Immunomodulating effects of food compounds:
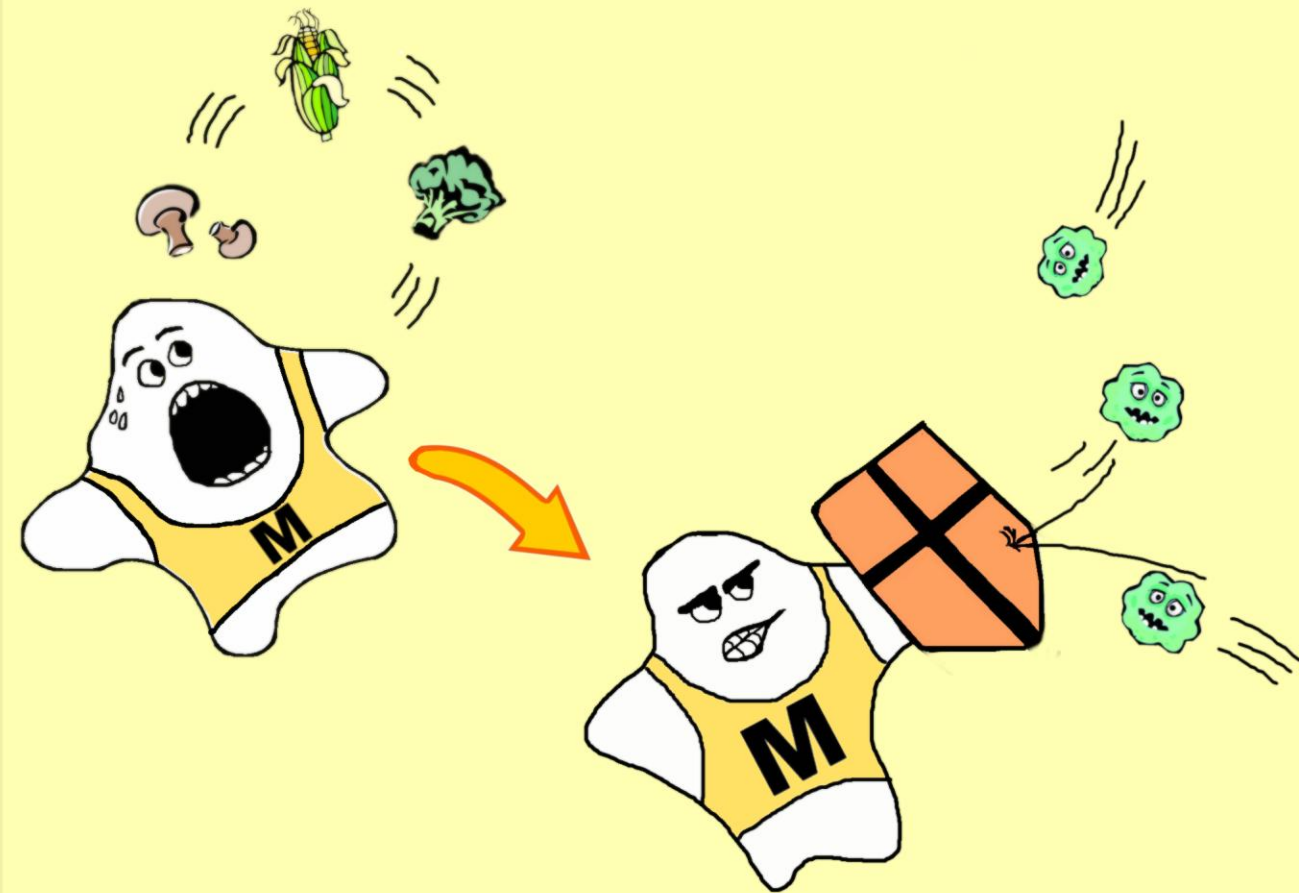
a study using the THP-1 cell line

