CHAPTER 7

GENERAL DISCUSSION AND FUTURE PERSPECTIVE

Leonie M. Vogt[1]

[1] *Department of Pathology and Medical Biology, Division Medical Biology, Groningen University, University Medical Center Groningen*

In a society in which the average lifespan is ever increasing, the importance of reaching old age in good health is evident. In this concept, well being is a natural result of healthy ageing, but there is also a large socio-economic advantage to be gained by reducing the annual health care costs. Nutrition is a very important component which can contribute to a healthy ageing process. Current opinions voice the essential role of sufficient fiber content in the daily diet to protect against a range of diseases. Although a collective health improving or protecting effect of different types of fibers is described, the individual mechanisms by which these types of fibers exert their health effects remain to be elucidated and warrant mechanistical studies into the individual structure-response relationships of different fiber types. The effects on the immune system are suggested to play an important role in dietary fiber mediated health effects. As a vital component of the body, the immune system is responsible for homeostasis by defending the body against pathogens. In addition, it is charged with the duty of maintaining a peaceful co-existence with the commensal microorganisms located in our intestines. The intestines can be considered as the most important immunological organ of the body as it harbors around 70 percent of our total immune cell population. It is constantly active in analyzing the status of the gut lumen by screening for 'good' or 'bad' signals in the form of commensal microbiota species vs. pathogenic organisms and toxins. Besides through continuous action of specialized immune cells, a tightly interconnected monolayer of epithelial cells prevents the external environment of the lumen from protruding into the surrounding tissue of the lamina propria. Continuous leakage of luminal content into surrounding tissue and into the periphery due to a defective epithelial barrier is an unwanted phenomenon, called 'leaky gut syndrome'. Several diseases have so far been linked to a leaky gut, and it is expected that more will follow, indicating the importance of maintaining the epithelial barrier function for disease prevention. Functional foods like prebiotic dietary fibers are aimed at stimulating a healthy gut microbiota composition by selectively stimulating lactobacilli and bifidobacteria, which coincides with stimulating host health. In addition to the well-known indirect health effects via these bacteria and their SCFA products, we propose that there is a third mechanism by which dietary fibers can cause immune modulation, i.e. by directly activating membrane receptors on intestinal immune cells and epithelial cells. In this thesis, the call for mechanistic studies on individual fiber types and their direct effects on the immune system and intestinal cell barrier function is addressed. In order to study

relevant parameters, first a technology platform needed to be established, which was done by using a well-studied type of dietary fiber, i.e. chicory root derived inulin-type fructans. After the extraction process of these fibers from chicory, the remaining root pulp is mainly used as a high fiber livestock feed. Due to the increased interest in dietary fibers, cellulose and pectins, two major fibers from this byproduct, were also studied using the established technology platform to evaluate their health effects for the potential use in functional food products.

7.1 Direct immunomodulatory effects of inulin-type fructans

In **Chapter 2** we demonstrate for the first time the proof of principle that inulin-type fructans directly activate immune cells. We found that signaling is dependent on the TLR adapter molecule MyD88, and therefore that this type of signaling is mediated via TLRs. In particular, TLR2 demonstrated dose-dependent activation upon cellular stimulation with inulin-type fructans, suggesting that this is the most important TLR by which inulin-type fructans induce immune signaling. Strikingly, the higher the average chain length of the formulation, the stronger the TLR2 response as measured by NF-κB activation. Although receptor studies specifically aimed at dietary fibers are still scarce, PRRs appear to be involved in these type of interactions, probably mimicking their responsiveness towards oligo-, or polysaccharides which occur in the form of pathogen associated molecular patterns. Examples of these interactions known so far are beta-glucans which are recognized by dectin-1 and complement receptor 3, and also cause immune modulation [1], beta-galactosides which are recognized by galectins [2], Mannose receptors on macrophages recognizing mannan oligosaccharides [3], and DC-sign which binds to Lewis Antigen glycans [4]. Broadening screening experiments by including these additional receptors and more types of dietary carbohydrate polymers and oligomers will provide additional information in future dietary fiber studies on their direct immunomodulatory properties. Blocking of TLR2 on human PBMCs was performed to study whether TLR2 was the most important receptor inducing the IL-10/ IL-12 cytokine responses in these cells (results not shown); however, the results showed much interindividual variation in the induced cytokine pattern, suggesting that the interactions of fructans with PBMCs could be more complex as compared to THP1 cells. PBMCs comprise several very different cell types, therefore a suggestion to get a better insight into the effects of fructans on individual cell types, may be to use cell sorting to

first separate the cell types and evaluate the induced immune effects per specialized cell type.

