Immune modulation by dietary glucans from oat and mushrooms; results from in vitro, animal and human studies

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**Immune modulation by dietary
glucans from oat and mushrooms;
results from *in vitro*, animal and human studies**

Julia Jennifer Volman

The studies in this thesis were performed within the Nutrition and Toxicology Research Institute Maastricht (NUTRIM), which participates in the Graduate School VLAG (Food Technology, Agrobiotechnology, Nutrition and Health Sciences), accredited by the Royal Netherlands Academy of Arts and Sciences.

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**Immune modulation by dietary glucans from oat and mushrooms;
results from *in vitro*, animal and human studies**

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CHAPTER 1

General introduction

Introduction

The human body is constantly threatened by invasive pathogens, like bacteria, viruses, fungi and parasites. A wide-ranging immune system, which is described into detail in **chapter 2**, is necessary to protect the body against these attacks. This system is comprised of an innate and an adaptive part, which work closely together. Inflammation is a natural immune response from the host against harmful stimuli. Although this is a protective mechanism, an excessive or prolonged inflammatory state (chronic inflammation) may be harmful and is associated with allergies, rheumatoid arthritis, and inflammatory bowel disease (IBD) [1-3]. In contrast to chronic inflammation, the inflammatory response may also be impaired, which may lead to an increased susceptibility for infections [4]. Evidence accumulates that the composition of the diet influences the functioning of our immune system. Therefore, changing dietary compositions as a tool to improve the immune function receives more and more attention.

Immune modulation

In general, a poor nutritional status of the body is related to suppressed immunity, which may increase the susceptibility towards infections [4]. Indeed, it has been reported that well-nourished patients had fewer complications and recover faster from infection and illness than malnourished patients [5]. Enhancing the immune response can be beneficial for people with an impaired functioning of the immune system, like for example the elderly [4, 6]. Besides optimizing nutritional status by improving the quality of the diet in general, also specific components of the diet can affect the immune response. For example β -glucan, as present in various foods like cereals and mushrooms, has immune stimulating properties [7-9]. β -Glucans are a highly heterogeneous group of linked sugar molecules with different structures. Besides β -glucans, there are also α -glucans. In this respect, mushrooms are interesting since in addition to β -glucans, they also contain α -glucans [10]. The immune modulating effects of some of these glucans from different sources are evaluated in this thesis.

The mechanism often mentioned in relation to immune stimulation in general and for β -glucans in particular relates to the skewing of the immune response into T helper (Th)1 direction. Th1 immunity is important for the defence against intracellular pathogens, like viruses, whereas Th2 immunity gives protection against extracellular pathogens [11]. In the normal healthy situation there is a

balance between the Th1 and Th2 responses and imbalance may lead to diseases. For example, people suffering from allergies have an immune system that is more oriented toward the Th2 response [1], whereas IBD is related to an increased Th1 phenotype [12]. Since β -glucans induce Th1 skewing, effects of β -glucans could theoretically be especially interesting for people suffering from allergies. Also HIV-infected patients who normally have a decreased Th1 response could benefit from the β -glucan-induced Th1 skewing [13]. Overall, there are several diseases that may benefit from immune modulation through the diet.

Dietary components handled in the intestine first of all come in contact with the intestinal mucosa. Therefore, it is likely that dietary components can affect the intestinal immune system. This highly specialized part of the immune system is described in the following paragraph.

Intestinal immune system

The intestinal mucosa is continuously exposed to antigens from harmful pathogens, as well as harmless commensal microflora and nutrients at the same time. To prevent invasion of pathogens from the intestinal lumen into the body, the epithelial lining is very selective in absorption on one hand, and initiation of an immune response on the other hand. There are several mechanisms present in the intestine to support the selective function of the intestine. The first one is the physical barrier, consisting of one single-cell layer of intestinal epithelial cells (enterocytes) that prevents the invasion of pathogens present in the intestinal lumen into the body. Enterocytes are closely connected to each other by junctional complexes, mainly consisting of tight junctions [14]. The formation of the mucus coat by intestinal goblet cells creates another barrier for pathogens as it forms a stream that draws bacteria away from the epithelial lining. In addition, mucus prevents adherence of bacteria to the epithelium [14]. Another way to limit adherence and invasion of pathogens is the secretion of antimicrobial peptides by enterocytes and paneth cells [15]. Moreover, adherence of the commensal microflora to the epithelial lining blocks the access for pathogens to the epithelial surface [14]. Another aspect of the intestinal immune system is the specialized gut-associated lymphoid tissue (GALT). This system is present in the lamina propria and consists of multiple lymphoid follicles [16]. These follicles in the small intestine are called Peyer's patches. Moreover, isolated lymphoid follicles and cryptopatches are other intestinal lymphoid aggregates [17]. Peyer's patches are covered by follicle-associated epithelium (FAE), which contains microfold (M)-cells. These specialized epi-

thelial cells together with dendritic cells continuously sample the intestinal lumen and transport antigens to the subepithelial region of the Peyer's patches, where presentation and activation of T and B lymphocytes will take place [18]. Activated T and B lymphocytes then migrate to the lamina propria, where they initiate an inflammatory response, like stimulation of IgA secretion by plasma cells [16]. In addition mesenteric lymph nodes (MLN) provide a second line of defence by filtering the mesenteric lymph vessels. **Figure 1.1** gives an overview of the intestinal immune system. Enterocytes together with cells of the GALT play an important role in orchestrating the mucosal immune response.

Figure 1.1: Simplified schematic view of the intestinal immune system

Two important components of the intestinal immune system are the enterocytes that form a physical barrier of one single cell layer and the gut-associated lymphoid tissue (GALT) system consisting of various immune cells (T-cells, B-cells, dendritic cells, and intestinal macrophages) in the small intestine clustered in follicles known as Peyer's patches (PP). The Peyer's patches are covered by follicle-associated epithelium (FAE), which contains microfold (M)-cells that sample antigens. Paneth cells are located at the base of the intestinal crypt and secrete antimicrobial peptides into the intestinal lumen together with enterocytes. Mesenteric lymph nodes (MLN) provide a second line of defence by filtering the mesenteric lymph vessels.

Outline of the thesis

In this thesis the effects of dietary glucans from oat and edible mushrooms on several parameters of the (intestinal) immune response are described. After setting out an comprehensive review on the immune modulating effects of dietary β -glucans in **chapter 2**, we examined in **chapter 3** the *ex vivo* immune modulating effects of fecal water prepared from ileostomy contents of patients who had consumed an oat β -glucan diet, on the response of different enterocyte cell lines as compared to fecal water prepared after a placebo diet.

Whether the observed effects as described in **chapter 3** were mediated via the β -glucan receptor dectin-1 is the topic of the study in **chapter 4**. In addition to oat, glucans (both alpha and beta) are also present in mushrooms and the effects of different mushroom-derived glucans on nitric oxide production by bone marrow-derived macrophages and nuclear factor- κ B (NF- κ B) transactivation enterocytes are evaluated in the study presented in **chapter 5**. Moreover, we evaluated the *in vivo* effects of dietary β -glucans from oat on mucosal immune responses in man and mice (**chapter 6**), while the study presented in **chapter 7** is focused on intestinal NF- κ B transactivation after oat β -glucans in mice. Moreover, in the study described in **chapter 8** we investigated the effects of α -glucans from the edible mushroom *A. bisporus* on peripheral blood mononuclear cell function in human subjects. Finally, the results of all experiments and possible future implications are discussed in **chapter 9**.

CHAPTER 2

Dietary modulation of immune function by β -glucans

Julia J. Volman, Julian D. Ramakers, Jogchum Plat

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Abstract

The immune response can be modulated by nutrients like β -glucans, which are glucose polymers that are major structural components of the cell wall of yeast, fungi, and bacteria, but also of cereals like oat and barley. There is a lot of structural variation in the β -glucans from these different sources, which may influence their physiological functions. In this review the current status concerning possibilities to modulate immune function by β -glucans is discussed. *In vitro* as well as *in vivo* studies in animals and humans show that especially β -glucans derived from fungi and yeast have immune modulating properties. Most frequently evaluated are effects on leukocyte activity, which has been suggested to contribute to the increased resistance against infections observed after β -glucan interventions. Although most studies supply the β -glucans parenteral (e.g. intravenous or subcutaneous), also enteral administered (dietary) β -glucan influence the immune response. Although more human studies are needed, it is tempting to suggest that dietary β -glucans may be a useful tool to prime the host immune system and increase resistance against invading pathogens.

Introduction

There is a constant threat of invasive pathogens attacking the human body. Our immune system composed of two mutually interactive systems, i.e. the innate and adaptive immune system, protects the body against these invasions. Evidence accumulates that the composition of the diet influences the functioning of our immune system. Therefore, changing dietary compositions as a tool to improve the immune function, currently receives a lot of attention. β -Glucans, as present in various foods such as cereals and mushrooms, are currently under investigation for this purpose. The objective of this review is to give an overview of the immune modulating properties of (dietary) β -glucans. Therefore we start with a brief overview of the immune system and β -glucans. Next, the results of immune modulation by *in vitro*, parenteral and enteral administrated β -glucans are described. After that the mechanism by which dietary β -glucans may work will be discussed followed by giving examples of potential applications.

Immune system

The immune system protects the human body against invasion of pathogens (i.e. bacteria, viruses and parasites), which is the result of two mutually interactive systems: the innate and adaptive immune system (**figure 2.1**). Epithelial barriers like the skin and the linings of the gastrointestinal tract, lungs and urinary tract are the first line of defence of the innate immune system. The innate immune response depends largely on the recognition of conserved microbial structures (pathogen associated molecular patterns, PAMPs) by so-called pattern recognition receptors (PRR). The toll-like receptors (TLRs) and dectin-1 are two examples of PRRs. Examples of PAMPs are cell wall components, such as lipopolysaccharide (LPS) from gram-negative bacteria, and β -glucan from fungi and yeast. Leukocytes that are involved in this early innate response are granulocytes (a.o. neutrophils), monocytes / macrophages and natural killer (NK) cells. These cells play an important role in phagocytosis of pathogens, free radical production (oxidative burst), cytokine production, and antigen presentation to lymphocytes. The innate immune response is rapid and functional, but non-specific. In contrast, the adaptive immune response is somewhat slower, but highly specific. This response depends on 1) the production of antibodies (immunoglobulin's (Igs)) by B-lymphocytes directed against specific antigens present on pathogens (called humoral immune response) and

on 2) the attack of infected body cells by cytotoxic and helper T-lymphocytes (called cell-mediated immune response). In addition, due to the presence of memory cells, the adaptive immune response is characterized by enhanced and fast responses after repetitive contacts with the same antigen. Ultimately, an immune response is the result of these two interacting systems. After leukocytes of the innate immune system are activated, they produce cytokines and present antigens to T- and B-lymphocytes, which in turn activates the adaptive immune system [19, 20].

Figure 2.1: Simplified schematic overview of the immune system
Abbreviations: Tc-cells, cytotoxic T-lymphocytes; Th-cells, helper T-lymphocytes.

Cytokines are signalling proteins secreted by a wide variety of cell types and used for inter-cell communication. The types of cytokines produced determine whether a naïve helper T-lymphocyte (Th0) develops into a type 1 helper T-lymphocyte (Th1) or a type 2 helper T-lymphocyte (Th2). For example, interleukin (IL)-12, produced by e.g. activated macrophages, stimulates Th1 cell development. This leads to the production of typical Th1 cytokines like IL-1 β , interferon (IFN) γ , IL-2, and tumour necrosis factor (TNF) α , which play an important role in cell-mediated immunity. In contrast, IL-4, results in the development of Th2 cells, producing IL-5, IL-6, IL-10, and IL-13, which are involved in the humoral immune response. Recently, also regulatory T-lymphocytes (also defined as Th3 cells) have been described. Their role is most likely to influence the

Th1/Th2 balance by inhibiting Th1 activity by producing anti-inflammatory cytokines like transforming growth factor (TGF) β and IL-10 [11].

The most important regulator in the immune response is the transcription factor nuclear factor- κ B (NF- κ B). As already mentioned immune cells can be activated directly by binding of PAMPs from pathogens to PRRs or indirectly by binding of cytokines produced by other cells to their respective receptors. After ligand receptor binding, a cascade of intracellular signalling events occurs, ultimately leading to activation of NF- κ B. In resting cells, NF- κ B is present in the cytoplasm, bound to inhibitor κ B (I κ B), a complex that inactivates NF- κ B. After cellular activation, I κ B kinase is activated, which in turn phosphorylates I κ B. This phosphorylation is followed by ubiquitination and degradation of I κ B. Next, I κ B-free NF- κ B migrates from the cytoplasm into the nucleus, binds to response elements and induces gene transcription. NF- κ B activation induces transcription of genes encoding for inflammatory proteins like cytokines and chemokines and induces free radical production [21].

β -Glucan

β -Glucans are carbohydrates consisting of linked glucose molecules, which are major structural components of the cell walls of yeast, fungi and some bacteria. Also cereals, such as barley and oat contain β -glucans as part of their endosperm cell walls. Depending on the source, there are clear differences in macromolecular structure between β -glucans (**figure 2.2**). The cell wall β -glucans of yeast and fungi consist of 1,3 β -linked glycopyranosyl residues with small numbers of 1,6 β -linked branches. In contrast, the oat and barley cell walls contain unbranched β -glucans with 1,3 and 1,4 β -linked glycopyranosyl residues, whereas β -glucans from bacterial origin are unbranched 1,3 β -linked glycopyranosyl residues [7, 8, 22]. Furthermore, besides differences in type of linkage and branching, β -glucans can vary in solubility, molecular mass, tertiary structure, degree of branching, polymer charge and solution conformation (triple or single helix or random coil). All these characteristics may influence their immune modulating effects. For example, Brown and Gordon [8] have recently suggested that high molecular weight (MW) and/or particulate β -glucans from fungi directly activate leukocytes, while low MW β -glucans from fungi only modulate the response of cells when they are stimulated with e.g. cytokines.

β -Glucan type	Structure	Description
Bacterial		Linear β 1,3 glucan (i.e. Curdlan)
Fungal		Short β 1,6 branched, β 1,3 glucan (i.e. Schizophyllan)
Yeast		Long β 1,6 branched, β 1,3-glucan (i.e. WGP β -glucan, Betafectin™)
Cereal		Linear β 1,3/ β 1,4-glucan (i.e oat, barley, rye)

Figure 2.2: Structure of cereal and yeast β -glucan and scheme of various β -glucan sources

With respect to the characteristics of the β -glucans, it should be noted that the isolation method may influence these characteristics. Consequently, differences can be expected between various β -glucans differentially isolated from the same source. **Table 2.1** lists various, common used, β -glucans from different sources and their structures.

Table 2.1: Various, commonly used, β -glucans from different sources and their structures^{ab}

Name	Source	Source type	Structure
Glomerellan	<i>Glomerella cingulata</i>	Fungus	1,3 1,6 branched
GRN (grifolan)	<i>Grifola frondosa</i> (Maitake mushroom)	Fungus/mushroom	1,3 1,6 branched
LNT (Lentinan)	<i>Lentinus</i> (lentinula) edode (shiitake mushroom)	Fungus/mushroom	1,3 1,6 branched
Pneumocytis carinii	<i>P. carinii</i>	Fungus/protozoan	1,3 1,6 branched
P-SG (polysaccharide from <i>Ganoderma lucidum</i>)	<i>G. lucidum</i>	Fungus/mushroom	1,3 1,6 branched
SPG (Sonifilan/ schizophyllan)	<i>Schizophyllum commune</i>	Fungus	1,3, 1,6 branched
SR (Scleroglucan)	<i>Sclerotium rolfsii</i> or <i>S. glaucicum</i>	Fungus	1,3, 1,6 single branched
SSG (Sclerotinia sclerotiorum glucan)	<i>S. sclerotiorum</i> (ascomycotma)	Fungus	1,3 1,6 highly branched
CSBG (<i>Candida</i> spp. β -glucan)	<i>Candida Albicans</i>	Yeast/fungus	1,3 1,6 branched
Glucan phosphate (GluP)	<i>Saccharomyces cerevisiae</i>	Synthetic modified	1,3
PGG (betafectin)	<i>S. cerevisiae</i>	(Genetically engineered) yeast	1,3 1,6 highly branched
<i>Saccharomyces cerevisiae</i>	<i>S. cerevisiae</i>	Yeast	1,3 and small numbers of 1,6 branches and 1,6 linked
WGP-glucan (whole glucan particle)	<i>S. cerevisiae</i> (baker's yeast)	Yeast	1,3 1,6
Zymocel	<i>S. cerevisiae</i>	Yeast	Crude β -glucan extract
Zymozan	<i>S. cerevisiae</i>	Yeast	Crude extract with β -glucan and mannan; non-uniform branches and backbone units
Barley, oat, wheat, rye, rice		Cereal (Gramineae (grasses))	1,3 1,4 mixed linkage, unbranched
Curdlan	<i>Alcaligenes faecalis</i>	(gram negative) bacteria	1,3 unbranched
LAM (Laminarin/ Laminaran)	<i>Laminaria species</i> (e.g. <i>digitata</i>)	algae e.g. brown seaweeds	1,3 unbranched (with some branching of 1,6)

^a Unbranched β -glucans are also regarded as linear^b Yeasts and mushrooms are specific types of fungi

Immune modulating effects of *in vitro* and parenteral administrated β -glucans

In vitro and animal studies have shown that 1,3 β -glucans from yeast and fungi are able to enhance the responsiveness or function of immune cells, whereas only a limited number of human studies have been carried out. In the next paragraphs the results of these studies will be discussed in more detail.

In vitro studies

β -Glucans can enhance the functional activity of macrophages and activate antimicrobial activity of mononuclear cells and neutrophils *in vitro* [23-25]. This enhanced immune response is accomplished by an increased pro-inflammatory cytokine production [26-28], oxidative burst, and chemokine production [23]. Olson *et al.* [26] demonstrated that fungal *Saccharomyces cerevisiae* β -glucan increased *in vitro* TNF α production by alveolar macrophages isolated from rats. Also the β -glucan rich yeast particle zymosan – that has often been used to study β -glucan effects – stimulated macrophages to secrete TNF α [22]. In addition, human whole blood incubated with soluble yeast β -glucan showed an enhanced production of TNF α , IL-6, IL-8 and monocyte tissue factor (TF). Furthermore, when LPS was added together with this β -glucan TNF α , IL-8, IL-10 and TF concentrations were strongly increased, whereas no further increase in IL-6 concentration was observed. In addition, pre-incubation with β -glucan increased the LPS-induced productions of these parameters [29]. Besides β -glucans from yeast and fungi, also β -glucans from oat show these immune modulating effects *in vitro*. Estrada *et al.* [7] have demonstrated an increased IL-1 α production by murine macrophages in the presence of oat β -glucan *in vitro*, while spleen cells showed an enhanced IL-2, IFN γ and IL-4 secretion.

Poly-[1-6]-D-glucopyranosyl-[1-3]-D-glucopyranose (PGG)-glucan from yeast increased the activity of leukocytes against various pathogens. Indeed, PGG-glucan increased migration of neutrophils *in vitro* toward C5a, whereas chemotaxis toward IL-8 was suppressed [30]. Furthermore, pre-incubation of whole blood with PGG-glucan increased the oxidative burst of *in vitro* activated isolated leukocytes. However, PGG-glucan induced immune stimulating responses without effects on inflammatory cytokine production [31]. In addition to macrophages and neutrophils, also the function of dendritic cells can be influenced by β -glucans, since it has been reported that the fungus β -glucan P-SG induced a Th1 type of cytokine response by human dendritic cells *in vitro* [32].

Moreover, besides leukocytes, also epithelial cells can respond to β -glucans, as alveolar epithelial cells isolated from rats secrete the chemokine macrophage inflammatory protein (MIP)-2 after *in vitro* culturing with *Pneumocystis carinii* β -glucan [33]. In contrast to the increased immune response as outlined above, Nakagawa *et al.* [34] reported a dampened LPS-induced IL-6, IL-2 and IFN γ production of cultured human peripheral blood mononuclear cells (PBMCs) when incubated with *Candida* spp. β -glucan (CSBG). However, when monocytes were depleted from the cultured PBMCs the decreased production of IL-2 and IFN γ disappeared. Therefore the authors suggest that the monocytes were responsible for the suppressed production of the Th1 type cytokines IL-2 and IFN γ . When cultured PBMCs were incubated with CSBG without lipopolysaccharide (LPS) stimulation, IL-6 production was only slightly increased [34]. Altogether, it can be concluded from these *in vitro* studies that β -glucans from various sources overall enhance the immune response of leukocytes and epithelial cells, mainly by modulating the cytokine production.

Animal studies

Besides *in vitro* studies, numerous animal studies have been conducted to evaluate the immune modulating effects of β -glucans. Most studies have been carried out with isolated leukocytes from animals that were treated with β -glucan supplied via various routes. Subsequently these isolated cells were challenged with LPS or pathogens (*ex vivo*). In general, these *ex vivo* stimulated leukocytes from the treated animals showed an increased pro-inflammatory cytokine production [28, 35], oxidative burst [36] and chemotaxis [37]. There are indications that the β -glucan induced response is in fact a Th1 specific response, since splenocytes from mice treated with bacterial 1,4 β -glucan, a polysaccharide from *Ganoderma lucidum* (PS-G), and *Sclerotinia sclerotiorum* glucan (SSG) show an increased ovalbumin-induced IFN γ production [32, 38, 39], whereas IL-4 [38, 39] and IL-5 [32] were decreased. Suzuki *et al.* [39] also reported that isolated splenocytes from SSG-administered mice showed an increase IgG2a production and a decreased IgG1 production. Since IgG2a responses are induced by IFN γ and suppressed by IL-4, whereas IgG1 production is inhibited by IFN γ and stimulated by IL-4, these antibodies responses are respectively regarded as Th1 and Th2. Overall, these studies indeed suggest that β -glucans induce Th1 specific immune responses. In agreement with *in vitro* studies, PGG-glucans showed no stimulating effects on *ex vivo* cytokine production, since LPS-stimulated lymphocytes and monocytes isolated from PGG-glucan treated mice produced less pro-inflammatory cytokines [40]. Further-

more, the oxidative burst activity of neutrophils was increased in rats, without effects on TNF α and IL-1 β levels [41].

Besides *ex vivo* effects of β -glucans, also *in vivo* effects of a variety of β -glucans have been reported on the response towards pathogen infections in animals. In general, these studies showed an increased microbial clearance and a reduced mortality of lethally infected animals [42-45]. For example, subcutaneous injection of yeast β -glucan (PGG-glucan) and whole glucan particle (WGP) β -glucan increased survival rate, diminished bacterial load in the lungs and increased the proportion of bacteria-free animals after infection with anthrax in mice [46]. In addition, bacterial counts in blood of *Staphylococcus aureus* challenged rats treated intramuscular with PGG-glucan were lower than in control rats, and also the number of monocytes and neutrophils were increased [41]. Bedirli *et al.* [45] found an increased survival rate after intramuscular β -glucan (Sigma) treatment of rats in an experimental model of sepsis, however the elevated TNF α , IL-1 β and IL-6 concentrations in the blood were blocked by β -glucan. The authors suggest that the protective effects of β -glucan were due to a dampened inflammatory response *in vivo* [45]. Whereas most studies showed an increased *in vitro* and *ex vivo* cytokine production after β -glucans, these cytokine effects *in vivo* might be dampened because of feedback mechanism, that protect the host against excessive inflammatory responses. Besides fungal and yeast β -glucans, intraperitoneal injection of β -glucans from oat also enhanced survival after bacterial and parasitic infection in mice [7, 47]. In contrast to the studies mentioned above, Dritz *et al.* [48] found no effect on neutrophil or macrophage function of weanling pigs and instead an increased susceptibility to bacterial infection after a β -glucan (MacroGard-STM) diet.

In general, *ex vivo* responses of leukocytes isolated from β -glucans treated animals are in line with *in vitro* results. When animals were treated with β -glucan this ultimately translated into an enhanced survival against pathogen infections.

Human studies

Effects of various β -glucans have been examined *in vitro* and in several animal models, while only a few human studies have been carried out. Three clinical studies demonstrated that pre-treatment of high-risk surgical patients with PGG-glucan supplied intravenously (1) decreased the infection incidence and need for antibiotics, (2) shortened intensive care unit length stay, and (3) ultimately improved survival compared to a saline placebo injection [49-51]. In another study, a soluble yeast β -glucan isolated from *S. cerevisiae* - also sup-

plied intravenously - decreased overall mortality and septic morbidity compared to a not further specified placebo injection in trauma patient's [52, 53]. Besides effects on clinical parameters, almost no effects on cytokine concentrations and immunoglobulin concentrations in humans have been reported. Only 3 days after trauma serum IL-1 concentrations were elevated in β -glucan treated trauma patients as compared to control treated patients, while TNF α concentrations remained unchanged [53]. Also Lehne *et al.* [54] found no difference in cytokine and immunoglobulin concentrations in the blood of humans after 5 days treatment with three different concentrations of yeast β -glucan (SBG) compared to baseline, although IgA concentrations were increased in saliva, only when using a high dose of β -glucan. To summarize, the protective effects of β -glucans on survival after pathogen infection in humans are in agreement with effects observed in animals. Furthermore, the finding that clinical parameters are improved without a severe elevation in concentrations of circulating cytokines and immunoglobulin's suggest an immune regulatory function of β -glucan that goes further than simply immune activation, as they may prime instead of activate leukocytes *in vivo* to combat invading pathogens.

Immune modulating effects of enteral administered β -glucans

Although the previous section may give the impression that only parenteral administration of β -glucans protects against pathogen infections, a number of reports have shown that enteral administration of β -glucans (further mentioned as dietary β -glucan) may also have biological effects. This implies that the addition of β -glucans to the diet may be used to modulate immune function and by that way might improve the resistance against invading pathogens in humans. Besides intraperitoneal or subcutaneous injection of oat β -glucans, also intragastric administration of oat β -glucans in mice enhanced resistance to bacterial and parasitic infections [47, 55, 56]. Furthermore, Davis *et al.* [57] demonstrated that daily ingestion of oat β -glucan counteracted the decrease in macrophage antiviral resistance induced by exercise stress in mice. Besides oat β -glucans also other β -glucans have been supplied orally and showed immune modulating activities. Rice *et al.* [58] found an increased survival in mice challenged with *S. aureus* or *Candida albicans* after oral administration of glucan phosphate (GluP). Furthermore, Kournikakis *et al.* [46] showed that orally administered WGP β -glucan increased survival in mice challenged with the anthrax bacteria.

Also after oral administration, effects on immune cell functions have been reported. Phagocytosis, bactericidal killing and oxidative burst of isolated heterophils (neutrophil like cells in chicken) were all increased in immature chickens fed a purified β -glucan of an unknown source, ultimately showing an increased protection against *Salmonella enterica* organ invasion [59]. The oxidative burst (NO) of *ex vivo* LPS-stimulated isolated peritoneal adherent cells from mice was also increased after oral administration of β -glucans from *S. cerevisiae* [36]. Li *et al.* [60] reported an increased LPS-induced IL-6 and TNF α production *in vivo* in the blood from weaned pigs after oral *S. cerevisiae* β -glucan supplementation. In contrast, another study by the same group with nearly the same design showed a decreased IL-6 and TNF α production in response to LPS, whereas IL-10 concentrations were increased. Moreover, the specific antibody response to ovalbumin in the first week after injection was increased [61]. This study does not specify the type of antibody that is produced, which is important regarding Th1 or Th2 phenotypes. In this respect, Saito *et al.* reported a decreased *in vivo* production of ovalbumin-specific IgG2a antibodies in mice after oral treatment with soluble branched bacterial 1,4 β -glucan, whereas the concentration IgE and IgG1 antibodies was increased [38]. This indicates that β -glucan induces a Th1 response, which is in agreement with the results of *ex vivo* animal studies. Intragastric administration of SSG showed an enhanced functionality of peritoneal macrophages in mice [62]. In addition, oral administration of SSG influenced the function of cells of the Peyer's patches, which in their turn stimulated the *in vitro* lysosomal enzyme activity of alveolar macrophages. Alveolar macrophages isolated from the SSG treated mice also showed increased phagocytic activity and IL-1 production, whereas hydrogen peroxide production was not affected. Moreover, also the number of alveolar macrophages increased. The authors suggested that after SSG administration via the oral route, the alveolar macrophages were stimulated by activation of cells of the Peyer's patches [63]. Also Tsukada *et al.* [64] reported an increased number of intraepithelial lymphocytes in the intestine after oral administration of β -glucan in mice.

It can be concluded that oral administration of β -glucan is as effective as parenteral administration to protect against pathogen infections. Moreover, the effect seems independent of the source of β -glucan used, although there have been no careful side-by-side comparisons of β -glucans obtained from different sources. Therefore, enriching our diet with β -glucans might be relevant in terms of enhancing our daily protection against pathogens, although further human studies are needed. In this respect it is important to mention that in animal feeding β -glucan is already applied as a food additive to strengthen the immune response [65]. In addition, the idea of β -glucan as a food additive is

supported by Ikewaki *et al.* who recently showed that an already available health food supplement (Sophy β -glucan) has immune modulating properties in human PBMCs *ex vivo*. [66]

Mechanism underlying the effect of dietary β -glucans

After oral administration β -glucans come in contact with the mucosal immune system (**figure 2.3**). The intestinal epithelial cells together with the immune cells of the Peyer's patches play an important role in regulating immune responses. As indicated in the previous section, oral administration of β -glucans can modulate the mucosal immune response by cells of the Peyer's patches as well as intestinal intraepithelial lymphocytes [62, 64].

Figure 2.3: Simplified schematic view of the intestinal immune system

Two important components of the intestinal immune system are the enterocytes that form a physical barrier of one single cell layer and the gut-associated lymphoid tissue (GALT) system consisting of various immune cells (T-cells, B-cells, dendritic cells and M-cells) in the small intestine clustered in follicles known as Peyer's Patches (PP).

It has been suggested that the protective effects of orally administered 1,3 β -glucans are mediated through receptor-mediated interactions with Microfold (M)-cells – specialized epithelial cells for the transport of macromolecules – in

the Peyer's patches, which lead to increased cytokine production and enhanced resistance to infection [62].

Besides these direct effects on immune function in the intestine it has also been suggested that orally administered barley and yeast WGP β -glucans are taken up by intestinal macrophages and then transported to lymph nodes, spleen and bone marrow. Whether the β -glucans are shuttled to these organs via the lymph or blood compartments has not yet been established. In the bone marrow, it has been described that β -glucans were degraded by macrophages and taken up by bone marrow granulocytes. Since *in vitro* experiments have shown that degraded β -glucan particles were released in the medium [67], this may imply that *in vivo* they could be released into the circulation to induce systemic effects. This assumption suggests that orally administered β -glucans reach the circulation in low amounts. However, the amount of dietary β -glucan that appears in the circulation by this route is lower than after subcutaneous administration [68]. Rice *et al.* [58] suggested that especially β -glucans with a high molecular mass (>2 MDa) may be taken up by M-cells from the intestinal lumen to the underlying lymphoid tissue. *In vivo* effects of β -glucan may depend on their molecular weight, which might be caused by differences in uptake from the intestinal lumen.

Also effects on non-immune cells have been shown [33]. Regarding dietary immune modulation particularly effects on enterocytes are interesting. In this respect we have earlier demonstrated in an *in vitro* study that various enterocyte cell lines showed an enhanced immune response when incubated with fecal water prepared from ileostomic contents obtained from subjects who had consumed an oat β -glucan diet versus a placebo diet [69].

Irrespective if β -glucans are absorbed or not, there seems to be general consensus that the reported immune modulating effects of fungal and yeast β -glucans are dectin-1 dependent although also other receptors might play a role. Alternative receptors involved may be TLR2 and TLR6, NO3 (CR3), scavenger receptors or lactosylceramide [70]. The dectin-1 receptor (in humans also called the β -glucan receptor (β GR)) is highly expressed on immune cells, such as dendritic cells, neutrophils, eosinophils, macrophages, monocytes, and some T-cells and in humans also on B-cells. Also intestinal cells might express dectin-1 in lower amounts, although this expression is under debate [42, 71].

