVA. LAB=Lactic acid bacteria, PBMC=peripheral blood mononuclear cells, IEC=intestinal epithelial cells.

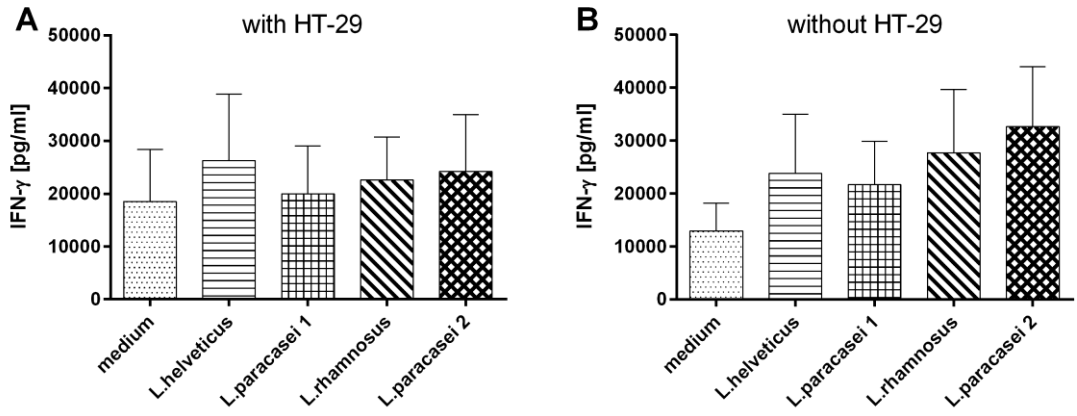


Figure 24. No effect of LAB on IFNγ release by PBMC. Human IEC were seeded into transwell-filter inserts and co-cultured with aCD3/aCD28 stimulated PBMC. Supernatants of PBMC in the basolateral compartment were generated in the presence (A) or absence (B) of apically stimulated IEC (HT-29 cells). IEC were stimulated with four different LAB or medium only (control) for 24 hours. Amount of IFNγ was measured by ELISA in cell-free supernatant of the basolateral compartment. Results are presented as mean ± SEM. LAB=Lactic acid bacteria, PBMC=peripheral blood mononuclear cells, IEC=intestinal epithelial cells.

IV. 2.2. Enhanced IL-10 release of PBMC induced by Lactobacilli in the presence of IEC

To analyze a potential immune-regulatory impact of LAB on IEC-PBMC co-cultures, HT-29 cells were stimulated apically with four LAB and IL-10 release of PBMC in the basolateral compartment was measured by ELISA. A significant increase in anti-inflammatory IL-10 production of PBMC could be measured concerning *L.rhamnosus* and the two *L.paracasei* strains in presence of IEC. For *L.helveticus*-stimulated IEC a trend (p=0.06) of enhanced IL-10 release by PBMC compared to medium control was observed (Fig. 25A). In absence of IEC these observed effects disappeared (Fig. 25B). An overall higher IL-10 release by PBMC could be determined in absence of IEC upon comparison of medium controls in absence and presence of IEC (Fig. 25A, B).

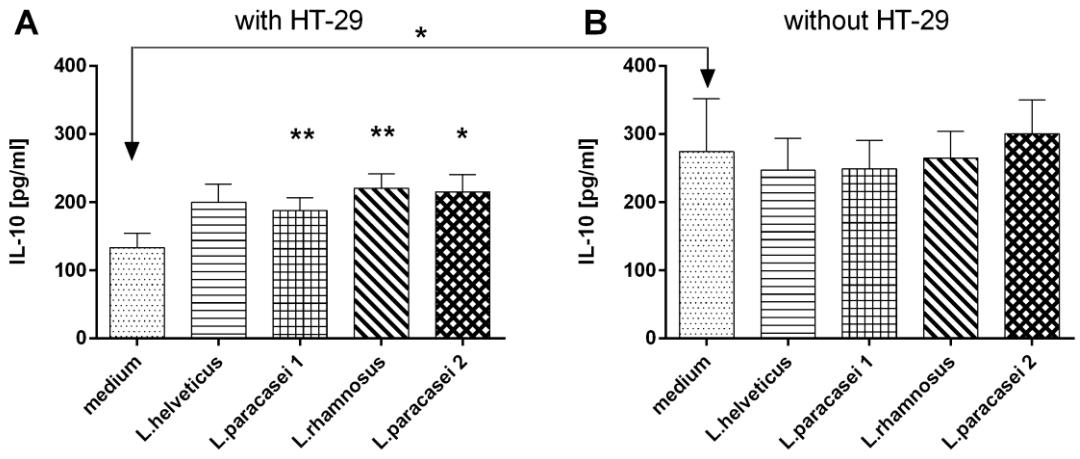


Figure 25. LAB induce enhanced IL-10 secretion by human PBMC in the presence, but not in absence of IEC. Human IEC were seeded into transwell-filter inserts and co-cultured with aCD3/aCD28 stimulated PBMC. Supernatants of PBMC in the basolateral compartment were generated in the presence (A) or absence (B) of apically stimulated IEC (HT-29 cells). IEC were stimulated with four different LAB or medium only (control) for 24 hours. Amount of IL-10 was measured by ELISA in cell-free supernatant of the basolateral compartment. Results are presented as mean ± SEM, ** $p<0.01$, * $p<0.05$, one-way ANOVA and paired student's t test. LAB=Lactic acid bacteria, PBMC=peripheral blood mononuclear cells, IEC=intestinal epithelial cells.

IV. 2.3. LAB lower IL-13 cytokine secretion of PBMC

To further investigate a possible influence of LAB in the described co-culture system, Th2 associated IL-13 release of PBMC was analyzed after stimulation of HT-29 cells with LAB. Although no statistical significance was reached compared to the medium control, a trend of dampened IL-13 release by PBMC after stimulation of IEC with *L.rhamnosus* (p=0.06) could be observed (Figure 26).

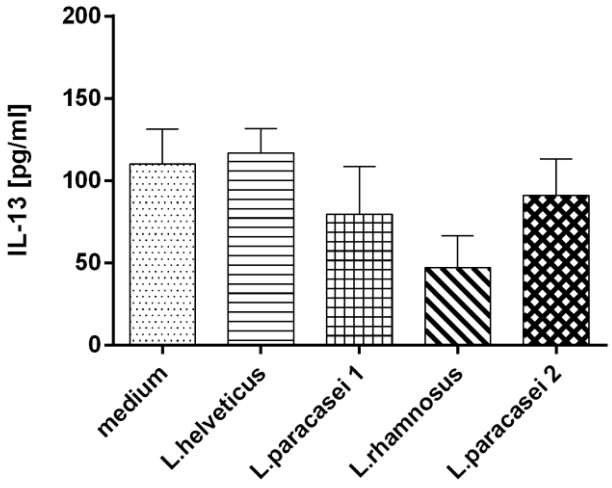


Figure 26. No evident effect of LAB on IL-13 secretion by human PBMC. Human IEC were seeded into transwell-filter inserts and co-cultured with aCD3/aCD28 stimulated PBMC. Supernatants of PBMC in the basolateral compartment were generated in the presence of apically stimulated IEC (HT-29 cells). IEC were stimulated with four different LAB or medium only (control) for 24 hours. Amount of IL-13 was measured by ELISA in cell-free supernatant of the basolateral compartment. Results are presented as mean ± SEM, one-way ANOVA. LAB=Lactic acid bacteria, PBMC=peripheral blood mononuclear cells, IEC=intestinal epithelial cells.

IV. 2.4. LAB enhance galectin-9 release of IEC

Galectin-9 release of IEC has been shown to play a role in polarization of T cells in direction of regulatory T cells (de Kivit 2012). Therefore galectin-9 release of HT-29 cells after 24 hours stimulation with LAB, washing and again 24 hours incubation in fresh medium was measured. A trend of enhanced galectin-9 release by IEC after LAB stimulation could be observed (Figure 27). Concerning one Lactobacillus strain (*L.paracasei 2*) a significant enhancement of galectin-9 release by HT-29 cells after 48 hours was measured.