7.2 Direct immunomodulatory effects of other chicory root dietary fibers

To address whether other dietary fibers are also capable of activating PRRs, we studied cellulose (**Chapter 4**) and pectins (**Chapter 5**), which are two major components of chicory root pulp. We found that both fiber types induced NF-κB activation in THP1 cells, but the activation by cellulose was partly mediated through TLR/MyD88-dependent and partly through TLR/MyD88-independent signaling pathways. Other PRRs expressed by the THP1 cells and also signaling to NF-κB are likely to cause the TLR-independent effects and require further mechanistic studies. Candidate PRRs to study would be in the categories of C-type lectin receptors, RIG-like receptors, galectins, and other innate immune receptors known to interact with carbohydrate polymers and oligomers. The activating capacity of cellulose in itself was striking however, as many researchers apply cellulose as a placebo under the assumption that it is an inert compound which passes through the gastrointestinal tract without affecting the intestines. In follow up of these unexpected results, we performed a microchip array and identified several genes in the NF-κB-, and TLR pathways which were modulated in human PBMCs upon stimulation with cellulose. Subsequent reporter experiments also confirmed TLR2-mediated NF-κB activation as well as a biphasic dose-related pattern of TLR4-mediated NF-κB activation. Compared to inulin-type fructans, the TLR activation by cellulose could be categorized as moderate, but these results show promise for further experiments and the set up of chicory derived cellulose supplementation studies.

The activation by pectins did prove to be fully MyD88-, and TLR-dependent, as the pectins induced no activation of the applied MyD88 deficient THP-1 cell line. Contrary to cellulose, pectins have been studied extensively for their health promoting and immunomodulatory effects, but also for pectic compounds the structure-function relationships and actual target receptors were not fully elucidated so far. Depending on their structural properties, pectins were capable of mounting a TLR2 response similar to long chain inulin-type fructans, indicating a strong and specific interation with this receptor. Pectins induced moderate TLR4 activation, however this was much less pronounced as compared to the activation of TLR2 and may have been due to interference of residual endotoxin traces in the original compound. On the other hand, if endotoxin is not selectively digested by the applied enzymes then the

complete abrogation of activation of THP1 cells by the pectins strongly suggests that the TLR4 activation as observed in the HEK cell lines was in fact not endotoxin related. To make a general statement about TLR4 activation by pectins, a broader range of pectin types should be studied, and the endotoxin content of the applied fibers should be carefully measured and documented in future studies.

Ongoing experiments comprise characterization of immune and barrier effects of the remaining dietary fibers of which chicory root is comprised, such as the hemicellulose xyloglucan and acetylated pectins, to reveal the full potential of individual chicory root pulp components, and also encompass the study of the chicory root pulp when it is applied as a whole in a nutritional supplement.

7.3 Structure-function relationships: Chain length

We aimed to elucidate whether and which structural properties of chicory root dietary fibers can modulate cellular immune responses (**Chapter 2**). One of these structural properties is fiber chain length. Upon studying inulin-type fructans, a specific chain length-dependency of immune responses was demonstrated, as the short chain-enriched inulin-type fructans induced a high, anti-inflammatory IL-10/IL-12 ratio in human PBMCs and the longer the average chain length of the inulin-type fructan formulation, the lower this ratio. A possible explanation for the difference in IL-10/IL-12 ratios can be deduced from our TLR reporter assays. Chain length-induced differences in activation, as observed in the experiments with inulin-type fructans, might be due to mechanistic differences in receptor interactions at the cellular surface by clustering smaller or larger numbers of the relevant receptors on the membrane, thereby creating a molecular complex which enhances signal transduction or alters the downstream outcome. This clustering mechanism has been described for LPS, clustering substantial numbers of TLR4 [5] and may be a relevant mechanism for other TLRs as well. A second explanation for the observed differences may be the interacting capacity of inulin-type fructans with cell membrane lipids in the membrane which was studied by Vereyken et al. [6]. Strikingly, the dynamics of these interactions are also chain length-dependent [7-9], and it may therefore be a plausible mechanism to explain different signaling outcomes.