Upon β -glucan binding to dectin-1, a cascade of intracellular signals is initiated which will ultimately lead to NF- κ B activation. Indeed several studies reported that various β -glucans activate NF- κ B *in vitro* as measured by EMSA in leukocytes [27, 31, 72, 73] and in alveolar epithelial cells [74]. NF- κ B activation initiates various processes – also described to be modulated by β -glucans – such as cytokine production, phagocytosis and respiratory burst. Confirmation of the

crucial role for dectin-1 in the effects of β -glucans comes from recent studies with dectin-1 deficient mice. Taylor *et al.* [75] showed that these mice are more susceptible to fungal infections with *C. albicans* than wild-type mice, whereas, Saijo *et al.* [76] demonstrated similar findings for protection against *P. carinii*. Interestingly, they found no difference in susceptibility between dectin-1 deficient mice and wild-type mice against *C. albicans*. This discrepancy may be explained by the use of different strains of *C. albicans* and the genetic background of the mice. These studies do however not provide insight which cells are obligatory for the dectin-1 mediated response towards β -glucans, and/or whether absorption of β -glucans into the circulation is an essential step in explaining their immune modulating characteristics. For this a tissue-specific dectin-1 knock-out approach seems warranted. These questions are important in light of dietary β -glucan to distinguish between effects on enterocytes and intestinal immune cells.

Conclusion and applications

β -Glucans are not only present as major structural components of the cell walls of yeast, fungi and some bacteria, but are also present in our daily diet as part the endosperm cell wall in cereals, such as barley and oat. Results both from *in vitro* studies as well as *ex vivo* stimulated leukocytes isolated from animals treated with β -glucans suggest that β -glucans enhance the immune response in leukocytes and epithelial cells. Since they influence the immune response of the host, 1,3 β -glucans are often described as biological response modifiers [77]. In the *in vivo* situation there is now substantial evidence that these effects ultimately translate into an enhanced survival after infection with pathogens. In this respect, effects are observed irrespective of the β -glucan source and/or route of administration. Therefore, it might be possible to modulate immune function by increasing the dietary β -glucan intake, for example by developing functional foods. This may have benefits for specific target populations like for example elderly or type II diabetic patients, who are both characterized by a suppressed (Th1) immune response [78-80]. Alternatively, subjects with an overactive Th2 immune response, such as observed in allergic reactions and asthma, may benefit in terms of restoring the balance by the described Th1 stimulation. Both concepts should however be confirmed in future well-controlled human intervention trials.

CHAPTER 3

Fecal water from ileostomic patients consuming oat β -glucan enhances immune responses in enterocytes

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Abstract

Yeast, fungal and dietary β -glucans have immune-modulating effects *in vitro* and *in vivo* as thought mainly by affecting leukocytes, however effects of oat β -glucan on enterocytes have never been studied. As recognized, supplying oat β -glucans as such to cells in culture directly is difficult because of solubility problems. Therefore, six ileostomic patients consumed in random order a control diet or an oat β -glucan enriched diet (5 g) and from the collected ileostomic content, fecal water was prepared and added to two small intestinal cell lines (INT407, Caco-2) and two colon cell lines (HT29, T84) together with a cytokine cocktail (interleukin (IL)-1 β + interferon γ + tumour necrosis factor α). Several parameters reflecting immune-modulation were measured. As compared to placebo fecal water, β -glucan enriched fecal water significantly increased IL-8 production in HT29 (5.0%; $p = 0.046$) and INT407 cells (22.0%; $p = 0.028$). Inter-cellular adhesion molecule (ICAM)-1 expression increased in T84 (11.0%; $p = 0.028$) and Caco-2 cells (20.4%; $p = 0.075$). These immune stimulating effects were confirmed by enhancement of inflammatory expression profiles, as determined with an antibody array. Our findings show immune enhancement by fecal water from ileostomic patients consuming oat β -glucan both in small intestinal and colon cell lines after stimulation, which is in agreement with documented effects in leukocytes. Whether these immune stimulating effects on enterocytes contribute to the enhanced protection of the host against invading pathogens as observed both in animals and in humans, as well as the underlying mechanism, needs further evaluation.

Introduction

β -Glucans are carbohydrates consisting of linked glucose molecules with a molecular mass between 50 and 2,300 kDa. They are major structural components of the cell walls of yeast and fungi, but also of some cereals such as barley and oat. In nature there are many different forms of β -glucans varying in length, molecular mass, tertiary structure, and degree of branching. In this respect, the cell wall glucans of yeast and fungi consist of β -(1 $\rightarrow$ 3)-linked glucopyranosyl residues with small numbers of (1 $\rightarrow$ 6)-linked branches, whereas the oat endosperm cell walls contain unbranched β -glucans with β -(1 $\rightarrow$ 3) and β -(1 $\rightarrow$ 4) linkages [7, 8, 22]. These characteristics influence their effects on physiological functions such as lipid and glucose metabolism, and inflammatory processes.

It is now well established that fungal β -glucans initiate - by a so far not completely understood mechanism - a very potent immune response in leukocytes [8]. Less is known about the immune-modulating effects of β -glucans from oats and barley. Estrada *et al.*, however, have demonstrated that culturing macrophages in the presence of oat β -glucans enhanced production of interleukin (IL)-1 β in a dose- and time-dependent manner [7]. Also the production of IL-2, interferon (IFN) γ and IL-4 in cultured spleen cells was increased by oat β -glucan [7]. *In vivo* studies demonstrated that oat β -glucan enhanced resistance towards bacterial challenges and intestinal parasitic infections [7, 47, 55, 56]. All together, these findings indicate that β -glucans not only from yeast and fungi but also from oat have immune stimulating effects *in vitro* and *in vivo*.

Moreover, Brown and Gordon, have recently suggested that high molecular weight (MW) and/or particulate β -glucans from fungi directly activate leukocytes, while low MW β -glucans from fungi only modulate the response of cells when they are stimulated with e.g. cytokines [81]. Whether effects of a low MW oat β -glucans are also indirect, i.e. comparable to those of low MW fungi β -glucans is unknown. In our hands, however, the fecal water with and without low MW oat β -glucans did not induce an immune-stimulation without the presence of an additional inflammatory trigger. Therefore, in this study we have chosen to evaluate immune-modulating effects of a low molecular weight oat (1 $\rightarrow$ 3), (1 $\rightarrow$ 4) β -glucan (60 kDa) not as only inducing agent, but particularly as modifier of a cytokine stimulated condition.

Most *in vitro* studies evaluated the effects of β -glucans in different leukocytes populations, however effects on enterocytes have never been examined. Since it is inevitable that enterocytes play an important role in the intestinal defence against pathogens the present study evaluated the immune modulating effects

of oat β -glucan on human enterocytes. It is however very difficult to study effects of β -glucans in cell cultures directly, because of problems with solubility [82]. Therefore, to study effects of oat β -glucan in a physiological matrix, ileostomic patients consumed in random order a control diet or an oat β -glucan enriched diet. From the collected ileostomic contents fecal water was prepared and added together with a cytokine cocktail (IL-1 β , IFN γ and tumour necrosis factor (TNF) α) to enterocyte cell lines. We have deliberately chosen to study effects in four different enterocyte cell lines (two small intestinal cell lines and two colon cell lines) to exclude the possibility that the presence or absence of the effects found were cell line specific. Next, the cells as well as the supernatant were analyzed for different inflammatory parameters. By this approach we were able to evaluate the effects of dietary oat β -glucan consumption under physiological conditions without making a distinction between direct (i.e. β -glucan-enterocyte interactions) or indirect effects (i.e. factors present in ileostomic content derived from effects of β -glucan in the intestinal tract of the patients). As far as we know, this is the first study showing immune enhancing effects of oat β -glucans in enterocytes by such a physiological approach.

Materials and methods

Study design

Six patients (3 males and 3 females) with an ileostomy participated in a double blind, placebo-controlled intervention trial with a cross-over design (**figure 3.1**). All subjects were proctocolectomized for ulcerative colitis. The median time since surgery was 6 years (range: 1-17 yr). The mean age of the subjects was 51 years (range: 38-74 yr). On the first day of the first period, all subjects received a standardized diet (3 main meals and 3 snacks). On the second day, three subjects received again a standardized diet, but in addition at breakfast and at lunch a beverage (250 mL) enriched with β -glucan (2.5 g, i.e. 5 g in total). The other three subjects received a placebo beverage enriched with rice starch. One week later, regimes were crossed over. The nutrient compositions of the two diets are presented for men and women in **table 3.1**. During the second day of each period ileostomic contents were collected 15 min before the standard meals at 7.45 h, 11.45 h, 15.45 h, 19.45 h, 21.45 h and at 7.45 h the following morning.

Figure 3.1: Study design

To study immune modulating effects of oat β -glucan on enterocytes in a physiological matrix, (A) ileostomic patients consumed a diet enriched with β -glucan or placebo and ileostomic contents were collected. These ileostomic contents were used to prepare fecal water, which (B) was used in an in vitro study of stimulated enterocytes. For the first in vivo part (A), all ileostomic patients (n = 6) received a standardized diet (diet composition see table 3.1) on the first day of the first period. On the second day, three subjects received a standardized diet and at breakfast and at lunch a beverage (250 mL) enriched with β -glucan (2.5 g, i.e. 5 g in total), while the other three subjects received a placebo beverage enriched with rice starch. One week later, regimes were crossed over. During this second day until the following morning (24 h in total) ileostomic contents were collected at fixed intervals. Fecal water was prepared by dissolving 24 h pooled freeze-dried ileostomic contents in PBS as described in the methods section and subsequently used for in vitro cell stimulation experiments. For this second in vitro part (B), placebo and β -glucan fecal water was added to 4 enterocyte cell lines (HT29, T84, INT407 and differentiated Caco-2) which were subsequently stimulated with a pro-inflammatory cytokine cocktail (IL-1 β , IFN γ and TNF α) for 16 hours. After 16 hours culture medium was collected for measuring interleukin (IL)-8 production and inflammatory protein expression profiles and living cells were used for cell membrane intercellular adhesion molecule (ICAM)-1 protein expression measurements.

The ileostomic contents was sealed in a plastic jar immediately after each collection and put on dry ice. The next morning the ileostomic contents were weighted, freeze-dried, weighted again, homogenized and stored at -20 °C. Ethical approval of the study protocol was obtained from the local Research Ethics Committee of Lund University. All subjects gave their written informed consent before participating.

Table 3.1: Nutrient composition of the diets

	Men (n = 3)		Women (n = 3)	
	placebo diet	β -glucan diet	placebo diet	β -glucan diet
Energy (kcal)	2170.0	2170.0	1813.0	1813.0
Protein (g)	97.5	97.5	89.2	89.2
Energy %	18.0	18.0	20.0	20.0
Fat (g)	65.1	65.1	53.3	53.3
Energy %	27.0	27.0	26.0	26.0
Carbohydrates (g)	299.5	299.5	240.8	240.8
Energy %	55.0	55.0	53.0	53.0
Fibres (g)	11.0	16.0	8.0	13.0
β -glucan (g)	0.0	5.0	0.0	5.0

β -Glucan was isolated from Swedish oats (oats var. Sang from Cerealia (Sweden)) at Cereal Base Ceba AB (Lund, Sweden), as described [83]. Briefly, oat bran was crushed and milled with water and treated with amylases and proteases, while at the end insoluble fibers were removed by ultra-filtration. The remaining β -glucan fraction was freeze-dried, mixed with water, and used for the production of the beverages with a β -glucan concentration of 1% (wt/wt). The $\beta(1\rightarrow3)$ -($1\rightarrow4$)-linked unbranched soluble glucan had a mean molecular weight of 60 kDa determined by VTT Biotechnology, Technical Research Centre of Finland (Espoo, Finland), by SEC-HPLC using μ Hydrogel columns, NaOH-eluent and post-column calcofluor staining with fluorescence detection, as described [84]. Control beverages were prepared using rice starch, while sucrose, glucose syrup and rapeseed oil were added to balance the nutrient composition of the two experimental products.

Ileostomic contents and fecal water

The ileostomic contents from each subject obtained at each period were freeze-dried, after which the molecular weights and the concentrations of the β -glucan in the ileostomic contents were determined, as mentioned above. The ileostomic contents from each subject used for the cell stimulations were pooled over the 24-h periods, hereby correcting for differences in volumes from each interval. Fecal water was prepared by dissolving the pooled freeze-dried ileostomic contents in phosphate buffered saline (PBS), which was subsequently rotated for 5 h. Next, the dissolved ileostomic contents were centrifuged for 11.5 min at 13,500 rpm, 17 °C. The supernatant (further called fecal

water) was carefully collected and used for the *in vitro* cell stimulation experiments. The pH of each fecal water sample was determined.

Intestinal cell cultures

Four different human derived intestinal cell lines - two small intestinal and two colon cell lines - were used. The human small intestinal cell line INT407 was obtained from the European Tissue Type Collection (Salisbury, UK) and the human cell line Caco-2 from the American Tissue Type Collection (Rockville, MD). INT407 cells were cultured in minimum essential medium (MEM) (Invitrogen, Breda, the Netherlands) supplemented with 10% heat inactivated fetal calf serum (FCS) (Invitrogen,) and 1% penicillin streptomycin (PS) (Invitrogen). Caco-2 cells were cultured in Dulbecco's Modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% FCS, 1% PS, 1% sodium pyruvate (Invitrogen) and 1% non-essential amino acids (Invitrogen). After 18 days culturing Caco-2 cells in normal DMEM they were differentiated into small intestinal enterocytes [85]. The human colon cell lines HT29 and T84 were kindly supplied by Prof. Dr. W.A. Buurman (Department General Surgery, Maastricht University, The Netherlands). HT29 cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% FCS and 1% PS. T84 cells were cultured in DMEM nut mix F-12 (DMEM/F12) (Invitrogen) supplemented with 10% FCS and 1% PS. All cells were cultured at 37 °C in a 5% CO₂ humidified atmosphere. Cells were refreshed every second day and were separated by trypsin-0.03% EDTA (Gibco BRL, Gaithersburg, MD) when they had reached 70-90% confluency.

Cell cytotoxicity

Cell cytotoxicity was measured to determine the maximum concentration of fecal water that could be added to the cell lines without causing cytotoxic effects. For this, different concentrations of fecal water were prepared and tested in two assays, i.e. (1) erythrocytes lytic activity and (2) phenol-red leakage across confluent Caco-2 cell monolayers.

Erythrocytes lytic activity assay

Cytotoxic activity of fecal water was determined by evaluating hemolysis of erythrocytes. For this, human blood with heparin as anti-coagulant - from the

same volunteer for all experiments - was centrifuged for 10 min at 3,500 rpm to obtain erythrocytes. Plasma was removed and the remaining erythrocytes were washed three times with PBS and centrifuged again for 10 min at 3,500 rpm. The cytotoxicity of fecal water was determined by adding different concentrations of fecal water - prepared by dissolving different amounts of the ileostomic content - to the washed human erythrocytes suspension. After incubation for 45 min at 37 °C, the tubes were centrifuged for 10 min at 3,500 rpm. The supernatant was transferred to a 96-wells plate and diluted 20 times. The hemoglobin concentration in the supernatant, released after lysis of the erythrocytes, was measured spectrophotometrically at 540 nm. Percentage hemolysis was calculated compared to a calibration curve, made from erythrocytes suspension incubated with ascending concentrations distilled water in PBS (0-100% hemolysis). The concentration at which 2% lysis occurred was defined as the critical lytic concentration (CLC) [86]. Concentrations below the CLC were determined as not cytotoxic and were used for further experiments.

Phenol-red leakage across confluent Caco-2 monolayers

Cytotoxicity of fecal water was also measured by phenol red leakage across confluent Caco-2 monolayers, which can be used as a good marker for cell confluence and tight junction formation and is a easy to use alternative for the electrical measurement of the transepithelial electrical conductance [87]. For this, Caco-2 cells were cultured in a polarized transwell system (Costar, Badhoevedorp, the Netherlands). The upper apical chamber contained DMEM (Invitrogen) with phenol red and the lower basolateral compartment DMEM without phenol red. Disturbance of the confluent Caco-2 cell monolayer due to cytotoxicity of the fecal water resulted in a flux of the small phenol red molecule (MW = 354) across the epithelium, which could be measured in the basolateral compartment by spectrophotometry at 479 nm [87]. When the cells were confluent as indicated by the absence of phenol red leakage, cells were refreshed with medium containing fecal water at different concentrations. Dimethyl sulfoxide (DMSO) (10%) and PBS were used as positive and negative controls, respectively. Cells were incubated for 6 hours with fecal water, 10% DMSO or PBS added apically. Next, the basolateral medium was collected, and refreshed after which the cells were incubated for another 18 hours with the same compounds. The phenol red concentration in the basolateral medium (at 6 and 18 hours) was measured. Values were corrected for phenol red free medium and compared to the 10% DMSO and PBS controls. Concentrations

without phenol red leakage were determined as not cytotoxic and were used for further experiments.

Cell stimulations

To evaluate the immune modulating effects of fecal water with β -glucan *in vitro* cell stimulation experiments were conducted as schematic represented in **figure 3.1b**. For this HT29 and T84 cells were plated in 6-well plates at an initial density of 1.0×10^6 cells/mL, INT407 at 0.6×10^6 cells/mL, and Caco-2 at 0.5×10^6 cells/mL in a total volume of 1.5 mL. When the HT29, T84 and INT407 cells had reached 70-90% confluency and the Caco-2 cells had differentiated for 18 days, the culture medium was replaced by medium containing fecal water (with or without β -glucan) and a cocktail consisting of the pro-inflammatory cytokines IFN γ (100 U/mL), IL-1 β (50 U/mL) and TNF α (10 ng/mL). After 16 h of incubation, culture medium was collected for analysis of IL-8 concentrations and inflammatory protein expression profiles, while the living cells were used to determine cell surface intercellular adhesion molecule (ICAM)-1 protein expression.

IL-8 enzyme-linked immunosorbent assay and inflammatory protein expression profiles

IL-8 concentrations in culture supernatants were determined using an enzyme-linked immunosorbent assay (ELISA) as described [88]. Briefly, plates (Greiner Bio-one, Frickenhausen, Germany) were coated with monoclonal murine anti-human IL-8 antibodies. Recombinant human IL-8 was used for standard curves. Immobilized IL-8 was detected using a specific biotinylated rabbit-anti-human IL-8 polyclonal antibody, followed by the addition of peroxidase-conjugated streptavidin (Zymed Laboratories, San Francisco, Canada) and tetramethylbenzidine (TMB) substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The detection limit of the ELISA was 5 pg/mL.

Cell culture supernatants were also used to evaluate expression profiles of multiple inflammatory proteins using the human cytokine antibody array III (Ray Biotech Inc., Norcross, GA). First, cell culture media of all six subjects after 16 hours incubation with fecal water containing β -glucan or placebo were pooled. Thus, eight arrays (HT29, T84, INT407, Caco-2 with β -glucan or placebo) were analyzed. One mL of the pooled samples was added to the array membranes. After incubating and washing, the cytokine-bound membrane was

incubated with a cocktail of biotin-labelled antibodies, followed by the addition of horseradish peroxidase-conjugated streptavidin. Array spot intensity was detected by using a LAS-3000 Lite Image reader (Raytest GmbH, Straubenhart, Germany) based on chemiluminescence imaging. Intensity of the spots was quantified in Arbitrary Units by densitometry using Aida software version 3.50 (Raytest GmbH), thereby correcting for background staining of the gel. Comparison of protein expression profiles was possible after normalization of each spot on an array using the positive controls, provided by the manufacturer. The cytokines used for stimulation (IFN γ IL-1 β and TNF α) were excluded from analysis.

Using the same protocol, this array was also used to determine the amounts of inflammatory proteins possibly present in fecal water. For this, fecal water containing β -glucan or placebo was prepared for each subject and pooled per dietary period. For analysis, the fecal water was not diluted.

Flow cytometry analysis of intercellular adhesion molecule-1 (ICAM-1)

ICAM-1 protein expression on the cell surface of living cells was measured by flow cytometry. After 16 hours incubation, cells were washed three times with PBS and detached with trypsin-0.03% EDTA. When the cells were detached, medium was added and cell suspensions were centrifuged for 5 min at 1,200 rpm at room temperature, followed by resuspending the pellets in 500 μ L PBS-1% BSA. Cells were counted and diluted to 1×10^6 cells/mL in PBS-1% BSA. Recombinant-phycoerythrin (R-PE)-conjugated mouse-anti-human CD-54 monoclonal antibody (anti-ICAM-1) or isotype-matched control antibody (Becton Dickinson Biosciences, San Diego, CA) 20 μ L/ 10^6 cells was added and incubated for 30 min on ice in the dark. Next, cell suspensions were centrifuged for 5 min at 1,500 rpm and pellets were resuspended in 500 μ L PBS-1% BSA. Because almost all cells were ICAM-1 positive, however greatly differed in amount of ICAM-1 expression, the mean fluorescence of 10,000 living cells was measured and analyzed with the FACSsort (Becton Dickinson) and CellQuest analysis software. Percent living cells were not different between the different samples and the isotype control antibody showed fluorescence below the threshold.

Statistical analysis

Effects on IL-8 production and ICAM-1 expression were examined with the non-parametric Wilcoxon signed ranks test. For data presentation, effects from the

-glucan enriched fecal water were also expressed as percentages relative to those of the placebo fecal water. All statistical analyses were performed using SPSS 11.0 (SPSS, Chicago, IL). p-values of less than 0.05 were considered statistically significant.

Results

Characteristics of ileostomic content and fecal water

The mean molecular weight of the β -glucan in the beverages and in the ileostomic content of four subjects was 60 kDa. For two subjects, only a very slight decrease in molecular weight was observed, indicating that overall the β -glucan was not degraded while passing the small intestine.

Fecal water concentrations that were not cytotoxic, as determined by erythrocyte lysis were in agreement with phenol-red leakage assays (data not shown). Experiments in all cell lines were therefore carried out with a concentration of 1.44 mg/mL re-dissolved freeze-dried ileostomic content in PBS. The calculated concentrations of β -glucan added to the cells varied from subject to subject between 0.12 and 0.18 mg/mL.

The mean pH of fecal water containing β -glucan was not significantly different as compared to placebo fecal water (7.4 ± 0.2 and 7.3 ± 0.2 (mean $\pm$ SD), respectively).

To exclude the possibility that the β -glucan enriched fecal water already contained pro-inflammatory proteins, which may have confounded the results, we measured inflammatory proteins with the antibody array. However, no detectable amounts of inflammatory proteins were present in the fecal water from the β -glucan or placebo period (data not shown).

IL-8 production

Addition of the β -glucan enriched fecal water without stimulation with cytokines did not induce IL-8 production in any of the four intestinal cell lines (data not shown). However, when β -glucan enriched fecal water was added in combination with a cytokine mixture we found enhancing effects as compared to placebo fecal water, illustrating that our low molecular weight oat (1 $\rightarrow$ 3), (1 $\rightarrow$ 4) β -glucan (60 kDa) indeed is not an inducing agent but a modifier of a

cytokine stimulated condition. **Figure 3.2a** shows the median IL-8 concentrations (and ranges) after placebo and β -glucan fecal water for all subjects together in the four cell lines, while in **figure 3.2b** percent changes for the β -glucan fecal water for each subject individually corrected for placebo fecal water are shown. β -Glucan containing fecal water from 5 subjects increased IL-8 production by HT29 cells. For one subject, no change was found. Overall, this resulted in a significant median increase in IL-8 production of 5.4% (range: -0.3%, 16.6%; $p = 0.046$). Comparable to HT29 cells, IL-8 production by INT407 cells was increased for all six subjects after addition of fecal water containing β -glucan, resulting in a significant increase of 22.0% (range: 6.5%, 29.4%; $p = 0.028$). In contrast, IL-8 production by T84 cells and Caco-2 cells was not significantly changed (median: 5.9%; range: -31.1%, 23.1%; $p = 0.917$ and median: 4.2%; range: -34.8%, 218.3%; $p = 0.463$, respectively).

Figure 3.2

β -Glucan containing fecal water increased IL-8 production by enterocytes. IL-8 production by HT29, T84, INT407 and Caco-2 cells after 16 h incubation with fecal water containing β -glucan or placebo and stimulation with cytokines (A, B). (A) The median (and ranges) of IL-8 concentrations (ng/mL) after β -glucan and placebo fecal water and (B) percent changes in IL-8 concentration of each individual after fecal water containing β -glucan corrected for placebo are shown for the 4 enterocyte cell lines. In panel B, each subject is represented by a dot and the median is represented by a line.

Cell surface ICAM-1 protein expression

In line with effects on IL-8 production, addition of the β -glucan enriched fecal water without stimulation with cytokines did not induce ICAM-1 expression in any of the four intestinal cell lines (data not shown), while stimulating effects on ICAM-1 expression were visible in the presence of cytokine stimulation. **Figure 3.3a** shows the ICAM-1 expression after placebo and β -glucan fecal water for all subject together in each of the four cell lines and in **figure 3.3b** percent changes for the β -glucan fecal water for each subject individually corrected for placebo fecal water are shown. These figures show that the β -glucan containing fecal water from all subjects increased ICAM-1 expression in T84 cells. This resulted in a significant increase of ICAM-1 expression for T84 (median: 11.0%; range: 3.8%, 31.1%; $p = 0.028$). ICAM-1 expression in Caco-2 cells and INT407 cells was increased for five out of the six subjects, with a median ICAM-1 increase of 20.4% (range: -8.8%, 40.5%; $p = 0.075$) and 16.7% (range: -19.0%, 30.0%; $p = 0.249$), respectively. ICAM-1 expression in HT29 cells, however, was not significantly changed (median: -0.4%; range: -8.4%, 10.8%; $p = 0.917$).

Figure 3.3

β -Glucan containing fecal water increased ICAM-1 expression in enterocytes. ICAM-1 expression in HT29, T84, INT407 and Caco-2 cells after 16 hours incubation with fecal water containing β -glucan or placebo and stimulation with cytokines (A, B). (A) The median (and ranges) of ICAM-1 expression (arbitrary units) after β -glucan and placebo fecal water and (B) percent changes in ICAM-1 expression of each individual after fecal water containing β -glucan corrected for placebo. In panel B, each subject is represented by a dot and the median is represented by a line.

Inflammatory protein expression profiles

In general, β -glucan enriched fecal water increased the expression of almost all detectable cytokines, chemokines, colony stimulating factors (CSF) and growth factors compared to placebo fecal water (figure 3.4).

Figure 3.4

β -Glucan containing fecal water mostly increased expression of various inflammatory proteins of enterocytes. Percent change in expression of chemokines, colony stimulating factors (CSF), growth factors and cytokines after β -glucan as compared to placebo fecal water in HT29, T84, INT407 and Caco-2 cells as measured by an antibody array. For experimental details see Materials and Methods. GRO, growth regulated protein; IL, interleukin; MCP, monocyte chemotactic protein; MDC, macrophage derived chemokine; MIG, monokine induced by gamma interferon; MIP, macrophage inflammatory protein; RANTES, regulated upon activation normal T-cell expressed and secreted; TARC, thymus and activation regulated chemokine; GM-CSF, granulocyte-macrophage colony stimulating factor; MCSF, macrophage colony stimulating factor; Tpo, thrombopoietin; EGF, epidermal growth factor; OSM, oncostatin M; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; TNF, tumour necrosis factor; Ang, angiogenin.

No bar means that the protein was not detectable in the culture medium of that particular cell type or that there was no change in protein expression after β -glucan compared to placebo fecal water. When examining the expression profiles into more detail, it can be seen that especially the changes in chemokines expressions were quite consistent. For example, in all cell lines expressions of all detectable MCPs were increased (MCP-2 and 3 expression not measurable in HT-29 cells and no MCPs measurable in Caco-2 cells), while that of MIG (monokine induced by interferon γ), a chemokine that attracts T-lymphocytes, was decreased (not measurable in T84 and INT407 cells). The increases in IL-8 expressions agreed very well with those observed with the ELISA technique, confirming the validity of the antibody array. It should be noted however that the relative change measured with the antibody array was higher in Caco-2. This can be explained by the fact that for the antibody array pooled samples were used and the results therefore represent a mean value.

For the ELISA, median values were presented. Changes in CSF and growth factor expressions were less consistent. The T84 cells showed an increased expression for all CSF and growth factors, whereas the HT29 cells showed a decreased expression for all these proteins except for GM-CSF. Also for INT407 and Caco-2 cells increases and decreases for these proteins were observed.

Discussion

Not only β -glucans from the cell wall of fungi and yeast, but also β -glucans from oats have been shown potent immune-stimulators, at least in macrophages and spleen cells [7]. Effects on enterocytes have however never been examined. Our results now show that fecal water prepared from ileostomic contents of patients consuming diets enriched with oat β -glucans induced an immune-stimulating response in enterocytes both from small intestinal as well as from colonic origin. The finding that not all four cells responded significantly regarding all parameters, but the fact that nearly all responses observed in the different cell lines were a stimulation strengthens our belief that oat β -glucans indeed enhanced immune responses in enterocytes. Since our primary goal was to see whether enterocytes responded to β -glucan enriched fecal water at all, we did not attempt to define the mechanism underlying this response in this phase. Therefore, as far as we know now, these findings could be ascribed to direct as well as indirect effects of oat β -glucan.

We measured immune modulating effects of β -glucan by examine IL-8 production and ICAM-1 expression of enterocytes. IL-8 is a chemokine and ICAM-1 is an adhesion molecule which expression is both increased in cause of an infection by pathogens. IL-8 production by enterocytes will induce recruitment of inflammatory cells such as neutrophils, basophils and lymphocytes to the inflamed tissue [89]. ICAM-1 expression on enterocytes may help to keep leukocytes that have transmigrated through the epithelial layer towards the intestinal lumen in close contact with the intestinal epithelium [90]. This will prevent the further invasion of the mucosa by pathogens. Fecal water with β -glucan increased the production of IL-8 and the cell surface expression of ICAM-1, which thus indicates an enhanced immune response. This agrees with the results of an *in vivo* study with C57BL/6 mice showing that oral administration of (1 $\rightarrow$ 3), (1 $\rightarrow$ 6)- β -glucan isolated from baker's yeast increased the number of intraepithelial lymphocytes in the intestine [64]. Although effects of yeast glucans and oat glucans may, because of structural differences, not as such be extrapolated to each other, the similarity in responses is suggestive.

Inflammatory signatures obtained from the antibody arrays suggest a potential role of other inflammatory players, because β -glucan elevated the expression of numerous inflammatory proteins. An important group of proteins that showed highly consistent elevations in all four enterocyte cell lines were the monocyte chemotactic proteins (MCPs). The elevation of these chemokines, which preferably attract monocytes, suggests a role of these cells (besides the granulocytes and lymphocytes attracted by IL-8) in the β -glucan induced improvement in immune function as well. Our results therefore suggest that the elevated IL-8 production and increased ICAM-1 expression and probably also the increased production of MCPs by enterocytes may contribute to the increased resistance to pathogens after β -glucan consumption, as shown in mice [7, 47] and humans [49, 50]. No correlation between the β -glucan concentration in the fecal water and the overall inflammatory response (in all cell lines) of each subject was present, probably due to the small β -glucan concentration interval (0.12-0.18 mg/mL) and the small number of subject. We showed that the four cell lines did not always respond in the same manner. For example, in T84 cells the IL-8 production was not significantly different, whereas ICAM-1 expression significantly increased. This suggests that individual inflammation markers are differently regulated in the various intestinal cell lines. Therefore we decided to measure a whole range of inflammation related markers in all four cell lines. Although there are differences between the cell lines and not all effects were significant, the overall conclusion is that they are nearly all in the same direction, indicating immune stimulating effects of β -glucan enriched fecal water in enterocytes.