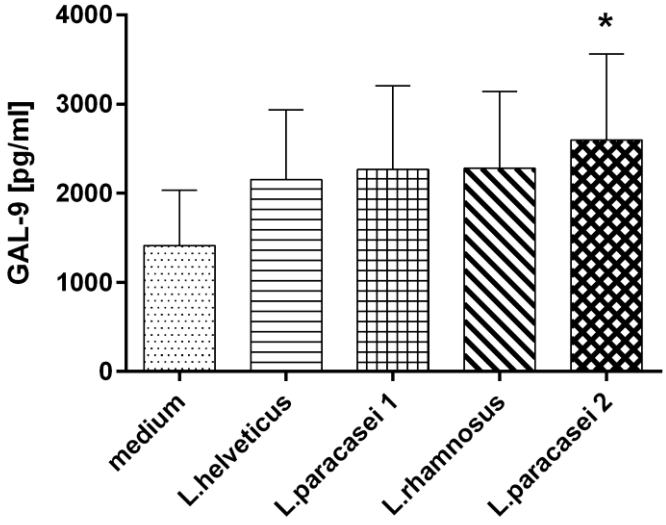


Figure 27. Enhanced induction of IEC derived galectin-9 by LAB. Human IEC were seeded into transwell-filter inserts and co-cultured with aCD3/aCD28 stimulated PBMC. IEC were stimulated apically with four different LAB or medium only (control) for 24 hours. After 24 hours IEC were washed and transferred to a new plate with fresh medium to determine galectin-9 cytokine release of IEC basolaterally by ELISA after another 24 hours of incubation. Results are presented as mean ± SEM, * $p<0.05$, one-way ANOVA. LAB=Lactic acid bacteria, PBMC=peripheral blood mononuclear cells, IEC=intestinal epithelial cells.

IV. 2.5. No significant effect of LAB-stimulated IEC on intracellular expression of IFNγ, IL10 and Foxp3 in $CD4^+$ cells

Although no effect of LAB or IEC on IFNγ release by PBMC could be observed, intracellular cytokine expression of $CD4^+$ T cells was investigated. Intracellular staining of PBMC after co-incubation with LAB-stimulated HT-29 cells showed no evident effect concerning IFNγ expression in PBMC (Fig. 28A, B).

An intracellular T cell staining for IL-10 was conducted to determine from which cell type among the PBMC the IL-10 measured in the supernatant could be derived. A trend for an enhancing effect of LAB, especially *L.helveticus* ($p=0.100$) on IL-10 expression by $CD4^+$ PBMC compared to medium control could be observed (Fig. 28C). No effect of LAB on IL-10 expression of T cells could be observed compared to the medium control without IEC present (Fig. 28D).

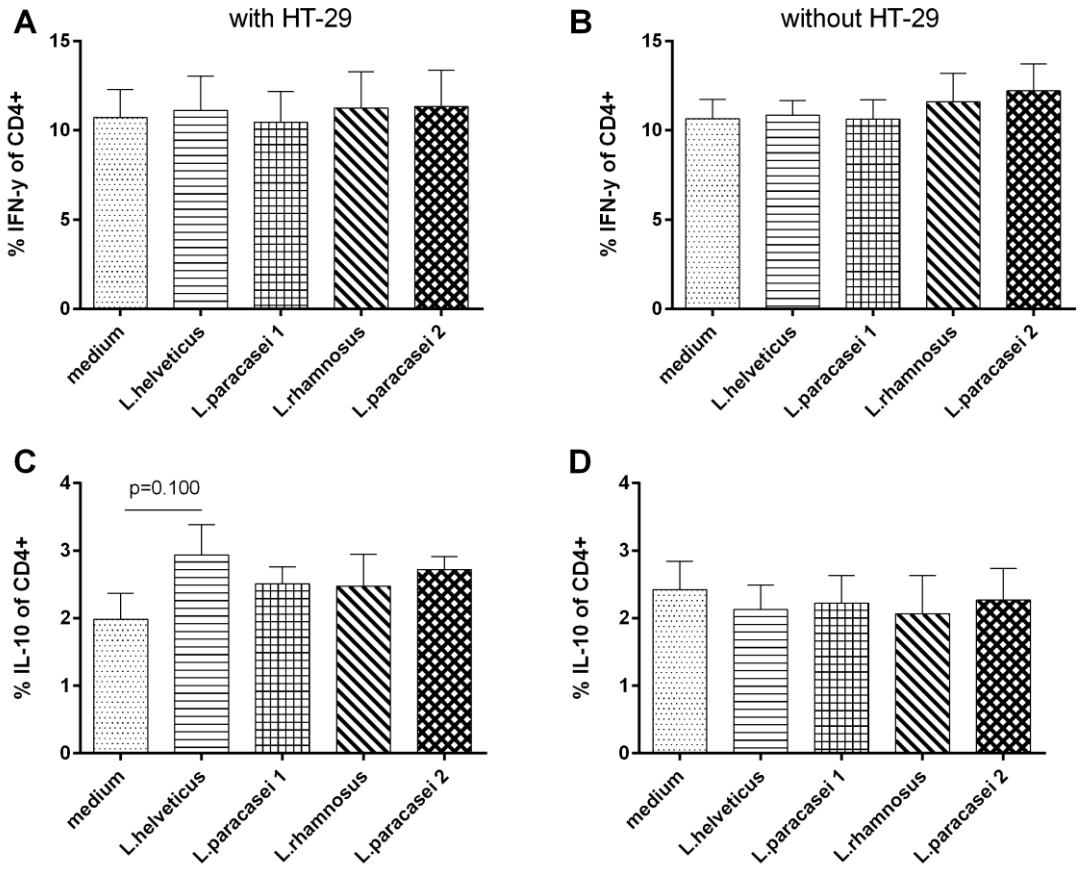


Figure 28. No effect of IEC or LAB on intracellular IFNγ and IL-10 expression of CD4⁺ T cells. Human IEC were seeded into transwell-filter inserts and co-cultured with aCD3/aCD28 stimulated PBMC. IEC were stimulated apically with four different LAB or medium only (control) for 24 hours. After 24 hours PBMC were harvested after co-incubation in either presence (A, C) or absence (B, D) of IEC and after restimulation stained extracellular for CD4 and intracellular for IFNγ (A, B) or IL-10 (C, D). Results are presented as mean ± SEM, one-way ANOVA.

Release of IL-10 and galectin-9 have been associated with the induction of Tregs (Boks 2012, de Kivit 2012). Therefore intracellular forkhead box protein 3 (Foxp3)-expression of $CD4^+CD25^+$ T cells was investigated. Foxp3 analysis showed no significant difference in induction by LAB compared to medium control (Fig. 29A, B). In absence of IEC however there was an overall higher percentage of $CD4^+CD25^+Foxp3^+$ cells observed upon comparison of medium controls with or without IEC present (Fig. 29B).

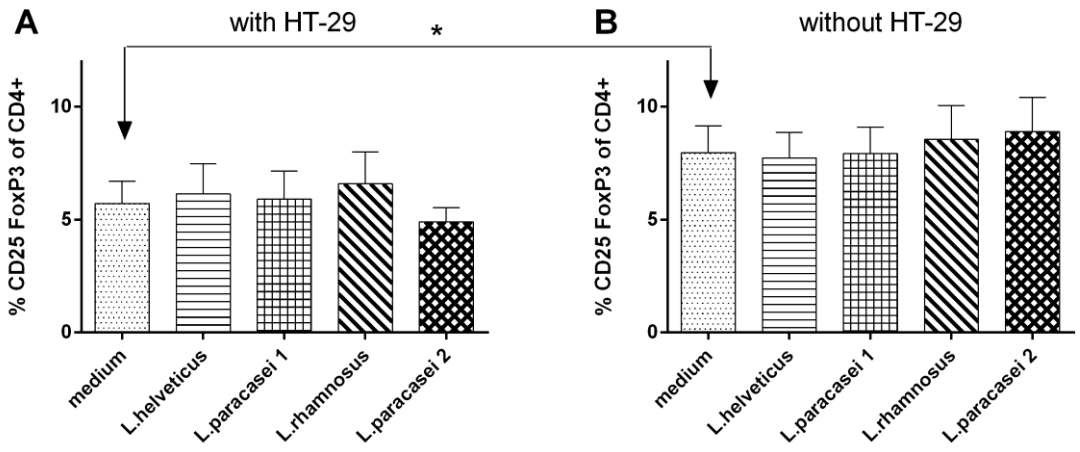


Figure 29. No difference in induction of Foxp3 expression by LAB, but higher percentage in absence of IEC. Human IEC were seeded into transwell-filter inserts and co-cultured with aCD3/aCD28 stimulated PBMC. IEC were stimulated apically with four different LAB or medium only (control) for 24 hours. After 24 hours PBMC were harvested after co-incubation in either presence (A) or absence (B) of IEC and after restimulation stained extracellular for CD4 and CD25 and intracellular for Foxp3. Results are presented as mean ± SEM, * $p<0.05$, paired student's t test.