Another explanation for differences in TLR2-mediated signaling can be that TLR2 is known to be a promiscuous receptor which can partner with a spectrum of different co-receptors [10]. As a consequence, the outcome of TLR2 activation can be highly dependent on the type of co-

receptor which is involved [10]. Two important co-receptors of TLR2 are TLR1 and TLR6, which are both present on the HEK reporter cells which were applied in our studies, as well as on human PBMCs. Activation of TLR2 which is dimerized to TLR1 is considered to induce mostly pro-inflammatory signals [11], whereas the activation of TLR2 which is dimerized with TLR6 is more likely to induce an anti-inflammatory response [12]. The applied reporter assays in our studies are based on PRR-mediated NF-κB activation because NF-κB is an essential transcription factor involved in TLR-mediated innate immune responses against pathogens [13,14]. As such, the activation of NF-κB is a reliable read out for TLR-mediated activation. Nevertheless, TLR2 is also known in some cases to not only activate NF-κB, but also other transcription factors, including cAMP response element (CREB), which is involved in IL-10 expression [15,16]. Our results tend to suggest that short chain inulin-type fructans induce NF-κB but also other, more anti-inflammatory transcription factors, and that long chain inulin-type fructans destinctly and very strongly activate NF-κB in particular. Future studies with immune cell transcription factor screens of in vitro stimulated cells could provide confirmation of this explanation. Although quite specific mechanistic results were already acquired, at the same time these results call for further biochemical characterization of the interactions and dynamics of inulin-type fructans with membrane receptors and lipids.

Literature reports regarding chain length dependent effects on the immune system or barrier function induced by other dietary fibers are relatively scarce. Oligosaccharides, which can be characterized as relatively short chain dietary fibers are often studied for their prebiotic and allergy-preventing properties in infants. The general idea behind the application of oligosaccharides in infant nutrition is to mimic the oligosaccharides which are present in breat milk, as these are strongly involved in the stimulation of a healthy intestinal microbiota and development of the immue system in infants. Oligosaccharides can be derived from a range of different backbone structures. Galactooligosaccharides (GOS) for example are often supplemented in a combination with inulin-type fructans and with or without (acidic) pectin oligosaccharides (pAOS). The typical mixture often applied in baby formula is comprised of a 9:1 ratio of GOS [17], which are short chain fibers with a DP ranging from 3 to 10, and long chain inulin, which ranges up to DP60. Since the allergy preventive effects of these infant supplements are aimed at stimulating the maturation of the immune system through beneficial bacteria, and skewing it into a relatively more Thelper1 oriented direction

and away from Thelper2 reactions which are allergy related [18,19], it would be highly interesting to know whether besides through prebiotic action, the TLR2 stimulation of immune cells and epithelial cells of infants is also involved in this beneficial skewing effects of dietary fibers, and inulin-type fructans in specific.