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Immunomodulating effects of food compounds :

a study using the THP-1 cell line

Wasaporn Chanput

For Prof.dr. Shuryo Nakai

Abstract

THP-1 is a human leukaemia monocytic cell line from the peripheral blood of a 1 year old human male. After exposure to phorbol-12-myristate-13-acetate (PMA), THP-1 cells in monocyte state start to adhere to culture plates and alter their morphology with an indication for differentiation into macrophages. In this thesis, the THP-1 cell line was used in both monocyte and macrophage state. The results obtained during this *in vitro* study show that THP-1 gene expression can be modulated by specific food compounds such as β-glucans, pectin, polyphenols and fungal immunomodulatory proteins (FIPs) in both activation and resting stage. In activation stage, these cells have been activated by LPS to mimic an inflammatory situation, while in resting stage, PMA-differentiated THP-1 macrophages without LPS challenged was used. The polarizing ability of the THP-1 cell line into either classically activated M1 or alternatively activated M2 macrophages was examined using stimuli applied *in vivo*. Based on the expression of M1 and M2 marker genes, THP-1 macrophages could be successfully polarized into both M1 and M2 stage. Thereby, they can be used as a new macrophage polarizing model to estimate the polarizing/switching ability of test compounds. The integration of results from this thesis with a review of recent publications leads to the conclusion that THP-1 cells present unique characteristics as a model to investigate/estimate immunomodulating effects of food-derived compounds in both activated and resting situations.

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Chapter 1

General Introduction

Immune system

The immune system is a network of cells, tissues and organs. Its role in defense against millions of bacteria, microbes, viruses, toxins and parasites that may invade the body is well known. In addition, the immune system is claimed to play a role in overall homeostasis in the body, and as such be able to respond not only to exogenous, but also endogenous 'danger' signals [1]. Improper immune functioning is involved in (i) immunodeficiencies (leading to increased susceptibility to infections and likely involved in carcinogenesis [2]), (ii) autoimmunity (such as e.g. coeliac disease, diabetes type I, rheumatoid arthritis and systemic lupus erythematosus (SLE) [3]), (iii) hypersensitivity (anaphylactic shock and allergies [4]). The immune system is basically composed of two compartments, innate and adaptive immunity. The innate immunity provides immediate but less-specific responses, while the adaptive immunity, which is activated by the innate response, gives stronger and very specific responses and develops a memory, by which it remembers specific invaders and mounts a faster and stronger response in later challenges [5, 6]. As such, this forms the basis of vaccination. Next to cellular immunity, humoral immune responses are critical to make the immune system function effectively. Humoral immunity is mediated by the secretion of antibodies produced by B cells with the aid of T cells [7, 8]. Figure 1 shows the innate and adaptive immune cells.
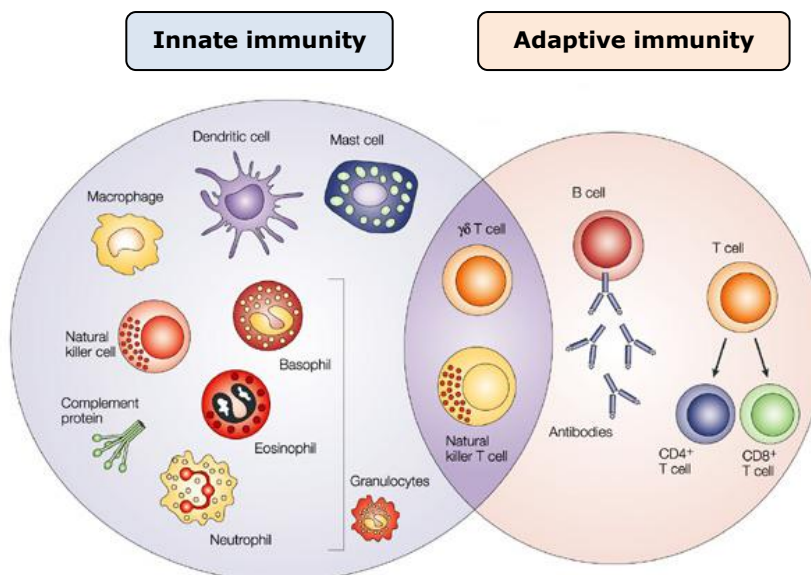