V. Discussion

Over the past decades chronic inflammatory diseases such as allergies or inflammatory bowel diseases are increasingly occurring worldwide. As genetic drift alone cannot explain this increase, epigenetic mechanisms that can regulate accessibility of genes for transcription by secondary, not mutation-based modification of DNA and DNA-associated proteins, as well as environmental factors are discussed as possible reason (Michel 2013, Renz 2011, Paparo 2014). Accumulating evidence exists that an altered composition of the microbiota and dysbalanced immune responses contribute to regulatory disorders like allergy. Taking into account that the composition of the host microbiota and microbes ingested from food products like Lactobacilli or Bifidobacteria as well as breast milk oligosaccharides or NDO from infant formula have been shown to influence immune responses and intestinal homeostasis, food based strategies involving these ingredients might be a promising and appropriate approach to prevent allergy (Romeo 2010, Candela 2010, Kau 2011, Prakash 2011, Tsai 2012, Nagpal 2012, Pfefferle 2013, Preter 2011, Jeurink 2013). Since therapy of allergies, affecting more than 80 million people in Europe, can be complex and costly, preventive, food based strategies are therefore preferable.

It has been reported in animal trials and human clinical studies that LAB and NDO like scGOS/lcFOS supplemented to infant formula can contribute to a decreased susceptibility to allergy (Grüber 2010, 2012, Osborn 2013, Rijnierse 2011, Dang 2013). However, the exact mechanisms are still unclear but might involve not only effects on the microbiota but, beyond that, also direct effects on host cells (Nauta 2013). Interactions with epithelial and immune cells are supposable. One aim of this thesis is therefore to investigate the immune-modulatory potential of non-digestible short chain galacto- and long chain fructo-oligosaccharides (scGOS/lcFOS) mimicking the natural distribution of oligosaccharides in human breast milk in presence or absence of certain LAB strains on human monocyte-derived dendritic cells (MoDC) and the ensuing T cell response. Furthermore the objective is to investigate an effect and a potential immune-modulatory impact of certain LAB in an *in vitro* co-culture model of human intestinal epithelial cells (IEC) and peripheral blood mononuclear cells (PBMC).

V. 1. Effect of lactic acid bacteria on monocyte-derived dendritic cells

Antigen-presenting cells like dendritic cells (DC) constitute an interface between the innate and adaptive immune system. DC play a crucial role by shaping adaptive immune responses after activation by secreting cytokines depending on the stimulus and dictating the T cell phenotype towards Th1, Th2, Treg etc. The determined release of cytokines and chemokines by MoDC investigated as part of this work can therefore provide

important information about the potential following adaptive immune response triggered by bacterial stimulation (Niers 2005, 2007). As MoDC are capable of promoting T cell polarization towards different effector T cell subtypes like Th1, Th2, Th17, Th22, Th9 or regulatory T cells, cytokine profiles after stimulation with lactic acid bacteria have been analyzed. *B.breve* induced a less pro-inflammatory cytokine-profile characterized by low IL-1, IL-12 and lower than *L.rhamnosus* in IL-6, CXCL10 (IP-10, interferon gamma induced protein 10) and TNFα. In contrast, *L.rhamnosus* induced a cytokine profile high in IL-1α and β, IL-6, IL-12, TNFα and IP-10 (CXCL10). The chemokines and cytokines CCL20 (MIP3α), CCL17 (TARC), CCL22 (MDC), IL-27, IL-23 and IL-10 were dose-dependently induced by LAB, but showed no strain dependent differences between *B.breve* and *L.rhamnosus*.

IL-1 is important for the inflammatory response against infection, it is an endogenous pyrogen, causes increased pain sensitivity and in presence of IL-23 leads to polarization of naïve T cells into Th17 cells (Dungan 2011, Dinarello 2014). IL-1β is produced upon stimulation by microbial and damage-associated signals and subsequent activation of the inflammasome, consisting of multiprotein complexes, leading to caspase-1 activation and following proteolytic maturation of IL-1β and IL-18 (Lu 2015). Not all tested bacterial strains had the capacity to induce IL-1α or β production. Its secretion was almost absent after stimulation with *B.breve*. IL-6 is known as pro-inflammatory cytokine with a role in acute phase responses. It has been postulated that IL-6 promotes terminal differentiation of B cells into plasma cells and has furthermore been found to be able to polarize naïve T cells to effector Th2 cells enhancing intestinal IgA responses after administration of probiotics (Christensen 2002). Many species of lactic acid bacteria induce IL-6 (Vinderola 2005). In the present study *L.rhamnosus* did induce higher IL-6 secretion compared to *B.breve*. IL-23, a member of the IL-12 family, has been described to induce polarization of naïve T cells into Th1 cells and the proliferation of memory T cells (Smits 2004). Its secretion was induced in comparable manner by the tested LAB. IL-12 is a pro-inflammatory cytokine that diverts the immune response toward Th1 differentiation through induction of IFNγ release which counteracts Th2 activity *in vivo*. Th1 responses are pro-inflammatory and lead to robust immunity against infections and other diseases (Manetti 1993). Certain LAB have been demonstrated to induce IL-12 production by DC, thus promoting IFNγ secretion and inflammatory Th1 responses (Mohamadzadeh 2005). In contrast to *B.breve, L.rhamnosus* did induce IL-12 release by MoDC.

TNFα induces inflammation and apoptosis and CXCL10 (IP-10) evokes upregulation of Th1 cytokines and enhanced inflammation (Kasama 2005). *L.rhamnosus* was a more potent inducer of these two analytes in the present study than *B.breve*. CCL17 (TARC, thymus and activation regulated chemokine) induces chemotaxis in T cells, interacting

with CCR4, as well as CCL22 (MDC, macrophage-derived chemokine). CCL20 (MIP3α, macrophage inflammatory protein 3α) is a strong chemotactic chemokine for T cells interacting with CCR6 (Penna 2002). CCL20 (MIP3α), CCL17 (TARC) and CCL22 (MDC) were dose-dependently induced but showed no strain dependent differences between *B. breve* and *L.rhamnosus* in this work, suggesting a potential comparable chemotactic capacity of MoDC stimulated with the two tested bacterial strains.

IL-10, induced by both tested LAB is an anti-inflammatory cytokine diverting the immune system to a regulatory mode and has been described to favor T cell polarization towards regulatory T cells (Boks 2012). Di Giacinto et al. (2005) could show that various lactic acid bacteria species were capable to ameliorate colitis in a murine model by stimulating the secretion of IL-10 by lamina propria mononuclear cells. DC exposed to LAB may acquire tolerogenic properties, involving IL-10 secretion, which provokes the generation of regulatory T cells (Smits 2005, Von der Weid 2001, Lyons 2010). Hart et al. (2004) observed that individual strains within VSL#3, a mixture of LAB strains, upregulated IL-10 production by human DC displaying distinct anti-inflammatory and immune-modulatory effects by inducing the differentiation of protective T regulatory cells. IL-27 also belongs to the IL-12 family, but has also been associated with the induction of regulatory T cells (Ilarregui 2009, Pot 2010, Awasthi 2007).

Upon analyzing the cytokine profile of MoDC after stimulation with lactic acid bacteria *L.rhamnosus* can be considered as a pro-inflammatory, immune-stimulatory bacterial strain favoring a Th1 response as it induced a cytokine profile high in IL-1, IL-6, IL-12, TNFα and IP-10. These results are in concordance with previous studies, reporting that Gram-positive bacteria are able to induce a cytokine pattern that promotes Th1 effector functions and Lactobacillus strains can be strong inducers of pro-inflammatory cytokines (Hessle 2000, Evrard 2011, Shida 2006). Since Lactobacilli have been shown to modulate the Th1/Th2 balance towards a Th1 response on the level of DC, these bacteria could contribute to a decreased susceptibility to atopy and allergy by counteracting Th2 skewed immune responses (Marschan 2008, Nermes 2011).