7.4 Structure-function relationships: Methyl esterification

In the study of pectins we applied commercially available lemon pectins to confirm the principle of immunomodulatory properties of pectin **(Chapter 5)**. In addition, these pectins are well characterized for their structure which is a linear backbone with different percentages of attached methyl groups. Literature on pectin-induced health effects describes that the amount and type of molecular groups esterified to the pectin backbone are of particular importance in determining the outcome of pectin challenges [20]. The observations that low DM (DM30) pectins only activated TLR2 at moderate concentrations, and high DM (DM56) pectins only activated TLR2 at higher concentrations confirm that pectin-induced activation is very sensitive to different experimental circumstances as also demonstrated and discussed by Popov et al. [20]. In addition, both DM30 and DM56 pectins only induced moderate activation, whereas DM74 pectins induced a relatively strong, and dose dependent TLR2 activation over the whole concentration range tested. When a comparison is drawn with the inulin-type fructan results for the strength of TLR2 activation and the anti-, or proinflammatory effects on the immune system, literature on pectin studies corroborated our results such that high DM pectins are generally characterized as immunostimulatory and low DM pectins tend to have immunoregulatory or anti-inflammatory properties [21]. A single study of pectins including cytokine analysis in pectin-stimulated PBMCs demonstrated opposite effects [22], suggesting that for a solid consensus more in vivo and in vitro experiments may still be required, however all circumstances of pectin experiments such as applied dose, duration of stimulation etc. should be carefully controlled, documented, and reported, as pectin studies can be very susceptible to these factors in changing the outcomes [20]. Other structural features of pectins which have been described in literature as an important determinants of the induced effects are the degree of acetyl groups attached to the pectin backbone or “Degree of Acetylation (DA)”, the branching and type of branches attached to the backbone, and also the molecular charge and acidity of the pectins [20]. These characteristics are interesting to include in future structure-response studies of pectins. Finally, to fully understand

the benefits of actual chicory root pectins for health, a detailed pectin content-, and structure profile of chicory root pulp is required as reported in the studies of Daas et al. [23,24], and ultra-pure pectin isolates of chicory can then be evaluated for their immunomodulatory and barrier protective effects using the technology platform we have described.

7.5 Barrier protective effects and TLR2 ligation

With the activation of TLR2 by dietary fibers in mind, and considering that TLR2 is an essential molecule for the regulation of a proper intestinal barrier function [25], we hypothesized that dietary fibers confer protection on intestinal epithelial cell barrier function via Toll-like receptor 2 (TLR2). We studied whether chain-length and methylation differences influence this process. Inulin-type fructans exerted time-dependent and chain length–dependent protective effects on the T84 intestinal epithelial cell barrier mediated via TLR2. These results suggest that TLR2 located on intestinal epithelial cells could be a target of dietary fiber–mediated health effects through modulation of the intestinal barrier function. For this particular type of fiber, the short chain formulations actually proved efficient in barrier protection whereas the long chain inulin-type fructans showed little or no protection against PMA. The effects on barrier function relating to different degree of methyl esterification (DM) of pectins were not as straightforward; 30DM and 74DM pectins induced a strong barrier protective effect, whereas 56DM pectins induced only moderate protection of T84 TEER. Cellulose was also included in these ECIS experiments, however it did not exert any effects on T84 barrier function. Results of these three fiber types raise the question why moderate activators of TLR2 such as short chain inulin-type fructans would protect the barrier function, but stronger TLR2 activators in the form of long chain inulin-type fructans did not show this effect. Moreover, as a moderate TLR2 activator, cellulose would be expected to induce protection, which was not observed. And finally, both low DM as well as high DM pectins protected the barrier function. For now, the only explanation for these apparently contradictive outcomes is that there are fundamental differences in the way these fibers interact with the cellular receptors and that they show very distinctive and compound-specific effects. Further mechanistic studies are required to explain these differences in barrier protective properties or lack thereof.

7.6 Human studies: Innate and adaptive immunomodulatory effects of inulin-type fructans

At the commencement of the first experiments documented in this thesis, The European Food Safety Authority (EFSA) described the status quo of inulin-type fructans as certainly being a prebiotic functional food, but that more studies were required to provide scientific evidence of related health benefits. Currently, anno 2015, the property of stimulating beneficial microbiota is supplemented with an accepted health claim stating that native chicory inulin beneficially effects blood glucose levels and bowel habit, but specific claims regarding stimulation of the immune system still require more scientific evidence and more studies are highly recommended. This evicence should comprise the stimulation of a functional parameter such as stimulation of vaccination efficacy. To address this call for in vivo evidence of immunomodulatory effects of inulin-type fructans we designed a human supplementation study with the aim to confirm these effects **(Chapter 6)**. The observed IL-10/IL-12 ratios suggested that application of short chain inulin-type fructans would be most suitable in situations which call for anti-inflammatory regulation of immune responses, and that supplementation with long chain-enriched inulin-type fructans would be suitable for applications where the immune system needs to be boosted in its response to pathogens. We hypothesized that long chain enriched inulin-type fructan supplementation in the critical 14 day period around the first injection of an anti-Hepatitis B surface antigen vaccination would be able to boost the adaptive immune responses against this vaccine.