To what extent our findings can be extrapolated to other types of β -glucans is not known. β -Glucans are a complex group of molecules with different degrees of branching, polymer lengths, tertiary structures and solubility's [22, 77]. We have used a soluble unbranched β -glucan with β -(1 $\rightarrow$ 3) and β -(1 $\rightarrow$ 4) linkages, that was extracted from oat with a mean molecular weight of 60 kDa. Brown and Gordon have recently suggested that high molecular weight and/or particulate β -glucans of fungi directly activate cells. In contrast, low molecular weight β -glucans might not directly activate cells, but may modulate the response to another challenge. Results of these low molecular weight β -glucans, however, are conflicting, as both primed and suppressed secondary responses have been reported [81]. There is no clear explanation for these different results, but it is known that small differences between β -glucan molecules besides molecular weight also have an impact on their immune-modulating effects [77, 81]. Unfortunately, characteristics of the β -glucans used in other studies have not always been reported [81]. In the present study, we have now shown that a low molecular weight oat (1 $\rightarrow$ 3), (1 $\rightarrow$ 4) β -glucan (60 kDa) in the

physiological matrix of fecal water induced an enhanced immune response in enterocytes after cytokine stimulation. Moreover, the finding that addition of the β -glucan enriched fecal water without stimulation with cytokines did not induce an inflammatory response (data not shown) supports the finding that low molecular weight β -glucans can modulate, but not evoke, an inflammatory response. However, despite numerous barriers and mechanisms counteracting bacterial translocation and too strong inflammatory responses, it is very likely that there is always some translocation also in a normal situation. [91] Therefore, it is very likely that a local inflammatory micro milieu is always present in the intestine [91], suggesting that adding cytokines to stimulate the enterocytes might be considered rather physiological. With respect to the physiological relevance of the β -glucan induced immune enhancement, the finding that numerous immune mediators all change in the same direction, suggest that this can have a strong physiologic effect. However, in contrast to the *in vivo* situation in the intestines, the *in vitro* cultured enterocytes do not produce mucus, however mucus is not expected to prevent direct contact between enterocytes and β -glucan since β -glucan is water soluble and is expected to easily pass the mucus layer.

Dectin-1 plays a central role to explain the immune-modulating effects of β -glucans [22]. Of the four epithelial intestinal cell lines we have used, dectin-1 mRNA expression has been shown in Caco-2 cells [42]. As far as we know, the expression of dectin-1 in the other cell lines is not examined yet. The expression of dectin-1 on intestinal epithelial cells *in vivo* is contradictory. Presence and absence on mRNA and protein level and on murine and human intestinal cells have been reported [58, 71, 92]. Because it is not evident if dectin-1 is expressed on enterocytes, we cannot exclude that the observed effects of β -glucan are not mediated by dectin-1. The present study was not initiated to mechanistically examine the effects of β -glucan. Whether our results are dectin-1 mediated or due to indirect changes in the fecal water composition induced by oat β -glucan will hopefully be addressed in future studies.

As suggested above, the observed effects in our *in vitro* model may also be caused by an increased or decreased production of another, yet unknown, factor. If true, this implies that effects of β -glucan fecal water on enterocytes are indirect and would in that case never have been found when - if possible - the oat β -glucans were applied to the cells directly. As an example of indirect effects, β -glucan could have induced changes in inflammatory proteins in the fecal water. However we could not identify any inflammatory proteins in the fecal water with the highly sensitive antibody array. Although the amount of LPS in the fecal water is not determined, effects of LPS as explanation for the observed changes in the intestinal cell lines used can be neglected since all cell

lines were found non-responsive to even high concentrations of LPS (data not shown). However, measuring LPS is not the solution since potential effects of other microbial products in fecal water can't be ruled out. However, since each volunteer was his own control, potential disturbing effects of these factors are not very likely. Another possible explanation for the observed effects is an increased production of antimicrobial peptides like defensins after consumption of the β -glucan. These peptides, which will then be present in elevated amounts in fecal water, may also influence intestinal inflammation by stimulating chemokine secretion and attracting leukocytes [93]. Another possible mechanism explaining the observed effects is degradation of β -glucan into short chain fatty acids (SCFA). SCFA, for example butyrate, are known to modulate the intestinal flora and intestinal inflammation [94]. However, in our study we worked with ileostomic patients and β -glucan did therefore not pass the colon, the only place where fibers can be degraded into SCFA by bacteria. Also the molecular weight of β -glucan in the ileostomic content was not substantially changed, which excludes the formation of degradation products. It is therefore not likely that the reported effects are caused by differences in SCFA composition of the fecal water. Finally, it is known that consumption of β -glucan enriched diets increases fecal bile acid excretion [48]. If anything, however, bile acids will have immune-suppressive effects [95].

In conclusion, we found that β -glucan enriched fecal water enhanced the immune response in stimulated epithelial intestinal cell lines, which should allow the host to better defend oneself against invading pathogens. This is to our opinion the first time that responsiveness of enterocytes towards β -glucans has been shown. Whether the reported effects of β -glucan enriched fecal water are direct effects of β -glucan (potentially via dectin-1) or indirect (via so far unknown mechanism) need further investigation. Finally, extrapolation of our findings to the *in vivo* situation remains to be established.

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CHAPTER 4

The absence of functional dectin-1 on enterocytes may serve to prevent intestinal damage

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Abstract

Enterocytes are exposed to antigens present in the intestinal lumen, like β -glucans that are carbohydrate structures present not only in the cell wall of yeast and fungi but also in cereals. β -Glucans are known for their immune modulating properties and we have earlier reported an increased immune response by enterocytes after addition of fecal water prepared from ileostomic contents obtained from participants consuming an oat β -glucan diet versus a placebo diet. We hypothesized that our observation of immune stimulating effects by oat β -glucan in enterocytes was mediated via the β -glucan receptor dectin-1.

Presence of dectin-1 in enterocytes was examined by reverse transcriptase PCR, western blot, and flow cytometry followed by an evaluation of the functional involvement of dectin-1 by using dectin-1 inhibitors during fecal water incubations.

Reverse transcriptase PCR and western blot analysis demonstrated dectin-1 presence in the INT407 and Caco-2 nuclear factor- κ B (NF- κ B) reporter enterocyte cell lines. Moreover, also human enterocytes isolated from ileum or colon biopsies contained dectin-1 protein. However, dectin-1 expression could not be confirmed by flow cytometry in INT407 cells, suggesting that in these cell lines dectin-1 is not expressed at the extracellular membrane. Furthermore, dectin-1 inhibitors did not suppress the β -glucan containing fecal water induced interleukin-8 production by INT407 cells and NF- κ B transactivation by Caco-2 NF- κ B reporter cells.

INT407 and Caco-2 NF- κ B reporter cells seem to express no functional dectin-1. The absence of this pattern recognition receptor may function to protect the intestine against inflammatory damage, as the dectin-1 ligand β -glucan is largely present in the intestinal lumen.

Introduction

Intestinal epithelial cells are located at the frontline of the mucosal immune system and play an important role in regulating the immune response [96]. The presence of various pattern recognition receptors (PRRs) at the luminal surface of enterocytes is an illustration of this involvement [97]. Binding of pathogen associated molecular patterns (PAMP) to these PRRs can lead to cytokine production, which affects surrounded intestinal immune cells and in that way modulate the mucosal immune response. Besides the production of cytokines, also antimicrobial peptides, like defensins, are secreted into the lumen as part of the innate immune response. These peptides belong to the innate immune response and deficiencies in antimicrobial peptides are associated with inflammatory bowel disease [98, 99]. The task of the gastrointestinal tract, given the presence of PRRs, is to discriminate between antigens, valuable nutrients, and potential harmful pathogens. This is challenging, especially as nutrients and pathogens can share comparable structures like β -glucans. These glucose polymers are the major structural components of not only the cell wall of yeast and fungi, but also of cereals like oat and barley. The structures of β -glucans from different sources are not identical. β -Glucans from yeast and fungi consist of β -(1 $\rightarrow$ 3) linked glucopyranosyl units with β -(1 $\rightarrow$ 6) linked side chains, while β -glucans from oat and barley are composed of unbranched β -(1 $\rightarrow$ 3) and β -(1 $\rightarrow$ 4) linkages.

It has been suggested that the various β -glucans give rise to different immune responses depending on structural properties of the β -glucans [100]. It is known that fungal β -glucans have clear immune stimulating effects on leukocytes [22]. They stimulate for example the production of proinflammatory cytokines like interleukin (IL)-1 β , IL-6, IL-8 and tumour necrosis factor α (TNF α) [8]. In addition to fungal β -glucans, also the unbranched β -glucans from oat have been reported to modulate the immune response. Estrada *et al.* [7] for example, have demonstrated an increased IL-1 α production by murine macrophages in the presence of oat β -glucan *in vitro*. Moreover, intragastric and intraperitoneal administration of oat β -glucans in mice enhanced resistance to bacterial challenge and parasitic infections [7, 47, 55, 56]. Concerning effects on the immune system it has been suggested that β -glucans mainly induce helper T-lymphocyte (Th)1 responses [32, 38, 39] by polarization of naïve intestinal T-cells.

It is known that the immune modulating effects of fungal β -glucans are mediated by the β -glucan receptor dectin-1 [22]. In man, dectin-1 is mainly expressed on dendritic cells and macrophages, but also on other cell types like

monocytes, neutrophilic and eosinophilic granulocytes, B-lymphocytes, and a subset of T cells. The expression of dectin-1 is high at sites with an increased exposure to pathogens, like the lung and intestine, which matches the role of dectin-1 as PRR in immune surveillance [70].

We have earlier reported an increased immune response by different human intestinal epithelial cell lines after addition of fecal water prepared from ileostomic contents obtained from participants who had consumed an oat β -glucan diet versus a placebo diet [69]. Here we evaluate whether our observation of immune stimulating effects by oat β -glucan could be explained by dectin-1 function on enterocytes. It has been reported by Rice *et al.* [58] that only 10% of intestinal epithelial cells interact with glucans. Therefore, we chose INT407 cells as cell type for the present study since it was highly responsive in our earlier studies. Caco-2 nuclear factor- κ B (NF- κ B) reporter cell were used as a second cell line. The primary aim of our study was to investigate dectin-1 presence in the enterocyte cell lines as reports concerning the presence of dectin-1 on enterocytes are not conclusive [42, 58]. The second aim was to determine whether dectin-1 was involved in our earlier observed immune stimulating effect of oat β -glucan enriched fecal water on human enterocytes. Therefore, we first examined the presence of dectin-1 in enterocytes followed by an evaluation of the functional involvement of dectin-1 by using dectin-1 inhibitors during β -glucan fecal water incubations.

Materials and methods

Intestinal cell culture

The human cell line INT407 was obtained from the European Collection of Cell Culture (Salisbury, UK) and the human cell line Caco-2 from the American Tissue Type Collection (Rockville, MD). INT407 cells were cultured in minimum essential medium (MEM) (Invitrogen, Breda, the Netherlands) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Invitrogen), 1% penicillin streptomycin (PS) (Invitrogen), 1% L-glutamine (Invitrogen) and 1% non-essential amino acids (NEAA) (Invitrogen). Caco-2 cells were grown in Dulbecco's minimum essential medium (D-MEM) (Invitrogen) supplemented with 10 % heat inactivated FBS, 1% PS, 1% sodium pyruvate (Invitrogen) and 1% NEAA. All cells were cultured at 37 °C in a 5% CO₂ humidified atmosphere. The cultures were refreshed every second day, and separated by trypsin-0.03% EDTA

(Gibco BRL, Gaithersburg, MD) when a confluence of 70-90% had been reached.

PBMC isolation

Peripheral blood mononuclear cells (PBMC) were used as positive control for RT-PCR, Western Blotting and flow cytometry. They were isolated from blood of healthy volunteers by means of gradient centrifugation using Lymphoprep according to the manufacturers' instructions (Axis-Shield PoC AS, Oslo, Norway).

Reverse transcriptase PCR

To verify the presence of dectin-1 in enterocytes, total RNA was extracted from INT407 cells and from isolated human PBMCs as a positive control with Trizol according the manufacturer's instructions (Gibco BRL). Next, cDNA synthesis was performed [101], and cDNA was used for PCR amplification. Primer sequences for dectin-1 targeting exon I and VI were identical to those described by Grunebach *et al.* [92]. The forward and reverse primers were 5'-TTTAGAAAATTTGGATGAAGATGGA-3' and 5' ATATGAGGGCACATACACA TTG-3' respectively [92] The PCR program was as follows: 5 min of pre-treatment at 94 °C, 45 cycles of 94 °C for 30 s, 60 °C for 45 s and 75 °C for 45 s, and annealing for 10 min at 72 °C. β -Actin was used as a housekeeping gene described by Kreuzer *et al.* [102]. The primer sequence and PCR temperature profiles for the β -actin gene were as follows: 5'-AGCCTCGCCTTTGCCGA-3' and 5'-CTGGTGCCTGGGGCG-3', 5 min of pre-treatment at 94 °C, 40 cycles of 94 °C for 30 s and 60 °C for 60 s and 75 °C for 45 s, and annealing for 2 min at 60 °C. PCR products were electrophorized and stained with GelStar nucleic acid gel staining (BioWhittaker Molecular Applications, Rockland, ME) for visualization under ultraviolet light. Preparing bands from the gels, and subsequent purification and sequencing using a BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) was carried out to confirm dectin-1 PCR products.

Western blot

To determine if the INT407 and Caco-2 NF- κ B reporter cell lines and isolated human enterocytes contained dectin-1 protein, protein extracts were pre-

pared. For this, the enterocytes and human PBMCs (as positive controls) were washed with ice-cold PBS and lysed on ice in ice-cold buffer containing 10 mmol/L, Tris pH 7.5, 150 mmol/L NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate containing protease and phosphatase inhibitors. Routinely, 15 μ g of protein was run on a dodecyl sulfate sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and then electrotransferred onto a polyvinylidene difluoride membrane (PVDF) membrane (Immun-Blot PVDF, Bio-Rad Laboratories, Hercules, CA). Resulting membranes were subjected to Ponceau S staining (Bio-Rad laboratories) to verify equal loading and proper transfer. The Ponceau S was washed and the membranes were probed with a polyclonal antibody raised against human dectin-1 (R&D systems, Wiesbaden-Nordenstadt, Germany) (1:1000 in 0.1% Tween, 5% nonfat dry milk in Tris-buffered saline (TBS) overnight). After incubation with horseradish peroxidase-conjugated secondary antibody (Sigma, St. Louis, MO) (anti-goat 1:5000 in 0.1% Tween, 5% nonfat dry milk in TBS for 2 h), the presence of antibody binding was visualized and recorded by enhanced chemo-luminescence using Super Signal West Pico ECL substrate (Pierce Biotechnology, Rockford, IL) on autoradiographic Super RX film (Fuji Photo Film Co., Tokyo, Japan).

Dectin-1 flow cytometry

Flow cytometry was used to detect extracellular cell surface dectin-1 protein expression on living INT407 cells. The monocyte fraction of isolated PBMCs was used as a positive control for dectin-1 expression. INT407 cells were washed with PBS and detached with trypsin-0.03% EDTA. Next, fresh complete medium was added and cell suspensions were centrifuged for 5 min at 1200 rpm at room temperature, followed by resuspending the cell pellets in PBS-1% BSA. Cells were counted and diluted to 1.25×10^6 cells/mL in PBS-1% BSA. Monoclonal murine anti-human dectin-1 IgG2b antibody or isotype-matched control antibody (R&D Systems) was added to the cells and incubated for 30 min on ice in the dark. Next, cell suspensions were centrifuged for 5 min at 1200 rpm and the pellets were resuspended in PBS-1% BSA. Washing was repeated and cells were incubated for 30 min on ice in the dark with the secondary antibody goat anti-murine IgG2b labelled with Alexa Fluor 488 (Molecular probes, Leiden, the Netherlands). Thereafter, cell suspensions were centrifuged again for 5 min at 1200 rpm, and pellets were resuspended in 200 mL PBS-1% BSA. The amount of fluorescence of 10 000 living cells was counted and analyzed with FACSsort and CellQuest analysis software (Becton Dickinson, Franklin Lakes, NJ). To distinguish monocytes from lymphocytes in the total population of PBMCs, a phy-

coerythrin-conjugated monoclonal antibody against CD14 was used (CLB, Amsterdam, the Netherlands). To evaluate the possible effects of trypsin treatment on dectin-1 expression by INT407 cells, PBMCs were incubated with trypsin-0.03% EDTA for 5 min under similar conditions as INT407 cells, and analyzed for dectin-1 expression as described above.

Fecal water preparation

Ileostomic contents were used to prepare fecal water as described previously [69]. Briefly, six patients with an ileostomy consumed a diet with or without oat β -glucan in random order. During the next 24h ileostomic contents were collected. The ileostomic contents were freeze-dried and pooled over the 24h periods. Differences in volumes from each time interval were corrected by adapting the relative contribution to the total volume of the pool sample. The pooled freeze-dried ileostomic contents were dissolved in PBS and centrifuged for 11.5 min at 13 500 rpm and at 17°C. The supernatant (further called fecal water) was carefully aspirated and used for the *in vitro* cell stimulation experiments. β -Glucan concentrations in the 24h pooled fecal water samples were calculated from the measured concentrations of β -glucan in the ileostomic content of each time interval, and amounted to be 120-180 $\mu\text{g/mL}$ [69]. We are aware that fecal water is a complex biological fluid. However, this is also the strength as by this approach we also include indirect effects. Moreover, as a consequence of the cross-over design each ileostomic patient is its own control making the interference of components other than the β -glucans less likely.

Cell stimulations

To evaluate dectin-1 involvement in the observed immune stimulating effects of β -glucan enriched fecal water, the effects of an accepted dectin-1 ligand zymosan (Sigma) [103] and different dectin-1 inhibitors were evaluated. For this, INT407 cells were plated in six-well plates at an initial density of 0.8×10^6 cells/mL in a total volume of 1.5 mL. After a confluence of 70-90% had been reached, culture medium was replaced by fresh medium with or without the indicated inhibitors, and fecal water or zymosan (100 $\mu\text{g/mL}$) always in the presence of an inflammation-inducing cocktail consisting of the cytokines interferon γ (IFN γ) (100 U/mL), IL-1 β (50 U/mL) and TNF α (10 ng/mL). After 16h of stimulation, culture medium was collected to determine IL-8 concentrations by enzyme-linked immunosorbent assay (ELISA) as described by Bouma *et al.* [88].

The inhibitors used were a goat anti-human dectin-1 polyclonal antibody raised against the extracellular domain of dectin-1 (R&D systems) and glucan phosphate at 0.2 $\mu\text{g}/\text{mL}$ and 100 $\mu\text{g}/\text{mL}$, respectively. Glucan phosphate was kindly provided by Professor Dr. D.L. Williams (Department of Surgery, James H. Quillen College of Medicine, East Tennessee State University, Johnson City, TN).

Stable transfection of NF- κ B in Caco-2 cells

To evaluate the effects dectin-1 inhibitors in another intestinal cell line, we used a stable NF- κ B reporter Caco-2 cell line. This cell line was chosen as we have previously shown immune stimulating effects of oat β -glucan fecal water in Caco-2 cells [69]. In addition, it is reported by others that Caco-2 cells contain dectin-1 transcripts [42]. The 6 κ B-TK-luciferase (NF- κ B reporter) plasmid and neomycin resistance plasmid were both kindly provided by Dr R.C. Langen (Department of Pulmonology, Maastricht University, The Netherlands). Cells were transfected using Lipofectamine 2000 (Invitrogen) according the manufacturers' instructions. Positive clones were selected by culturing with geneticin (1 mg/mL). For the experiments, Caco-2 NF- κ B reporter cells were plated in six-well plates at an initial density of 0.5×10^6 cells/mL in a total volume of 1.5 mL and used for stimulation experiments when they had reached 70-90% confluence. Culture medium was replaced by fresh medium containing fecal water with or without dectin-1 antibody (0.2 $\mu\text{g}/\text{mL}$) and glucan phosphate (200 $\mu\text{g}/\text{mL}$) in combination with an inflammation-inducing cytokine cocktail consisting of IFN γ (100 U/mL) and IL-1 β (50 U/mL). After 3 hours of incubation, cells were lysed in luciferase lysis buffer (Promega, Madison, WI) and stored at -80 °C. Luciferase activity was measured according to the manufacturers' instructions (Promega) and expressed relative to total protein (Bio-rad assay; Bio-rad, Hercules, CA).

Results

Dectin-1 expression on enterocytes

To evaluate dectin-1 involvement in the earlier observed immune stimulating effects in INT407 and Caco-2 cells [69], we first investigated the presence of dectin-1 in these cells. INT407 cells contained the mRNA products of the receptor. Both the isoforms A (697 bp) and isoform B (559 bp) of dectin-1 were

present. Sequencing confirmed that the PCR products from INT407 cells were indeed dectin-1 transcripts (data not shown). As expected, the same dectin-1 transcripts were present in PBMCs, which were used as a positive control (**figure 4.1a**).

Figure 4.1

A) Reverse transcriptase PCR (RT-PCR) expression analysis of dectin-1 in peripheral blood mononuclear cells (PBMCs) (positive control) and INT407 cells shows dectin-1 RT-PCR products in INT407 cells of both isoform A (697 bp) and isoform B (559 bp). Lane M, 100 bp DNA ladder.

B) Western blot analysis of dectin-1 expression showed the presence of dectin-1 in PBMCs (positive control), INT407 cells, Caco-2 NF- κ B reporter cells, and human enterocytes isolated from ileum and colon biopsies.

Next, the presence and cellular location of dectin-1 protein was evaluated by Western blot and flow cytometry. **Figure 4.1b** shows that dectin-1 protein is present in INT407 and Caco-2 NF- κ B reporter cells as well as in PBMCs. Moreover, enterocytes isolated from human ileum and colon also contained dectin-1 protein. Flow cytometry showed dectin-1 expression on the surface of monocytes, which were identified as CD14-positive cells in the PBMC mixtures (data not shown). Importantly, expression on PBMCs (including monocytes) was independent of trypsin treatment (**figure 4.2**), and allowed to analyze expression on INT407 cells after trypsinization. In contrast to the Western blot, dectin-1 expression on the extracellular membrane of INT407 cells was not detected by flow cytometry (data not shown). Altogether, these results suggest that dectin-1 protein is present in the enterocytes, but most likely not functionally expressed on the cell surface.

Figure 4.2
A) Dectin-1 expression on PBMCs was visualized by flow cytometry with a polyclonal goat anti-human dectin-1. B) Dectin-1 expression on PBMCs was independent of 5 min trypsin treatment.

Dectin-1 inhibitors do not counteract the immune stimulating effect of β -glucan fecal water

The dependence of dectin-1 for INT407 cells to respond to β -glucan enriched fecal water was determined using dectin-1 inhibitors. For this, the cells were pre-incubated with the polyclonal goat anti-human dectin-1 antibody during 1h, after which fecal water was added for a period of 16h. **Figure 4.3a** shows that the antibody was unable to inhibit the enhanced IL-8 production by INT407 cells after β -glucan fecal water as compared to placebo fecal water. The observed increase in IL-8 production in the pooled β -glucan fecal water sample (19.8 % and 23.4 %) was comparable with the median of all six individual samples (22.0 %) as previously shown [69]. Similar results were found when the dectin-1 inhibitor glucan phosphate was added to INT407 cells in combination with fecal water (**figure 4.3b**), that is, addition of glucan phosphate even en-

hanced IL-8 secretion by INT407 cells in β -glucan as well as in placebo fecal water by 35% and 34% respectively.

Figure 4.3

Dectin-1 inhibitors did not affect the increased IL-8 production after β -glucan by INT407 cells. The culture supernatant of INT407 cells was analyzed for IL-8 production after 16h incubation with oat β -glucan-enriched or placebo fecal water in presence of an inflammation inducing cocktail consisting of interferony (100 U/mL), interleukin-1 β (50 U/mL) and tumour necrosis factor α (10 ng/mL). Cells were pre-incubated for 1h with 0.2 μ g/ml polyclonal anti-human dectin-1 (A) or 100 μ g/ml glucan phosphate (GluP) (B). Values are means and SD of duplicate cultures.

Altogether, these data suggest that the enhanced IL-8 production by INT407 cells induced by oat β -glucan-enriched fecal water was independent of dectin-1 at the extracellular membrane. We confirmed these results in another enterocytes cell line as shown in **figure 4.4**. The dectin-1 inhibitors glucan phosphate and dectin-1 antibody were unable to inhibit the oat β -glucan-enriched fecal water-induced NF- κ B transactivation in Caco-2 NF- κ B reporter cells.

Figure 4.4

Dectin-1 inhibitors did not affect the increased NF- κ B transactivation after β -glucan by Caco-2 NF- κ B reporter cells. The cell lysates were analyzed for luciferase activity as measurement for NF- κ B transactivation after 3h incubation with oat β -glucan-enriched or placebo fecal water in presence of an inflammation inducing cocktail consisting of interferony (100 U/mL) and interleukin-1 β (50 U/mL). Cells were pre-incubated for 1h with 0.2 μ g/ml polyclonal anti-human dectin-1 (A) or 200 μ g/mL glucan phosphate (GluP) (B). Values are means and SD of duplicate cultures. RLU, relative light unit.

The dectin-1 ligand zymosan has no immune stimulating effect in INT407 cells

In line with the absent effects of the dectin-1 inhibitors on IL-8 production in INT407 cells after β -glucan enriched fecal water, zymosan (an accepted dectin-1 ligand) also did not increase IL-8 production in INT407 cells (data not shown).

Discussion

In addition to β -glucans from yeast and fungi, also β -glucans from cereals have been found to induce immune stimulating effects [7]. The influence of β -glucans from yeast, fungi and cereals, all present simultaneously within the intestinal lumen as part of our daily diet, on the intestinal immune response is of interest.

In this respect, we have earlier reported an increased immune response of INT407 and Caco-2 cells after addition of fecal water prepared from ileostomic contents obtained from subjects who had consumed an oat β -glucan-enriched diet as compared to placebo [69]. It was tempting to speculate that the effects of oat β -glucan fecal water on these enterocytes cell lines were mediated via the β -glucan receptor dectin-1. To investigate the possible role of dectin-1 we first checked dectin-1 presence on enterocytes. Caco-2 cells are already reported to contain dectin-1 transcripts [42] and our results showed dectin-1 at mRNA level in INT407 cells. Moreover, we demonstrated that dectin-1 protein is present in the both cell lines, although not expressed on the cell surface. Besides cell lines, we also showed that isolated human enterocytes from ileum and colon contained dectin-1 protein. The second aim of our study was to verify the functional involvement of the β -glucan receptor dectin-1 in the increased immune response of INT407 and Caco-2 cells to β -glucan enriched fecal water. However, here we present data showing that these effects are most likely independent of extracellular dectin-1.

Dectin-1 is described as the main β -glucan receptor on leukocytes and generally considered to be responsible for the cellular responses of β -glucan [22]. Northern blots have shown the presence of dectin-1 in the murine intestine [71]. Other studies, however, reported that besides immune cells, other cell types also contain β -glucan receptors, including lung epithelial cells (A-549) [104] and vascular endothelial cells [105]. In line, our results show dectin-1 both at mRNA and protein level in small intestinal INT407 cells. This is in correspondence to the findings of Saegusa *et al.* [42], who showed dectin-1 tran-

scripts in Caco-2 cells, another human derived enterocyte cell line. Moreover, also human isolated enterocytes from ileum and colon contained dectin-1 protein. However, when using flow cytometry, we were unable to demonstrate the presence of dectin-1 on the cell surface of INT407 cells. Dectin-1 expression at the outer membrane, which is considered obligatory for a functional role in macrophages, seems unlikely [106]. A function for intracellular dectin-1 has up till now not been described, but cannot be excluded. Similarly, other PRRs like Toll-like receptors 3, 7, 9 and also nucleotide-binding oligomerization domains (NODs) exert their function intracellular [107, 108]. Moreover, experiments in which dectin-1 was blocked supported our conclusion that INT407 and Caco-2 NF- κ B reporter cells do not express functional dectin-1 on the cell membrane. Two different dectin-1 inhibitors did not counteract the immune stimulating effects of β -glucan-enriched fecal water. Glucan phosphate is a specific dectin-1 antagonist and often used to block the β -glucan-induced immune effects [22]. Furthermore, the murine antibody has been described to block the action of dectin-1 [71]. In addition, also the generally accepted dectin-1 ligand zymosan could not stimulate the immune response in INT407 cells as measured by IL-8 production. This can also be interpreted as an absence of the zymosan receptor dectin-1.

As the immune stimulating effects of oat β -glucan-enriched fecal water seems to be dectin-1 independent, an alternative explanation might be that the consumption of a β -glucan-enriched diet has influenced *in vivo* immune responses within the GI tract of the ileostomy patients. This *in vivo* response may have changed the composition of the *in vitro* used fecal water, which resulted in an increased *in vitro* immune response of enterocyte cell lines exposed to the fecal water *in vitro*. It is of course entirely speculative to talk about potential candidates that could be present in higher or lower amounts in the fecal water and as such responsible for the observed effects, but changes in antimicrobial peptides or short chain fatty acid concentrations are a possibility. Next to the possibility that effects of oat β -glucan-enriched fecal water are indirect, there might be an alternative β -glucan receptor involved. Possible candidates may be complement receptor (CR)3 and lactosylceramide, which have been described as β -glucan receptors present on leukocytes [8]. Evens *et al.* [74] have shown that the chemokine responses of alveolar epithelial cells in response to *Pneumocystis carinii* cell wall β -glucan were indeed dectin-1 independent and were more likely mediated by lactosylceramide. We did, however, not further explore the identification of other receptors involved in the effects of oat β -glucan enriched fecal water.

The question why dectin-1 is not functionally expressed on enterocytes is intriguing. The intestinal wall is continuously exposed to antigens from the micro-

biota and of nutritional origin. Therefore, silencing of the intestinal immune response to these structures is important, as continuous stimulation of the epithelial lining results in an inflammatory bowel. The absence of functional dectin-1 on INT407 and Caco-2 NF- κ B reporter cells, as models of enterocytes, may be an example of this tolerance against these antigens as dietary β -glucans are present in the intestinal lumen.

In summary, we conclude that INT407 and Caco-2 cells - as models for enterocytes - do not contain functional dectin-1 at the cell membrane. This may have a protective function as the luminal surface of the gastrointestinal tract is abundantly supplied with β -glucans both from nutritional and potentially harmful sources. These dectin-1 ligands would otherwise induce continuous PRR activation with intestinal damage as a consequence. Finally, the finding that extracellular dectin-1 does not play a functional role in INT407 and Caco-2 cells, indicates that the previously found immune stimulating effects of β -glucan-enriched fecal water in enterocyte cell lines should be explained by another mechanism.

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CHAPTER 5

Effects of mushroom derived β -glucan-rich polysaccharide extracts on NO production by bone marrow-derived macrophages and NF- κ B transactivation in Caco-2 reporter cells: can effects be explained by structure?

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Abstract

Mushrooms are known for their immune modulating and anti-tumour properties. The polysaccharide fraction, mainly β -glucans, is responsible for the immune-modulating effects. Fungal β -glucans have been shown to activate leukocytes, which depend on structural characteristics of β -glucans. As edible mushrooms come in contact with the intestinal immune system, effects on enterocytes are also interesting. Our aim was to evaluate the effect of mushroom polysaccharide extracts varying in β -glucan structure, on nitric oxide production by bone marrow-derived macrophages (BMMs) from mice and on nuclear factor- κ B transactivation in human intestinal Caco-2 cells. We demonstrated that extracts from *Agaricus bisporus* stimulated nitric oxide production by BMM, whereas extracts from *Coprinus comatus* and spores of *Ganoderma lucidum* had only minor effects. Furthermore, extracts of *A. blazei* Murill and *Phellinus linteus* had no effect at all. Almost all mushroom extracts lowered nuclear factor- κ B transactivation in Caco-2 cells. Structural analysis of *A. bisporus* compared with *A. blazei* Murill suggests that branching of the β -glucan chain is essential for immune-stimulating activity. In conclusion, extracts from *A. bisporus* activate BMM, without activating enterocytes. These characteristics make *A. bisporus* an attractive candidate as a nutritional compound to stimulate the immune response in depressed states of immunity.

