CHAPTER 8

GENERAL SUMMARY

Current consensus on a healthy diet is that sufficient daily dietary fiber intake can protect us from developing several diseases. The exact mechanisms by which this protection occurs are currently unknown but the effects on the immune system are suggested to play an important role. In addition to the indirect immune effects elicited by lactid acid 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 immune cells and epithelial cells. 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. The studies described in this thesis were aimed specifically to identify and characterize direct effects of different dietary fibers from chicory root. The acquired knowledge of chicory root dietary fibers can be applied in designing a healthy daily nutritional regimen and provides us with the tools to design chicory-derived functional foods for specific immune boosting purposes.

Chicory root has been known for its high content in one of the best characterized prebiotic fibers, inulin-type fructans. **Chapter 1** provides a literature overview of the immune mediated health effects of inulin-type fructans. From studies that applied these fibers in colon cancer, chronic inflammatory diseases, vaccination efficacy, and prevention of infection and allergy, a large body of evidence with a range of immunomodulatory effects was gathered. These fructans are potent immunomodulating food components that hold many promises for prevention of disease. Recommended for future studies on immunological effects of inulin-type fructans and other dietary fibers is that the choice of immune markers is correlated with the particular condition that is being assessed, and the relevant clinical end-points should be clear. In case of patient studies, it should be mentioned whether immune markers are differentially expressed in disease and control populations. Due to the lack of experimental evidence, studies aimed at identifying direct effects of immune modulation through activation of gut DCs or other gut-residing immune cells are warranted. We hypothesize that by ligating receptors of the innate immune system such as TLRs, and NODs, dietary fiber supplementation can have substantial effects on the intestinal as well as the peripheral immune system. In addition, dietary fibers may also ligate PRRs expressed on gut epithelium, which could influence its barrier function.

In **Chapter 2** we describe that inulin-type fructans exert direct signaling upon contact with immune cells, and we discuss the role played by TLRs and NODs in this process. Human PBMCs were studied as model immune cells as they express many PRRs including TLRs and NODs. These PBMCs were stimulated with four inulin-type fructan formulations with different chain length profiles. Not only direct activation was observed by significant production of cytokines upon stimulation, but also that the produced cytokine patterns were dose-, and chain length dependent. Strikingly, short chain enriched inulin-type fructans induced a regulatory cytokine balance compared to long chain enriched inulin-type fructans as measured by higher vs. lower IL- 10/IL-12 ratios respectively. TLR reporter cell experiments demonstrated that fructan-induced signaling is highly dependent on TLRs and their adapter MyD88. From the follow up experiments in reporter cells overexpressing a single type of PRR per cell line, we concluded that TLR2 was prominently activated, while TLR4, 5, 7, 8, and NOD2 were mildly activated. These results indicate that inulin-type fructans indeed possess direct signaling capacity on human immune cells, and that this activation is dose-, chain length-, and predominantly TLR2 dependent.

TLR2 is an important receptor involved in intestinal barrier homeostasis. The observation that TLR2 is prominently involved in inulin-type fructan recognition prompted us to investigate whether these fibers improve or protect the epithelial barrier function. We hypothesized that receptor interactions with TLR2 on the epithelial surface are involved in inulin-type fructan mediated barrier modulation and that fructan chain length is also a factor which determines the effect on the epithelium. In **Chapter 3** we demonstrate a model for disrupted barrier function using PMA, which causes a considerable decrease in TEER. TEER is a parameter studied as a measure for tight junction–mediated barrier function. Short-chain inulin-type fructans strongly attenuated the PMA-induced decrease in TEER, whereas long chains did not exert protective effects. These findings confirmed that chain length is a clear determinant in experimental outcome in these barrier studies. Timing also proved en essential factor in the protective capacity as no effect on recovery was observed during addition of the fructans when PMA was applied first. By blocking TLR2 with an antibody, the protective effect of short chain inulin-type fructans on barrier protection was abrogated, which confirmed the involvement of TLR2 in barrier modulation by inulin-type fructans. Thus, in addition to ligating TLR2 and modulating cytokine expression in immune

cells, inulin-type fructans exert 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 both intestinal epithelial cells as well as immune cells could be a target of inulin-type fructan–mediated health effects in vivo.