Figure 1 The innate and adaptive immune cells (modified from [9])

Factors influencing immune function

Functions of immune cells are shown in Table 1. Final immune functioning is the resultant of many parameters, for instance, age, gender, endocrine factors, stress, environment and health condition etc. Age is seen as a highly critical factor, which determines immune function. In human adults, cellular and humoral immunity are generated in the adaptive immune responses, whereas, newborns rely preliminary on innate immunity which transfers maternal immune factors because the adaptive immunity is not yet fully developed [10, 11]. Also gender-based differences in human immune responses have been evidenced. Females are, e.g., generally more resistant to viral infections but tend to have more autoimmune diseases than males [12]. Both female and male hormones are shown to have a big impact on immune function. Oestrogens (female hormone) enhance immune function, whereas androgens and testosterones (male hormone), exert suppressive effects on both humoral and cellular immune responses [13].

Psychological stress is also believed to influence immune function, at least partly via an endocrine mechanism [14]. For example, chronic psychological stress lowers salivary IgA levels, as examined in students under academic examination stress [15]. Chronic exercise (i.e. exercise training) modifies immune function, particularly when training loads are high [16]. It has been addressed that acute short periods of exercise cause a temporary depression of various aspects of immune function, for example, respiratory burst by neutrophils, lymphocyte proliferation and monocyte MHC class II expression. All of these events last approximately 3-24 h after exercise depending on the intensity and duration of the exercise, and create a 'window' of immune-related susceptibility [17].

Table 1 Functions of immune cells

Immune cells	Function
Innate immune cells	
- Neutrophils	Engulfing and inflammation
- Basophils	Release histamine and inflammation
- Eosinophils	Destroy worms; hypersensitivity reactions
- Monocytes	Engulfing invading pathogens and differentiate into macrophages
- Macrophages	Engulfing invading pathogens and activate T cells
- Mast cells	Trigger inflammatory response
- Natural killer cells	Kill cells with guidance from antibodies
- Dendritic cells	Potent antigen presenting cells to T cells
Adaptive immune cells	
- B cells	Recognize foreign antigens, differentiate to plasma cells and memory cells, secrete antibodies to guide attack
- Cytotoxic T cells	Recognize and attack cancerous and infected cells
- Helper T cells	Help activate B cells and cytotoxic T cells

Pathologic conditions related to immune function

1. Autoimmune diseases

Autoimmune diseases (AIDs) are a well-known consequence of a malfunction of immune responses of the body against substances and tissues normally present in the body. Basically, our immune system mistakes recognizing own cells and tissues as foreign substances, therefore it attacks the wrong targets. AIDs include more than 70 different disorders [18]. Examples of AIDs are e.g. coeliac disease, diabetes type 1 and multiple sclerosis.

2. *Inflammatory disorders*

Inflammation is caused by a complex immune response of cells or tissues in response to harmful stimuli, such as pathogens, damaged cells or irritants [19]. It can be classified as acute or as chronic inflammation, depending on duration and type of cells involved. During inflammation, a variety of immune cells, such as monocytes, neutrophils, eosinophils, basophils, T and B lymphocytes, are critically involved in the initiation and sustain the inflammation. These cells must be able to get to the site of injury from their usual location in the blood, so called chemotaxis [20]. Examples of disorders in which inflammation is critically involved are asthma, autoimmune diseases, atherosclerosis, coeliac disease, inflammatory bowel diseases (IBDs), rheumatoid arthritis and transplant rejection. Anti-inflammatory medicines are sometimes used to diminish extreme inflammation [21].