Bifidobacteria have also been described to favor Th1 responses (Niers 2007), however *Bifidobacterium breve* can also be a potent inducer of IL-10 production in DC and has been shown to provide more immune-regulatory properties (Latvala 2008, Baba 2008). This is in agreement with the results of this study. *B.breve* induced a profile characterized by low IL-1, IL-12 and lower than *L.rhamnosus* in IL-6, IP-10 and TNFα, but IL-10, IL-27 in a comparable manner. Lopez et al. (2010) reported that some *B.bifidum* strains seem to promote Th17 polarization. There is evidence that IL-1β, in the absence of IL-12 and IL-4 could drive T cell polarization towards Th17 differentiation (Annunziato 2008). Therefore, the IL-1β/IL-12 balance present in the stimulation experiments could be an

indicator for a subsequent Th17 differentiation. As *B.breve* didn't induce IL-1β secretion by MoDC, this is probably not the case in this work. *L.rhamnosus* did induce IL-1β, IL-6 and TNFα secretion by MoDC, therefore a potential skewing of T cell responses in direction of Th22 cells might be possible. However the induction and therefore presence of IL-12 release points to polarization towards Th1 cells. Other authors described that some Bifidobacterium strains drive Treg responses based on their low inflammatory potential promoting a tolerant state with decreased Th1 immune responses in the gut (Konieczna 2012, Bermudez-Brito 2013, Zheng 2014). IL-27 and IL-10, two cytokines that have been associated with a possible induction of regulatory T cells were released in comparable manner by MoDC after stimulation with either *L.rhamnosus* or *B.breve.* Therefore also a contribution to maintenance of homeostasis of the tested LAB could be possible.

Taken together, different bacterial strains can either stimulate or suppress immune responses by determining T cell polarization via the induction of various cytokine profile patterns of MoDC. High IL-10, low IL-12 and low IL-1β production drives T cell differentiation towards regulatory T cells, high IL-12, low IL-10 secretion instructs T cells to convert into Th1 types. Low IL-12 and high IL-6 release determines polarization towards Th2 and a combination of both high IL-1β and IL-23 production leads to Th17 differentiation. IL-6 and TNFα secretion of MoDC induces Th22 cells and IL-4 and TGFβ drive differentiation of Th9 cells. This study shows strain-specific immune-modulatory effects of Bifidobacteria and Lactobacilli on the human immune system.

The exact mechanisms underlying the beneficial effects of LAB are not yet completely understood, but may involve pattern recognition molecules such as TLR. The described strain specific effects of LAB on cytokine release of MoDC could be linked to specific interactions between bacterial cell wall components and pattern recognition receptors. There is evidence that some lactic acid bacteria induce an anti-inflammatory response and appear to preferentially evoke regulatory T cells, mainly via interaction with TLR9 (Gómez-Llorente 2010). Plantinga et al. (2011, 2012) elucidated receptors involved in the interaction by specific blocking of particular PRRs. The first important observation of their study was that cytokine induction by both Bifidobacteria and Lactobacilli was strongly dependent on TLR9. Another TLR described as a mediator of certain activities of lactic acid bacteria strains is TLR2. Interestingly, no involvement of TLR2 could be demonstrated concerning *L. rhamnosus* by Plantinga et al, indicating that these bacteria either lack TLR2 ligands in their cell wall or potential TLR2 ligands are masked.

Previously, an immune-regulatory role of TLR2 in recognition of LAB has been described as recognition of *B.breve* by TLR2 had an anti-inflammatory effect and TLR2 has also been implicated in the induction of regulatory T cell responses (Lebeer 2008, Hoarau

2006, van Bergenhenegouwen 2013, 2014). Teichoic acids, a cell wall component of Gram-positive bacteria, are considered to be one of the main immune-stimulatory components of these bacterial strains through their activation of TLR2 (Schröder 2003). There exist considerable differences between the wall teichoic acid and lipoteichoic acid molecules of different Lactobacillus or Bifidobacterium strains, both in composition and quantity, which may influence the immune stimulating properties of the different strains (Grangette 2005). Closely related bacteria strains of the same species may have different physiological effects. Each mechanism of action is strain-dependent, making it important to select and scientifically compare LAB for their intended purpose. However, recently Sagar et al. could show in a murine model for chronic asthma that *Bifidobacterium breve* as well as *Lactobacillus rhamnosus* treatment was as effective as budesonide in reducing inflammation (Sagar 2014). Strain specific protective effects of LAB can vary depending on the area of application, but the differential effects of LAB can also work together in some clinical outcomes and confer beneficial effects independent of bacterial strain specificities.

Another mechanism by which LAB could improve health outcomes could imply short-chain fatty acids (SCFA) that are produced by fermentation of NDO by anaerobic bacteria in the large bowel. An increased proportion of Bifidobacteria in the stool has been associated with a reduction in stool pH. This effect could be explained by an enhanced occurrence of SCFA metabolized by Bifidobacteria, causing a SCFA pattern containing a higher proportion of acetate and a lower content of propionate (Fukuda 2011). SCFA are on the one hand essential nutrients for IEC. On the other hand, *in vitro*, acetate and propionate increased anti-inflammatory IL-10 production and butyrate inhibited Th1 type T cell responses (Cox 2009). A combination of three SCFA has been reported to cause a shift in the T helper lymphocyte phenotype towards a more anti-inflammatory subset (Cavaglieri 2003). Recently, clinical effects of dietary fiber and the gut microbial-derived SCFA like systemic effects on metabolism or hematopoiesis have been described (Trompette 2014, Kasubuchi 2015, Fernandes 2014).

V. 2. Effects of non-digestible oligosaccharides on human monocyte-derived dendritic cells and the ensuing T cell response

In this work direct, immune-modulatory effects of an unique combination of oligosaccharides on human MoDC and the ensuing induced T cell response are shown for the first time. The specific mixture of scGOS/lcFOS, as used in this study, was previously described by de Kivit et al. to modulate cytokine release from CD3/CD28 stimulated PBMC both directly and through effects on epithelial cells in 2D co-cultures (de Kivit 2013). This suggests that scGOS/lcFOS might stimulate cells directly. MoDC

treated with scGOS/lcFOS released IL-10 in the absence of IL-12p70. This effect could be abrogated via blocking of TLR4, suggesting that this effect is mediated via activation of TLR4. In contrast, TLR4 activation through LPS stimulation of MoDC induced both IL-10 and IL-12 release, which would argue against a potential bacterial product contamination of the scGOS/lcFOS preparation. In line with this, upon measuring the endotoxin content of the scGOS/lcFOS mixture using a LAL-assay, endotoxin content was measured to be very low (<3 ng/ml).

Moreover, upon analysis of additional soluble mediators (chemokines, as well as cytokines) the profile of scGOS/lcFOS-induced release was markedly different from LPS, suggesting that other PRR besides TLR4 may play a role. Additionally, the maturation marker profile of MoDC induced by scGOS/lcFOS was different compared to LPS. scGOS/lcFOS did induce a slight upregulation of maturation markers CD80, CD83, CD86 and CD40, however lower compared to LPS-triggered MoDC.

Lin et al. (2014) showed an induction in phenotypic maturation of murine DCs by feruloylated oligosaccharides. In TLR4/2 deficient mice this effect was abrogated, suggesting an involvement of TLR4/2 in the effect of oligosaccharides. Tsai et al. (2013) revealed that oligosaccharides isolated from wheatgrass modulate monocytes via toll-like receptor 2 signaling. Recently He at al. (2014) described that human milk oligosaccharides modulate CD14 expression in human enterocytes and thereby attenuated LPS-induced inflammation. Zhou et al. (2015) suggested that oligosaccharides obtained by oxidative degradation from alginate may reduce the LPS-stimulated inflammatory responses through blocking the activation of NF-κB and MAP kinases. Several other studies described a potential immune modulation by different NDO on cellular level via the interaction with specific sugar receptors including DC-SIGN, TLRs or C type lectin receptors (Capitan-Canadaz 2014, Ortega-Gonzalez 2014, Kurakevich 2013, Vogt 2013).

The presented data gives rise to further studies investigating the role of other types of receptors, including PRR crosstalk involved in scGOS/lcFOS-mediated, direct mechanisms on human MoDC. Binding assays need to be conducted to reveal the intensity of the potential TLR-epitope interaction of scGOS/lcFOS and TLR4 on human MoDC and it is necessary to analyze if NDO can be taken up by MoDC. Possible signaling pathways involved need to be investigated by analyzing the down-stream signaling of TLR4 after stimulation of DC with scGOS/lcFOS (Figure 30).