Evidence confirming this hypothesis was best observed at the final sampling time point T35, which was 35 days after the start of fiber intake, 28 days after receiving the vaccination, and 21 days after the final supplement intake. Anti-HBsAg titers in plasma of supplemented subjects confirmed that in the long chain enriched inulin-type fructan group, the titer response was significantly enhanced as compared to short chain inulin-type fructans, and showed a clear increased trend ($p=0.01$) as compared to the placebo group. Moreover, the category of responders, i.e. subjects with an antibody titer above 10IU/mL counted two subjects in the long chain inulin-type fructan group vs. no responders in the placebo group or the short chain-enriched inulin-type fructan group. Inulin-type fructans have been studied in several infant vaccination trials, but in the majority the fructans were only studied in combination with GOS and/or pectic oligosaccharides. In one study though, long term supplementation with a mixture of oligofructose/inulin, i.e. short chain inulin-type fructans

combined with long chain inulin-type fructans, enhanced vaccination responses. In this study Saavedra *et al.* [26] observed an increase in blood IgG levels after measles vaccination in a 10 week supplementation study with OF/inulin (7/3, 0,2 g/kg BW/d) in 7-9 months old infants. In a study by Duggan *et al.* [27] in which 6-12 month old infants were supplemented with OF, i.e. only short chain inulin-type fructans (0.7g/d), no effect was observed on antibody response after vaccination with *H. influenza* type B vaccine. Strikingly, other studies in infants with prebiotic mixtures did not induce vaccine potentiating effects [28,29]. It should be noted that the applied fructans in these mixtures are often - if not always - of a short chain nature. Although this body of evidence is still relatively small, it is tempting to speculate that the long chain inulin-type fructans are indeed more suitable to apply for the purpose of potentiating vaccination programs.

To relate our findings of increased antibody titers to changes in peripheral lymphocytes, we analyzed B cell-, T cell-, and NK cell-subsets of supplemented individuals for their percentage of the total lymphocyte population, percentage of the relevant subpopulations, and for differences in activation marker expression. Supplementation with long chain inulin-type fructans induced striking differences in T cell populations in time, the increased titer response for the long chain group on T35 was associated with an increased percentage of TBET+ Thelper1 cells compared to basal samples, and this percentage was also significantly increased compared to all time point measurements of the placebo group. Although increased in time, at T35, the percentage of CD294+ Thelper2 cells was smallest in the long chain enriched inulin-type fructan group, compared to the short chain enriched inulin-type fructan group, and placebo group. These results suggest that in the long chain group a shifted Th1/Th2 balance was induced which was skewed towards Th1 cell responses. These results are corroborated by literature reports mentioned in **Chapter 1**, which demonstrate that inulin-type fructans can exert stimulation of Thelper 1 reactions and prevent Thelper2 mediated allergic responses.

The induction of memory cells is important in the efficacy of vaccinations, as these cells are responsible for fast recognition of a second encounter with the relevant antigen and mounting an adequate immune response [30]. The percentage of memory $CD45RO^{hi}$ Th cells was increased for the long chain inulin-type fructan group as well as for the placebo group at T35, but not for the short chain inulin-type fructan group. Although the in vitro results from our group described in **Chapter 2**, and

results from literature [31] led to expect increased Treg percentages at T35 in the short chain inulin-type fructan group as compared to the long chain inulin-type fructan group and the placebo group, no changes in regulatory T-cell populations were observed. Th17 populations also demonstrated no changes in time or differences due to treatment. Thus, long chain inulin-type fructans as well as the placebo stimulate Th memory cells, but this result could also be regarded from a different viewpoint, in the way that the induction of Memory cells was actually inhibited in the short chain group. The significant increase in Thelper1 cells appears to be the parameter which can be linked directly to the increased vaccination reponse as it was significantly increased compared to both the short chain group and the placebo group.