Introduction

Mushrooms, which belong to the basidiomycetes and ascomycetes classes of the kingdom fungi, contain many different biologically active compounds, e.g. polysaccharides, small proteins, lectins, and polyphenols. The polysaccharides are mainly β -glucans that are present in the cell walls [109]. It is believed that these compounds have immune-modulating effects [110-112]. Indeed, it has been reported that fungal β -glucans increase the phagocytic activity and the production of reactive oxygen intermediates [113], inflammatory mediators, and cytokines by leukocytes [22]. For *Pleurotus ostreatus* (Oyster mushroom), it was reported that a water extract from fruit bodies and mycelia played a role in increasing the production of reactive oxygen species (ROS) from neutrophils and has also additional immune-modulating properties involving all immuno-competent cells [114].

As part of the human diet, compounds derived from digested edible mushrooms come in contact with enterocytes, which are located at the frontline of the intestinal immune system. Enterocytes have to discriminate between harmful and harmless antigens. To perform this task they play an important role in orchestrating the intestinal immune response and can induce an inflammatory response if necessary. Therefore, it is likely that mushrooms might also affect the immune response of enterocytes. It has been demonstrated that intestinal macrophages [67] and enterocytes (presumably microfold (M)-cells) [42, 58] can take up and distribute β -glucans after oral intake. However, one study suggested that β -glucans from a fungal extract did not enter the blood as the effect *in vivo* was strongly diminished as compared with *in vitro* [115]. To examine whether the mushroom polysaccharide extracts have an effect on enterocytes we used a nuclear factor- κ B (NF- κ B) reporter Caco-2 cell line. NF- κ B is a transcription factor that plays a key role in the immune response. In inactive form it is present in the cytoplasm bound by its inhibitor I κ B. Upon stimulation I κ B is degraded by I κ B kinase and NF- κ B is free to move to the nucleus where it can bind to response elements of NF- κ B target genes. One of the consequences of NF- κ B activation is activation of the numerous NF- κ B target genes, which results in the production of cytokines like tumour necrosis factor α (TNF α) and ROS. Responsiveness of macrophages towards β -glucan treatment has already been reported by many different groups [26, 62, 116, 117]. More into detail, β -glucans have been classified as biological response modifiers, which can influence the activity of macrophages [26, 62, 117]. For that reason bone marrow-derived macrophages (BMM) have been used by others to examine the systemic immune modulating properties of β -glucans.

For this nitric oxide (NO), which is produced by the NF- κ B regulated enzyme NO synthase, was chosen as one of the outcome parameters in the BMM experiments as fungal β -glucans have been reported to modulate NO production [118-120].

β -Glucans consist of a backbone formed by linear $\beta(1\rightarrow3)$ -, $\beta(1\rightarrow4)$ - or $\beta(1\rightarrow6)$ -linked glucose molecules, which in addition can contain branches. The type of linkages of the backbone and branches differs for the different β -glucans. For example, it has been suggested that β -glucans from yeast and fungi consist of $\beta(1\rightarrow3)$ -linked glucopyranosyl units with $\beta(1\rightarrow6)$ -linked side chains, whereas β -glucans from oat and barley are composed of unbranched $\beta(1\rightarrow3)$ and $\beta(1\rightarrow4)$ linkages [7, 8, 22]. The activity of β -glucans may depend on their structural properties, like polymer length, degree of branching, tertiary structure, and molecular weight [100]. Besides the structure, the method of isolation can also affect the activity of β -glucans [109].

The aim of this study was to investigate the effect of several β -glucan-rich mushroom polysaccharide extracts, which were a priori based on the literature expected to vary in β -glucan structure, on NO production by BMMs from mice as well as on NF- κ B transactivation in a human small intestinal enterocytes-like cell line, the Caco-2 cell. As besides the structure, the method of isolation can also affect the immune-modulating activity of β -glucans, we also evaluated the effect of the mushroom extract with the highest *in vitro* activity in the BMM cells, e.g. the extract from *Agaricus bisporus*, when isolated with a different methodology. Additionally, we tried to find a structure-response relationship by evaluating some structural characteristics of two extracts with opposite effects, e.g. the extracts from *A. bisporus* and *A. blazei* Murill. We hypothesized that the branched β -glucans are the most potent immune stimulating extracts.

Materials and methods

Preparation of mushroom polysaccharide extracts

Different mushroom polysaccharide extracts were isolated and used for BMM and Caco-2 NF- κ B reporter cell stimulations. Extracts were isolated from spores (A) and fruit body tissue (B) of *Ganoderma lucidum*; from different parts of the *A. bisporus*, e.g. the caps and stems; and from *A. blazei* Murill, *Coprinus comatus*, and *Phellinus linteus*. In addition, α -glucans were extracted from the stems of *A. bisporus* to distinguish between α -glucan and β -glucan effects.

Semi-purified polysaccharides were obtained from fruiting body material by hot water extraction followed by ethanol precipitation as described before [113]. A final solution was made in phosphate buffered saline (PBS) at 900 µg/mL, which was then heated for 1h at 100 °C and stored at 4 °C. The extracts will be further referred to as fruit body polysaccharide extracts. $\alpha(1\rightarrow4)$ -Glucan was obtained from *A. bisporus* stipes by passage of the polysaccharide extract over a diethylaminoethyl-cellulose column. The colourless non-binding polysaccharide has a molecular weight (MW) > 200 kDa, which can be completely degraded by α -amylase, and is characterized as an α -glucan [113].

A. bisporus mushroom polysaccharide extract was also isolated from culture mycelium by centrifugation and filtration. To this extract four volumes of ethanol were added and precipitation was allowed to proceed overnight at 4 °C. The resulting precipitate was redissolved in water. To obtain the extract, the above-described procedure was repeated twice. The resulting fluid was sterilized by a 0.45 µm filter and adjusted to a concentration of 900 µg/mL and further called culture mycelium extracts.

Chemical analysis of glucans from *Agaricus* species

To try and explain the physiological effects by differences in structure, we determined the monosaccharide composition and analyzed the glycosidic linkages of *A. bisporus* and *A. blazei* Murill mushroom polysaccharide extracts.

Monosaccharide composition

To evaluate the monosaccharide composition, a 5 µL aliquot of the glucan solutions or suspension was diluted in 0.3 mL 2M trifluoroacetic acid (TFA) and hydrolyzed overnight at 100 °C. Solutions were evaporated to dryness at 40°C under a N₂ stream. Residual TFA was removed by two evaporation cycles in 0.5 mL methanol, as earlier. The final residue was dissolved in 0.5 mL water and analyzed for monosaccharides on a Dionex HPLC, equipped with pulsed amperometric electrochemical gold detector. A PA1-column (250 x 4 mm), fitted with a PA-1 guard column (10 x 32 mm), was used as the stationary phase and 0.020 M NaOH as the mobile phase at 1.0 mL/min (isocratic elution). Quantification of monosaccharides was performed on the basis of external standards.

Linkage analyses via gas chromatography-mass spectrometry of partially methylated alditol acetates

To determine the distribution of glycosidic linkages in the polysaccharides gas chromatography-mass spectrometry (GCMS) analysis of partially methylated alditol acetates (PMAAs) derived from these compounds was performed. Permethylation was performed according to one of the protocols described in Kang *et al.* [121] where 0.7% water was added during the incubation. Permethyated products were separated by phase partitioning after adding 0.5 mL cold (4 °C) chloroform and 2.5 mL cold water. The chloroform phase was washed 3 times with 2.5 mL water to remove all NaOH, evaporated to dryness and hydrolysed overnight in 0.3 mL 0.5M TFA at 100 °C. Further processing to PMAAs was performed as described [122]. PMAAs were quantified by GCMS using a Hewlett Packard HP 5890 gas chromatograph connected to a HP 5972A mass spectrometer, which was equipped with a ZB-5 column (30 m, i.d. 0.25 mm, layer thickness 0.25 µm), run at the following gradient: 3 min 170°C, 2 °C/min to 210 °C, then 10 °C/min to 230 °C and a final time of 4 min.

BMM stimulation

BMMs were isolated from the tibiae and femurs of C57BL/6 mice. Cells were cultured in 15 cm bacterial plastic petri dishes in standard RPMI with L-glutamine and HEPES (Invitrogen, Breda, the Netherlands), 10% heat-inactivated fetal calf serum (Bodinco BV, Alkmaar, the Netherlands), and 100 U/ml penicillin streptomycin (Invitrogen) with the addition of 15% L929 cell-conditioned medium kindly provided by Danielle Curfs (Department of Molecular Genetics, Maastricht University, the Netherlands). After 1 wk differentiation, cells were detached with lidocaine (Sigma Chemicals, St Louis, MO) and plated at a concentration of 1×10^6 cells/mL in a final volume of 0.5 mL. Cells were primed with 100 U/mL IFN γ during 24h to obtain a maximal effect on NO production, as earlier described [123]. Then the medium was refreshed and cells were incubated with the mushroom polysaccharide extracts in a concentration range of 50-200 µg/mL for 24 h. These concentrations are physiological as we reported earlier that the β -glucan concentration in the 24 h pooled fecal water obtained from ileostomic patients who consumed an oat β -glucan-enriched diet varied between 120 and 180 µg/mL [69]. Besides mushroom polysaccharide extracts we also evaluated the effects of cellulose, a linear molecule which consists of $\beta(1 \rightarrow 4)$ linkages (Sigma-Aldrich, Steinheim, Germany) on BMM-derived NO production to further substantiate our assumptions

regarding a possible structure-effect relationship. Supernatants from the BMM were removed after 24 h incubation and NO concentrations were determined. To evaluate whether the effects of the mushroom polysaccharide on NO production were mediated by the β -glucan receptor dectin-1, we used the dectin-1 inhibitor laminarin. For this, cells were incubated with 250 or 500 μ g/mL laminarin (Sigma Chemicals) 1h before stimulation with 200 μ g/mL *A. bisporus* stems, *A. bisporus* stems α -glucans, *A. blazei* Murill, and cellulose.

Measurement of NO and TNF α concentrations

NO concentrations in the supernatants of BMM were determined as total nitrite by the Greiss method [124]. In short, 50 μ l supernatant was combined with 50 μ l Greiss reagents (2.5% v/v phosphoric acid, 0.1% w/v naphthylethylenediamine dihydrochloride and 1.0% w/v sulfanilamide in distilled water). After incubation for 10 min at room temperature, absorbance was determined at 540 nm. Sodium nitrate (Sigma Chemicals) was used to prepare a standard curve (0-200 mM). Besides NO production we also determined TNF α production by BMM after stimulation with *A. bisporus* stems, *A. bisporus* stems α -glucan, and *A. blazei* Murill preincubated with 500 μ g/mL laminarin. TNF α concentrations were measured as described [125].

Intestinal cell culture

We also determined the effect of all mushroom polysaccharide extracts on enterocytes. For this, we used the human cell line Caco-2, which was obtained from the American Tissue Type Collection (Rockville, MD). Caco-2 cells were cultured in DMEM (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 1% penicillin streptomycin (Invitrogen), 1% sodium pyruvate and 1% non essential amino acids (Invitrogen). Cells were cultured at 37 °C in a 5% CO₂ humidified atmosphere, refreshed every second day and separated by trypsin-0.03% EDTA (Gibco BRL, Gaithersburg, MD) when they had reached 70-90% confluence.

Stable transfection of NF- κ B in Caco-2 cells

To evaluate the effects of the various mushroom-derived polysaccharide extracts on transcriptional activity of NF- κ B, a stable NF- κ B reporter Caco-2 cell

line was created. This cell line was chosen as we have previously shown immune-stimulating effects of oat β -glucan fecal water in Caco-2 cells [69]. The 6 κ B-TK-luciferase (NF- κ B reporter) plasmid and neomycin resistance plasmid were both kindly provided by Dr. R.C. Langen (Department of Pulmonology, Maastricht University, The Netherlands). Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturers' instructions. Positive clones were selected by culturing with geneticin (1 mg/mL). For the experiments, Caco-2 NF- κ B reporter cells were plated in 24-well plates at an initial density of 0.3×10^6 cells/mL in a total volume of 0.5 mL and used for stimulation experiments when they had reached 70-90% confluence. Culture medium was replaced by fresh medium containing different mushroom polysaccharides at physiological concentrations in combination with an inflammation-inducing cytokine cocktail consisting of IFN γ (100 U/mL) and IL-1 β (50 U/mL). After 3 h of incubation, cells were lysed in luciferase lysis buffer (Promega, Madison, WI) and stored at -80 °C. Luciferase activity was measured according to the manufacturers' instructions (Promega) and expressed relative to total protein (Bio-rad assay; Bio-rad, Hercules, CA).

Statistical analysis

All parameters are presented as means $\pm$ SD and were analyzed for statistical differences by analysis of variance (ANOVA). When a significant difference in concentration was found, the four concentrations were compared pairwise using a Tukey post hoc test for multiple comparison of observed means. Differences were considered significant at $p < 0.05$ (corrected for four groups by SPSS). All statistical analyses were performed using SPSS 15.0 (SPSS, Chicago, IL).

Results

Effects of mushroom polysaccharide extracts on NO and TNF α production by BMM

As demonstrated in **figure 5.1**, extracts isolated from *A. bisporus* fruit bodies significantly increased NO production by BMM. These effects were almost identical for extracts isolated from caps and stems. In addition, NO production was also increased ($p < 0.05$) when BMM were incubated with mycelium extract

isolated from *A. bisporus* indicating that these effects were independent of the method of culturing and isolation. Interestingly, the effects on NO production were not observed for all fruit body extracts. Spore extracts from *G. lucidum* and *C. comatus* also stimulated NO production but only significant at the highest concentration and not as prominent as extracts from *A. bisporus*. The polysaccharide extracts from *A. blazei* Murill, *P. linteus* and *G. lucidum* fruiting bodies had no NO-inducing activity.

Figure 5.1

Increased NO production by IFN γ (100 U/mL) primed BMM after 24 h stimulation with *A. bisporus* mushroom polysaccharide extracts. Extracts isolated from *C. comatus* and *G. lucidum* mushroom polysaccharide extracts had minor effects. Values are means and SD of duplicate cultures. Experiments have been conducted three times and a representative example is shown. Bars with different superscripts differ ($p < 0.05$). ^a Reference for the other mushroom polysaccharide extracts.

As the cell wall of mushroom consists for a large part of the polysaccharide β -glucan, it is tempting to suggest that these polysaccharides increased NO production. Therefore, to test whether effects of *A. bisporus* were β -glucan-specific, we also evaluated effects of *A. bisporus* derived α -glucans from the stems on BMM-derived NO production. In line with β -glucans isolated from *A. bisporus*, *A. bisporus* stem α -glucans dose-dependently increased NO production to a maximum of 12 mM (figure 5.2).

Figure 5.2
Mushroom polysaccharide extracts isolated from *A. bisporus* alpha dose- dependently increased NO production by IFN γ (100 U/mL) primed BMM after 24 h stimulation, whereas cellulose had no effect. Values are means and SD of duplicate cultures. Bars with different superscripts differ ($p<0.005$).

However, the maximum NO concentration that was reached was only 50% of the concentration observed after incubation with the *A. bisporus* polysaccharide extract containing merely β -glucans. Cellulose, consisting of only $\beta(1\rightarrow4)$ linkages had no effect on NO production by BMM (**figure 5.2**). For TNF α production we observed a comparable doses-response relationship as for NO (**figure 5.3**).

Figure 5.3
Mushroom polysaccharide extracts isolated from *A. bisporus* alpha dose- dependently increased TNF α production by IFN γ (100 U/mL) primed BMM after 24 h stimulation, whereas cellulose had no effect. Values are means and SD of duplicate cultures. Bars with different superscripts differ ($p<0.001$).

To evaluate the potential involvement of the β -glucan receptor dectin-1 in the observed NO production by BMM, the dectin-1 inhibitor laminarin was used. As shown in **figures 5.4 and 5.5**, laminarin had no effect on the *A. bisporus* stems β -glucan induced NO and TNF α production.

Figure 5.4

Dectin-1 inhibitor laminarin was unable to inhibit the increased NO production by BMM caused by 200 μ g/mL *A. bisporus* mushroom polysaccharide extracts. Values are means and SD of duplicate cultures. Experiments have been conducted to two times and a representative example is shown.

Comparable with the results described earlier, also in the presence of laminarin, *A. bisporus* stems α -glucan stimulated the NO and TNF α production to 50% beyond the level of *A. bisporus* stems β -glucans, whereas extracts isolated from *A. blazei* Murill and cellulose again showed no effect (**figures 5.4 and 5.5**). Dectin-1 inhibitor laminarin was unable to inhibit the increased NO production by BMM caused by 200 μ g/mL *A. bisporus* mushroom polysaccharide extracts. Values are means and SD of duplicate cultures. Experiments have been conducted to two times and a representative example is shown.

Figure 5.5
Dectin-1 inhibitor laminarin was unable to inhibit the increased TNF α production by BMM caused by 200 μ g/mL *A. bisporus* mushroom polysaccharide extracts. Values are means and SD of duplicate cultures. Experiments have been conducted two times and a representative example is shown.

Effects of *A. bisporus* stems mushroom polysaccharide extracts on NF- κ B transactivation in enterocytes

To evaluate whether the polysaccharide extracts isolated from the edible mushroom *A. bisporus* and the other mushrooms also modulated the immune response of enterocytes, we measured NF- κ B transactivation in our Caco-2 NF- κ B reporter cell line. **Figure 5.6a** demonstrates that the effects of extracts isolated from the fruit bodies were highly variable; however, almost all extracts lowered the transactivation of NF- κ B in Caco-2 cells as compared to buffered saline. This decreased NF- κ B transactivation was most obvious after incubation with the highest concentration of the extracts, whereas at 100 μ g/mL some extracts even showed an increased NF- κ B transactivation. The magnitude of the effect differed between the mushroom extracts. The extract isolated from *A. blazei* Murill and *C. comatus* fruit bodies showed the most pronounced decrease in NF- κ B transactivation (35.3 and 41.4% ($p=0.074$) at the highest concentration, respectively), whereas *P. linteus* had no effect at all. Furthermore, extracts from both spores and fruiting bodies of *G. lucidum* reduced NF- κ B transactivation at 50 μ g/mL and 200 μ g/mL. Finally, extracts isolated from two different parts of *A. bisporus* (stems or caps) showed similar effects at the highest concentration (reductions of 32 and 33%, respectively). Surprisingly, in contrast to extracts isolated from the *A. bisporus* fruiting bodies, extracts iso-

lated from *A. bisporus* culture medium did not lower NF- κ B transactivation (figure 5.6b).

Figure 5.6

a) Most of the different mushroom polysaccharide extracts decreased NF- κ B transactivation by Caco-2 NF- κ B reporter cells after 3 h stimulation in presence of IL-1 β (100 U/mL) and IFN γ (50 U/mL). Values are means and SD of duplicate cultures. Experiments have been conducted three times and a representative example is shown. ^a $p=0.074$, 200 μ g/mL versus control.

b) NF- κ B transactivation by Caco-2 NF- κ B reporter cells after 3 h stimulation with *A. bisporus* isolated from the culture medium in presence of IL-1 β (100 U/mL) and IFN γ (50 U/mL) is increased. Values are means and SD of duplicate cultures. Experiments have been conducted three times and a representative example is shown.

Chemical analysis of glucans from *A. bisporus* versus *A. Blazei* Murill

As the activity of β -glucans depends on the chemical structure, we determined the monosaccharide composition and linkage type to explain the differences in immune-modulating properties of *A. bisporus* and *A. blazei* Murill. The monosaccharide compositions of *A. bisporus* and *A. blazei* Murill are shown in **table 5.1**. The polysaccharides of *A. bisporus* consisted of mainly glucose (96.3%) with some minor proportions of galactose (2.9%) and xylose (0.8%). This polysaccharide can thus be considered as consisting of mainly glucans. *A. blazei* Murill polysaccharide was more complex as it contained in addition to glucose (57.7%) considerable proportions of galactose (27.7%); mannose and xylose (not separated, together 7.3%); fucose (4%); and some rhamnose, arabinose, fructose and GlcNH₂ (each about 1%). GlcNH₂ was probably present in the native oligosaccharide as GlcNAC, which was degraded by TFA-hydrolysis.

Table 5.1: Monosaccharide composition of *A. bisporus* and *A. blazei* Murill as determined by HPLC

Monosaccharide	<i>A. bisporus</i>	<i>A. blazei</i> Murill
Glucose	93.3	57.7
Galactose	2.9	27.7
Mannose/xylose	0.8	7.3
Fucose	n.d.	4.0
Rhamnose	n.d.	1.2
Arabinose	n.d.	1.2
GLcNH ₂	n.d.	0.9
Fructose	n.d.	0.7

n.d., not detected

In addition to the monosaccharide composition, we also measured the glycosidic type of linkage and branching. *A. bisporus* glucan contained predominantly 1→4 and a few 1→3 linkages, while no 1→6 linkages were measured (**table 5.2**). In agreement with the presence of 1→3 and 1→4 linkages 1→3,4-branching glucose residues were also observed at about 1.5%. The relative high number of terminal glucose residues and low number of branch points indicate a short-chain molecule. The high proportion of 1→4 glucan suggests that the main chain contains predominantly this type of linkage.

Table 5.2: Linkages of *A. bisporus* and *A. blazei* Murill as determined via GCMS of PMAAs

Linkage type	<i>A. bisporus</i>	<i>A. blazei</i> Murill
Terminal glucose	9.5	10.8
β(1 → 3)	3.5	5.1
β(1 → 4)	85.5	43.3
β(1 → 6)	n.d.	40.8
β(1 → 3, 4) branch	1.6	n.d.
β(1 → 3, 6) branch	n.d.	n.d.
β(1 → 4, 6) branch	n.d.	n.d.

n.d., not detected

For *A. blazei* Murill the linkage was different as more than 80% of the glucose residues in the glucan fraction were linked by either 1→4 or 1→6-linkages at a ratio of about 1:1, while a small proportion of 1→3 glucosidic linkages were also observed and with no branch points. The observations from this PMAA analysis may be interpreted by either a single main chain with alternating 1→4 and 1→6 linkages or by a mixture of more glucan chains with a single linkage type.

Discussion

Fungal β -glucans are known for their immune-modulating and tumour growth-inhibitory effects [110-112]. It has been suggested that the polysaccharides present in mushrooms are the most likely candidates for these effects. As edible mushrooms are present in our daily diet, effects of these mushrooms on (intestinal) immune responses are of potential interest for optimizing the functioning of our immune system. We here show that extracts isolated from *A. bisporus* – either from caps and stems from *A. bisporus* fruit bodies or from *A. bisporus* mycelium – stimulated the production of NO (a NF- κ B target gene) of BMM. Furthermore, purified α -glucan from the stems of *A. bisporus* dose-dependently stimulated the NO production also, although the maximum effect observed was only 50% of that achieved with *A. bisporus* β -glucan. When using cellulose, consisting of only $\beta(1\rightarrow4)$ linkages, no NO was produced. All results were identical if TNF α production by BMM was analyzed instead of NO. Immune-modulating effects of β -glucans are generally accepted, whereas the effects for α -glucan are debated. However, the fact that *A. bisporus* α -glucans stimulated BMM to a lesser extent as compared to *A. bisporus* β -glucans at least illustrates that effects are glucan-type dependent. Interestingly, effects were not identical for all mushroom extracts. *C. comatus* and *G. lucidum* A extracts had only minor effects on NO concentrations as compared with the effects of *A. bisporus*, whereas *A. blazei* Murill and *P. linteus* had no effect at all. Earlier, immune-stimulating effects of mycelium and fruit body extracts from *A. blazei* Murill, although not for all fractions, have been reported [116, 126]. It has been shown that the method of isolation and chemical modification can influence the activity of β -glucans, which may explain the difference in results between the studies [25, 109, 111]. The largest difference in effects on BMM behaviour was found for *A. bisporus* and *A. blazei* Murill, in which *A. bisporus* was the most potent immune-stimulating extract, whereas *A. blazei* Murill extract showed almost no effect. To explain these differences in activating potential we determined some structural characteristics of both extracts and tried to define a sort of structure-effect relationship. Chemical analysis demonstrated that the polysaccharide from *A. bisporus* consists of mainly glucose, whereas that from *A. blazei* Murill is a mixture of different monosaccharides. This may suggest that a β -glucan almost exclusively composed of glucose exerts a greater immune-stimulating activity as compared with glucans containing other monosaccharides also. However, cellulose consisting solely of glucose molecules has no NO-inducing activity on BMM. This implies that other factors besides monosaccharide composition play a role. A second pronounced structural difference between both extracts we observed was the type of lin-

linkages. *A. bisporus* was mostly composed of 1→4 linkages and some 1→3 linkages, while *A. blazei* Murill contains 1→4, 1→6 and some 1→3 linkages. It has been reported that 1→3 linkages are essential for immune-modulating activity and both extracts contain approximately the same amount of 1→3 linkages so this could not explain the difference in immune-modulating properties. Moreover, *A. bisporus* is in our hands free of 1→6-linkages, although earlier reports describe the presence of such linkages in glucans from this fungus [127]. The absence of 1→6-linkages in our extract may be due to the origin and developmental stage of the fungal tissue used for the isolation of glucans. Moreover, our method of extraction mainly isolates the mucilage layer of the cell wall, which has been reported to consist mostly of 1→4 linked polysaccharides with minor portions of 1→6 linkages [128]. In contrast, hot water extracts of only lamellae consist of polysaccharides with relatively high numbers of 1→6 linkages [129], whereas polysaccharide extracts made with KOH instead of hot water, consist of mainly 1→3 and 1→6 linkages. Despite the fact that our *A. bisporus* lacks 1→6-linkages, the immune-stimulating activity as described for other *A. bisporus* extracts remains [116]. Furthermore, *A. blazei* Murill has no branch points, which indicates a single chain molecule, where *A. bisporus* is branched with a few 1→3 side chains. It is generally believed that branches are important for immune-modulating activity of glucans [25], so this may explain the different immune-stimulating activities of *A. bisporus* and *A. blazei* Murill. This is further supported by the observation that oat β -glucan, which also lacks branches, showed comparable results on both BMM derived NO and TNF α as *A. blazei* Murill (data not shown). Oat β -glucan is composed of 1→3 and 1→4 linkages and the difference compared to *A. blazei* Murill is the absence of 1→6 linkages. However, this is not essential for immune-stimulating activity as these linkages are also absent in *A. bisporus*. Moreover, unbranched cellulose has no NO-inducing activity on BMM, although this could also be explained by the fact that this β -glucan consist only of 1→4 linkages. Overall, we conclude from this study that 1→3 branches are essential for immune-stimulating activity of mushroom polysaccharides as this is the main difference between *A. bisporus* and all other glucans tested. Nevertheless, we must remark that immune-modulating effects of other compounds present in the mushroom polysaccharide extracts, like polyphenols, cannot be excluded. Unfortunately, we do not have detailed information concerning the type or concentration of polyphenols as present in the mushroom polysaccharide extracts.

There seems to be a general awareness that the β -glucan receptor dectin-1 mediates the β -glucans induced immune stimulating effects of macrophages [22]. However, in our hands dectin-1 seems not to be involved in the observed NO production after *A. bisporus* by BMMs, as preincubation with the dectin-1

antagonist laminarin did not affect the response. This implies that the mushroom polysaccharide extracts exert their effect via another mechanism or receptor. This is in agreement with Wei *et al.* [113], who found no competing effects of laminarin on ROS production after addition of *A. brasiliensis* and *G. lucidum* by peripheral blood mononuclear cells (PBMCs). Other potential candidate receptors for the observed effects that have been suggested in this perspective are CR3, lacosylceramide, scavenger receptors, Toll-like receptor 2 or 4 [70, 113].

Since the mushroom polysaccharide extracts isolated from the edible *A. bisporus* showed the most potent immune-stimulating effect on BMM, we were also interested in its immune-modulating effects on enterocytes as the intestine is the place where enterocytes have to discriminate between potential harmful pathogens and vulnerable nutrients. These cells are located at the frontline of the intestinal immune system and orchestrate the immune response. Therefore, it has been suggested that oral administration of mushroom extracts is an easy way of increasing immunological tolerance, anti-tumour effect and the innate immunity against intracellular pathogens. In this respect, it has been shown that the consumption of mushroom derivatives can influence the (intestinal) immune response in mice. When extracts isolated from *Lentinula edodes* were dispersed with lecithin micelles and administered to mice, the number of intestinal epithelial lymphocytes in the small intestine increased as well as TNF α and IL-2 in serum [122]. Furthermore, mice fed with water soluble extracts isolated from *G. lucidum* and *A. blazei* Murill showed prolonged survival time after X-irradiation and the small intestinal crypt survival was increased [130], which is another example of immune modulation through consumption of mushrooms. To evaluate the effects of the mushroom polysaccharide extracts, and especially those from *A. bisporus*, we used NF- κ B transactivation as outcome measurement since this transcription factor plays a central role in the immune response. We demonstrated that the different mushroom polysaccharide extracts isolated from the fruit body tissue lowered NF- κ B transactivation in Caco-2 NF- κ B reporter cells, including extracts from the highly consumed *A. bisporus*. Although almost all extracts showed a decreased luciferase activity, the effects were highly variable between the extracts, which may be caused by biological diversity in our cell model or different threshold concentrations. However, when the extract from *A. bisporus* was isolated from the mycelium instead of the fruit body, no reduced NF- κ B transactivation was seen. The contrast can be explained by the method of isolation, which has been described to influence the activity of β -glucans [109].

Thus, the observed effects of the mushroom polysaccharide extracts, especially those isolated from *A. bisporus*, on transactivation of NF- κ B in Caco-2 NF- κ B

reporter cells were opposite to the activating effects seen in BMM. This discrepancy may be explained by the fact that intestinal cells are constantly exposed to pathogens from the intestinal lumen where they mostly dampen the local immune response to prevent severe intestinal inflammation, which is reflected by a decreased transactivation of the central inflammation marker NF- κ B. However, systemic immune cells like BMM, are not continuously confronted with pathogens and if so an effective response is desirable. Moreover, we must remark that the macrophage data were obtained by using mouse macrophages. However, similar effects of human and mice primary macrophages on pro-inflammatory cytokine production after exposure to zymosan, a crude β -glucan extract, have been reported [131].

In summary, we demonstrated that effects of mushroom polysaccharides on NO production by BMM depend on structural characteristics of the polysaccharides. *A. bisporus* showed a strong immune-stimulating effect on BMM, whereas *A. blazei* Murill has almost no effect. In contrast to the exclusive activating effects of *A. bisporus* in BMM almost all mushroom extracts lowered NF- κ B transactivation in Caco-2 enterocyte cells. Altogether, the edible mushroom *A. bisporus* seems an interesting nutritional compound in situations where the activity of the immune system needs to be stimulated, as *A. bisporus* activates macrophages without activating enterocytes. This might, for example, be beneficial in depressed states of immunity. The functionality of this assumption needs to be confirmed in studies especially designed for this research question.

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CHAPTER 6

In vivo effects of dietary β -glucans from oat on mucosal immune responses in man and mice

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Submitted

Abstract

Antimicrobial peptides and tight junction proteins are crucial for mucosal immunity. As oat β -glucan may affect intestinal immunity the aim of the present study was to evaluate its effects on the presence of antimicrobial peptides and the tight junction protein zonula occludens (ZO)-1.

For this we analyzed antimicrobial peptide levels in fecal water prepared from 24 h ileostomic bag contents collected from ileostomic patients consuming oat β -glucan enriched or control diets in a cross-over design. In addition, intestinal sections of mice, which received oat β -glucan via oral gavages for 3.5 days, were analyzed for lysozyme and ZO-1 expression.