3. *Allergy*

Allergies occur as a reaction of the immune system to innocuous substances from the environment. One of the most dangerous allergies is food allergy, e.g. for peanut, egg, milk, soy, fish, spices, fruits and vegetables etc. Non-food substances that cause allergies are, for instance, latex, poison ivy, birch pollen and house-dust mites. Organs in which allergic symptoms may manifest are sinuses, eyes, upper (nose) and lower (lungs) airways, ears, skin and gastrointestinal tract [22-24].

4. *Obesity*

Substantial evidence at the cellular and molecular level indicates that obesity causes a chronic low-grade inflammatory disease [25]. Monocytes infiltrate the adipose tissue and differentiate to adipose tissue macrophages (ATMs). ATMs from lean mice express many genes characteristic for the M2 phenotype, anti-inflammatory macrophage, which may protect adipocytes from inflammation. The fat resulting from diet-induced obesity leads to a shift from the M2 activation state to the M1, pro-inflammatory state that contributes to low-grade inflammation and to insulin resistance, which underlies diabetes type II [25-28]. More details on M1-M2 polarization can be found in the "polarization of macrophages" section in this chapter.

Innate immunity

Innate immunity can be found in all multi-cellular organisms, whereas adaptive immunity is found only in vertebrates. Epithelial barriers like the skin and lining cells in the gastrointestinal tract, lungs and urinary tract can be considered to be part of this immune system. The innate immune system provides immediate defence against infection within 4-96 h and is based on general molecular recognition mechanisms to detect the presence of bacteria, fungi or viruses. This response does not lead to long term immunity to that particular pathogen [29, 30]. However, it can lead to the activation of long lasting pathogen-specific response of the adaptive immune system, which consists of B and T-lymphocytes. These cells can provide pathogen specific immunity to the host through an enormous versatility of receptors encoded by different genes [31]. Cells belonging to innate immunity are, for instance, monocytes, macrophages, neutrophils, dendritic cells and natural killer cells as shown in Figure 1. The major roles of innate immune cells are (i) recognition of foreign pathogens such as bacteria, fungi and viruses by different types of Pattern Recognition Receptors (PRRs), (ii) proliferation to increase the amount of cells to be able to eliminate pathogens, (iii) production of pro-inflammatory cytokines to recruit effector cells to the site of infection and anti-inflammatory cytokines when the infection is under control and (iv) phagocytosis to engulf and digest pathogens [30].

Macrophages

Macrophages are part of the innate immunity which detect and eliminate invading microbes and toxic molecules. Their responses towards these targets are rapid and triggered by specific molecular structures, commonly referred to as Pathogen-Associated Molecular Patterns (PAMPs) [32]. Macrophage pathogen recognition is mediated by a series of germ-line encoded PRRs as described in Table 2. Circulating monocytes differentiate into macrophages once they arrive in tissues, for instance, intestine, adipose tissue, alveolar space, which then constrains the functional properties of macrophages [33-37]. There are two major macrophage populations; tissue macrophages which can survive by self-renewal for long periods and monocyte-derived macrophages which loose proliferative capacity [38]. The main functions of macrophages are involvement in wound healing, resolution of inflammation, coordinating cell migration and tissue remodelling [32]. In an inflammatory situation, monocytes adhere to vascular endothelial cells,

migrate towards the site of infection and differentiate into exudate macrophages locally. The destroyed pathogens or infectious agents are taken by monocytes and macrophages through the flow of lymph and blood from infected site to the secondary lymphoid tissue where they are processed and presented to T-lymphocytes [39].

Table 2 Macrophage receptors implicated in PAMPs recognition (adapted from [40])

Receptors	Functions
SR-A	Phagocytosis of bacteria and apoptotic cells, endocytosis of modified LDL, adhesion
CD36	Phagocytosis of apoptotic cells, diacyl lipid recognition of bacteria
TLRs	Response to peptidoglycan and LPS
CD14	LPS-binding protein/interaction MD2/MYD88, TLR signalling, apoptotic cell recognition
CR3 (CD18/11b)	Complement receptor (C3b) mediated phagocytosis, Adhesion to endothelium
CCR2	Receptor for MCP-1
Dectin-1	β-glucan receptor, fungal particle ingestion, interaction with TLR-2
DC-SIGN	Mostly in dendritic cells, pathogen recognition, ICAM adhesion
MINCLE	Induction of inflammatory signalling in response against yeast infection and pathogenic fungus [41, 42]