Changes in B cell populations were significant, but not as straightforward as for the T cell populations. Class-switching is one of the markers indicating activation and maturation of B cells. Strikingly in the B cell compartment, the percentage of IgM+ cells indicating the non-class switched memory B cell population was decreased for the long chain group at T35 compared to basal samples. Upon class switching, B cells can become IgD^- and IgA^+ or IgG^+. And although no significant changes were observed for the total class switched IgD^- subpopulation in the long chain group and the placebo group, the relative contribution of IgG^+ cells within the IgD^- population in fact were increased for the long chain enriched inulin-type fructan group at time point T21 as compared to basal samples. Finally, the percentage of transitional B cells which are in the process of maturation ($CD38^{hi}$ IgM^{hi}) was significantly increased in the long chain group and the placebo group for time points T14 and T21 compared to the basal samples but not in the short chain group. Increased percentages of this population indicates that B cells are activated and stimulated to differentiate into antibody producing plasma cells, which is one of functional objectives of vaccination. The fact that this population was stimulated by long chain supplementation and not by short chain supplementation is a strong evidence of the effective differences of these two supplements. NK and NKT cells did not demonstrate clear supplement dependent effects, indicating that B cells and especially T cells are more involved in the boosting of the vaccination response via dietary inulin supplementation. Future vaccination studies may also include regulatory B cells in flow cytometry analysis, as these cells may also play a role in the success or absence of anti-HBsAg vaccination reponses [32]. It would be interesting to study whether these Bregs are stimulated by short chain

inulin-type fructans as opposed to the Tregs which did not show increases in this study.

We observed clear results from this human study, and long chain inulin-type fructans are now established to have in vivo immunostimulatory properties in humans. This pioneer study can provide important clues for further studies into the mechanisms how long chain inulin-type fructan supplementation leads to potentiation of the response against the hepatitis B vaccine. Generally, the antigens of an intramuscular vaccination such as the applied hepatitis B vaccination, are thought to be detected by circulating dendritic cells, which then recruit other immune cells and migrate towards a draining lymph node, where antigen is presented to B-, and T cells followed by a primary immune response [30]. The exact mechanism by which inulin-type fructan supplementation boosts this process remains to be studied, but can include effects on the gut microbiota and SCFA, as well as effects on dendritic cells circulating through the intestine and the periphery.

Because of the natural surveillance function exerted by DCs, they circulate through the body and mount immune responses against antigens which are encountered. Ligation of innate immune receptors on DCs followed by antigen presentation toward effector cells, such as B cells, T cells, and NK cells locally, or in specialized lymfoid structures could be a mechanism which explains the induction of pro- and anti-inflammatory cytokines as observed in many prebiotic studies. Due to the fact that DCs can ‘sample’ the gut lumen and they are migratory cells, they are one of the candidate cell types to mediate dietary fiber-induced immune effects occurring in the periphery. The role of dendritic cells in these processes are still to be confirmed, which should start with in vitro culture of cell lines which are differentiated into cells with a dendritic phenotype and analysis of the effects of inulin-type fructans on the cytokine production, the antigen presentation potency of these cells towards effector cells and the skewing of effector cell responses. In addition, barrier measurements in vivo are neccesary to corroborate the statements made in **Chapter 3**; these experiments are relatively easy and non-invasive for test subjects, as they can be performed by means of lactulose-mannitol tests during dietary supplementation trials. These tests are highly recommended for future dietary fiber supplementation studies.

7.7 Future perspective

The studies presented in this thesis demonstrate that chicory root dietary fibers exert direct effects on immune cells and intestinal epithelial cell

barrier function. We demonstrate that not only the primary product of chicory root, i.e. inulin-type fructans, posses valuable nutritional value but that this also holds true for fiber components of the remaining chicory root pulp after extraction of the inulin. Moreover, the effects of inulin-type fructans do not only comprise modulation of innate immune responses but can range as far as adaptive responses in the periphery. We describe a technology platform of in vitro studies and human studies which can be applied for future evaluation of beneficial effects of many more dietary fibers, or other nutritional compounds of interest.

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