We observed a trend to lower lysozyme (-17%; $p=0.076$) and bactericidal/permeability-increasing protein (-23%; $p=0.098$) levels in oat β -glucan enriched fecal water as compared to placebo. Also mice receiving oat β -glucan showed a lower lysozyme expression in stained distal small intestinal sections ($p=0.011$). Staining of ZO-1 was decreased in β -glucan treated mice indicating disruption of the tight junction integrity.

In conclusion, the consumption of oat β -glucan decreased the levels of antimicrobial peptides in fecal water from human ileostomy patients and its expression in distal small intestine sections in mice. The decreased intestinal integrity in mice could be explained by the drop in antimicrobial peptides.

Introduction

β -Glucans are glucose polymers that are major structural components of the cell wall of yeast, fungi and cereals. Both oat and fungal β -glucans have frequently been reported to modulate immune function [7, 22, 23, 26]. In addition, we have previously reported that fecal water prepared from ileostomy contents of patients who had consumed an oat β -glucan diet, increased the *in vitro* immune response of enterocytes as compared to fecal water prepared after a control diet [69]. We have previously suggested that the observed effects *in vitro* were not explained by the presence of β -glucans themselves in the fecal water [132]. It might be that the consumption of a β -glucan enriched diet has influenced *in vivo* immune responses already in the gastrointestinal tract of the ileostomic patients. This suggests that consumption of β -glucan influences the *in vivo* immune response in the gastrointestinal tract. This *in vivo* response may subsequently have changed the composition of the prepared fecal water, which could explain the increased *in vitro* immune response of enterocytes. To test this hypothesis we examined in the present study whether β -glucans affect the intestinal expression of antimicrobial proteins and tight junction proteins. This is not unlikely since nutritional components present in the lumen may affect the expression of antimicrobial [133-135] and tight junction proteins [136].

Antimicrobial peptides are mainly produced by paneth cells, which are located at the base of the intestinal crypts. These secretory cells produce and secrete numerous antimicrobial peptides, like defensins, lysozyme and secretory phospholipase A2 into the lumen. It is postulated that the luminal presence of antimicrobial peptides keeps intestinal crypts free of pathogens [137] to prevent translocation of pathogens over the epithelial lining as they may cause undesirable and harmful inflammatory responses or even sepsis. The importance of antimicrobial peptides was shown by the observation that the expression of these proteins relates to the resistance of man and mice against bacterial infections [135, 138, 139]. Of all antimicrobial peptides, defensins are by far most abundantly secreted. In addition to paneth cells, also enterocytes produce and secrete antimicrobial peptides, like bactericidal/permeability increasing protein (BPI), human β -defensins (hBD) and the human cathelicidin peptide LL-37 [140]. To test whether oat β -glucan consumption affects the mucosal immune response, we first analyzed the antimicrobial peptide content of fecal water obtained from ileostomy subjects that had consumed an oat β -glucan enriched or control diet. In addition, we measured in an animal study the effects of dietary oat β -glucans on the expression of antimicrobial protein marker lysozyme.

Besides secretion of antimicrobial substances, also an intact epithelial barrier plays an important role to prevent translocation. The epithelial barrier consists of enterocytes connected by junctional complexes, like tight junctions, adherens junctions, gap junctions and desmosomes [141]. Zonula occludens protein 1 (ZO-1) is an intracellular tight junction protein which connects the actin cytoskeleton to the paracellular tight junction proteins occludin and claudins [142]. Changes in expression of ZO-1 have been postulated as valid markers for changes in intestinal integrity [143]. Therefore, besides analyzing lysozyme expression we also evaluated the effects on ZO-1 in the β -glucan fed animals.

Material and methods

Experimental design human study and fecal water preparation

Fecal water was prepared from ileostomic contents obtained as described previously [69]. Briefly, six patients with an ileostomy were randomly assigned to a diet containing either 5 gram oat β -glucan or rich starch (control fiber). Next, ileostomic contents were collected during the next 24 h. The ileostomic contents of each subject were freeze-dried and pooled over the 24 h periods, thereby correcting for differences in volumes from each interval. Fecal water was prepared by dissolving the pooled freeze-dried ileostomic contents in phosphate buffered saline (PBS). Afterwards the ileostomic contents were centrifuged for 11.5 min at 13,500 rpm at 17 °C. The supernatant (further called fecal water) was carefully aspirated. By this approach we tried to mimic the physiological situation as much as possible in which β -glucan has passed the small intestine.

Antimicrobial peptide enzyme linked immuno-sorbent assays (ELISA)

Antimicrobial peptide concentrations were determined in fecal water. Concentrations of lysozyme, LL-37 and HBD-2 were determined using the Lysozyme ELISA kit (Immun Diagnostik Bensheim, Germany), the Human LL-37 ELISA test kit (Hycult biotechnology, Uden, The Netherlands) and the human BD-2 ELISA development kit (PeproTech, London, United Kingdom), respectively, according to the manufacturers' instructions. BPI was measured using a sandwich ELISA as described by Dentener [144].

Experimental design of the mice intervention study

Sixteen 6-8 week-old NF- κ B luciferase C57BL/6 reporter mice (Cgene, Oslo, Norway) generated by Carlsen *et al.* [145] were used in a placebo controlled dietary intervention study. The rationale behind using these mice was that we had also determined the effect of oat β -glucan on NF- κ B activation as described elsewhere (Volman *et al.*, in press). The mice were randomly assigned to two groups (both 4 males and 4 females), which received either 3 mg highly purified oat β -glucan of medium viscosity (20-30 cSt) (Megazyme International, Bray, Ireland; n=8) dissolved in 200 μ L sterile endotoxin free PBS or 200 μ L PBS (placebo; n=8). The oat β -glucan or the PBS was administered twice a day (at 8 a.m. and 5 p.m.) during 3.5 day via an oral gavage. Body weights of the mice were recorded daily. On day 4, all mice were anesthetized with pentobarbitone i.p. 3 h after the last gavage (8 a.m.) and sacrificed via a cardiac puncture. The small intestines were removed, cleaned after a midline incision and divided into two parts: the first (proximal) half for immunofluorescence of ZO-1 and the second (distal) half for immunofluorescence of lysozyme. From each part 5 cm was taken from the middle of the section for analysis. We measured the expression of lysozyme in the distal part of the small intestine, as the expression of paneth cell defensins increases in the distal direction of the small intestine. All parts were rolled up, frozen in liquid nitrogen and stored at -80°C until analysis. The mice were housed with 4 animals per cage under standard conditions (temperature $20-23^{\circ}\text{C}$, normal day/night rhythm) in wire-topped Macrolon type-1 cages with a layer of maize fibers as bedding. The mice were fed regular mouse diet (Hope Farms, Woerden, the Netherlands) and had free access to water and food during the entire experiment. The experiment was approved by the Animal Studies Ethics Committee of Maastricht University, The Netherlands (DEC 2006-115).

Immunofluorescence of zonula occludens protein-1 (ZO-1) and lysozyme

To determine the effects of oat β -glucan administration on the distribution of tight junctions and expression of antimicrobial proteins, we stained sections from the small intestine for ZO-1 and lysozyme from mice that received oat β -glucan and control mice. Frozen sections of rolled-up intestines were cut at 6 μm . For ZO-1 staining, frozen sections of 5 cm from the middle of the proximal part of the small intestine were allowed to air dry, fixed with 4% paraformaldehyde, and then washed three times with cold PBS. The sections were blocked

with 1:10 normal goat serum and then incubated for 1h at room temperature with rabbit anti-ZO-1 polyclonal antibody (Zymed, San Francisco, CA). As a negative control, sections were incubated with PBS-0.1% BSA. Thereafter, sections were washed three times with cold PBS, incubated with Texas Red conjugated anti-rabbit antibody (Jackson, West Grove, PA) for 50 min at room temperature and washed three times with PBS. Next, sections were incubated with 4',6-diamino-2-phenyl indole (DAPI) (0.5 mg/mL, Sigma) for 2 min, washed three times with PBS, dehydrated in ascending ethanol series (100-70%), and mounted in mounting solution (Dako Cytomation, Ely, United Kingdom). Distribution of tight junctions was recorded with Metasystems Image Pro System (Metasystem, Sandhausen, Germany) mounted on a Leica DM-RE fluorescence microscope (Leica, Wetzlar, Germany). Photomicrographs were recorded at a 200x magnification and evaluated in a blind manner. The loss of epithelial lining was scored on a scale ranging from 1 to 5 as follows: 1—normal, 2—some damage, 3—damage, 4—strong damage, and 5—severe damage.

For lysozyme staining, frozen sections of 5 cm from the middle of the distal part of the small intestine were allowed to air dry and fixed with 4% paraformaldehyde. Sections were incubated with rabbit anti-lysozyme antiserum (1:250; Dako) was incubated for 1h at room temperature and lysozyme was visualized with detected using a Texas red-conjugated anti-rabbit secondary antibody (1:50; Jackson ImmunoResearch Laboratories). Counterstaining was performed with fluorescein-conjugated wheat germ agglutinin at a dilution of 1:50 (Vector Laboratories) to identify morphology. Slides were mounted in DAPI containing Vectashield (Vector Laboratories) and visualized using a ApoTome-equipped microscope (Axioplan 2) connected to a digital camera (AxioCam Mr; all purchased from Carl Zeiss, Inc.). Photomicrographs were recorded at a 630x magnification and evaluated in a blind manner. The presence of lysozyme was scored on a scale ranging from 1 to 5 as follows: 1—no expression, 2—low expression, 3—normal expression damage, 4—strong expression, and 5—very strong expression.

Statistical analysis

Antimicrobial protein concentrations in fecal water are expressed as means $\pm$ SD. Differences between BPI and lysozyme concentrations in fecal water obtained at the end of the β -glucan and control period were tested by the paired t-test. A one-tailed probability value of less than 0.05 was considered to be significant. Scores of ZO-1 staining were expressed as median values with ranges. Differences in scores between β -glucan and placebo groups were

tested by the non-parametric Mann-Whitney test. A two-tailed p-value of less than 0.05 was considered to be statistically significant. Statistical analyses were performed using SPSS 15.0 (SPSS, Chicago, IL).

Results

A trend to lower antimicrobial peptide concentrations in β -glucan fecal water

To evaluate whether consumption of oat β -glucan affected the secretion of antimicrobial peptides, we evaluated a potential change in fecal water composition. As shown in **figure 6.1**, the concentration of lysozyme tended to be lower in the fecal water of subjects who consumed the β -glucan diet as compared to the placebo diet. Concentrations after β -glucan consumption (1.04 ± 0.36 ng/mL) were 17% lower as compared to the control diet (1.25 ± 0.51 ng/mL). This difference nearly reached statistical significance ($p=0.076$).

Figure 6.1: Lysozyme concentrations in fecal water

Fecal water was prepared from ileostomic content from subjects who had consumed a β -glucan or placebo diet and lysozyme concentrations were determined by ELISA. Panel A represents the concentrations for individual subjects, whereas panel B shows the average concentration for all subjects together. Indicated values are means of duplicates. * $p=0.076$ versus control fecal water.

A similar pattern was observed for BPI levels, which also tended ($p=0.098$) to be lower in fecal water after β -glucan consumption (2.36 ± 0.54 ng/mL) as compared to control (3.10 ± 1.30 ng/mL) (**figure 6.2**). The levels of HBD-2 and LL-37 were in all fecal water samples below their detection limits of 8 pg/mL and 0.14 ng/mL, respectively.

Figure 6.2: BPI concentrations in fecal water
Fecal water was prepared from ileostomic content from subjects who had consumed a β -glucan or placebo diet and BPI concentrations were determined by ELISA. Panel A represents the concentrations for individual subjects, whereas panel B shows the average concentration for all subjects together. Indicated values are means of duplicates. * $p=0.098$ versus control fecal water.

Oral administration of oat β -glucan decreased expression of lysozyme in the distal part of the small intestine of mice

The expression of the paneth cell marker lysozyme in the crypt of the distal small intestine was decreased in mice that received an oat β -glucan gavage as compared to mice in the control group, though not significant (figure 6.3).

Figure 6.3: Oral administration of oat β -glucan decreased expression of lysozyme in the distal part of the small intestine of mice
Ten mice received twice daily a gavage containing 3 mg/200 μ L oat β -glucan (N=5) or a placebo gavage (N=5) (PBS) during 3.5 days. Immunolocalization of lysozyme (red) showed a decreased expression in the intestine of mice that received an oat β -glucan gavage (A) as compared to control (B). Sections were counterstained for the nuclei (blue) and wheat germ agglutinin (green). Photographs are representative for all tissue sections studied. (magnification 630x).

Oat β -glucan consumption decreased intestinal integrity in the proximal small intestine of mice

Besides the secretion of antimicrobial peptides, we also evaluated the effects of oat β -glucan consumption on the expression of tight junction protein ZO-1. Mice who received oat β -glucan gavages showed a lower ZO-1 expression indicating a less intact epithelial lining as compared to control (**figure 6.4**). Moreover, the small intestine tissue was more disrupted and disorganized, illustrated by an irregular distribution of nuclei.

Figure 6.4: Oat β -glucan consumption caused intestinal damage in the proximal small intestine of mice

Fifteen mice received twice daily a gavage containing 3 mg/200 μ L oat β -glucan (N=7) or a placebo gavage (N=8) (PBS) during 3.5 days. Immunolocalization of ZO-1 (red) showed some loss of ZO-1 in the upper part of enterocytes from the proximal small intestine of control mice (panel B). However, oat β -glucan administration leads to significant more loss of ZO-1 (panel A). Moreover, the small intestine tissue was more disrupted and disorganized, illustrated by an irregular distribution of nuclei (blue). Photographs are representative for all tissue sections studied. (magnification 200x).

The median score for oat β -glucan was 4 (range 2 to 4), whereas mice that had been administered a control gavage scored 2 (range 1 to 3) (**table 6.1**). These scores were statistically different ($p=0.011$). Moreover, the expression of ZO-1 showed a tendency towards a negative correlation with the concentration of lysozyme ($r=-0.629$; $p=0.094$).

Table 6.1: Intestinal tight junction protein ZO-1 staining after oat β -glucan and PBS administration

Proximal small intestine of mice that received an oat β -glucan gavage (N=8) or a control gavage (N=8) during 3.5 day were stained for ZO-1. The loss of epithelial lining was scored on a scale ranging from 1 to 5 as follows: 1—normal, 2—some damage, 3—damage, 4—strong damage, and 5—severe damage.

β -glucan	placebo
2	1
2	1
4	2
4	2
4	2
-	2
4	3
3	2
4 (2 - 4)*	2 (1 - 3)

*p=0.011 versus placebo

Discussion

The luminal secretion of antimicrobial peptides as well as the maintenance of epithelial barrier integrity is crucial to prevent translocation of luminal pathogens across the epithelial lining. In this study we have now evaluated the effect of oat β -glucan consumption on the expression of antimicrobial peptides and tight junction proteins. We observed a tendency towards a decreased expression of lysozyme and BPI in fecal water obtained from ileostomic patients who had consumed an oat β -glucan enriched diet as compared to a control diet. Additionally, the expression of lysozyme was lowered in mice after administration of oat β -glucan for 3.5 days, whereas the expression of ZO-1 was decreased. To the best of our knowledge, this is the first study to evaluate the effects of dietary oat β -glucans on these proteins reflecting mucosal immunity and gut barrier integrity. Based on these findings we conclude that oat β -glucans lowered the intestinal expression and luminal secretion of antimicrobial peptides, which as such might have induced a decrease of ZO-1 indicating a loss of barrier function.

In general, β -glucans are known to enhance immune responses, but its effects on intestinal immune and barrier function are not understood in detail. It has already been shown that enriching diets with specific functional ingredients can affect intestinal barrier function. For example, consumption of probiotics by humans may modulate barrier function by increasing tight junction expression, activating leukocytes and modulating cytokine secretion [146, 147]. Moreover,

immune-enhancing formula ingredients (containing albumin, arginine and isoleucine) increased *in vitro* hBD-1 expression by human colon cells [133]. We specifically measured the expression of the paneth cell marker lysozyme in the distal part of the mice ileum, as the expression of paneth cell defensins increases distally in the small intestine with no expression in the colon [148, 149]. An early decrease in the gene expression of antimicrobial peptides including lysozyme has been reported after *Salmonella* infection in the ileal mucosa of rats followed by an elevation of cytokines in the mucosa and Peyer's patches [150]. In contrast to our results, Atlantic salmon who were administered yeast β -glucan i.p., showed an elevated intracellular lysozyme activity in the intestine [151]. Although speculative, it is possible that the β -glucan source (oat versus yeast) explained the difference in immune activity between our and the study of Paulsen *et al.* In fact, there are numerous examples of β -glucan structure specific effects on immune function related parameters [8, 25, 100]. It is also possible that the routes of administration (oral versus i.p.) effect has contributed to the differences in results.

Besides lysozyme, also BPI concentrations tended to be lower in fecal water obtained from ileostomic patients that had consumed an oat β -glucan enriched diet. BPI competes with pathogens for binding to pattern recognition receptors [152]. Antimicrobial peptides can dampen the endotoxin-stimulated production of pro-inflammatory cytokines (tumour necrosis factor α (TNF α) and IL-6) [153]. Therefore, this may explain our earlier observation that oat β -glucan fecal water, containing less antimicrobial peptides, increased the inflammatory response of intestinal cell lines. However, we also observed an increased production of the chemotaxis-inducing chemokine IL-8 [69], whereas antimicrobial peptides secreted at sites of infection normally induce chemotaxis [153, 154]. It should be realized however that enterocytes not only secrete IL-8, but also other chemokines, which were unfortunately not measured in our earlier *in vitro* study.

In general, we observed low levels of all measured antimicrobial peptides in fecal water. This may be due to dilution of these peptides when preparing the fecal water, as these peptides exert their antimicrobial activity mainly at the mucus surface and minimally in the intestinal lumen [155]. In line with this observation, we were unable to detect hBD-2 and LL-37 in fecal water, which may also be explained by the fact that these peptides are mainly produced by colonic epithelial cells [156], while the β -glucan had only passed the small intestine of the ileostomic patients. A possibility for the diminished antimicrobial peptide expression after oat β -glucan consumption may be that β -glucans blocked the carbohydrate receptors where otherwise pathogens would adhere. Such a competitive mechanism to prevent bacterial adherence is com-

parable to the defending mechanism of antimicrobial peptides [15]. We hypothesize that as a result the need for antimicrobial peptides might decrease, reflected by attenuated concentrations in the intestinal lumen. However, the diminished antimicrobial peptide expression was not accompanied by an increased intestinal protection as mice that had received oat β -glucan gavages showed more disruption of the epithelial lining as compared to placebo. Therefore, it might also be that the damaged mucosa has caused the drop in antimicrobial peptides, which made it easier for the pathogens present to modulate tight junctions. Indeed we observed a trend towards a correlation between the concentration of lysozyme and the degree of damage. On the other hand, antimicrobial peptides can also influence the expression of tight junction proteins as Mahar and colleagues showed that the antimicrobial peptide melittin increased the permeability of a Caco-2 monolayer via alterations in tight junction proteins [157]. Moreover, it has been reported that commensals [158] and also bacterial infections [159] can induce changes *in vitro* in the distribution of ZO-1. Additionally, ZO-1 protein has been shown to redistribute away from the tight junction during times of increased permeability [160]. Furthermore, the expression of ZO-1 is decreased in response to pro-inflammatory cytokines like $\text{TNF}\alpha$ and $\text{IL-1}\beta$ [161, 162]. Moreover, these changes were reported to be mediated partly by activation of the transcription factor nuclear factor- κB (NF- κB) [161]. Indeed, we showed increased NF- κB transactivation in the small intestine of mice that received oat β -glucan, which correlated with the ZO-1 expression in the proximal part of the small intestine in the oat β -glucan group (p 0.797; $N=7$). However, the secretion of pro-inflammatory cytokines was not affected (Volman *et al.*, in press). Another possibility could be that the loss of ZO-1 resulted from direct interaction of oat β -glucan with the epithelium. Nevertheless, the resulting loss of epithelial integrity might result in translocation of pathogens into the circulation. This is by definition undesirable since an increased permeability has been associated with the development of sepsis and multiple organ failure [163]. However, we did not observe any signals of illness in the mice that received oat β -glucan. In addition, there were no signs of difference in food consumption or bodyweight between the oat β -glucan and placebo group (data not shown). Moreover, there were no indications of systemic inflammation like sepsis due to intestinal damage, as we observed no elevated concentrations of pro-inflammatory cytokines in spleens of mice that received oat β -glucan as compared to placebo, but we have to realize that the follow-up was only 3.5 days. Also, when extrapolating to the human situation, no such side effects in longer term human intervention studies evaluating effects of β -glucans on for example cholesterol metabolism have been reported to our knowledge.

In summary, fecal water prepared from ileostomic contents of patients who had consumed an oat β -glucan diet contained lower levels of the antimicrobial peptides lysozyme and BPI as compared to fecal water from control diets. In addition, intestinal sections from mice, which were administered oat β -glucan via oral gavages for 3.5 days showed lower levels of lysozyme. We suggest that the diminished antimicrobial peptides in oat β -glucan fecal water may have caused the previously reported increased inflammatory response of intestinal cell lines. Moreover, the expression of the tight junction marker ZO-1 suggests disruption of the epithelial lining of mice that had received oat β -glucan gavages. We speculate that the indications of a decreased intestinal integrity in mice could be explained by the drop in antimicrobial peptides.

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CHAPTER 7

Dietary oat (1→3), (1→4)-β-D-glucans activate intestinal NF-κB in mice

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Abstract

The intestinal immune response can be modulated by dietary components, like β -glucans. We previously showed that fecal water enriched with oat β -glucan stimulated the cytokine-induced immune response of enterocytes. It is, however, not clear whether β -glucans activate the nuclear factor- κ B (NF- κ B) pathways in the intestine *in vivo* and if so, whether enterocytes, intestinal leukocytes, or both respond to the presence of β -glucans.

Therefore, we evaluated the effects of an oral gavage of 3 mg dietary oat (1 $\rightarrow$ 3), (1 $\rightarrow$ 4)- β -D-glucans that were administered twice daily during 3.5 days on intestinal NF- κ B transactivation and subsequent cytokine production of intestinal leukocytes and enterocytes in sixteen NF- κ B reporter mice. We hypothesized that oat β -glucan activates the central immune transcription factor NF- κ B followed by an increased intestinal cytokine secretion.

We found that mice that were administered oat β -glucans (N=8) showed an increased intestinal NF- κ B transactivation in leukocytes ($p=0.021$) and enterocytes ($p=0.012$), particularly in the proximal part of the small intestine (ileum), as compared to placebo mice (N=8). Surprisingly, NF- κ B was not activated in the colon. Furthermore, the level of IL-12 was increased in intestinal lysates from all compartments, whereas the concentration of IFN γ was decreased in the proximal small intestine ($p=0.046$). Finally, TNF α showed a trend towards a reduced production in the colon ($p=0.057$).

Since we have earlier shown that human enterocyte cell lines do not express functional dectin-1 *in vitro*, we now conclude that after consumption dietary oat β -glucans most likely firstly activate the intestinal leukocytes, which in turn increases cellular activation of enterocytes.

Introduction

The intestine not only absorbs nutrients, but also forms a crucial barrier to prevent invasion of pathogens and commensal bacteria into the body. For this, several mechanisms are present. The mucus coat and the secretion of anti-microbial proteins create a first line of defence, whereas pathogens that cross this barrier are confronted with the (gut-associated lymphoid tissue) GALT. The enterocytes (including Microfold (M)-cells) and intestinal lymphocytes, like dendritic cells, play an important role in these processes as they orchestrate the mucosal immune response [164].

There are numerous examples of dietary components affecting the intestinal immune response, which are therefore interesting candidates to influence intestinal mucosal processes related to various diseases [20, 165]. A typical example of a dietary component with such an effect is the viscous soluble fiber β -glucan. In natural products such as oat, barley and mushrooms, β -glucans are the major structural components of the cell wall. β -Glucans consist of a backbone formed by linear $\beta(1\rightarrow3)$, $\beta(1\rightarrow4)$ or $\beta(1\rightarrow6)$ linked glucose molecules, which in addition can contain branches. The type of linkages of the backbone and branches can differ for different β -glucans from various sources. For example, it has been suggested that β -glucans from yeast and fungi consist of $\beta(1\rightarrow3)$ linked glucopyranosyl units with $\beta(1\rightarrow6)$ linked side chains, whereas β -glucans from oat and barley are composed of unbranched $\beta(1\rightarrow3)$ and $\beta(1\rightarrow4)$ linkages [7, 8, 22]. With respect to their effects on the immune system, β -glucans can be considered as biological response modifiers [23]. As oat $(1\rightarrow3)$, $(1\rightarrow4)$ - β -D-glucans (oat β -glucan) are part of the human diet, they come in direct contact with enterocytes, M-cells and dendritic cells (DC), which are all located at the frontline of the intestinal immune system. It is, however, not clear whether specifically the enterocytes, the leucocytes or both respond to the presence of β -glucans in the lumen. In this respect, we have previously reported that fecal water prepared from ileostomy contents of patients who had consumed an oat β -glucan enriched diet, increased the immune response of *in vitro* cultured enterocyte cell lines [69]. In a second comprehensive series of experiments, we concluded that these effects were not mediated by the described β -glucan receptor dectin-1, since dectin-1 did not seem to be functionally expressed in enterocytes [132]. It can however not be excluded that an alternative β -glucan receptor is involved in the *in vitro* experiments with the enterocytes cell lines. Possible candidates may be complement receptor (CR)3 and lactosylceramide, which have been described as β -glucan receptors present on leukocytes [8]. Another possibility is that in the ileostomy patients the leucocytes instead of enterocytes had responded to dietary β -glucans,

which changed fecal water composition. Therefore, in this study we aimed to unravel which cells from the intestinal immune defence system respond to the presence of dietary β -glucan in the lumen.

In this respect, a number of *in vivo* studies focused on the role of enterocytes in response to β -glucan consumption. Saegusa *et al.* [42] speculated that β -glucans as part of intact yeast cells are taken up and degraded by enterocytes. However, Hong *et al.* [67] suggested that instead of enterocytes, gastrointestinal macrophages played a major role in the uptake and distribution of yeast and barley β -glucans after oral intake [67]. Overall, from the literature and our previous results there is no clear answer whether enterocytes or leukocytes are responsible for the immune modulating effects after β -glucan consumption. Therefore, the objective of this study was to evaluate the effects of dietary oat β -glucans that were administered via oral gavages on intestinal translocation of the transcription factor nuclear factor- κ B (NF- κ B) and subsequent cytokine production in intestinal leukocytes and enterocytes of NF- κ B reporter mice. NF- κ B is a transcription factor that plays a key role in the immune response. We hypothesize that the consumption of oat β -glucan activates intestinal NF- κ B, which may be favourable to alert the immune system, resulting in better resistance against attacks by pathogens. Since the consumption of oat β -glucan is easy to incorporate into the diet of people, this might be a approach to enhance the immune response of people with an impaired functioning of the immune system, like for example the elderly.

Materials and methods

Experimental design

Sixteen 6-8 week-old NF- κ B luciferase C57BL/6 reporter mice (Cgene, Oslo, Norway) stratified by gender (8 male/8 female) generated by Carlsen *et al.* [145] were used in a placebo controlled dietary intervention study. The mice were randomly assigned to two groups (both 4 males and 4 females), which received either 3 mg highly purified linear oat (1 $\rightarrow$ 3), (1 $\rightarrow$ 4)- β -D-glucan of medium viscosity (20-30 cSt) (Megazyme International, Bray, Ireland; n=8) dissolved in 200 μ L sterile endotoxin free phosphate buffered saline (PBS) or 200 μ L PBS only (placebo; n=8). The oat β -glucan or the PBS was administered twice a day (8 a.m. and 5 p.m.) during 3.5 days via a gavage. The rationale behind the administration during 3.5 days is to evaluate more acute effects.

The amount of 6 mg/day was based on studies from the group of Yun, who used 3 mg/day oat β -glucan [47, 55, 56]. Body weights of the mice were recorded daily. On the last day, all mice were anesthetized with pentobarbitone i.p. 3 h after the last gavage (8 a.m.) and sacrificed via a cardiac puncture. The intestines were removed, cleaned after a midline incision and divided into three parts: the first (proximal) and second (distal) half of the small intestine, and the colon. In addition, each section was divided into three parts for 1) luciferase activity measurement reflecting NF- κ B transactivation, 2) immunofluorescent NF- κ B staining and 3) cytokine protein measurement by means of a cytometric bead array (CBA) (see **figure 7.1**).

Figure 7.1: Schematic overview of division of the intestines

The intestines were divided into three parts: the first (proximal) and second (distal) half of the small intestines, and the colon. Next, each section was divided into three parts: 1) luciferase activity measurement (luciferase), 2) immunofluorescent NF- κ B staining (IF) and 3) cytokine detection by means of a cytometric bead array (CBA).

The intestinal parts for luciferase activity measurements were placed in ice-cold PBS for further proceeding the same day. The parts for immunofluorescence staining of the NF- κ B subunit p65 were rolled up, frozen in liquid nitrogen and stored at -80°C until analysis. Finally, the parts to be used for the CBA were frozen in liquid nitrogen and stored at -80°C until analysis. Besides the three intestinal parts, spleens were also removed, weighed, and used for cytokine detection by means of CBA. The mice were housed under standard conditions (temperature $20\text{--}23^{\circ}\text{C}$, normal day/night rhythm) in wire-topped Macro-lon type-1 cages with a layer of maize fibers as bedding with 4 animals per cage. The mice were fed regular mouse diet (SNIFF, Soest, Germany) and had free access to water and food during the entire experiment. The feed (pellets) contained 9 percent of energy (en%) fat, 58 en% carbohydrates, and 33 en% protein. Of the crude material, 4.9 % consist of fiber. The experiment was approved by the Animal Studies Ethics Committee of Maastricht University, The Netherlands (DEC 2006-115).

Luciferase activity in intestines of NF- κ B reporter mice

The enterocyte fractions of the three parts of the intestines (proximal small intestine, distal small intestine and colon) were used to determine luciferase activity as measure for NF- κ B transactivation. For this, the intestinal parts were removed from ice-cold PBS and incubated in 10 mL 30 mM EDTA on ice for 20 min. Afterwards, the intestines were transported into tubes containing 0.3 U/mL dispase at 37 °C for an additional 20 min. Next, intestines were washed with PBS in a Petri dish and the mucosal layer was scraped off the intestines using a blade. The PBS containing the scraped epithelial cells centrifuged at 1000g for 2 min, lysed in luciferase lysis buffer (Promega, Madison, WI) and stored at -80 °C. Luciferase activity was measured according to the manufacturers' instructions (Promega) and expressed relative to the amount of total protein (Bio-rad assay; Bio-rad, Hercules, CA).