Proof of principle studies confirmed direct mechanisms for inulin-type fructans in model cell types, and the established technology platform was then applied to study different fiber types. Because of renewed interest to apply fibers from chicory root byproduct (pulp) for health promoting nutrition, two major root pulp components, cellulose and pectins, were studied. In **Chapter 4** the immunomodulatory and barrier modulating capacity of cellulose is described. The transcriptome of cellulose-stimulated PBMCs was studied by gene chip array and barrier function measurements were performed with human intestinal epithelial cells. Reporter assays confirmed activation through TLR/MyD88 dependent-, and independent pathways. Cellulose induced upregulation of three NF-κB related genes, i.e. cluster of differentiation 40 (CD40) molecule, interleukin 1 receptor antagonist (IL-1Ra), and interleukin-1 receptor-associated kinase 1 (IRAK1). Five upregulated genes related specifically to TLR signaling were identified, i.e. interleukin 1 receptor antagonist (IL-1Ra), interleukin-1 receptor-associated kinase 1 (IRAK1), jun proto-oncogene, mitogen-activated protein kinase kinase 3 (MAP2K3), and mitogen-activated protein kinase 13 (MAPK13). Cellulose did not improve or protect T84 resistance. From these experiments we concluded that cellulose does not directly affect intestinal cell barrier function. However, it alters gene expression in human immune cells and activates TLR and non-TLR related pattern recognition pathways, indicating the immunomodulatory potential of cellulose as major component of root pulp byproduct.

In **Chapter 5** pectin, was studied for its immunogenic and barrier protective effects, as this is another major component of chicory root pulp. In addition, since pectins can differ in their chain length but also in the degree of methyl esterification, this was also studied as a structural feature, to evaluate possible structure-response properties of this molecular characteristic. Due to the lack of standardized commercially available chicory root pectin, and the similarity of the pectin profiles which can be extracted from lemon, these lemon pectins were studied as model pectins. Stimulation of TLR reporter cells with lemon pectins with different

degrees of methyl esterification (30DM, 56DM and 74DM), demonstrated TLR dependent activation, and an increasing TLR activation with increasing DM. The applied pectins induced TLR2 activation, and moderately activated TLR4. Upon enzymatically digesting the pectin polymers into oligomers we found that this treatment abrogated their TLR activating potential. In experiments testing the barrier function of human intestinal epithelial cell, 30DM and 74DM pectins induced a strong protective effect, while 56DM pectins induced moderate protection of T84 TEER. From these experiments we can conclude that activation of immune cells by lemon pectins is TLR2 dependent, and may also involve TLR4 ligation, and that the intact polymer backbone is indispensable for activation. Besides in activation potential, the degree of methyl esterification is also a determining factor for epithelial barrier protective effects, which were strongest for 30DM and 70DM lemon pectins.

Building on the collected in vitro results in our studies with inulin-type fructans, we hypothesized that the long chain-enriched inulin-type fructans would stimulate vaccine responses in vivo due to their predominant induction of proinflammatory cytokines, including a relatively low IL-10/IL-12 ratio, in combination with their strong ability to activate TLR2. In **Chapter 6** we describe the results of a human hepatitis B vaccination and supplementation study with different inulin-type fructan supplements. By comparing the effects of long chain fructan supplementation with short chain fructans and a placebo group on anti-HBsAg titer development in the 28 days following the first injection we observed that supplementation with long chain fructans significantly enhanced the titer response (T35 of the study) as compared to the short chain supplemented group, and that a strong increased trend was present as compared to the titer development in the placebo group. Another striking observation was the identification of two responders, i.e. with a titer above 10 IU/mL plasma, in the long chain fructans group vs. no responders in either the short chain or the placebo group.

Flow cytometry analysis of peripheral blood lymphocyte subsets of the supplemented individuals demonstrated that 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 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 a shifted Th1/Th2 balance for the long chain group towards Th1 cell responses.

Strikingly in the B cell compartment, the percentage of IgM+ cells in the non-class switched memory B cell population was decreased for the long chain group at T35 compared to basal samples. 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. 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. In conclusion, this study confirms the in vivo immunostimulatory potential of long chain enriched inulin-type fructans, and subscribes an important nutritional health claim that these fibers can be beneficial for the immune system.

The conclusions of this thesis are discussed in **Chapter 7**.