Polarization of macrophages

Macrophages are dynamic and heterogeneous cells due to different mechanisms involving their differentiation, responsiveness to stimuli and tissue distribution. The distinct CD4+ T cell subsets, among other cell types, play major roles during the differentiation of macrophages into distinct phenotypes. IFN-γ induces M1, or classically activated macrophages, while IL-4, IL-13 and IL-10 induced-macrophages are referred as alternatively activated macrophages or M2 [33, 36, 43]. The characterization is based on observations that M1-like macrophages play a role in producing pro-inflammatory cytokines and mediators, providing host defence against microorganisms and in tumour regression by stimulating a Th1-driven immune response. Expression of TNF-α, IL-1β, IL-6, IL-8 and IL-12 genes, and expression of PRRs, such as Toll like receptors (TLRs) and NOD-like receptors (NLRs), have been reported to be up-regulated during M1 activation [33, 44]. Alternatively activated macrophages or M2 actively participate in the resolution of parasite infection, tissue modelling, immunoregulation, allergy and tumour progression by stimulating a Th2-driven immune response [45, 46]. A reverse orchestration of the activated Th1 and Th2 cytokines backwards macrophage type switching has been also known as described in [47]. Three subsets of M2 macrophages have been described: M2a induced by IL-4 or IL-13, M2b induced by exposure to immune complexes (IC)/TLRs agonist or IL-1Receptor and M2c induced by IL-10 [48]. These characteristic M2 subtypes are hypothesized to relate to specific functions as shown in Figure 2.
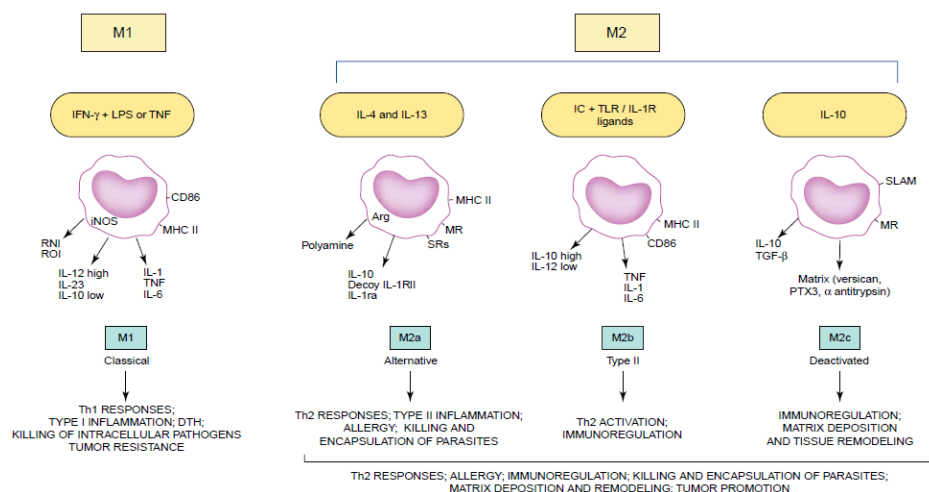

Figure 2 Stimuli, secreted molecules, receptors and functional properties of different polarized macrophage states (modified according to [48]).