Immunofluorescence of NF- κ B subunit p65

To examine the effects of oat β -glucan administration on the cellular distribution of NF- κ B in enterocytes and intestinal leukocytes, we stained the intestinal sections (proximal small intestine, distal small intestine and colon) for localization of the NF- κ B subunit p65. The degree of nuclear translocation was measured by analysis of the amount of NF- κ B p65 present in the nucleus. To differentiate between enterocytes and leukocytes, we counterstained the sections with the leukocyte marker CD45. Frozen sections of rolled-up proximal and distal small intestines, and colons were cut (6 μ m), and allowed to air dry. Next, sections were fixed with 3% paraformaldehyde, dehydrated in ascending ethanol series (100-50%), and washed with PBS. Sections were then incubated at room temperature with rabbit anti-NF- κ B p65 (2 μ l/mL, SC-372; Santa Cruz Biotechnology, Santa Cruz, CA) and rat anti-CD45 (0.5 μ g./mL; BD Biosciences, Heidelberg, Germany) in PBS-1% BSA for 45 min at room temperature. Thereafter, sections were washed three times with PBS and incubated with goat anti-rabbit Alexa 555 and chicken anti-rat Alexa 488 labelled secondary antibodies (Molecular Probes, Leiden, the Netherlands) in PBS for 30 min. Sections were counterstained with 4',6-diamino-2-phenyl indole (DAPI) (0.5 mg/mL, Sigma, St. Louis, MO) to label nuclei. As a negative control, sections were incubated with isotype control antibodies in PBS-1% BSA. Slides were then washed three times with PBS, mounted with Mowiol (Noechst, Frankfurt, Germany) and cover slipped. Distribution of NF- κ B p-65 and CD45 was recorded with a Nikon E800 fluorescence microscope (Uvikon, Bunnink, the Netherlands) coupled to a laser

A101C progressive scan colour CCD camera with identical gain settings. Digitally captured images were processed and analyzed using LUCIA laboratory image processing and analysis software (LUCIO G/F v. 4.8, build 099, Laboratory Imaging, Prague, Czech Republic). Nuclear translocation of NF- κ B p65 was defined as Alexa 555 intensity in nuclei stained by DAPI. Intestinal leukocytes were identified by DAPI and CD45 membrane staining (Alexa 488), whereas enterocytes were identified by DAPI staining only. To calculate NF- κ B translocation in leukocytes, Alexa 555 intensity was measured in all nuclei that were also counterstained for CD45. NF- κ B translocation in enterocytes was determined in all other nuclei that were not counterstained for CD45. The average intensity of four different images of the same slide was used to calculate NF- κ B translocation for each intestinal part of all mice. Negative control stainings were negative (data not shown).

Homogenisation and CBA

For analysis of cytokine concentrations, homogenates were made from sections of the proximal and distal parts of the small intestine, the colon, and spleens with a tissue homogenizer in 9 volumes (w/v) Greenberger lysis buffer (300 mmol/L NaCl, 15 mmol/L Tris, 2 mmol/L MgCl₂, 2 mmol/L Triton X-100 (Sigma), Pepstatin A, Leupeptin, Aprotinine (Roche, Mannheim, Germany), all 20 ng/mL; pH 7.4). The tissues were lysed in the Greenberger lysis buffer for 1 h on ice and centrifuged for 7 min at 3000 rpm and for 10 min at 14,000 rpm. The supernatant was collected and stored at -80 °C until cytokine analysis using a CBA. The mouse inflammation and Th1/Th2 CBAs (BD Biosciences) were used to determine simultaneously the concentrations of various cytokines i.e. interleukin (IL)-2, IL-4, IL-6, IL-10, IL-12p70, monocyte chemotactic protein (MCP)-1, interferon (IFN) γ , and tumour necrosis factor (TNF) α according to the instructions of the manufacturer as described earlier [166]. Briefly, 5 μ L of sample or the cytokine standard mixture was mixed with 5 μ L of the mixed capture beads and 5 μ L of the detection antibody-phycoerythrin (PE) reagent and incubated at room temperature for 2 h in the dark. Two-colour flow cytometric analysis was performed using a FACScan[®] flow cytometer (Becton Dickinson Immunocytometry Systems (BDIS), San Jose, CA). Data were acquired and analyzed using Becton Dickinson CBA software.

Statistical analysis

Luciferase activity, NF- κ B subunit p65 staining and cytokine levels are expressed as medians with interquartile ranges in the tables and as individual dots with medians in the figures. Differences between β -glucan and placebo groups were tested by the non-parametric Mann-Whitney test. Differences between luciferase activity of enterocytes and leukocytes were tested by the non-parametric Wilcoxon signed rang test. Correlations between NF- κ B translocation within leukocytes and enterocytes were calculated using the Spearman correlation test. A p-value less than 0.05 was considered to be statistically significant. Statistical analyses were performed using SPSS 15.0 (SPSS, Chicago, IL).

Results

NF- κ B translocation was increased in the proximal part of the small intestine of NF- κ B reporter mice

NF- κ B translocation in leucocytes and enterocytes from the proximal part of the small intestine was increased after oat β -glucan gavages as compared to placebo gavages (p=0.021 and p=0.012, respectively; **figure 7.2**).

Figure 7.2: Oat β -glucan gavage increased NF- κ B p-65 translocation in enterocytes and leukocytes in the proximal part of the small intestine in NF- κ B reporter mice as compared to PBS administration

Sixteen mice received twice daily a gavage containing 3 mg/200 μ L oat β -glucan (N=8) or a placebo gavage (PBS) (N=8) during 3.5 days. Intestinal nuclear NF- κ B p-65 staining showed a higher intensity in nuclei of enterocytes (panel A) and leukocytes (panel B) in the proximal part of the small intestine in the oat β -glucan group as compared to the control group. NF- κ B translocation was defined as NF- κ B p65 intensity (Alexa 555) in nuclei stained by DAPI, whereby intestinal leukocytes were identified by CD45 staining. The average intensity of four different images of the same slide was used to calculate NF- κ B translocation for each intestinal part of all mice. Values are expressed as individual dots with the median. * $p < 0.05$ versus control.

The elevated NF- κ B translocation in the oat β -glucan group as compared to the control group is also shown in **figure 7.3**. NF- κ B translocation in leukocytes and enterocytes of the proximal part of the small intestine after oat β -glucan gavages strongly correlated (Spearman's $\rho=0.905$, $p=0.002$). No effects of oat β -glucan gavage on NF- κ B translocation were observed in the distal part of the small intestine and colon in leukocytes as well as in enterocytes. Interestingly, the average NF- κ B translocation was higher in enterocytes as compared to leukocytes in all groups ($p=0.001$, $p=0.001$ and $p=0.000$ for the proximal and distal part of the small intestine, and the colon, respectively).

Figure 7.3: Increased NF- κ B translocation in enterocytes and leukocytes of the proximal part of the small intestine in NF- κ B reporter mice after oat β -glucan gavage as compared to PBS administration

Sixteen mice received twice daily a gavage containing 3 mg/200 μ L oat β -glucan (N=8) (1) or a placebo gavage (PBS) (N=8) (2) during 3.5 days. Immunostaining of cytoplasmatic and nuclear cellular distribution of NF- κ B subunit p65 (panel D; overlay of panel A, B and C). Intestinal nuclear NF- κ B p-65 (red, panel B) was more intense in nuclei (blue, panel A) in the oat β -glucan group as compared to the placebo in the proximal part of the small intestine. Leukocytes were counterstained with the marker CD45 (green, panel C) to discriminate them from enterocytes. Photographs are representative for all slides studied of the proximal small intestine. (magnification 40x)

Next, we evaluated luciferase activity as a measurement for NF- κ B transactivation in lysates of intestinal scrapings. Here we could not differentiate between enterocytes and leukocytes. As shown in **figure 7.4**, there was a slight increase after oat β -glucan gavages in the distal part of the small intestine, although this did not reach statistical significance ($p=0.141$). Moreover, NF- κ B transactivation as measured by the luciferase activity did not change in the proximal small intestine and the colon.

Figure 7.4: Increased intestinal NF- κ B translocation in distal part of the small intestine of NF- κ B reporter mice after oat β -glucan administration

Sixteen mice received twice daily a gavage containing 3 mg/200 μ L oat β -glucan (N=8) or a placebo gavage (PBS) (N=8) during 3.5 days. Intestinal NF- κ B translocation, determined by luciferase activity was increased in lysates of intestinal scraping from the distal part of the small intestine of NF- κ B luciferase reporter mice that had received an oat β -glucan gavage as compared to placebo mice. NF- κ B translocation is corrected for protein content and expressed as individual dots with the median.

Cytokine expression in the intestine and spleen

Besides NF- κ B activation, also cytokine concentrations in homogenates of the three intestinal parts and spleen were evaluated by means of a CBA. As shown in **table 7.1**, especially the concentration of Th1 cytokine IL-12 was elevated in the distal part of the intestine after the oat β -glucan gavages as compared to placebo ($p=0.014$). Also in the proximal part of the intestine an increased IL-12 production was observed after oat β -glucan, which nearly showed a trend ($p=0.102$). Most importantly, the overall pattern showed an increased IL-12 level in all locations (intestines and spleen) analyzed after oat β -glucan gavage as compared to placebo. Remarkably, despite the elevated IL-12 concentrations a decreased level of IFN γ was observed in the proximal part of the small intestine after oat β -glucan gavages as compared to placebo ($p=0.046$), whereas the

levels were similar in the distal part of the small intestine and for both groups. Moreover, TNF α levels were elevated in the colon of mice that had received oat β -glucan gavages as compared to placebo, which nearly reached statistical significances ($p=0.057$). Finally, IL-10 and MCP1 levels in the three parts of the intestine and the spleen did not differ between the two groups. The cytokine expression levels of IL-2, IL-4, IL-5 and IL-6 were all below the detection limit.

Table 7.1: Cytokine concentrations in homogenates of the proximal and distal part of the small intestine, colon and spleen of NF- κ B reporter mice that have received an oat β -glucan gavage (N=8) or a placebo gavage (N=8) during 3.5 days

Cytokine	Placebo	β -Glucan
<i>Proximal small intestine</i>		
TNF α (pg/mL)	27 (0-47)	6 (0-42)
IFN γ (pg/mL)	19 (12-99)	12 (0-23)*
MCP1 (pg/mL)	101 (0-122)	88 (64-200)
IL-10 (pg/mL)	130 (0-456)	165 (0-283)
IL-12 (pg/mL)	0 (0-76)	28 (0-45)
<i>Distal small intestine</i>		
TNF α (pg/mL)	34 (17-56)	37 (0-75)
IFN γ (pg/mL)	28 (13-73)	25 (16-61)
MCP1 (pg/mL)	97 (46-120)	95 (71-200)
IL-10 (pg/mL)	130 (52-200)	166 (102-283)
IL-12 (pg/mL)	0 (0-26)	39 (0-45)*
<i>Colon</i>		
TNF α (pg/mL)	0 (0-18)	13 (0-24)**
IFN γ (pg/mL)	0 (0-0)	0 (0-0)
MCP1 (pg/mL)	78 (44-158)	72 (49-139)
IL-10 (pg/mL)	126 (89-321)	141 (76-359)
IL-12 (pg/mL)	0 (0-85)	14 (0-72)
<i>Spleen</i>		
TNF α (pg/mL)	51 (41-65)	54 (47-72)
IFN γ (pg/mL)	31 (21-41)	29 (20-42)
MCP1 (pg/mL)	101 (61-127)	102 (88-149)
IL-10 (pg/mL)	175 (79-261)	140 (87-213)
IL-12 (pg/mL)	0 (0-35)	10 (0-24)

Cytokine concentrations were determined with CBA and are expressed as medians with ranges.

* $p<0.05$ versus placebo, ** $p<0.10$ versus placebo

Discussion

We here show that dietary oat 1 $\rightarrow$ 3, 1 $\rightarrow$ 4- β -D-glucan that was administered via oral gavages increased intestinal NF- κ B translocation in both leukocytes and enterocytes. A more pronounced NF- κ B activation may be favourable to alert the immune system against potential pathogens. Indeed, oat β -glucans have been reported to increase resistance towards bacterial challenges and intestinal parasitic infections pathogens [7, 47, 55, 56]. It is well accepted that intestinal leukocytes (e.g. DC) and specialized enterocytes (e.g. M-cells) sense the content of the intestinal lumen and influence the intestinal immune response. The increased NF- κ B translocation in enterocytes and leukocytes indicates that both cell types were activated by oat β -glucan. We have, however, earlier shown that *in vitro* enterocyte cell lines do not functionally express the β -glucan receptor dectin-1 [132]. Therefore, it is most likely that in this *in vivo* situation, intestinal DCs have sensed the oat β -glucan by extending dendrites through the intestinal epithelium and present β -glucan to naïve T cells [167]. However, in contrast to the human derived enterocyte cell lines, we did not evaluate whether C57BL/6 mice-derived enterocytes contain functional dectin-1. Alternatively, M-cells could have taken up oat β -glucan from the intestinal lumen and deliver it to immune cells in the underlying Peyer's patches [168]. We here demonstrate that the production of IL-12 was elevated in all compartments of the intestines after oat β -glucan as compared to placebo gavages, suggesting activation of the antigen presenting cells. Since IL-12 is predominantly secreted by leukocytes and hardly by enterocytes [169], combined with the possible lack of functional dectin-1 on enterocytes, it is tempting to speculate that the oat β -glucans most likely activated the intestinal leukocytes first. Next, the increased immune response of the intestinal leukocytes may in turn have activated NF- κ B in the enterocytes. Further evidence comes from our observation that direct *in vitro* administration of the same purified oat β -glucan decreased NF- κ B transactivation in enterocyte-like Caco-2 NF- κ B reporter cell line (unpublished data), which may suggest that cellular activation of enterocytes depends on the presence of (activated) leukocytes. Moreover, it has been reported that Caco-2 cells produced less cytokines [170] or even none [171] in absence of leukocytes, indicating the important immune orchestrating role for interacting intestinal leukocytes and enterocytes supporting our hypothesis. Hong *et al.* [67] have demonstrated that intestinal macrophages played a major role in the uptake and distribution of yeast and barley β -glucans after oral intake. They showed *in vitro* that degraded β -glucan particles were released in the medium by macrophages [67], which may imply that *in vivo* they can be

released into the circulation to induce systemic effects. However, we observed no differences in cytokine concentrations in the spleen between the two groups, suggesting that the oat β -glucan gavage did not affect the systemic immune response [172]. We must remark that this is no conclusive evidence for the absence of systemic effects, as we did not analyze for example systemic cytokine levels. Others have shown that β -glucans were taken up by enterocytes (presumably M-cells) instead of intestinal macrophages *in vivo* [42, 58]. Besides an increase in IL-12, we also demonstrated a trend towards an elevation in TNF α concentrations in the colon of mice in the oat β -glucan group. Since NF- κ B was not activated in this segment, other transcription factors, like interferon regulatory factor 3/7 (IRF3/7) or activator protein-1 (AP-1), which collaborate to induce transcription of cytokines [173] might also be involved. The same might hold for the reduction of the Th1 cytokine IFN γ in the proximal part of the small intestine while NF- κ B transactivation was increased.

We used two different techniques to determine NF- κ B translocation, showing only activation of NF- κ B in the proximal part different segments of the small intestine with immunofluorescence. This outcome may relate to the location of the intestinal samples used for the different analysis. The sections used for immunofluorescence were more distal than those for luciferase activity measurements (**figure 7.1**). As a result we have more or less used the duodenum for luciferase activity measurement of the proximal small intestine, which is known to contain a low number of Peyer's patches. These nodules play an important role in generation of a local immune response, which may be reflected by activation of NF- κ B. We indeed observed no elevated NF- κ B transactivation in enterocytes from the proximal part (duodenum) by luciferase activity measurement, whereas there was a slight increased NF- κ B transactivation in enterocytes from the distal segment of the small intestine. This may result from the fact that the number of Peyer's patches increases along the tract with highest density in the terminal ileum [174, 175]. Moreover, the proximal intestinal sections used for immunofluorescent analysis of NF- κ B translocation were sampled more distal than those used for luciferase activity measurements and these sections showed an increased NF- κ B transactivation. Finally, effects were specific for the small intestine, since no activation of NF- κ B was observed in the colon. This local NF- κ B sensitivity to inflammatory agents has also been reported for NF- κ B DNA binding activity and *in vivo* NF- κ B imaging in endotoxin-injected mice [176, 177]. An explanation for the fact that we did not observe an increased NF- κ B transactivation in the colon after β -glucan feeding may relate to the suggested formation of short chain fatty acids (SCFA) from β -glucans. Undigested oat β -glucans that reach the colon, can be fermented by anaerobic bacteria leading to SCFA. It has been shown that the increased SCFA concentra-

tion may inhibit NF- κ B transactivation [178, 179]. It has indeed been shown that *in vitro* fermentation of the same oat β -glucan as we used in our this study, modulates the microbiota composition and resulting SCFA profiles when compared with a no treatment control [180]. On the other hand, we only administered the oat β -glucan for 3.5 days, which might have been too short to induce immune modulating effects in the colon. Furthermore, as β -glucans are dietary fibers, they can modulate peristaltic movement, and lead to increased stool bulk and feces weight [165]. As the placebo gavage contained no dietary fiber, the presence of fibers as such may have influenced the outcomes. However, when functioning as a dietary fiber we would rather expect anti-inflammatory intestinal responses in mice administered oat β -glucan as described [165], while we showed that NF- κ B translocation was increased in the small intestine.

In summary, we found that dietary oat β -glucan administered via oral gavages to NF- κ B reporter mice increased NF- κ B transactivation of intestinal leukocytes as well as enterocytes in the proximal part of the small intestine as compared to placebo. This elevated NF- κ B activation may serve to prevent inflammation by preconditioning the cells against stronger attacks when necessary upon infection with pathogens. Interestingly, there was an elevated level of IL-12 present in intestinal homogenates. We therefore propose the following model; upon consumption dietary oat β -glucans activate intestinal leukocytes, which in turn induce a mild inflammatory phenotype of enterocytes.

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CHAPTER 8

Effects of α -glucans from *Agaricus bisporus* on cytokine production by LPS and PHA stimulated PBMCs; a placebo-controlled study in slightly hypercholesterolemic subjects

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Submitted

Abstract

Mushrooms are known for their immune modulating effect for which the polysaccharide fraction, mainly glucans, seems to be responsible. Fungal β -glucans have been studied extensively, whereas little is known about mushroom α -glucans. We have earlier shown that the polysaccharide fraction from the mushroom *Agaricus bisporus*, consisting for 90% of α -glucans, induced *in vitro* tumour necrosis factor (TNF) α and nitric oxide production. The purpose of the present study was to evaluate the effects of consuming *A. bisporus* α -glucan on *ex vivo* cytokine production by human peripheral mononuclear blood cells (PBMCs).

A double-blind randomized trial was designed in which fifty-six mildly hypercholesterolemic subjects consumed a control fruit juice with no added α -glucans (200 mL/day) for a 2 weeks run-in period. For the next 5 weeks, the control group (N=30) continued consumption of the control fruit juice, while the intervention group (N=26) consumed the same fruit juice enriched with α -glucans from *A. bisporus* (5 g glucans/day). Changes in interleukin (IL)-1 β , IL-6 and TNF α cytokine production by lipopolysaccharide (LPS)-stimulated PBMCs were evaluated, as well as changes in T-helper (Th)1/Th2 cytokines by phytohemagglutinin (PHA)-stimulated PBMCs.

Consumption of *A. bisporus* α -glucans lower LPS-induced TNF α production by 69% ($p=0.017$) as compared to the control group, whereas no effect on IL-1 β and IL-6 was observed. No obvious Th1-Th2 skewing by PHA-stimulated PBMCs was observed. However, we observed a trend towards a decreased production of IL-12 and IL-10.

Our current finding suggests that *in vivo*, α -glucans have lost their efficacy to stimulate the immune response as observed *in vitro*.

Introduction

Mushrooms have been studied extensively for their immune modulating and anti-tumour properties [22, 110-113]. Indeed, there are many potentially biologically active compounds present in mushrooms, e.g. small proteins, lectins, polyphenols and polysaccharides. It is assumed that the polysaccharides are a main bioactive part of the mushrooms, at least in relation to their immune modulating effects [109]. These polysaccharides are mainly β -glucans that are present in the cell walls. In addition, also α -glucans are present in the polysaccharide fraction of mushrooms [10]. Whereas the effects of β -glucans have been studied extensively, little is known about the immune modulating effect of mushroom α -glucans. We have reported earlier that a polysaccharide fraction from *Agaricus bisporus*, which contained 90% α -glucan increased nitric oxide and tumour necrosis factor (TNF) α production by bone marrow-derived macrophages from mice *in vitro* (Volman *et al.*, in press). Furthermore, Bittencourt *et al.* [181] have reported that α -glucans from *Pseudallescheria boydii* stimulated the *in vitro* secretion of TNF α and interleukin (IL)-12 by mice macrophages and dendritic cells. This increased secretion of IL-12 indicates a polarization of naïve T-cells into T-helper (Th) type 1 skewed responses, which is generally attributed to mushroom β -glucans [32, 38, 39, 182]. In addition, animal studies have also shown that consumption of *A. bisporus* stimulated the immune response [183, 184], but the bioactive fraction from these extracts is not known. It is possible that these immune-modulating effects were due to *A. bisporus* derived α -glucans extracts, which have been studied to some extent *in vitro* and in animals. Effects of consuming these extracts on *ex vivo* cytokine production by isolated and stimulated human peripheral mononuclear blood cells (PBMCs) have however, to the best of our knowledge, not been studied in well-controlled human trials. Therefore, the purpose of the present study was to evaluate PBMC cytokine secretion patterns after consumption of α -glucan rich fractions from *A. bisporus* incorporated into fruit drinks in humans. For this, PBMCs were isolated and stimulated *ex vivo* by two approaches. First, we measured IL-1 β , IL-6 and TNF α production after lipopolysaccharide (LPS) stimulation and secondly we evaluated a potential shift in the Th1/Th2 balance of secreted cytokines (IL-2, IL-4, IL-5, IL-12, IL-13, IL-17 and interferon (IFN)- γ) after phytohemagglutinin (PHA) induced T-cell proliferation of isolated PBMCs.

Methods

Subjects

Fifty-six apparently healthy men and women with slightly elevated serum total cholesterol concentrations (between 6.0 – 8.0 mmol/L) were included in this study. Volunteers were recruited from Maastricht and surrounding villages by advertisements in local newspapers. In addition, subjects who had participated in earlier studies at our department were approached. Inclusion criteria were stable body weight (weight gain or loss <3 kg in the past 3 months); Quetelet index <32 kg/m²; systolic blood pressure <160 mmHg, and diastolic blood pressure <95 mmHg; no presence of glucosuria, proteinuria or anemia; no use of medication or a prescribed diet known to affect lipid or glucose metabolism; no history of coronary heart disease, cancer, diabetes, kidney, liver, pancreatic disease, or malignancies <5 y ago; no abuse of drugs and/or alcohol; and no pregnant or breast-feeding women, serum triacylglycerol concentrations <3.0 mmol/L and serum total cholesterol concentrations between 6.0 and 8.0 mmol/L. Baseline characteristics are shown in **table 8.1**.

Table 8.1: Baseline characteristics of the subjects before start of the study

	Control	<i>A. bisporus</i>
Age (years)	57 ± 9	57 ± 7
Sex (males/females)	17/13	15/11
Smokers (yes/no)	2/28	2/24
BMI (kg/m ²)	25.4 ± 3.6	26.4 ± 2.8
Total cholesterol (mmol/L)	6.55 ± 0.89	6.65 ± 0.70
Systolic blood pressure (mmHg)	129 ± 12	127 ± 12
Diastolic blood pressure (mmHg)	84 ± 7	84 ± 7

Values are means ± SD

There were no statistical differences between the two groups in these baseline characteristics. The subjects were requested not to change their habitual diets, levels of physical exercise, smoking habits, or use of alcohol during the study. Blood donation or participation in another biomedical trial was not allowed 30 days before the start and during the study. The Medical Ethical Committee of the University of Maastricht approved the study and all subjects gave written informed consent before entering the study. Eight subject dropped out during the study - all during the run-in period - because of various reasons not related to the experimental products, e.g. holidays, difficulties during venipunctures.

Study design

The study had a placebo-controlled, double-blind, parallel design. Before the study started, all 56 subjects were randomly divided into two groups. During the first two weeks of the study (run-in period), all subjects consumed a fruit juice (200 mL/day) without added α -glucans from *A. bisporus*. The 200 mL was consumed at two occasions divided over the day, i.e. ± 100 mL around lunch and ± 100 mL around dinner. During the following 5 weeks intervention period, the control group (N=30) continued consumption of the control fruit juice, while the intervention group (N=26; α -glucan group) consumed the same fruit juice but now enriched with α -glucans from *A. bisporus* (*A. bisporus* juice providing 5 g glucans/day). The glucan extracts were especially for this study prepared by Innerlife (Venlo, the Netherlands) and subsequently incorporated into the fruit juices. The fruit juice was a blend containing ten different fruits and contained per 100 mL 47.2 kcal, 11.3 g carbohydrate, 0.01 g fat and 0.47 g protein. During the study, subjects recorded in diaries about their illnesses, medication used, alcohol consumption, any deviations to the study protocol, and any side effects experienced.

At the end of the run-in period (week 2) and the end of the intervention period (week 7) blood was sampled after an overnight fast of at least 10 h. Subjects were not allowed to consume alcohol 24 h before blood sampling and were not allowed to smoke the morning before blood sampling. There were two smokers in both the control and the α -glucan group. Blood was sampled in endotoxin-free heparinized tubes and serum tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) for PBMC isolation and clinical chemistry, respectively.

PBMC stimulation

To examine *ex vivo* cytokine production by PBMCs, cells were isolated from 16 mL heparinized whole blood by means of gradient centrifugation under sterile conditions using Lymphoprep according to the manufacturers' instructions (Nycomed Ltd, Birmingham, United Kingdom). After isolation, PBMCs were immediately plated in 24-well flat-bottom culture plates.

For evaluation of IL-1 β , IL-6 and TNF α production, cells were diluted to 2.5×10^6 cell/mL and seeded. Cells were mixed with LPS (E Coli 055:B5, Sigma, St Louis, MO) or polymyxin B (Sigma) to a final concentration of 10 ng/mL. Polymyxin B was used as a negative control as it binds to LPS. Cells were cultured in RPMI-1640 with HEPES and L-glutamine (Invitrogen, Breda, The Netherlands) supplemented with 1% heat-inactivated human serum pool, 1% penicillin strepto-

mycin (Invitrogen) and 1% sodium pyruvate (Invitrogen). The cells were incubated for 4 h at 37 °C in a 5% CO₂ humidified atmosphere. After incubation, the culture media were aspirated and centrifuged at 1,500 RPM for 5 min to obtain cell-free media, which were stored at -80 °C until further analysis.

For analysis of Th1/Th2 cytokine production, PBMCs were diluted to 1×10^6 cells/mL and seeded in 24-well flat-bottom culture plates. Next, 50 µg/mL PHA (Roche, Mannheim, Germany) was added to initiate proliferation of the CD4⁺ T cells. Medium was aspirated after 52 h proliferation and centrifuged at 14,000 RPM for 2 min. Culture media were stored at -80 °C until further analysis for cytokines.

Cytokine analysis

IL-1 β , IL-6 and TNF α concentrations in supernatant of 4 h stimulated PBMC were assessed by commercially available MSD multiplex ELISAs (MSD, Gaithersburgh, MD), as described by the manufacturers. Also IL-2, IL-4, IL-5, IL-12, IL-13, IL-17 and IFN- γ concentrations were analyzed in supernatant of 52 h stimulated PBMC, by custom panel MSD multiplex ELISAs (MSD).

Clinical chemistry

Serum samples collected at the end of the run-in and intervention period were analyzed to determine parameters reflecting kidney and liver function (creatinine, alanine transaminase, aspartate aminotransferase, γ -glutamyl transpeptidase, alkaline phosphatase, and total bilirubin) by routine procedures.

Statistics

Normality was tested by the Shapiro-Wilk test. Baseline characteristics are presented as means $\pm$ SD and were analyzed for statistical differences by an independent-samples t-test. The baseline cytokine concentrations were tested by the non-parametric Mann-Whitney test, as these were not normally distributed. For each subject, responses in amounts of cytokines produced were calculated as the change between the concentration at the end of the run-in period (week 2) and those at the end of the intervention period (week 7). The same was done for clinical chemistry parameters. Cytokine concentrations and clinical chemistry parameters are presented as medians with ranges. Since the

changes in concentrations of the cytokines, liver and kidney function parameters were not normally distributed, the differences in changes were tested by the non-parametric Mann-Whitney test. Differences were considered statistically different at $p < 0.05$. All statistical analyses were performed using SPSS 15.0 (SPSS, Chicago, IL).

Results

Effects of α -glucans from *A. bisporus* on cytokine production by LPS stimulated PBMCs

Concentrations of the cytokines IL-1 β , IL-6 and TNF α produced by LPS stimulated PBMCs both at the end of the run-in period as well as at the end of the experimental period were analyzed (table 8.2).

Table 8.2: Effects of α -glucan consumption (5 g/d) from *A. bisporus* on median IL-1 β , IL-6 and TNF α concentration after ex vivo LPS stimulation of PBMCs

	Control (N=30)		<i>A. bisporus</i> (N=26)	
<i>IL-1β</i> (pg/mL)				
Run-in	391	(151 to 1189)	488	(217 to 1030)
Test period	455	(126 to 1427)	425	(158 to 1364)
Change	15	(-412 to 346)	-81	(-387 to 334)
<i>IL-6</i> (pg/mL)				
Run-in	1760	(535 to 4093)	2249	(466 to 4108)
Test period	2310	(776 to 5378)	2334	(894 to 4710)
Change	396	(-695 to 1768)	212	(-834 to 1633)
<i>TNFα</i> (pg/mL)				
Run-in	3053	(885 to 8203)	3465	(944 to 6688)
Test period	3795	(895 to 8414)	3677	(863 to 7499)
Change	792	(-1071 to 1675)	249 ^a	(-656 to 2025)

Values are medians with ranges. PBMCs were stimulated for 4h with 10 ng/mL LPS from E Coli 055:B5. ^a $p=0.017$ versus control

At baseline there were no differences between both groups. The median change in TNF α production by PBMCs was 69% lower in the *A. bisporus* group (249 pg/mL, range -656 to 2025 pg/mL) as compared to the change in the control group (792 pg/mL, range -1071 to 1675 pg/mL; $p=0.017$). No statistical

differences were found for the changes in IL-1 β and IL-6 between both groups. All polymixin B controls were negative (data not shown).

Effects of α -glucan rich fractions from *A. bisporus* on cytokine production by PHA stimulated PBMCs

The effects of the consuming α -glucan enriched drinks on cytokine production by PHA stimulated PBMCs as compared to the effects of PBMCs isolated from the control group are shown in **table 8.3**. At baseline there were no differences between both groups. The change in IL-12 production after the intervention period showed a trend towards a decreased production after *A. bisporus* consumption (-10 pg/mL, range -79 to 37 pg/mL) as compared to control drinks (4 pg/mL, range -51 to 53 pg/mL; $p=0.059$). Also the production of IL-10 showed a trend towards a lower production after *A. bisporus* consumption (-159 pg/mL, range -1454 to 918 pg/mL) as compared to the control group (56 pg/mL, range -644 to 1343 pg/mL; $p=0.053$). There was no effect of the α -glucan fractions from *A. bisporus* on the production of IFN γ , IL-2, IL-13, IL-4 and IL-17 of PHA stimulated PBMCs (p -values for diet effects: IFN γ , $p=0.451$; IL-2, $p=0.274$; IL-13, $p=0.961$; IL-4, $p=0.791$ and IL-17, $p=0.173$) (**table 8.3**).

Effects of α -glucan enriched drinks on kidney and liver function parameters

Parameters reflecting kidney and liver function were determined in serum samples from all subjects at end of the run-in period as well as at the end of the intervention period. Median creatinine, alanine transaminase, aspartate aminotransferase, γ -glutamyl transpeptidase, alkaline phosphatase, and total bilirubin concentrations were all within normal ranges at both occasions (**table 8.4**). Moreover, the changes were not different between the intervention and control groups, except for creatinine, which showed a trend towards a lower concentration in the *A. bisporus* group ($p=0.081$).