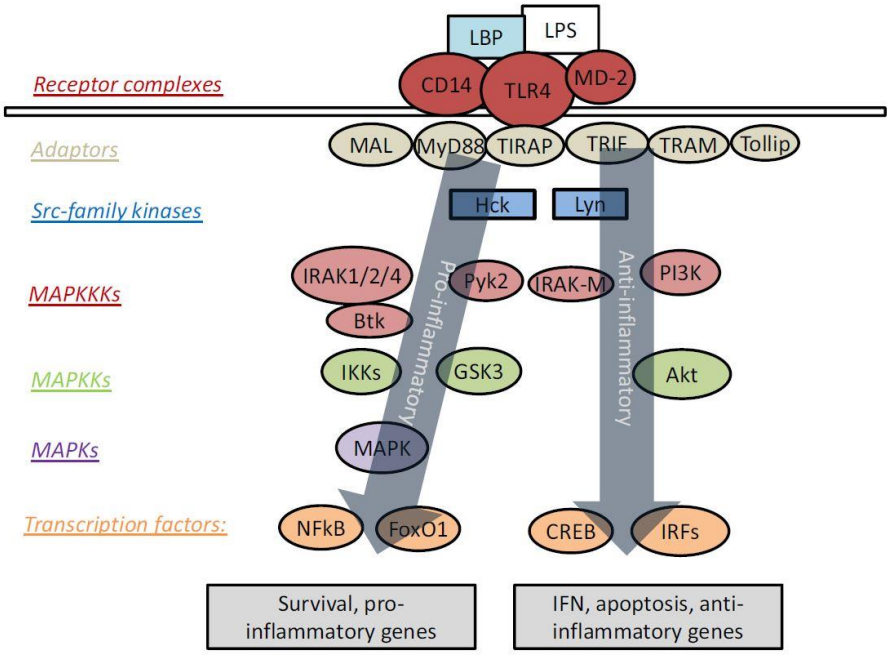


Figure 30. Dynamic complexity of intra-cellular signaling circuits downstream of the Toll-like receptor 4 (TLR4). LBP=lipopolysaccharide-binding protein, LPS=lipopolysaccharide, TLR=toll-like receptor, K=kinase (Morris 2015).

Possibly signaling transduction factors and kinases such as PI3K, Akt and IRFs might be involved as MoDC stimulated with scGOS/lcFOS released IL-10 in the absence of IL-12. The possibility that scGOS/lcFOS bind to TLR4 might also be involved in better protection against enteric infections by competition with pathogens for adhesion sites. Further research is necessary following the hypothesis that NDO probably use the same TLR as Gram-negative pathogens but elicit different immune responses. Studies using TLR4 deficient mice could provide additional information and confirm a mode of action of the specific mixture of scGOS/lcFOS used in this study via TLR4.

The different effects of oligosaccharides have been primarily directed toward the gut, but accumulating evidence demonstrates that oligosaccharides exert impact beyond the gastrointestinal tract (Candela 2010). Recently a possible systemic effect of dietary fibre on allergic airway disease and hematopoiesis has been reported (Trompette 2014), suggesting systemic immunological effects of NDO. Furthermore Ruhaak et al. (2014) described that HMO can be detected in plasma of infants and also Goehring et al. (2014) provided evidence for the presence of HMO in the circulation of breastfed infants. The blood concentration of human milk oligosaccharides comprises around 100-200 µg/ml (0.01-0.02%), while in this study 5 mg/ml (= 0.5%) were used. In infant formulas NDO are used at a concentration of 8 mg/ml, suggesting higher concentrations of NDO locally

in the gut as infant formula is the only source of nutrition for bottle-fed infants. Therefore it could be hypothesized that 5 mg/ml as used in the present study might be in the physiological range in an infants' intestine. Studies showing that supplementation with galacto-oligosaccharides reduces not only stress-induced gastrointestinal dysfunction but also reduces days of cold or flu (Hughes 2011) underline possible systemic and immune-regulatory effects.

DC production of IL-10 in the absence of IL-12p70 could contribute to the development of regulatory T cells (Rutella 2004, Maldonado 2010, Palomares 2014). Expression of the transcription factor Foxp3 is a phenotypical characteristic of Tregs (Linston 2014, Peterson 2012). Results obtained in this study indicate that oligosaccharides only slightly upregulate maturation markers CD80, CD83 and CD86 in comparison to LPS-matured DC. It has been postulated that partially or semi-mature intestinal DC producing little IL-12 in the presence of IL-10 are involved in induction of tolerance (Boks 2012, Hubo 2013). In the present study exposure of MoDC to scGOS/lcFOS and subsequent co-culture of MoDC and naïve T cells led to an increase in Foxp3 expressing T cells, which is associated with Tregs. Experiments of this study indicate a suppressive function of the induced $Foxp3^+$ T cells. Treg induction by scGOS/lcFOS in the present study analyzing human immune cells would be in accordance with previous studies in mice where a diet containing scGOS/lcFOS decreased allergic symptoms in a mouse model of food-allergy (Schouten 2010, 2012). Recently Hartog et al. (2015) revealed a potential role for regulatory T cells in the amelioration of DSS (dextran sodium sulfate) induced colitis by dietary non-digestible polysaccharides and Hogenkamp et al. (2015) could show that supplementing pregnant mice with a specific mixture of NDO reduced symptoms of allergic asthma in male offspring.

V. 3. Combinations of lactic acid bacteria and non-digestible oligosaccharides

Combinations of LAB and NDO are used as ingredients of infant milk formulas (Romeo 2010). Here the oligosaccharides are thought to act as substrates for the bacteria thereby assisting in their function. Herein, the contribution of the mixture scGOS/lcFOS on the bacteria-induced MoDC cytokine profile was investigated. Stimulation with *L.rhamnosus* or *B.breve* strain-dependently induced release of IL-10 and IL-12 by MoDC. This is in accordance with previous data showing that different bacterial strains have various, strain-dependent effects on cytokine release of human immune cells potentially through the differential use of PRR (Snel 2011, Smits 2005, Latvala 2008, Plantinga 2011, 2012).

In line with the data observed on the direct effects of scGOS/lcFOS only on MoDC, scGOS/lcFOS addition enhanced dose-dependent *B.breve*- and *L.rhamnosus*-induced

IL-10 release while IL-12 release was unaffected. This effect could be blocked using a TLR4 antagonist indicating a role for TLR4 in the observed effect. According to van der Aa et al. (2011) a combination of *B.breve* and scGOS/lcFOS induces Treg cell development through modulation of DC function, preventing asthma-like symptoms in infants with atopic dermatitis. A reduced allergen induced Th2 response and an improved peak expiratory flow in allergic asthmatics have been described after treatment with a combination of scGOS/lcFOS and *B.breve* (van den Pol 2011). An intervention study in a mouse model for orally induced cow's milk allergy revealed a reduced acute allergic skin response and anaphylactic scores after dietary intervention with scGOS/lcFOS in combination with *B.breve*. Enhanced whey-specific Th1 type serum IgG2a, fecal IgA and reduced mast cell degranulation were further results of this animal study (Schouten 2009). The authors suggested a possible involvement of whey-specific $CD25^+$ regulatory T cells in induction of tolerance by NDO and confirmed a role for regulatory T cells in suppressing casein allergy by NDO (Schouten 2010, 2012).

An important role of regulatory T cells as well as anti-inflammatory cytokine profiles in the prevention of allergies and related disorders has been affirmed by several studies (Cavani 2001, Fyhrquist 2012, Romagnani 2006, Niers 2007, Feleszko 2007). Hougee et al. (2010) showed that a combination of *B.breve* with the mixture scGOS/lcFOS was more effective than several other Bifidobacterium and Lactobacillus strains alone to suppress airway hyper responsiveness and pulmonary inflammation in a murine model for asthma. Combinations of LAB and NDO have also been shown to reduce allergen induced Th2 responses and to promote allergy-preventive immunologic effects like a reduction of incidence of eczema (Dang 2013, Pfefferle 2013). Effects on microbiota composition, like an enhanced Bifidobacteria count and systemic immune response in infants with short bowel syndrome have been reported after one year of therapy including Bifidobacteria, Lactobacilli and GOS (Uchida 2007). Further clinical studies demonstrate that a regular, long-term intake of various combinations of LAB and NDO may improve health by reducing the incidence and severity of respiratory diseases during the cold season, infectious diseases and increasing growth of infants (Picaud 2010, Sazawal 2010, Pregliasco 2008).

Despite various promising results for beneficial effects of combinations of LAB and NDO, certain other studies showed no effect e.g. regarding infection prevention, only gut-related effects like increased stool frequency were revealed (Jain 2004, Mugambi 2012). Although beneficial outcomes concerning eczema prevention and wheezing have been reported, the respective studies failed to show effects on other allergy outcomes or total IgE (Pfefferle 2013, Dang 2013, van der Aa 2011). Among others, the considered population, symptoms, clinical endpoints, the time point and duration of application, as

well as the composition or dose of LAB/NDO are important variables differing between studies, making general conclusions difficult.