The THP-1 cell line as monocyte and macrophage response model

Tsuchiya *et al.* [49] established the human THP-1 cell line in 1980 from the peripheral blood of a 1 year old human male with acute monocytic leukemia [http://en.wikipedia.org/wiki/THP1_cell_line]. Early studies indicated that THP-1 cells resemble primary monocytes and macrophages in morphological and differentiation properties. THP-1 cells are round suspension cells expressing distinct monocyte markers [49]. After exposure to phorbol-12-myristate-13-acetate (PMA), THP-1 cells in monocyte state start to adhere to culture plates and alter their morphology which is an indication for differentiation into macrophages; flat and amoeboid in shape with well-developed Golgi apparatuses, rough endoplasmic reticula and large numbers of ribosomes in the cytoplasm [50]. Apart from PMA used as a differentiating stimulus, 1α, 25-dihydroxyvitamin D3 (vD3) is another reagent used to differentiate monocytic cells towards the macrophage state. However, PMA and vD3 regulate different signalling pathways, in which PMA recruits protein kinase C to the intracellular side of the plasma membrane, whereas vD3 up-regulates expression of MAPK phosphatase-1 (MKP-1) [51]. PMA treatment leads to a more mature phenotype with higher levels of adherence, a lower rate of proliferation, a higher rate of phagocytosis and higher cell-surface CD11b and CD14 [51]. Therefore, PMA-differentiated THP-1 macrophages are widely used to study macrophage functions and responses.

THP-1 cells exposed to LPS

Exposure of THP-1 monocytes to bacterial products such as lipopolysaccharide (LPS) results in activation of the NF-κB transcription factor, which orchestrates a gene expression program leading to the activation of inflammation, cell proliferation, differentiation, migration and cell survival, which are mediated through the release of chemokines and cytokines [52, 53]. Sharif *et al.* [53] showed that THP-1 cells exposed to LPS responded with a change in expression of a number of inflammation-related genes. These responses can already be identified within 1 h of incubation and some of them maintained their expression after 4 h of incubation.

THP-1 cells exposed to food/non-food compounds

THP-1 cells have been widely used to study monocyte and macrophage responses to various stimuli. Food derived or non-food derived compounds have been tested in this cell model either at the resting or inflammation-activated state. Table 3 shows examples of studies using THP-1 cells tested with various compounds.

Table 3 Examples of studies using THP-1 cell model

	Compound	Read out	Reference
THP-1 monocytes	Besifloxacin (antibacterial agent)	Pro-inflammatory cytokine	[54]
	Aloe vera gel	Suppression of bacteria-induced pro-inflammatory cytokine	[55]
	IL-10	Autocrine and exocrine regulation of IL-10 production	[56]
	Monascus-fermented metabolites	Suppression of inflammation	[57]
THP-1 macrophage	Korean traditional prescription	Anti-inflammatory effects	[58]
	Nobiletin (citrus flavonoid)	Suppression of scavenger receptors	[59]
	Astragalus polysaccharide	Inhibition of TNF-α and IL-1β production in LPS-induce status	[60]
	Ethanolic extracts of Brazillian red propolis	ABCA1 expression and cholesterol efflux	[61]
	Curcumin	Apoptosis	[60]
	25-hydroxycholesterol-3-sulfate	Inflammatory responses	[62]
	β-glucans	Inflammatory responses	[63-65]
	Long-chain saturated fatty acid	Pro-inflammatory cytokine	[66]
	Titanium and polyethylene particle	NF-κB signalling and TNF-α expression	[67]
	Pulmonary surfactant protein A +/- LPS	Inflammation-related cytokine gene expression	[68]
	Shiga Toxin 1 +/- LPS	Inflammatory cytokine gene expression and production	[69, 70]

Studies on co-culture of THP-1 cells with other cell types

Apart from studying responses of THP-1 cell in the monocyte or macrophage state as a single cell type, this cell model is also used in co-culture systems with other cell types, for instance vascular smooth muscle cells, adipocytes, T-lymphocytes, platelets and intestinal cells.

Vascular smooth muscle cells Co-culture of THP-1 cells and human umbilical vein endothelial cells (HUVECs) [71] or human aortic endothelial cells (HAECs) [72] were used to study attractions of THP-1 monocytes by the atherosclerotic plague formation of endothelial cells in the inflammatory areas. The adhesion ability of fluorescently labelled THP-1 cells was determined [73-75]. It was concluded that THP-1 monocytes are chemically attracted by activated endothelial cells.