Table 8.3: Effects of α -glucan consumption (5 g/d) from *A. bisporus* on median IFN γ , IL-12, IL-2, IL-13, IL-4, IL-10 and IL-17 concentration after *ex vivo* PHA stimulation of PBMCs

	Control (N=25)		A. bisporus (N=23)	
Th1 cytokines				
IFN γ (pg/mL)				
Run-in	2549	(918 to 9444)	2432	(525 to 7484)
Test period	2439	(453 to 7935)	1882	(667 to 9036)
Change	-291	(-4068 to 1392)	141	(-1534 to 3106)
IL-12 (pg/mL)				
Run-in	59	(37 to 108)	57	(47 to 148)
Test period	55	(30 to 125)	55	(24 to 106)
Change	4	(-51 to 53)	-10 ^a	(-79 to 37)
IL-2 (pg/mL)				
Run-in	6362	(1442 to 18194)	8618	(741 to 31607)
Test period	6206	(1513 to 15881)	4776	(600 to 28082)
Change	-937	(-8112 to 5177)	-2073	(-10866 to 7649)
Th2 cytokines				
IL-13 (pg/mL)				
Run-in	3185	(946 to 6631)	3064	(1323 to 7254)
Test period	3019	(942 to 6450)	3157	(1258 to 7980)
Change	-181	(-1835 to 1857)	-201	(-3281 to 1147)
IL-4 (pg/mL)				
Run-in	179	(94 to 468)	190	(90 to 668)
Test period	154	(76 to 453)	180	(56 to 418)
Change	-12	(-205 to 171)	-12	(-292 to 73)
Other cytokines				
IL-10 (pg/mL)				
Run-in	1310	(399 to 3095)	1106	(618 to 2783)
Test period	1422	(455 to 3956)	1057	(631 to 2127)
Change	56	(-644 to 1343)	-159 ^b	(-1454 to 918)
IL-17 (pg/mL)				
Run-in	993	(402 to 3651)	1414	(451 to 2921)
Test period	741	(391 to 2470)	934	(259 to 1902)
Change	-159	(-1362 to 487)	-246	(-1958 to 256)

Values are medians with ranges. PBMCs were stimulated for 52 h with 50 μ g/mL PHA.

^a p=0.059 versus control, ^b p=0.053 versus control

Table 8.4: Effects of α -glucan consumption (5 g/d) from *A. bisporus* on median serum creatinine, alanine transaminase (ALAT), aspartate aminotransferase (ASAT), γ -glutamyl transpeptidase (γ -GT), alkaline phosphatase, and total bilirubin concentrations

	Control (N=30)		<i>A. bisporus</i> (N=26)	
<i>Creatinine (μmol/L)</i>				
Run-in	85	(63 to 136)	85	(61 to 119)
Test period	86	(60 to 140)	80	(60 to 125)
Change	-1	(-12 to 15)	-4 ^a	(-32 to 12)
<i>ALAT (U/L)</i>				
Run-in	26	(12 to 58)	27	(18 to 54)
Test period	26	(10 to 77)	29	(16 to 81)
Change	2	(-12 to 45)	1	(-12 to 52)
<i>ASAT (U/L)</i>				
Run-in	21	(11 to 37)	22	(12 to 32)
Test period	24	(9 to 43)	24	(10 to 41)
Change	2	(-6 to 26)	1	(-11 to 20)
<i>γ-GT (U/L)</i>				
Run-in	22	(12 to 46)	27	(15 to 75)
Test period	24	(14 to 66)	25	(14 to 76)
Change	-1	(-9 to 20)	0	(-13 to 28)
<i>Alkaline phosphatase (U/L)</i>				
Run-in	61	(20 to 101)	69	(45 to 133)
Test period	60	(24 to 101)	73	(48 to 130)
Change	4	(-9 to 15)	3	(-24 to 30)
<i>Bilirubin (μmol/L)</i>				
Run-in	14	(7 to 33)	12	(8 to 25)
Test period	12	(5 to 25)	12	(6 to 28)
Change	-2	(-10 to 5)	-1	(-10 to 6)

Values are medians with ranges. ^ap=0.081 versus control

Discussion

In this study we have evaluated the effects of fruit juice enriched with the α -glucan fraction from *A. bisporus* on cytokine production patterns by LPS and PHA stimulated PBMCs. The use of fungal β -glucans, and perhaps also of α -glucans, has been proposed to skew the Th1-Th2 balance into the Th1-direction. This would be useful to ameliorate Th2-skewed allergies, and on the other hand promoting Th1 responses in depressed states of immunity. In contrast to the expectation, we here show no skewing into the Th1 direction by *A. bisporus* derived α -glucans. On the contrary, we found that the consumption of α -glucans from *A. bisporus* rather tended to lower the production of the Th1 cytokine IL-12 as compared to control in PHA-induced proliferating PBMCs. IL-12 is produced by monocytes/macrophages and, besides stimulating NK cells, it plays a crucial role in promoting the differentiation of naïve T-cells to a Th1 phenotype. The decreased level of IL-12 after *A. bisporus* is in line with the fact that there was no change in the concentration of the Th1 cytokine IFN γ . In contrast, in mice an elevated IFN γ production by splenocytes after dietary supplementation with *A. bisporus* has been reported [184]. Moreover, the consumption of an α -glucan-protein complex (94.3% sugar) from another fungal source (*Tricholoma matsutake*) recovered the stress-induced decrease in IL-12 serum concentration in mice [185]. This suggests that α -glucans could have *in vivo* effects on Th1 cytokines, at least in mice.

Besides IL-12, also the production of IL-10 showed a trend towards decreased production after *A. bisporus* consumption as compared to control drinks. In contrast, Yu *et al.* [186] found that oral administration of *A. bisporus* had no effect on *ex vivo* IL-10 concentrations in mice. Moreover, Wu *et al.* [184] demonstrated that dietary supplementation of dried *A. bisporus* powder did not affect IL-10 production by mice splenocytes. Therefore, the observation that IL-10 tended to be lower in the *A. bisporus* group is more or less in line with other studies. Finally, the α -glucan fractions from *A. bisporus* did not affect the production of IL-2, IL-13, IL-4 and IL-17.

Besides the immune modulating effect of *A. bisporus* α -glucan rich fractions on the Th1-Th2 balance of cytokines produced by PBMCs, we also evaluated the behaviour of PBMC towards LPS. We showed that α -glucans from *A. bisporus* reduced TNF α production by LPS stimulated PBMCs as compared to control. This effect is in contrast to our *in vitro* findings in which TNF α secretion by bone marrow-derived macrophages from mice was increased by *A. bisporus* α -glucan extract. This *in vitro* finding was in line with the elevated TNF α and IL-12 secretion by mice macrophages and dendritic cells treated with α -glucans from

Pseudallescheria boydii *in vitro* [181]. However, our current finding suggests that in the *in vivo* situation, α -glucans have lost their efficacy to stimulate the response to LPS.

There is a vast amount of data from *in vitro* and animal studies showing immune stimulating effects of mushrooms. Comparing our data with that of others, who demonstrated immune stimulating effects, is difficult since earlier studies have used whole mushroom extracts [183, 184], while we have specifically evaluated the effect of the α -glucan rich polysaccharide fraction from the mushrooms, which might have contributed to observed differences in results. On the other hand, by comparing results of studies evaluating effects of whole mushroom extracts with our data may provide further insight in effective components since we now know that *in vivo*, α -glucans are not effective in promoting Th1 responses by PHA-stimulated PBMCs, as well as stimulating LPS induced cytokine production by PBMCs.

The question remains why the *A. bisporus* α -glucan rich fractions did not stimulate human PBMC behaviour in response to *ex vivo* LPS and PHA treatments, while there are suggestions from the literature, not only from cell experiments but also from animal studies, that α -glucans have immune stimulating capacity. First, the immunological response of mice and humans differs [187], so the use of different experimental models may have influenced the outcomes. There are several examples in the literature of treatments that were effective in mice, whereas they failed to show effects in humans. For example, IFN γ has been reported to exacerbate disease in an experimental autoimmune model for multiple sclerosis (MS) in mice [188], whereas human clinical trials showed a beneficial effect [189]. Additionally, more knowledge regarding intestinal α -glucan handling is essential to explain the lack of effect. When the α -glucans are orally administered, the question arises whether they are absorbed in the gastrointestinal tract and reach the PBMCs as such. For β -glucans it has been demonstrated that mice intestinal macrophages [67] and enterocytes (presumably Microfold (M)-cells) [42, 58] can take up and distribute them after oral intake. Whether this also holds for α -glucans needs to be determined. On the other hand, it might be that absorption of the α -glucans is not crucial to exert their effects since intestinal dendritic cells sense the intestinal lumen and by this deliver fragments of the α -glucans to the underlying cells of the intestinal immune system [167, 168]. Consequently, activated intestinal lymphocytes may migrate to the mesenteric lymph nodes and come back via lymphatic vessels and blood to intestinal lamina propria. Hypothetically, as food antigen specific T cells have been reported in peripheral blood [190, 191], the isolated PBMCs might contain α -glucan activated lymphocytes that normally would return to the intestine. Nevertheless, we did not observe a clear effect on cyto-

kine production by isolated PBMCs, suggesting that if lymphocytes are activated by the mushroom α -glucans, these effects remained local. However, we only analyzed effects in circulating PBMCs and we did not evaluate the intestinal immune response as such. Besides the idea that the immune modulating effects only occurred locally, a final explanation for the lack of a clear immune modulating effect of the *A. bisporus* derived α -glucans might relate to the comparable structure with amylose. *In vivo* these compounds can be digested by α -amylases present in saliva and pancreatic juice. Furthermore, we investigated the effect of 90% pure α -glucan extract, which does not rule out that other components of *A. bisporus*, like β -glucans, can have an immune stimulating effect. They were, however, only in minor fractions present in our drinks.

Safety of the mushroom α -glucan enriched drinks is important as three cases of cancer patients who consumed extracts from another edible mushroom (*Agaricus blazei*) for several days to months during conventional chemotherapy reported severe hepatic damage [192]. We found no effects of the *A. bisporus* α -glucan drinks on parameters reflecting liver and kidney function. We even observed a trend towards a lower concentration of creatinine in the *A. bisporus* group as compared to the control group. Therefore we conclude that in this population of healthy subjects with slightly elevated serum total cholesterol concentrations, consumption of 5 g (dry matter) of a α -glucan rich polysaccharide fraction does not affect liver or kidney function, at least within this 5-weeks follow up period.

In summary, this is the first large-scale placebo controlled intervention study in humans to evaluate the immune modulating effects of α -glucan from *A. bisporus*. In contrast to the expected immune stimulation into a Th1 direction, we here demonstrated no obvious Th1-Th2 skewing effects of α -glucans from *A. bisporus* by PHA-stimulated PBMCs as compared to the control group. Furthermore, we found that the consumption of α -glucans from *A. bisporus* lowers TNF α production by LPS stimulated PBMCs as compared to the control group. The absence of strong immune modulating effect on PBMCs might relate to the comparable structure of α -glucan with amylose. It is known that these compounds can be digested *in vivo* by α -amylases that are present in saliva and pancreatic juice. Therefore, our current finding suggests that in the *in vivo* situation, α -glucans have lost their efficacy to stimulate the systemic immune response observed *in vitro*. Furthermore, it cannot be excluded that α -glucans have only local effects in the intestine, which are not reflected in systemic PBMC behaviour.

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CHAPTER 9

General discussion

Introduction

The intestinal mucosa forms a barrier for harmful pathogens to prevent the luminal microflora from penetrating the intestinal mucosa and from spreading systemically, whereas at the same time nutrients need to be absorbed [164]. To perform this difficult task, the epithelial lining is very selective in absorption on one hand, and on initiation of an immune response on the other hand. There are several mechanisms present in the intestine to maintain the balance in this process. The formation of the mucus coat, presence of tight junction proteins and the secretion of anti-microbial proteins are examples of the first line of defence, whereas pathogens that cross this barrier are directly confronted with the gut-associated lymphoid tissue (GALT) [148]. Enterocytes and intestinal lymphocytes play an important role as they orchestrate the mucosal immune response after contact with the intestinal lumen. Dietary components can affect the intestinal immune response [20, 165] and in this context β -glucans are certainly interesting candidates in the potential prevention of various diseases. β -Glucans are the major structural components of the cell wall and present in bacteria, fungi and yeast. However, β -glucans are also present in food sources including various grains and edible mushrooms. Immune stimulating effects of β -glucans from fungi on leukocytes have been reported in several studies [8, 23, 111]. In addition to β -glucans, also α -glucans are present in the polysaccharide fraction of mushrooms [10]. Besides present in our diet, α -glucans can also be found in the cell wall of yeast [193] and bacteria [194]. Whereas the effects of β -glucans have been studied extensively, little is known about the immune modulating effects of mushroom α -glucans.

Not only α - and β -glucans from fungi, but, also β -glucans from oats have been shown to modulate the immune response [7, 47, 55, 56, 195]. As oat β -glucans, and mushroom α - and β -glucans are part of the human diet, they come in contact with enterocytes, which are located at the frontline of the intestinal immune system. Therefore, it is likely that dietary glucans might also affect the intestinal immune response. In this thesis we evaluated the effects of dietary glucan from oat and mushrooms on several parameters of the intestinal and systemic immune responses.

Immune modulation by dietary oat β -glucan

In the first study (**chapter 3**) we examined the immune modulating effects of oat β -glucan on enterocytes. For this, six patients with an ileostomy consumed

a diet enriched with oat β -glucan or a control fiber (rice starch). During the next 24 h ileostomic contents were collected. From these ileostomic contents fecal water was prepared and added *in vitro* to four different enterocyte cell lines in presence of inflammation-inducing cytokines. By this approach we tried to mimic the physiological situation as much as possible in which the β -glucan was added to the enterocytes after it has passed the small intestine. We found that fecal water enriched with oat β -glucan stimulated the cytokine-induced immune response of enterocytes. This finding suggests that the consumption of oat β -glucan might result in a stronger immune response, which may ultimately lead to a greater resistance against pathogens. If true, this finding is in line with the assumed role of β -glucans as biological response modifiers (BMR), which means that they belong to the group of polysaccharides that have the ability to enhance the immune system [23].

How does the mucosal immune system “sense” the glucans

Enterocytes transduce signals from luminal antigens to adjacent immune cells of the intestinal immune system via specific germ line-encoded pattern recognition receptors (PRRs), like Toll-like receptors (TLRs) and cytoplasmic receptors. PRRs are able to discriminate between the normal commensal microbiota and nutrients on one hand, and pathogens on the other hand. Pathogen-associated molecular patterns (PAMPs) present on diverse microbes are initially recognized by PRRs and their interaction results in the activation of intracellular signalling pathways, nuclear translocation of transcription factor (NF- κ B), and the transcription of pro-inflammatory cytokines [70]. In principle, glucans can also be considered as a PAMP, since they are conserved structures in the cell wall of yeast, fungi and bacteria. We hypothesized that our observed immune stimulating effects of oat β -glucan enriched fecal water in enterocytes were mediated via the β -glucan receptor dectin-1, a PRR that has been identified as the major β -glucan receptor on macrophages [196]. Human dectin-1 is expressed on many cells of the immune system including monocytes, macrophages, dendritic cells (DCs), and granulocytes, as well as B cells and a subset of T cells. It has been demonstrated by competitive ELISA that barley β -glucan, which also consist of an unbranched chain like oat β -glucan, can be recognized by dectin-1 [197]. However, it has to the best of our knowledge not explicitly been described that oat β -glucans bind to dectin-1. Therefore, in **chapter 4** we investigated the possible role for dectin-1 on the observed immune stimulating

effects of β -glucan fecal water in INT407 and Caco-2 NF- κ B reporter cells. We have chosen for these two human enterocyte cell lines as they were highly responsive in the study described in **chapter 3**. Reverse transcriptase PCR and western blot analysis demonstrated the presence of dectin-1 in INT407 cells. Moreover, also human enterocytes isolated from ileum and colon biopsies, as well as our Caco-2 NF- κ B reporter cells contained dectin-1 protein. For Caco-2 cells, these data are in line with observations from Seagusa *et al.* [42], who already reported dectin-1 mRNA transcripts in Caco-2 cells. However, in the INT407 cells, dectin-1 expression could not be confirmed by flow cytometry, suggesting that dectin-1 is most likely not expressed at the extracellular membrane. Furthermore, two different dectin-1 inhibitors (glucan phosphate and anti-human dectin-1 antibody) did not suppress the β -glucan containing fecal water induced interleukin (IL-8) production by INT407 cells and NF- κ B transactivation by Caco-2 NF- κ B reporter cells. We therefore concluded that immune stimulating effects of oat β -glucan fecal water described in **chapter 3** are most likely independent of extracellular dectin-1. We therefore speculated that the absence of functional dectin-1 on enterocytes may have a protective function *in vivo* as the luminal surface of the gastrointestinal tract is abundantly supplied with β -glucans, especially from nutritional and also to a certain extend from potentially harmful sources. These dectin-1 ligands would otherwise induce continuous PRR activation with potential intestinal damage as a consequence. It also implies that the immune stimulating effects earlier observed with β -glucan containing fecal water must have been mediated via another process.

When using fecal water it is difficult to distinguish between direct and indirect effects of β -glucan on enterocytes. To investigate whether the immune stimulating effects of oat β -glucan fecal water were directly caused by β -glucan in the fecal water we now added purified oat β -glucan instead of the oat β -glucan enriched fecal water to Caco-2 NF- κ B reporter cells, again in presence of an inflammation-inducing cytokine cocktail. We now observed a decreased NF- κ B translocation after oat β -glucan as compared to control (PBS) (**figure 9.1**), indicating that the earlier observed effects with the β -glucans in fecal water were indeed more likely indirect. However, we do realize that we now had used another oat β -glucan than the ileostomy patients had consumed. This is an important and relevant remark because the immune modulating activity of β -glucans may depend on their structural properties [100] and method of isolation [109]. Another possibility to explain the immune stimulating effects of β -glucan enriched fecal water as described in **chapter 3** is that the consumption of a β -glucan enriched diet has influenced *in vivo* immune responses already in the gastrointestinal tract of the ileostomic patients. This *in vivo* response may

have changed the composition of the prepared fecal water, which could explain the increased *in vitro* immune response of enterocytes. If true, this would imply that the effects we observed in the enterocyte cell lines exposed to fecal water were indirect to consuming β -glucans, and not directly caused by the presence of β -glucans in the fecal water. Our hypothesis was now that the consumption of β -glucans modulated the *in vivo* secretion of antimicrobial peptides in the gastrointestinal tract, reflected by different levels of these peptides in fecal water obtained from ileostomic patients that had consumed an oat β -glucan enriched diet as compared to a placebo diet.

Figure 9.1

NF- κ B transactivation by Caco-2 NF- κ B reporter cells after 3 h stimulation with different concentrations oat β -glucan (50-200 $\mu\text{g/mL}$) in presence of IL-1 β (100 U/mL) and interferon (IFN) γ (50 U/mL) is decreased. Values are means and SD of duplicate cultures.

Antimicrobial peptides are part of the first line of defence against pathogens and are produced by epithelial cells and paneth cells [198]. To test our hypothesis, we analyzed the concentration of four antimicrobial peptides (BPI, hBD-2, LL-37 and lysozyme) in oat β -glucan and placebo fecal water (**chapter 6**). We found a trend towards lower concentrations of the antimicrobial peptides BPI and lysozyme in fecal water of ileostomic patients that consumed a diet enriched with β -glucan as compared to control. We were unable to detect hBD-2 and LL-37 in fecal water, which could have been expected since these peptides are mainly produced by colonic epithelial cells [156], while the fecal water had only passed the small intestine of the ileostomic patients. Next, we tried to confirm the assumption of a changed composition of fecal water from patients participating in the dietary intervention trial in an mice study in which oat β -glucans were administered via oral gavages during 3.5 days. Indeed, we observed that the presence of lysozyme (paneth cell marker) was decreased in mice that were administered oat β -glucan as evaluated by immunohistochemistry on intestinal sections. However, these mice also showed indications of a

decreased intestinal integrity as compared to placebo. We speculate that this may have been caused by the drop in antimicrobial peptides, which made it easier for pathogens to modulate tight junctions. This was further supported by a trend towards a correlation between the concentration of lysozyme and the degree of damage. The resulting loss of epithelial integrity may lead to translocation of pathogens into the circulation. However, we did not observe any signals of illness in the mice that received oat β -glucan. In addition, there were no signs of difference in food consumption or body weight between the oat β -glucan and placebo group (**figure 9.2**). Regarding our hypothesis, it might be that oat β -glucan fecal water, containing less antimicrobial peptides, increased the inflammatory response of intestinal cell lines. Indeed, it has been reported that antimicrobial peptides dampen the endotoxin-stimulated production of pro-inflammatory cytokines (tumour necrosis factor (TNF) α and IL-6) [153], so a diminished level of these peptides may indeed stimulate the immune response.

Figure 9.2

The bodyweight of mice that received twice daily a gavage containing 3 mg/200 μ L oat β -glucan (N=8) did not change during 3.5 days as compared with mice that received a control gavage (PBS) (N=8). Values are mean and SD

Effect of oat β -glucan on NF- κ B activation

Activation of PRRs results in nuclear translocation of transcription factor NF- κ B [199]. It has been reported that β -glucans can activate NF- κ B *in vitro* as measured by EMSA in leukocytes [27, 31, 72] via dectin-1 [73], and in alveolar epithelial cells [74]. Food substances with the ability to modulate NF- κ B activity can be of both nutritional and pharmacological interest [200]. Therefore, we examined the effects of oat β -glucan consumption on intestinal NF- κ B activation. From literature it was not clear whether enterocytes, intestinal leuko-

cytes, or both, were responsible for the immune modulating effects after β -glucan consumption. For that reason, we evaluated the effects of oat β -glucan consumption on both cell types (**chapter 7**). For this we used intestinal samples of the same NF- κ B reporter mice as described in **chapter 6**. We observed that dietary oat β -glucans that were administered via oral gavages increased NF- κ B translocation as compared to placebo gavage, both in leukocytes as well as enterocytes. Effects were specific for the small intestine, since activation of NF- κ B was not observed in the colon. This local NF- κ B sensitivity to inflammatory agents has also been reported for NF- κ B DNA binding activity and *in vivo* NF- κ B imaging in endotoxin-injected mice [176, 177]. An explanation for the absence of NF- κ B activation in the colon after β -glucan feeding may relate to the suggested formation of short chain fatty acids (SCFA) from β -glucans. Undigested oat β -glucans that reach the colon, can rapidly be fermented by anaerobic bacteria leading to SCFA. It has been shown that the increased SCFA concentration may inhibit NF- κ B activation [178, 179]. The increased NF- κ B translocation in enterocytes and leukocytes indicates that both cell types were activated by oat β -glucan, but we were interested if enterocytes or leukocytes were activated first. Concerning effects on the immune system it has been suggested that β -glucans mainly induce Th1 responses [32, 38, 39] by polarization of naïve intestinal T-cells. Interestingly, the level of the Th1 cytokine IL-12 present in intestinal lysates was clearly increased. We hypothesized that after consumption, dietary oat β -glucans firstly activate the intestinal leukocytes, which in turn increase cellular activation of enterocytes. Unexpectedly, the concentration of the Th1 cytokine IFN γ , which is mainly produced by CD4⁺ T helper cells in response to increased IL-12 production by the antigen presenting cells (APCs) was decreased in the proximal small intestine. Finally, TNF α was increased in the colon. Since NF- κ B was not activated in this segment, other transcription factors, like interferon regulatory factor 3/7 (IRF3/7) or activator protein-1 (AP-1), which collaborate to induce transcription of cytokines [173] might also be involved.

As mentioned above, we have also determined the production of antimicrobial peptides in our mice model, which has been reported to depend on NF- κ B activation [138, 147, 201]. However, we observed a decreased production of the antimicrobial product lysozyme, whereas NF- κ B translocation was elevated. Moreover, antimicrobial peptides have been described to modulate DC function and to induce polarization into Th1 or Th2 direction [138]. Mouse β -defensin-2 matured DCs secrete pro-inflammatory (Th1-polarizing) cytokines IL-12, IL-1 α , IL-1 β and IL-6 [202]. As we observed a decreased level of antimicrobial peptides, whereas IL-12 was increased, this may indicate that DCs were not involved. On the other hand, intranasally co-administration of defensins

with ovalbumin (OVA) skewed *in vitro* OVA-induced cytokine secretion by mice splenocytes to Th2 direction [203].

How should the increased intestinal NF- κ B activation in response to oat β -glucan be interpreted

NF- κ B plays a central role in chronic (intestinal) inflammation and activated NF- κ B has been demonstrated in inflammatory bowel disease (IBD) patients [204, 205]. However a protective function for this transcription factor in intestinal immune cells has also been described [206]. NF- κ B activation can be seen as an early event in the detection of pathogens and the innate host response. A more pronounced NF- κ B activation may be favourable to alert the immune system, resulting in better resistance against attacks by pathogens. This has been shown using genetically engineered mice with deletions in the NF- κ B pathway. For example, p50^{-/-},p65^{+/+} mice showed a worsened *Helicobacter hepaticus*-induced typhocolitis as compared to wildtype mice [207]. Furthermore, it was shown that NF- κ B directly affects the intestinal clearance of pathogens. For example, p50^{-/-},p65^{+/+} mice failed to clear *Campylobacter jejuni* and developed gastric and duodenal inflammation compared with wildtype mice [208]. As we observed increased intestinal NF- κ B activation after oat β -glucans administration in mice, it is worth to mention that oat β -glucans have been reported to increase resistance towards bacterial challenges and intestinal parasitic infections pathogens [7, 47, 55, 56]. Additionally, NF- κ B is important to help maintain intestinal barrier integrity by inducing keratinocyte chemokine [209]. However, we observed that mice that were administered oat β -glucan showed indications of a decreased intestinal integrity as compared to placebo. Nevertheless, we did not observe a correlation between the intestinal integrity and NF- κ B activation in these mice, so it is unlikely that the decreased intestinal integrity directly relates to the effects of oat β -glucan on NF- κ B. However, as earlier mentioned, the degree of damage showed a trend towards a correlation with the concentration of lysozyme.

Immune modulation by dietary mushroom α - and β -glucans

Mushrooms have been studied extensively for their immune modulating and anti-tumour properties [22, 110-113]. There are many potentially biologically active compounds present in mushrooms, e.g. small proteins, lectins, polyphenols, and polysaccharides. It is assumed that the polysaccharides are a main bioactive part of the mushrooms, at least in relation to their immune modulating effects [109]. These polysaccharides are mainly β -glucans that are present in the cell walls, but also α -glucans are present in the polysaccharide fraction of mushrooms [10]. Whereas the effects of β -glucans have been studied extensively, little is known about the immune modulating effect of mushroom α -glucans. Therefore, following the experiments described for oat β -glucans we also investigated the effects of glucan-rich fractions from edible mushrooms on intestinal and systemic immune function (**chapters 5 and 8**). Therefore we used several glucan-rich mushroom polysaccharide extracts, which were a priori based on the literature expected to vary in glucan structure (**chapter 5**). We found that extracts from *Agaricus bisporus* strongly stimulated nitric oxide (NO) production by bone marrow-derived macrophages (BMM), whereas extracts from *Coprinus comatus* and spores of *Ganoderma lucidum* had only minor effects. In addition, also purified α -glucan from the stems of *A. bisporus* dose-dependently stimulated the NO production, although the maximum effect observed was only 50% of that achieved with *A. bisporus* β -glucan. Immune stimulating effects of whole extracts of *A. bisporus* have been reported earlier [184]. Furthermore, extracts of *A. blazei* Murill and *Phelinus linteus* had no effect at the NO production by BMM at all. However, immune stimulating effects of *A. blazei* Murill extracts, although not for all fractions, have been reported [116, 126, 210, 211]. It has been shown that the method of isolation and chemical modification can influence the activity of β -glucans, which may in theory explain the difference with our *A. blazei* Murill extract [25, 109, 111]. In contrast to the exclusive activating effects of *A. bisporus* in BMMs, almost all mushroom extracts lowered NF- κ B transactivation in Caco-2 cells. However, we observed in our mice study that oat β -glucans activated intestinal NF- κ B. This may be explained by the difference in structure between oat and mushroom β -glucans, though we found that oat β -glucans directly added to Caco-2 cells also decreased NF- κ B activation (**figure 9.1**). Therefore, it is more likely that the method of administration accounts for these outcomes. β -Glucans directly added *in vitro* to Caco-2 cells lowered NF- κ B activation, whereas *in vivo* administration of the β -glucans stimulated intestinal NF- κ B activation. This may be

another indication that β -glucans *in vivo* firstly activate intestinal leukocytes, which on their turn stimulate the enterocytes as they are not directly stimulated by β -glucans *in vitro*.

Structural analysis between the most and least potent immune stimulating extract on BMMs (*A. bisporus* and *A. blazei* Murill, respectively), suggested that branching of the β -glucan chain is essential for immune stimulating activity, at least *in vitro* in BMMs. We therefore concluded that the edible mushroom *A. bisporus* seems an interesting nutritional compound to stimulate the immune response in depressed states of immunity. To evaluate whether the consumption of *A. bisporus* indeed stimulates systemic immune function we evaluated in **chapter 8** the peripheral blood mononuclear cell (PBMC) cytokine secretion patterns after consumption of α -glucan rich fractions from *A. bisporus* incorporated into fruit drinks in humans. However, in contrast with the increased TNF α secretion of BMM *in vitro*, we observed that α -glucans from *A. bisporus* reduce TNF α production by lipopolysaccharide (LPS) stimulated PBMCs *ex vivo* as compared to the control group. The use of fungal β -glucans, and perhaps also α -glucans, has been proposed to skew the Th1-Th2 balance into the Th1-direction. This would be useful to ameliorate Th2-skewed allergies, and on the other hand promoting Th1 responses in depressed states of immunity. However, no obvious Th1-Th2 skewing effects of α -glucans from *A. bisporus* were observed, although we observed a trend towards a decreased production of IL-12 and IL-10. Nevertheless, the *a priori* hypothesized Th1 shift after mushroom extracts would suggest an increase in IL-12 concentrations. The lower level of IL-12 after *A. bisporus* α -glucans is in line with the fact that there was no change in the concentration of the Th1 cytokine IFN γ . In contrast, in mice an elevated IFN γ production by splenocytes after dietary supplementation with *A. bisporus* has been reported [184]. The lack of a clear immune modulating effect of the *A. bisporus* derived α -glucans might relate to the comparable structure with amylose. *In vivo* these compounds can be digested by α -amylases present in saliva and pancreatic juice. Furthermore, it cannot be excluded that glucans have only local effects in the intestine, which are not reflected in systemic PBMC behaviour. Also oat β -glucan did not influence systemic inflammatory parameters of LPS stimulated PBMCs in hypercholesterolemic subjects [212]. Though, we have not evaluated the systemic immune effects of *A. bisporus* β -glucan consumption, which might have the potential to induce immune stimulation based on our *in vitro* results.

Mechanism of immune modulation by dietary glucans

When glucans are orally administered the question arises whether they stay in the intestinal lumen and trigger responses via specified receptors from the outside or otherwise are absorbed in the gastrointestinal tract and enter into the circulation to influence the systemic immune system. The potential uptake of β -glucans may depend on the molecular weight and it has been suggested that especially high molecular mass glucans (>2 MDa) are taken up [58]. The oat β -glucans that were used in our mice study had a lower molecular weight (150 kDa) [180], which makes it more likely that they have not been taken up from the intestinal lumen. This may explain the absence of a systemic effect reflected by the unchanged cytokine concentrations of the spleen (**chapter 7**). Furthermore, it has been reported that low molecular weight yeast β -glucans (20 kDa) were also not detected in serum of supplemented individuals in a clinical study [54]. Moreover, the consumption of *A. bisporus* α -glucan rich fractions did not induce systemic effects, as cytokine secretion by LPS and phytohemagglutinin (PHA) stimulated human PBMC *ex vivo* was not stimulated (**chapter 8**). On the other hand, as already discussed, it might be that absorption of the glucans is not crucial to exert their effects since intestinal DC sense the intestinal lumen and by this deliver fragments of the glucans to the underlying cells of the intestinal immune system [167, 168]. Moreover also M-cells can take up glucans from the lumen and deliver them to immune cells in the underlying Peyer's patches [168]. Consequently, activated intestinal lymphocytes migrate to the mesenteric lymph nodes (MLN) and come back via lymphatic vessels and blood to intestinal lamina propria. Hypothetically, as food antigen specific T cells have been reported in peripheral blood [190, 191], the isolated PBMCs in the human intervention trial might contain α -glucan activated lymphocytes that normally would return to the intestine. It has been reported that commensal loaded DCs do not penetrate further than the mesenteric lymph nodes and were not found in systemic immunological organs like the spleen. This is most likely to ensure that immune responses to commensal bacteria are induced locally, without potentially damaging systemic immune responses [148, 164, 213]. Nevertheless, as we did not observe a clear stimulating effect on cytokine production by isolated PBMCs, this suggests that if lymphocytes are activated by the *A. bisporus* α -glucans, these effects remained local. However, in the study described in **chapter 8** we only analyzed effects in circulating PBMCs and we did not evaluate the intestinal immune response as such. Nevertheless, fecal water prepared from ileostomic contents of patients who had consumed an oat β -glucan enriched diet, increased the immune response of *in vitro* cultured enterocyte cell lines (**chapter 3**). The

conclusion that dectin-1 did not seem to be functionally expressed in enterocytes (**chapter 4**), whereas the functionality of dectin-1 on leukocytes (mainly macrophages and dendritic cells) has been reported [196], is another indication that the enterocytes are not the main responsive cells to orally applied β -glucan. This supports our earlier proposed hypothesis that the oat β -glucans most likely activated the intestinal leukocytes first. Next, the stimulated intestinal leukocytes may in turn have activated the enterocytes (**figure 9.3**).