Further research is necessary investigating specific interactions between LAB and NDO on the molecular level. In the current study direct immune-regulatory effects of NDO on MoDC as well as immune-modulatory effects of LAB on MoDC have been shown. Upon combining LAB und NDO an enhancement of cytokine release by MoDC mainly concerning the anti-inflammatory IL-10 could be observed (Overview results see Figure 31).

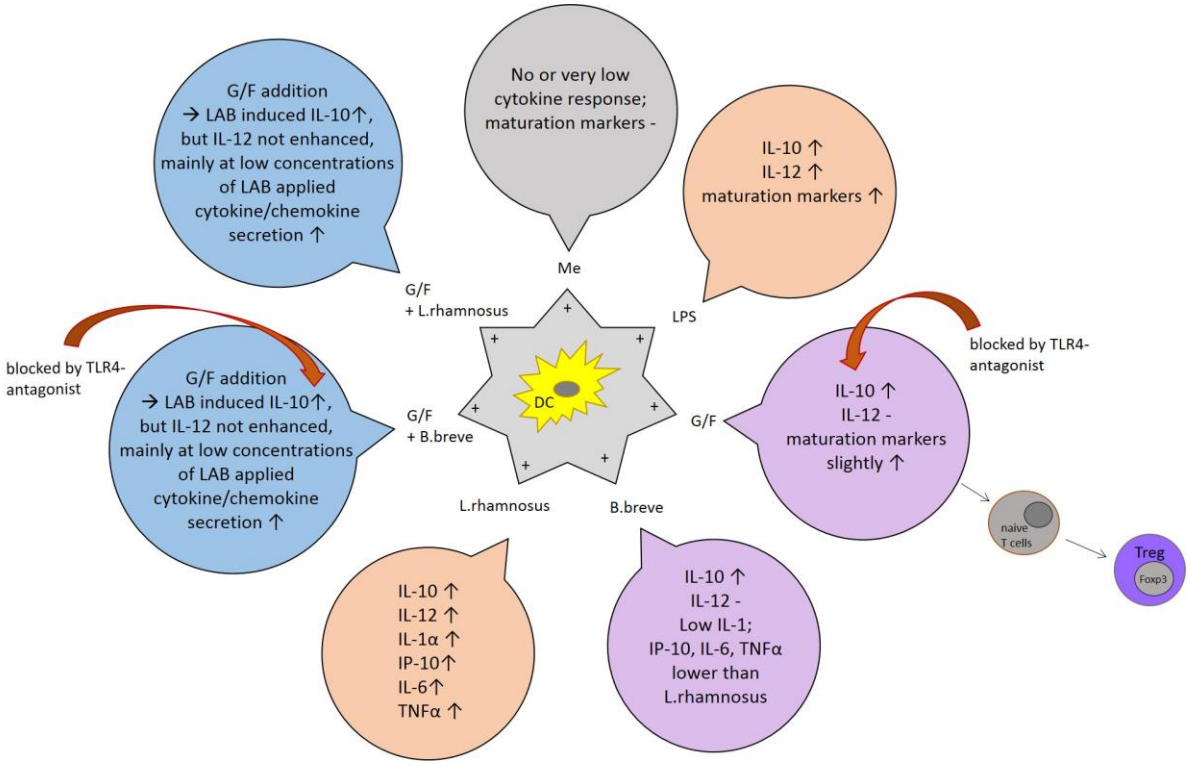


Figure 31. Overview results of this study on effects of LAB and NDO on human MoDC and the ensuing T cell response. DC=dendritic cell, Me=medium, LAB=lactic acid bacteria, LPS=lipopolysaccharide, G/F=scGOS/lcFOS, B.=Bifidobacterium, L.=Lactobacillus, NDO=non-digestible oligosaccharides.

It is however still unclear if NDO and LAB exert their effects directly on immune cells independent of each other or if there are other mechanisms involved whereby NDO alter the constitution or metabolic activity of LAB. Also an alteration of the constitution of MoDC induced by NDO and thereby influencing the subsequent response of MoDC to LAB could be supposable. Future experimental settings could be transwell experiments where DC and LAB/NDO mixtures are separated by inserts of certain pore sizes to investigate a potential role of soluble factors. To analyze such metabolites produced by

bacteria in response to NDO and characterize bacteria exactly after co-incubation with oligosaccharides, cell culture medium without antibiotics could be used for the possibility of an active metabolism of bacteria. To explore possible independent influences of bacteria and oligosaccharides directly on MoDC, co-incubation experiments of MoDC with subsequently LAB or NDO could be part of future analysis. After stimulation with either LAB or NDO, MoDC could be washed extensively to remove the first stimulant, subsequently incubated with the corresponding other stimulant and analyzed.

V. 4. Effect of lactic acid bacteria in a human *in vitro* co-culture model of intestinal epithelial cells and PBMC

It is well known that IEC play an important role as immune-modulatory mediators between commensal or pathogenic bacteria and immune cells beyond their function as simple borderline between gut lumen and GALT (Artis 2008, Iliev 2009, Rescigno 2014, Haller 2008). Therefore dietary intervention using Lactobacilli could be an adequate strategy to modulate and shape immune responses in the gut in a beneficial direction via interaction with IEC. Another objective of this thesis is therefore to investigate the immune-modulatory potential of certain LAB in an *in vitro* co-culture model of human intestinal epithelial cells (IEC) and peripheral blood mononuclear cells (PBMC).

In the present study effects and the immune-modulatory potential of four specific Lactobacillus strains in a human transwell co-culture model of HT-29 intestinal epithelial cells and PBMC developed previously by van Hoffen et al. (2010) was investigated. In concordance with van Hoffen et al. and de Kivit et al. (2011), production of cytokines by not activated PBMC (not stimulated with aCD3/aCD28) was not detectable, even in co-culture with LAB stimulated HT-29 cells. This result suggests that a certain activation status of immune cells has to be present in order to be able to trigger a reaction of IEC to LAB or other stimuli. Literature reports that IEC express toll-like receptors (TLR) and thereby can recognize LAB and commensals (de Kivit 2014, Abreu 2010, Viljanen 2005, Vinderola 2005, Zeuthen 2008, Marschan 2008). TLR are pattern recognition molecules that distinguish a wide variety of structural components of microbes, enabling IEC to transmit information about luminal stimuli (microbes) to underlying immune cells IEC and therefore can orchestrate critical immune responses (Hörmannsperger 2009, Ratajczak 2007). A certain hypo-responsiveness of IEC to commensals has been investigated by several studies (Parlesak 2004, Haller 2002).

Results of the present study indicate lower levels of IL-10 secretion and intracellular Foxp3 expression by PBMC when IEC were present upon comparing the medium controls in presence and absence of IEC. This could suggest a possible induction of regulatory T cells when IEC are absent. Additionally, an enhancing effect of LAB on

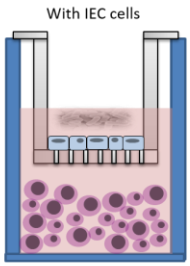
IL-10 secretion by PBMC was observed only in the presence of IEC, in the absence of HT-29 cells this effect disappeared. This indicates an important role of IEC in transmitting information of apical stimuli to immune cells and thereby influencing the effects exerted by LAB and following immune responses. In general it has been shown that Lactobacilli differ in their capacity to induce cytokine secretion like IL-10, IFNγ and TNFα by PBMC (Christensen 2002, Niers 2005, Vissers 2010). Haller et al. (2001) demonstrated in a co-culture model of Caco cells and PBMC a discriminate activation between *E.coli* and LAB. Campeau et al. (2012) confirmed that murine intestinal epithelial cells have the capacity to modulate subsequent antigen-presenting and T cell responses to bacterial DNA (bifidobacteria and salmonella) with pathogenic but not commensal bacterial DNA inducing effector CD4 T lymphocytes. Zoumpopoulou et al. (2009) revealed in a murine IEC and DC co-culture model that IEC respond poorly to Gram-positive bacteria. However, van Hoffen et al. (2010) could show that IEC exposed to *Lactobacillus GG*, but not *Bifidobacterium breve* enhanced an inflammatory Th1 type response.

In the present study an enhanced IL-10 and TNFα release of PBMC induced by LAB-stimulated HT-29 was determined. *L.paracasei 1 and 2* enhanced epithelium dependently immune-regulatory IL-10 secretion by PBMC and *L.rhamnosus* and *L.paracasei 2* elicited TNFα release by PBMC after co-incubation with LAB-exposed IEC cells. No significant difference between LAB- or medium-treated HT-29 concerning IFNγ release of PBMC could be detected. A tendency ($p=0.06$) was observed for reduced IL-13 release of PBMC co-cultured with *L.rhamnosus*-stimulated IEC, indicating a possible change in the Th2/Treg ratio. A cytokine profile involving TNFα and IFNγ could point to the induction of a pro-inflammatory Th1 T cell response, while IL-13 has been associated with Th2 responses. De Kivit et al. (2011, 2013) studied apical stimulation of IEC with genomic DNA from *B.breve* or a synthetic TLR9 ligand in absence or presence of non-digestible oligosaccharides and also effects of IEC exposure to TLR4 and TLR9 ligands on human PBMC in a human co-culture system. The authors revealed an induction of Treg/Th1 polarization of T cells involving a role of endogenous glycan binding proteins like galectin-9 analyzing the levels of secreted galectin-9 by HT-29 cells. In contrast to our results on Lactobacilli they observed enhanced IFNγ release by PBMC induced by *B.breve* or a TLR9 ligand.

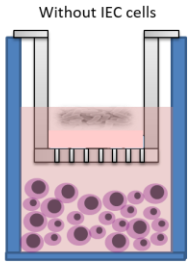
In concordance with this study, in the current work, increased amounts of galectin-9 released by IEC after stimulation with *L.paracasei 2* were observed. Galectin-9 is discussed to be a promising target for the induction of regulatory T cells (de Kivit 2012). Other studies described tolerogenic signals delivered by DC to T cells through a galectin-1 driven immune-regulatory circuit and a function of galectin-9 as autocrine regulator of mast cell function to suppress excessive degranulation, thus anti-allergic

effects (Ilarregui 2009, Kojima 2014, Niki 2009). Expression of the transcription factor Foxp3 is associated with a potential induction of regulatory T cells, which could be beneficial regarding diseases that occur with dysbalanced immune responses, like allergy (Boks 2012, Smits 2005, Palomares 2014, Fyhrquist 2012). Interestingly, in the current study a tendency of *L.rhamnosus* and *L.paracasei 1* to block steady state IL-13 release compared to medium control and the other tested LAB was observed. However this result did not reach statistical significance. These findings are in concordance with de Kivit et al. (2011, 2013) showing that Th2-associated IL-13 was significantly reduced upon apical exposure of IEC with synthetic CpG DNA of a *Bifidobacterium breve*. TGFβ, under certain conditions an immune-regulatory cytokine, could play a role in counteracting dysbalanced immune responses next to IL-10 and galectin-9. Mileti et al. (2009) demonstrated in a co-culture model of caco cells and DC that *L.paracasei* induced TGFβ release by DC, pointing to a potential differentiation of non-inflammatory DC. Therefore further investigations have to be conducted to reveal other factors which could be involved in LAB-mediated effects observed in the current study.

Results of this part of this thesis could point to an epithelium derived galectin-9 dependent immune-dampening or modulating capacity of certain Lactobacilli with strain-specific differences needed to be taken into account (Overview, see Figure 32).



	TNFα (PBMC)	IFNγ (PBMC)	IL-10 (PBMC)	IL-13 (PBMC)	galectin-9 release of IEC
L.helveticus	-	-	(↑)	-	(↑)
L.paracasei 1	(↑)	-	↑	(↓)	(↑)
L.rhamnosus	↑	-	↑	(↓)	(↑)
L.paracasei 2	↑	-	↑	-	↑



	TNFα	IFNγ	IL-10
L.helveticus	-	-	-
L.paracasei 1	(↑)	-	-
L.rhamnosus	↑	-	-
L.paracasei 2	↑	-	-

↑ = significant enhanced release in comparison to medium control
(↑) = not statistical significant

medium control without intestinal epithelial cells (IEC) present → IL-10 cytokine release ↑ and % CD25foxp3⁺ T cells↑ in comparison to medium control with IEC present

Figure 32. Overview results on effects of LAB in a human *in vitro* co-culture model of intestinal epithelial cells and PBMC. IEC=intestinal epithelial cells, L.=Lactobacillus, PBMC=peripheral blood mononuclear cells.

However the setup of these experiments does not allow specific conclusions for a potential application of these LAB as dietary intervention or prevention against allergy. First of all cell lines like HT-29 cells may not reflect the *in vivo* situation as clearly as freshly isolated cells or polarized cell lines. Furthermore *in vitro* systems may provide valuable information about potential mechanisms of action on cellular level but do not take into account the complexity of the intestinal ecosystem involving microbiota, nervous system, etc., making the interpretation of *in vitro* data difficult. Additionally age, gender, health status, activity and dietary habits of blood donors have to be taken into account. Due to obvious limitations of test systems on cellular level, *in vitro* studies should be limited to investigation of mechanism of action and screening. Substantiation and final evaluation by *in vivo* experiments and randomized, placebo-controlled human clinical trials is inevitable before applying LAB as dietary intervention or prevention also depending on the respective area of application.

V. 5. Conclusion

Accumulating evidence exists that an altered composition of the microbiota and dysbalanced immune responses, including loss of tolerance to harmless antigens, contribute to the establishment of regulatory disorders like allergies. It has been reported in animal studies and human clinical trials that LAB and NDO like scGOS/lcFOS supplemented to infant formula can contribute to a decreased susceptibility to allergy (Grüber 2010, 2012, Osborn 2013, Rijnierse 2011, Dang 2013). However, the exact mechanisms are still unclear but might involve beyond effects on the microbiota direct effects on host cells (Nauta 2013, Jeurink 2013).

Results of this work indicate anti-inflammatory and direct, microbiota independent, immune-modulatory properties of scGOS/lcFOS mixtures displaying similar functionality as HMO supplemented to infant formula on human MoDC. A direct effect of scGOS/lcFOS on human dendritic cells involving TLR4 and subsequently induction of regulatory T cells has been revealed. These results provide a possible explanation for clinical observed anti-inflammatory, immune-modulatory effects of these NDO in presence or absence of certain LAB involving a mechanism at least partly via TLR4.

Furthermore certain tested LAB exhibited several effects in the used human *in vitro* co-culture system. IEC dependent immune-regulatory and anti-inflammatory IL-10 release of PBMC and galectin-9 secretion of IEC as well as enhanced pro-inflammatory TNFα secretion could be observed. This cytokine profile could point to induction of a putative T cell phenotype in direction of Treg/Th1 cells which could be potentially beneficial by counteracting Th2 skewed conditions like allergies. Interactions of LAB with epithelial cells and the ensuing immune response are supposable. Therefore LAB might be

considered as potential tool to influence dysbalanced immune responses by dietary supplementation.

Nutritional intervention with NDO and/or LAB to restore immune balance and to establish (or re-establish) a protective intestinal microbiota could be a promising concept for prevention of disorders associated with loss of tolerance. The tested combinations of LAB and scGOS/lcFOS might represent a useful dietary ingredient for the maintenance of intestinal homeostasis via the induction of potential suppressive $Foxp3^+$ T cells. *In vitro* results obtained in this study have certainly to be further substantiated with *in vivo* efficacy in human clinical and animal studies. The question where and when in the human gut MoDC would encounter NDO and if they are able to take NDO up, as well as the exact signaling pathways remain to be answered. Finally, next to all limitations of *in vitro* systems, the results presented in this work on cellular level could contribute in part to the establishment of NDO and LAB as potential food based strategy in case of dysbalanced immune responses with their exact mechanisms remaining to be further investigated and *in vivo* efficacy and spectrum of effects to be expanded.

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Material

	Supplier	City, country
Buffy coats	Sanquin	Amsterdam, The Netherlands
Human colon adenocarcinoma cell line HT-29 (HTB-38)	American Type Culture Collection	Manassas, USA

Instrument	Supplier	City, country
AutoMACSpro	Miltenyi biotec	Bergisch Gladbach, Germany (Ger.)
Bio Photometer	Eppendorf	Hamburg, Ger.
Centrifuge	Thermo scientific	Schwerte, Ger.
ELISA-Reader Mrx tc revelation	Thermo Labsystems	Chantilly, USA
FACS Fortessa	Becton Dickinson	Heidelberg, Ger.
FACS Canto	Becton Dickinson	Heidelberg, Ger.
Incubator	Heraeus Instruments	Hanau, Ger.
Microscope	Zeiss	Jena, Ger.
Multichannel pipet (30-300µl)	Brand	Wertheim, Ger.
Pipettes with disposable tips	Eppendorf	Hamburg, Ger.
Pipetus-akku	(a)bimed	Langenfeld, Ger.
Precision balance	Kern 770	Witten, Ger.
Sterile hood	Heraeus Instruments	Hanau, Ger.