Figure 9.3: Simplified overview of hypothesis

Intestinal DC and macrophages sense the intestinal lumen by extending dendrites through the intestinal epithelium and by this deliver fragments of the glucans to the underlying cells of the intestinal immune system. Moreover, also M-cells can take up glucans from the lumen and deliver them to immune cells in the underlying Peyer's patches. The glucans most likely activate the intestinal leukocytes first. Next, the stimulated intestinal leukocytes may in turn activate the enterocytes.

This hypothesis suggests that intestinal DCs and macrophages have sensed the oat β -glucan by extending dendrites through the intestinal epithelium and present β -glucan to naïve T cells [167]. The idea that oral administration of β -glucan modulates intestinal leukocytes has been proposed earlier [183]. Moreover, it has been reported that Caco-2 cells produced less cytokines [170] or even none [171] in absence of leukocytes, indicating the important immune orchestrating role for intestinal leukocytes and supporting our hypothesis. Moreover, Hong *et al.* [67] have demonstrated that intestinal macrophages played a major role in the uptake and distribution of yeast and barley β -glucans after oral intake. Therefore, we suggest that the consumption of glucans has only local immune modulating effects in which leukocytes are the primary responsive cells.

Can β -glucans be considered as prebiotics?

Much of the literature that features intestinal immune modulation by nutrition focuses on pro- and prebiotics. Prebiotics can be defined as ‘non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or limited number of bacteria in the colon’. The traditional targets for prebiotics are species of *Bifidobacterium* and *Lactobacillus* [214]. Most dietary fibers that are fermentable carbohydrates could also be considered as prebiotics. As glucans are in fact “dietary fibers” they may have several characteristics in common with prebiotics, which will be discussed in the following paragraphs.

Prebiotics can change the bacterial composition and bacterial products, which in turn can modulate (local) cytokine and antibody production. Indeed, oat β -glucan administration in mice altered the intestinal cytokine expression (**chapter 7**). In addition, intragastric applied oat β -glucan was reported to increase intestinal IgA responses as compared with controls in a mice infection model [56]. However, we as well as the group of Yun did not examine whether these effects were achieved via changes in (colonic) bacterial composition. The fermentation of prebiotics leads to the formation of SCFA like acetate, propionate and butyrate. It has been reported that β -glucans reaching the cecum and colon were rapidly fermented [215, 216]. Furthermore, dietary curdlan, a linear 1 $\rightarrow$ 3 β -glucan, selectively increased the total *bifidobacteria* population and SCFAs in rats, compared with cellulose fed controls [217]. Moreover, *in vitro* fermentation for 48 h in pH-controlled and stirred batch cultures of the same oat β -glucan as we used in our mice study, modulates the microbiota composition and resulted in higher amounts of acetate, butyrate and mainly propionate when compared with a no treatment control. However, counts of the classical prebiotic bacteria *Bifidobacteria* and *Lactobacilli* were not increased [180]. Barley β -glucan, having structural similarities with oat β -glucan, has been shown to selectively stimulate the growth of *Lactobacilli* species in a rat model [218]. Besides fibers from cereals, fibers from mushrooms (*Lentinus edodes* and *Flammulina velutipes*) also have been reported to increase concentrations of SCFAs in the cecum of rats that were fed a diet enriched with 50 g/kg mushroom fibers for 4 weeks [219]. Moreover, *in vitro* fermentation of the mushroom *Wolfiporia cocos* leads to the production of SCFAs, although two other mushrooms in that study had no effect. The authors suggested that structural characteristics of the glucans may explain the variation in fermentability [220]. The only study evaluating *in vivo* SCFA formation after *A. bisporus* consumption reported no effect as compared to cellulose fed rats [221]. It has been shown that the increased SCFA concentration may inhibit NF- κ B transactivation [178,

179]. Indeed, when mice were administered oat β -glucan for 3.5 days we observed an increased NF- κ B transactivation in the small intestine of mice, though not in the colon (**chapter 7**). The absence of effect in the colon may be caused by the formation of SCFA. Butyrate is the most studied SCFA and appears to have the most potent modulating effects on immune cells, like inhibiting lymphocyte proliferation and suppressing the production of the Th1 cytokines IFN γ and IL-12 [222]. However, we observed an increased production of IL-12 in the intestines of mice that were administered oat β -glucan (also slightly in colon), although we observed a decreased level of IFN γ in the distal small intestine. On the other hand it has been suggested that prebiotics can lead to changes in several regions of the GALT as supplementation with fructooligosaccharide (FOS) in mice increased immune responses in the small intestine [223, 224]. Moreover it has been reported that butyrate stimulated colonic antimicrobial cathelicidin expression [225], while we observed a decreased level of the antimicrobial marker lysozyme in the distal small intestine of the mice (**chapter 6**). This can be explained by the fact that we evaluated the effects of β -glucan on antimicrobial peptides only in the small intestine. On the other hand, antimicrobial peptides can also modulate the microbial composition of the intestine [226]. Whether the effects of oat β -glucans consumption in our mice study were mediated by changes in the microbiota is an alternative possibility, which needs to be investigated.

Besides modulating the bacterial composition and bacterial products, prebiotics may also directly influence the intestinal immune system by binding to receptors as certain prebiotics contain structures that are similar to those found on pathogens. Enterocytes express specific receptors which mediate various cellular reactions when activated, that might be affected by prebiotics [227]. Probiotics have been reported to attenuate the pro-inflammatory responses of enterocytes on pathogens, rather than stimulating the immune response [228, 229]. In addition, prebiotics have been shown to induce anti-inflammatory effects in rats with hapten-induced [230, 231] and DSS-induced colitis [232]. Even without activating, prebiotics can modulate the receptor function by effective blocking of the receptor from its normal ligand [233, 234]. This mechanism prevents the attachment of pathogenic bacteria, which may protect the gut from infection and inflammation. It was shown that prebiotics (galactooligosaccharides) reduced the adherence of pathogens to HEp-2 and Caco-2 cells [234], and the colonic cell line HT29 [235]. Moreover, it has been suggested that the presence of luminal carbohydrates can influence binding and uptake of soluble and particulate (tiny suspended particles) molecules by M-cells [236], which may also be the case with α - and β -glucans. However, we previously showed that the β -glucan receptor dectin-1 did seem not to be

functionally expressed in enterocytes (**chapter 4**), which may function to prevent β -glucan-induced inflammatory responses. Although enterocytes have been reported to contain TLRs [237], which transduce signals from the intestinal lumen. In addition to receptors on enterocytes, prebiotics may also interact with carbohydrate receptors present on leukocytes, like DCs, monocytes and macrophages. There are multiple receptors described on leukocytes to bind to β -glucan, like dectin-1, complement receptor 3, lactosylceramide, scavenger receptors, TLR2 or TLR4 [70, 113] [8]. However, binding of β -glucan to its receptor on leukocytes rather induces pro-inflammatory processes than attenuating them as reported for prebiotics. So, it seems not likely that the immune modulating effects of β -glucan is due to receptor blocking. Overall, there are indications that glucans have prebiotic properties. However, from our studies we cannot conclude whether glucans are prebiotics as we did not measure if the immune modulating effects were achieved via changes in bacterial composition. In addition, the studies described in this thesis were mainly focused on the effects of glucans in the small intestine instead of the colon, although we included two colonic cell lines in the study described in **chapter 3** showing immune stimulating effects of oat β -glucan enriched fecal water. However, the role of microbiota-dependent mechanisms in the oat β -glucan induced immune stimulation were not investigated in this study.

Conclusions and recommendations

The immune response can be modulated by dietary components and from this respect glucans as present in oat and mushrooms may be beneficial in preventing immune related diseases.

We conclude from our studies that the consumption of oat β -glucan stimulates the *ex vivo* human and *in vivo* mice intestinal immune response, but has no systemic immune effects. Enterocytes play a major role in the response to dietary oat β -glucan, although our observed effects in enterocytes are not mediated by the β -glucan receptor dectin-1. Blocking studies are needed to identify which receptors are involved in the effects of dietary β -glucan. Based on our studies, we suggest that most likely, the intestinal leukocytes are firstly activated by β -glucan, which in their turn stimulate enterocytes. This hypothesis should be further investigated in, for example, an *in vitro* co-model of leukocytes and enterocytes. Furthermore, our findings show that the consumption of β -glucan from oat diminished the secretion of antimicrobial peptides in human and mice. Whether this directly relates to the increased immune re-

sponse of enterocytes and observed decreased intestinal integrity in mice warrants future study.

Whether the consumption of oat β -glucan results in a greater resistance against pathogens should be evaluated in properly designed human trials. A possible study design might be by analyzing symptoms of diarrhea caused by *Escherichia coli* infection in healthy volunteers after oat β -glucan consumption. In addition to oat, also mushroom contain glucans. Our results showed that both α and β -glucans from *A. bisporus* stimulated *in vitro* systemic immune cells in mice. However, the consumption of α -glucans from *A. bisporus* reduced TNF α production by LPS stimulated PBMCs in humans. Since we propose that intestinal leukocytes are firstly activated by β -glucans, future studies should focus on the effects of α -glucans on intestinal immune function. Also additional studies on the systemic immune effects of β -glucan from *A. bisporus* instead of α -glucans should be performed, since they showed promising *in vitro* results.

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SUMMARY

The human body is constantly threatened by various types of pathogens like bacteria, viruses, fungi and parasites. A wide-ranging immune system protects the human body against these attacks. Evidence accumulates that the immune response can be modulated by specific dietary components and in this respect glucans are interesting candidate compounds to modulate disease risk. In this thesis we have evaluated the effects of dietary glucans from oat and mushrooms on several parameters of primarily intestinal and to a lesser extend also of systemic immune responses.

β -Glucans are the major structural components of the cell wall and present in bacteria, fungi and yeast. Immune modulating effects of β -glucans from yeast and fungi have been studied extensively. However, β -glucans are also found in foods including grains and edible mushrooms. Effects of these β -glucans on the immune response have received much less attention. In addition to β -glucans, also α -glucans are present in mushrooms. As oat β -glucans and mushroom α - and β -glucans are part of the human diet, they come in contact with enterocytes, which are located at the frontline of the intestinal immune system. Therefore, it is likely that dietary glucans affect the intestinal immune response. In the study described in **chapter 3** we showed that fecal water prepared from ileostomy bag contents from ileostomic patients consuming an oat β -glucan enriched diet stimulated the immune response of enterocytes as compared fecal water after a control diet. We hypothesized that the observed immune stimulating effects were mediated via the β -glucan receptor dectin-1. However, we demonstrated in **chapter 4** that enterocytes do not express functional dectin-1. We therefore concluded that immune stimulating effects of fecal water containing oat β -glucan as described in **chapter 4**, were most likely independent of dectin-1. The absence of functional dectin-1 on enterocytes may have a protective function as the luminal surface of the gastrointestinal tract is abundantly supplied with β -glucans both from nutritional and potentially harmful sources. These dectin-1 ligands would otherwise induce continuous immune activation with intestinal damage as a consequence.

Besides direct effects of oat β -glucan on enterocytes, it may also be that the consumption of an oat β -glucan enriched diet has influenced *in vivo* immune responses already in the gastrointestinal tract of the ileostomic patients. This *in vivo* response may have changed the ultimate composition of the prepared fecal water, which could relate to the increased *in vitro* immune response enterocytes exposed to fecal water. We demonstrated in **chapter 5** a trend towards lower concentrations of the antimicrobial peptides BPI and lysozyme in fecal water of ileostomic patients that had consumed a diet enriched with β -glucan as compared to control. Antimicrobial peptides are part of the first line of defense against pathogens. Moreover, we confirmed the lower lysozyme

levels in an mice study in which 6 mg/day dietary oat β -glucans were administered via oral gavages for 3.5 days. Regarding our hypothesis, it might be that oat β -glucan fecal water, containing less antimicrobial peptides, increased the inflammatory response in the intestinal cell lines as described in **chapter 3**. Interestingly, the β -glucan fed mice not only showed a lower lysozyme expression in enterocytes, but also tended to show a decreased intestinal integrity as compared to placebo. We speculated that this might have been caused by the drop in antimicrobial peptides, which made it easier for pathogens to modulate tight junctions.

Enterocytes and intestinal lymphocytes play an important role as they orchestrate the mucosal immune response after contact with the intestinal lumen. It is, however, not entirely understood whether enterocytes, intestinal leukocytes, or both are responsible for the suggested immune modulating effects after β -glucan consumption. Therefore, we evaluated at the same time the effects of oat β -glucan consumption on both cell types in one study with mice (**chapter 6**). We observed that dietary oat β -glucans that were administered via oral gavages increased intestinal NF- κ B activation as compared to placebo gavages, both in leukocytes as well as enterocytes. A more pronounced NF- κ B activation may be favourable to alert the immune system more profoundly against attacks by pathogens. Effects were specific for the small intestine, since activation of NF- κ B was not observed in the colon. An explanation for the absence NF- κ B activation in the colon after β -glucan feeding may relate to the suggested formation of short chain fatty acids (SCFA) from β -glucans as SCFA may inhibit NF- κ B activation. The increased NF- κ B activation in enterocytes and leukocytes indicated that both cell types were activated by oat β -glucan. Interestingly, the level of the Th1 cytokine IL-12 present in intestinal lysates was clearly increased, suggesting activation of the antigen presenting cells whereas the concentration of the Th1 cytokine IFN γ was decreased in the proximal small intestine. Since IL-12 is predominantly secreted by leukocytes and hardly by enterocytes combined with the possible lack of functional dectin-1 on enterocytes (**chapter 4**), we hypothesized that after consumption, dietary oat β -glucans firstly activate the intestinal leukocytes, which in turn increases cellular activation of enterocytes.

Besides oat β -glucan we also investigated the effects of glucan-rich fractions from edible mushrooms on intestinal and systemic immune function (**chapters 7 and 8**). For this, we used several glucan-rich mushroom extracts, which were a priori expected to vary in glucan structure (**chapter 7**). We incubated these extracts with differentiated bone marrow-derived macrophages (BMM) from mice to evaluate changes in NO production, which is produced by the NF- κ B regulated enzyme NO synthase. Furthermore, we examined the effects of the

extracts on NF- κ B transactivation in Caco-2 NF- κ B reporter cells. We found that extracts from *Agaricus bisporus* strongly stimulated NO production by BMM, whereas extracts from *Coprinus comatus* and spores of *Ganoderma lucidum* had only minor effects. Furthermore, extracts of *A. blazei* Murill and *Phellinus linteus* had no effect at all. In contrast to the exclusive activating effects of *A. bisporus* in BMMs, almost all mushroom extracts lowered NF- κ B transactivation in Caco-2 cells. Structural analysis between the most and least potent immune stimulating extract on BMMs (*A. bisporus* and *A. blazei* Murill, respectively), suggested that branching of the glucan chain is essential for immune stimulating activity. We therefore concluded that the tested glucan fraction from the edible mushroom *A. bisporus* might be an interesting nutritional possibility to stimulate the immune response in depressed states of immunity. To evaluate whether the consumption of this *A. bisporus* derived glucan fraction indeed stimulates systemic immune function in the *in vivo* situation, we evaluated in **chapter 8** the PBMC cytokine secretion patterns after consumption of α -glucan rich fractions from *A. bisporus* incorporated into fruit drinks in humans. However, in contrast with the increased TNF α secretion of BMM *in vitro*, we observed that α -glucans from *A. bisporus* reduced TNF α production by lipopolysaccharide (LPS)-stimulated PBMCs *ex vivo* as compared to the control group. Furthermore, no obvious Th1-Th2 skewing effects of α -glucans from *A. bisporus* by phytohaemagglutinin (PHA)-stimulated PBMCs as compared to the control group were observed. The lack of a clear immune modulating effect of the *A. bisporus* derived α -glucans might relate to its comparable structure with amylose which is digested in the gastrointestinal tract. Furthermore, it cannot be excluded that glucans have only local effects in the intestine, which are not reflected in systemic PBMC behavior.

We conclude from our studies that the consumption of oat β -glucan stimulates the *in vitro* human and *in vivo* mice intestinal immune response, but has no systemic immune stimulating effects. Although enterocytes play a major role in the response to dietary oat β -glucan, our results do not suggest that these effects are mediated by the β -glucan receptor dectin-1. Based on our studies, we suggest that the intestinal leukocytes are firstly activated by β -glucan, which in turn stimulate enterocytes. Furthermore, our findings showed that the consumption of β -glucan from oat diminished the secretion of antimicrobial peptides in human and mice. We finally showed that both α - and β -glucans from *A. bisporus* stimulated *in vitro* systemic immune cells in mice. However, the consumption of α -glucans from *A. bisporus* reduced TNF α production by LPS-stimulated PBMCs in humans, whereas no obvious Th1-Th2 skewing effects by PHA-stimulated PBMCs were observed.

SAMENVATTING

Het menselijk lichaam wordt constant bedreigd door verschillende soorten ziekteverwekkers, zoals bacteriën, virussen, schimmels en parasieten. Een omvangrijk immuunsysteem beschermt het menselijk lichaam tegen deze bedreigingen. Er komt steeds meer bewijs dat de immuunreactie, en daarmee het risico op bepaalde ziekten, door voedingsstoffen wordt beïnvloed. Een voorbeeld van zo'n voedingsstof zijn de glucanen, die onder andere voorkomen in haver en paddenstoelen. In dit proefschrift hebben we dan ook de effecten onderzocht van deze glucanen op parameters die met name een rol spelen bij immuunreacties in de darm en in mindere mate bij systemische immuunreacties.

β -Glucanen zijn belangrijke structurele bestanddelen van de celwand en aanwezig in bacteriën, schimmels en gisten. De immuunmodulerende effecten van β -glucanen uit schimmels en gisten zijn uitgebreid bestudeerd. Echter, β -glucanen zijn ook aanwezig in granen en paddenstoelen. De effecten van deze β -glucanen hebben veel minder aandacht gekregen. Behalve β -glucanen, zijn er ook α -glucanen aanwezig in paddenstoelen. Aangezien de β -glucanen uit haver en de α - en β -glucanen uit paddenstoelen in de voeding voorkomen, komen ze in contact met de darmcellen, die een belangrijk onderdeel zijn van het immuunsysteem. In de studie die in **hoofdstuk 3** is beschreven, toonden wij aan dat fecaal water, bereid uit de inhoud van stomazakjes van ileostoma patiënten die een voeding hadden gegeten verrijkt met β -glucan uit haver, *in vitro* ("in de reageerbuis") de immuunreactie van darmcellen verhoogde in vergelijking met fecaal water van dezelfde patiënten na een voeding zonder β -glucanen. Wij stelden hierna de hypothese op dat deze verhoogde immuunreactie werd gemedieerd via de β -glucan receptor dectin-1. Echter, in **hoofdstuk 4** toonden we juist aan dat darmcellen geen functioneel dectin-1 bevatten. Dit kan mogelijk komen, omdat de afwezigheid van functioneel dectin-1 in darmcellen juist een beschermende werking kan hebben, aangezien het oppervlak van het maagdarmkanaal continu in aanraking komt met β -glucanen uit de voeding of uit de celwanden van bijvoorbeeld bacteriën. Deze dectin-1 liganden zouden anders onafgebroken een immuunrespons opwekken, met darmschade als gevolg.

Naast de mogelijkheid dat haver β -glucanen rechtstreeks de immuunrespons van darmcellen beïnvloeden, is het ook mogelijk dat de haver β -glucanen *in vivo* ("in het lichaam") de secretie van stoffen stimuleren die op hun beurt de immuunrespons beïnvloeden. Deze *in vivo* reactie zou de samenstelling van het bereide fecaal water kunnen hebben veranderd, hetgeen weer de verhoogde *in vitro* immuunreactie van de darmcellen na blootstelling aan fecaal water zou kunnen verklaren. In **hoofdstuk 5** laten wij zien dat de concentraties van de antimicrobiële eiwitten BPI and lysozym in fecaal water van ileostoma patiën-

ten, die een voeding verrijkt met β -glucan uit haver hadden gegeten in vergelijking in vergelijking met fecaal water van dezelfde patiënten na een voeding zonder β -glucanen, lager waren. Dit verschil was bijna significant. Antimicrobiële eiwitten maken deel uit van de eerstelijns afweer tegen ziekteverwekkers. De verlaagde lysozym waarden werden ook gevonden in een studie, waarin muizen gedurende 3,5 dagen dagelijks via orale gavages 6 mg haver β -glucan kregen. De bevinding dat fecaal water met haver β -glucanen minder antimicrobiële eiwitten bevatte zou kunnen verklaren dat de ontstekingsreactie van darmcellen *in vitro*, zoals beschreven in **hoofdstuk 3**, verhoogd was. Naast lagere lysozyme waarden, waren er ook aanwijzingen dat de darmintegriteit van de muizen, die haver β -glucan hadden gekregen, verlaagd was. Dit zou het gevolg kunnen zijn van de daling in antimicrobiële eiwitten, waardoor het voor ziekteverwekkers gemakkelijker wordt om de verbindingen tussen de darmcellen kapot te maken.

Darmcellen en de witte bloedcellen in de darm spelen beiden een belangrijke rol bij de immuunreactie, die in het darmlumen optreedt door contact met de voeding. Echter, het is niet duidelijk of de darmcellen, de witte bloedcellen in de darm, of beiden verantwoordelijk zijn voor deze immuunreactie na de consumptie van β -glucan. Om deze reden hebben wij de effecten van haver β -glucan op de immuunrespons van beide celtypen onderzocht in muizen (**hoofdstuk 6**). Wij zagen dat haver β -glucan, die via orale gavages werden toegediend, de NF- κ B activering in witte bloedcellen en in darmcellen verhoogde, in vergelijking met controle gavages. Als gevolg van de verhoogde NF- κ B activering zou het immuunsysteem alerter kunnen zijn op ziekteverwekkers. De gevonden effecten waren specifiek voor de dunne darm, aangezien er geen NF- κ B activering in de dikke darm werd waargenomen. De afwezigheid van NF- κ B activering in de dikke darm na de consumptie van β -glucanen zou kunnen worden verklaard door de vorming van korte keten vetzuren uit β -glucanen, waarvan bekend is dat ze de NF- κ B activering kunnen remmen. De verhoging van de NF- κ B activiteit in de darmcellen en de witte bloedcellen in de darm wijst erop dat beide celtypen door haver β -glucanen werden geactiveerd. Het was opvallend dat de concentratie van de Th1 cytokine ("signaalstof") IL-12 duidelijk verhoogd was in de darmpreparaten, hetgeen de aanwezigheid van antigeen presenterende cellen suggereert. Echter, de concentratie van Th1 cytokine IFN γ was toegenomen in het begin van de dunne darm. IL-12 wordt hoofdzakelijk door witte bloedcellen en nauwelijks door darmcellen uitgescheiden. Tevens hadden we al aangetoond dat functioneel dectin-1 mogelijk niet aanwezig is op darmcellen. Op basis van deze bevindingen stelden wij de hypothese op dat na consumptie van haver β -glucan eerst de witte bloedcellen in de darm worden geactiveerd, die dan vervolgens de darmcellen stimuleren.

Naast haver β -glucan hebben wij ook gekeken naar de effecten van glucan-rijke fracties verkregen uit paddenstoelen op de immuunreactie van de darm en op de systemische immuunreactie (**hoofdstukken 7 and 8**). Hiervoor hebben wij gebruik gemaakt van paddenstoelextracten, die rijk waren aan glucanen met verschillende structuren (**hoofdstuk 7**). Wij incubeerden deze extracten met gedifferentieerde macrofagen, die waren verkregen uit het beenmerg van muizen (BMM). Vervolgens keken wij naar de veranderingen in stikstofoxide (NO) productie door de BMM, welke wordt gereguleerd door het NF- κ B afhankelijke enzym NO synthase. Daarnaast onderzochten wij de effecten van de extracten op NF- κ B activering in Caco-2 NF- κ B reporter cellen. We vonden dat extracten uit *Agaricus bisporus* de NO productie van BMM sterk stimuleerden, terwijl de extracten uit *Coprinus comatus* en de sporen van *Ganoderma lucidum* slechts geringe effecten hadden. De extracten uit *A. blazei* Murill and *Phellinus linteus* hadden helemaal geen effect. Bijna alle paddenstoelextracten verminderden de NF- κ B activering in Caco-2 cellen. Na een structuuranalyse van de glucanfractie afkomstig van het meest en minst krachtige immuunstimulerende extract (*A. bisporus* en *A. blazei* Murill, respectievelijk), suggereerden wij dat de vertakkingen van de glucanketen essentieel zijn voor de immuunbevorderende activiteit. De glucanfractie van *A. bisporus* leek dan ook geschikt om de systemische immuunreactie te bevorderen. Om na te gaan of consumptie van de glucanfractie van *A. bisporus* inderdaad de immuunreactie *in vivo* bevordert, onderzochten wij in mensen de cytokine productie van perifere bloed mononucleaire cellen (PBMC) na consumptie van α -glucan uit *A. bisporus*, welke was toegevoegd aan fruitdrankjes (**hoofdstuk 8**). Terwijl de α -glucanen uit *A. bisporus* de *in vitro* TNF α secretie van BMM verhoogde, bleek de *ex vivo* TNF α productie door PBMCs gestimuleerd met lipopolysaccharide (LPS) verlaagd te zijn na consumptie van deze glucanen. Daarnaast zagen we geen duidelijke verschuiving in de Th1-Th2 balans door α -glucanen uit *A. bisporus* bij PBMCs die waren gestimuleerd met phytohaemagglutinin (PHA). Een verklaring voor de afwezigheid van een duidelijk immuunstimulerende werking van α -glucanen uit *A. bisporus* zou de vergelijkbare structuur met amylose kunnen zijn. Amylose kan in het maagdarmkanaal worden verteerd door enzymen en hetzelfde zou met de α -glucanen gebeurd kunnen zijn. Daarnaast kan niet worden uitgesloten dat de glucanen alleen een effect bewerkstelligen op de immuunrespons in de darm, maar niet op de systemische immuunreactie van PBMCs.

Wij concluderen uit onze studies dat de consumptie van β -glucan uit haver de immuunreactie in de darm *in vitro* bij mensen en *in vivo* bij muizen bevordert, terwijl het de systemische immuunreactie niet stimuleert. Darmcellen spelen een belangrijke rol in de reactie op haver β -glucanen, maar wij zagen niet dat

deze effecten afhankelijk waren van de β -glucan receptor dectin-1. Daarnaast suggereren wij dat haver β -glucanen eerst de witte bloedcellen in de darm activeren, die vervolgens de darmcellen stimuleren. Verder toonden onze bevindingen aan dat de consumptie van β -glucan uit haver de secretie van antimicrobiële eiwitten in mensen en muizen verlaagde. Tot slot toonden onze resultaten aan dat zowel α - als β -glucanen uit de paddenstoel *A. bisporus* de systemische immuuncellen van muizen *in vitro* stimuleerden. Echter, de α -glucanen uit *A. bisporus* hadden geen duidelijke effecten op de systemische immuunrespons bij mensen.

APPENDIX

Dankwoord

Curriculum Vitae

Publications

Abbreviations

DANKWOORD

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Julia, 26 februari 2010.

CURRICULUM VITAE

Julia Jennifer Volman was born on November 6th, 1980, in Zevenaar, the Netherlands. She completed secondary school at the Candea College in Zevenaar in 1999. In the same year she started her academic education at the Eindhoven Technical University, where she studied Biomedical Technology for 6 months. In 2000 she continued academic education and studied Biological Health Sciences at the Faculty of Health Sciences of the Maastricht University. During her internship in 2003 at the department of Human Biology of the Maastricht University, she examined the immune modulating effects of β -glucan. She graduated cum laude in 2004, and was appointed as a PhD-student at the department of Human Biology from September 2004 until February 2009. During this period she conducted cell-based, animal as well as human studies to examine the effects of dietary β -glucans on processes related to intestinal and systemic inflammation, which are described in this thesis. Currently she is working as a Clinical Research Specialist at Medtronic Bakken Research Center in Maastricht.

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tin-1. Eur J Gastroenterol Hepatol 2007; 19(10): A46.

ABBREVIATIONS

ANOVA	analysis of variance
AP-1	activator protein-1
β GR	β -glucan receptor
BMM	bone marrow-derived macrophage
BPI	bactericidal/permeability increasing protein
Caco-2	human colon adenocarcinoma cell line; differentiated small intestinal cell line
CBA	cytometric bead array
CLC	critical lytic concentration
CR	complement receptor
CSBG	<i>Candida</i> spp. β -glucan
CSF	colony stimulating factor
DAPI	4',6-diamino-2-phenyl indole
DC	dendritic cell
DMSO	Dimethyl sulfoxide
ELISA	enzyme-linked immunosorbent assay
FAE	follicle-associated epithelium
FBS	fetal bovine serum
FCS	fetal calf serum
GALT	gut-associated lymphoid tissue
GCMS	gas chromatography-mass spectrometry
GluP	glucan phosphate
GM-CSF	granulocyte/macrophage colony stimulating factors
hBD	human beta-defensin
HT29	human colon adenocarcinoma cell line
IBD	inflammatory bowel disease
ICAM-1	intercellular adhesion molecule-1
IEL	intestinal epithelial lymphocytes
IFN	interferon
I κ B	inhibitor κ B
IL	interleukin
INT407	human small intestinal cell line
IRF3/7	interferon regulatory factor 3/7
LPS	lipopolysaccharide
M-cell	microfold cell
MCP	monocyte chemotactic protein
MIG	monokine induced by interferon gamma

APPENDIX

MIP	macrophage inflammatory protein
MLN	mesenteric lymph node
MMP	matrix metalloproteinase
MW	molecular weight
ND	not determined
NEAA	non-essential amino acids
NF- κ B	nuclear factor- κ B
NK cell	natural killer cell
NO	nitric oxide
PAMP	pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PGG	Poly-[1-6]-D-glucopyranosyl-[1-3]-D-glucopyranose
PHA	phytohemagglutinin
PMAA	partially methylated alditol acetates
PP	Peyer's patches
PRR	pattern recognition receptor
PS	penicillin streptomycin
PS-G	polysaccharide from <i>Ganoderma lucidum</i>
RLU	relative light units
ROS	reactive oxygen species
SCFA	short chain fatty acids
SD	standard deviation
SP	sodium pyruvate
SSG	<i>Sclerotinia sclerotiorum</i> glucan
T84	human colon carcinoma cell line
TBS	Tris buffered saline
Tc cell	cytotoxic T-lymphocytes
TF	tissue factor
TFA	trifluoroacetic acid
TGF	transforming growth factor
Th cell	helper T-lymphocyte
TLR	toll-like receptor
TNF	tumor necrosis factor
WGP	whole glucan particle
ZO	zonula occludens