Material	Supplier	City, country
AnaeroGen packages	Oxoid Ltd.	Basingstoke, Hampshire, UK
Maxi sorp plates (96 well)	Nunc	Roskilde, Denmark
Perfusor syringes	Braun	Melsungen, Ger.
Petri Dish	Greiner bio-one	Frickenhausen, Ger.
Pipette tips	Sarstedt Eppendorf	Newton, USA
Pipettes (1, 5, 10 and 25 ml)	Greiner bio-one	Frickenhausen, Ger.
Sterile filter (0.22; 0.45 µm)	Millipore express	Cork, Ireland
Tissue culture flask (175 cm^2, 650 ml)	Greiner bio-one	Frickenhausen, Ger.
Tissue culture plates (96 well) U-bottom	Sarstedt	Newton, USA
Tissue culture plates (96 well) flat-bottom	Nunc	Roskilde, Denmark
Tissue culture plates (96 well) flat-bottom	BD	Heidelberg, Ger.
Transwell plates (12 well, 0.4 µm pore size)	Costar, Corning	NY, USA
UVette 50 – 2000 microliter; 220-1600 nm	Eppendorf	Hamburg, Ger.

Reagent	Supplier	City, country
2-Propanol	Merck	Darmstadt, Ger.
2-mercaptoethanol	Fluka	München, Ger.
10x D-PBS without Ca/Mg	Gibco/Invitrogen	Paisley, Scotland
ABTS	Roche Diagnostics	Mannheim, Ger.
Albumin from bovine serum (BSA)	Sigma-Aldrich	München, Ger.
Aqua ad injectabilia	Laboratori Diaco Biomedicali	Trieste, Italy
autoMACS rinsing solution	Miltenyi Biotech	Bergisch Gladbach, Ger.
autoMACS running buffer	Miltenyi Biotech	Bergisch Gladbach, Ger.
Brefeldin A	BD Biosciences	Heidelberg, Ger.

Cell trace Violet Cell Proliferation Kit	Molecular Probes, Life technologies	Darmstadt, Ger.
CD14+ micro-beads (human)	Miltenyi Biotech	Bergisch Gladbach, Ger.
$CD4^+CD45RA^+$ naïve T Cell Isolation Kit II (human)	Miltenyi Biotech	Bergisch Gladbach, Ger.
$CD4^+$ T cell isolation Kit II (human)	Miltenyi Biotech	Bergisch Gladbach, Ger.
Citrat-monohydrate	Merck	Darmstadt, Ger.
Citric acid (0.1 M)	Merck	Darmstadt, Ger.
DEPC treated water	Invitrogen	Paisley, Scotland
Dimethylsulfoxid (DMSO)	Merck	Darmstadt, Ger.
D-PBS – MgCl	Gibco /Invitrogen	Paisley, Scotland
EDTA (0.5 M, pH 8.0)	Gibco/Invitrogen	Paisley, Scotland
Ethanol absolute	Merck	Darmstadt, Ger.
FBS	Hyclone	Uppsala, Sweden
FCS	Invitrogen	Paisley, Scotland
Ficoll-Isopaque	GE Healthcare Life Sciences	Uppsala, Sweden
Foxp3 staining kit	eBioscience	Alasdar Stewart, UK
Gentamycin	Gibco /Invitrogen	Paisley, Scotland
Glutamin	Gibco /Invitrogen	Paisley, Scotland
Glycerol	Sigma	München, Ger.
Golgiplug Leucocyte activation cocktail	BD Pharmingen	Heidelberg, Ger.
H_2O_2	Sigma-Aldrich	München, Ger.
H_2SO_4	Merck	Darmstadt, Ger.
Heparin-natrium-250000	Ratiopharm	Ulm, Ger.
Human serum	Lonza	Basel, Switzerland
Ionomycin	Sigma	München, Ger.
LIVE/DEAD Fixable Aqua dead cell stain kit	Life Technologies	Darmstadt, Ger.
LPS	Sigma	München, Ger.

LPS-RS Ultrapure TLR4 Antagonist	InvivoGen	San Diego, USA
Lymphoprep	FreseniusKabiNorge Axis-Shield	Oslo, Norway
McCoy's 5A medium	Invitrogen	Carlsbad, USA
Monensin	eBioscience	Alasdar Stewart, UK
MRS Bouillon	Oxoid	Basingstoke, Hampshire, UK
MRS Agar	Oxoid	Basingstoke, Hampshire, UK
NaN_3 (sodium azide)	Merck	Darmstadt, Ger.
Nonessential amino acids	Gibco	Paisley, Scotland
Penicillin-streptamycin	Gibco	Paisley, Scotland
Penicillin-streptamycin	Sigma	Gillingham, UK
PMA	Sigma-Aldrich	München, Ger.
Propidiumiodide (PI)	Sigma	München, Ger.
PyroGene Recombinant Factor Endotoxin Detection System	Lonza	Walkersville, USA
rh IL-2	Novartis	München, Ger.
rh IL-4 ($5x10^5$)	ImmunoTools/Promocell	Heidelberg, Ger.
rh GM-CMSF ($5x10^5$)	ImmunoTools/Promocell	Heidelberg, Ger.
RPMI 1640	Gibco /Invitrogen	Paisley, Scotland
RPMI 1640	Lonza	Verviers, Belgium
Sodium pyruvate	Gibco	Paisley, Scotland
Streptavidin-horseradish peroxidase	R&D GE Healthcare UK limited	Wiesbaden, Ger.
Tetramethylbenzidin (TMB)	Fluka (Sigma-Aldrich)	München, Ger.
TGFβ	Promokine	Heidelberg, Ger.
Trypanblue	Invitrogen	Paisley, Scotland
Tween 20 detergent	Calbiochem	San Diego, USA

Antibody/ELISA-Kit	Supplier	City, country
Anti-CD1a PE	eBioscience	Alasdar Stewart, UK
Anti-CD4 FITC	BD Biosciences	Heidelberg, Ger.
Anti-CD4-PerCP-Cy5.5	eBioscience	San Diego, USA
Anti-IL-10-PE	eBioscience	San Diego, USA
Anti-IFNγ-APC	eBioscience	San Diego, USA
Anti-CD14 FITC	eBioscience	Alasdar Stewart, UK
Anti-CD25 PE	BD Biosciences	Heidelberg, Ger.
Anti-CD25-AlexaFluor488	eBioscience	San Diego, USA
Anti-Foxp3-APC	eBioscience	San Diego, USA
Anti-CD40 FITC	BD Biosciences	Heidelberg, Ger.
Anti-CD80 FITC	BD Biosciences	Heidelberg, Ger.
Anti-CD83 PE	eBioscience	Alasdar Stewart, UK
Anti-CD86 APC	BD Biosciences	Heidelberg, Ger.
Anti-HLA-DR APC	eBioscience	Alasdar Stewart, UK
Anti-CD3	BD Biosciences	Heidelberg, Ger.
Anti-CD28	BD Biosciences	Heidelberg, Ger.
Anti IL-12	eBioscience	Alasdar Stewart, UK
Anti IFNγ	eBioscience	Alasdar Stewart, UK
CD4 APC-Cy7	BD Biosciences	Heidelberg, Ger.
IL-10 ELISA-Kit	BD Biosciences	Heidelberg, Ger.
IL-10 ELISA-Kit	U-CyTech Biosciences	Utrecht, The Netherlands
IL-12 ELISA-Kit	eBioscience	Alasdar Stewart, UK
IL-13 ELISA-Kit	R&D Systems	Wiesbaden, Ger.
Galectin-9 ELISA-Kit	R&D Systems	Wiesbaden, Ger.
TNFα ELISA-Kit	Biosource, Life technologies	Darmstadt, Ger.
IFNγ ELISA-Kit	Biosource	Darmstadt, Ger.
Mouse IgG1 FITC	BD Biosciences	Heidelberg, Ger.
Mouse IgG1 PE	BD Biosciences	Heidelberg, Ger.
Mouse IgG1 APC	BD Biosciences	Heidelberg, Ger.

Publications

(published peer-reviewed original manuscripts)

Sarah Lehmann*, Julia Hiller*, Jeroen van Bergenhenegouwen, Leon M.J. Knippels, Johan Garssen, Claudia Traidl-Hoffmann.

In vitro evidence for immune-modulatory properties of non-digestible oligosaccharides: direct effect on human monocyte derived dendritic cells.

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*these authors contributed equally to this study.

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Human IL-31 is induced by IL-4 and promotes TH2-driven inflammation.

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