Adipocytes Obesity-associated inflammation enhances macrophage infiltration in adipose tissue by inflammatory cytokine production, such as TNF-α and IL-6 [76]. Keuper *et al*. [77] established an *in vitro* model system for human adipose tissue by either incubation of SGBS adipocytes (pre-adipocyte cell line) with THP-1 cells in indirect incubation with conditioned medium from THP-1 cells or direct co-culture of SBGS adipocytes with THP-1 cells. Spencer *et al*. [78] co-cultured primary human adipocytes and pre-adipocytes with THP-1 cells using a transwell method to examine the gene expression pattern of M1, M2a and M2c macrophages. The results indicated that co-culture of adipocytes with either M1 or M2 macrophages led to an overall shift of macrophage gene expression. Because THP-1 cells and adipocytes did not come into contact with each other in the co-culture system, these findings hint that adipocyte secrete soluble factors which promote a shifting of the M1- to the M2-phenotype cell during co-culture.

T-lymphocytes Jurkat T cells are an immortalized T-lymphocyte cell line that is often used to study T cell signalling. Fuentes *et al.* [79] used co-culturing of THP-1 cells with Jurkat cells to study the interaction between macrophages and T-cells.

Platelets Leukocyte-platelet interaction is important in the initiation and progression of atherosclerosis. Aslam *et al.* [80] incubated human platelets labelled with fluorescent dye with THP-1 cells. The result led to the conclusion that this co-culture can be used as a model to mirror the interaction between platelets and monocytes.

Intestinal cells Watanabe *et al.* [81] found that Caco-2 cells, a human epithelial colorectal adenocarcinoma cell line, grown in transwells could be disrupted by co-culturing with THP-1 macrophages due to the secretion of TNF-α by THP-1. A similar finding was reported by Moyes *et al.* [82], Caco-2 cells in transwell system with underlying THP-1 macrophages produced a lower, less organised epithelium and greater microparticle uptake. Thereby, indirect co-culture of these two cell lines might help to prevent the disruption of the Caco-2 monolayer.

Immunomodulation by food

As mentioned earlier, there are numerous diseases caused by malfunction of the immune system. Some of these are caused by consumption of a suboptimal diet, while others are related to the malfunction of immune system against a genetic, environmental, gender and age background. It is believed that diet affects the immune system at multiple levels [83, 84]. Firstly, nutrition serves to provide the immune system with diet-derived factors, such as protein, vitamins and minerals. Lack of such components e.g. zinc, vitamin D3 can negatively influence immune functioning. Secondly, it is known that using effective immunomodulating compounds that target PAMPs can consequently alter several signalling pathways and biological functions [85]. A major focus of recent research is to understand the role of specific food components or nutrients in supporting the human immune system to maintain or improve health or to reduce risks for diseases. Target groups are the general population, as well as certain vulnerable groups with particular sensitivity towards diseases. Examples of well-known immunomodulatory compounds showing effects *in vivo*, *ex vivo* and *in vitro* research are listed below.

β-Glucan

β-Glucans exert their immunomodulating effects in a variety of species, including humans, other mammals (ruminants, pigs), fish and poultry. Their effects are likely mediated via the innate immune system, causing release of cytokines which later on stimulate the humoral and cell mediated parts of the adaptive immune system [86-90]. The immunomodulating properties of β-glucans, in innate and adaptive immunity, and their anti-inflammatory properties have been widely

investigated over many years. β-Glucan induces NF-κB gene expression via binding to dectin-1 in association with TLR-2 and -4 in a MyD88 –dependent signalling cascade [91]. Up-regulation of the NF-κB gene is considered significant as an important regulator of cellular activation, for instance, cycle progression, cell survival, cell adhesion, invasion and inflammatory responses [92].

Best analysed β-glucans are those derived from mushrooms and yeast which have shown to increase phagocytosis activity, nitric oxide production, catalase activity of macrophages and reduced tumour formation in a mouse model [93, 94]. The production of inflammatory cytokines and mediators induced by mushroom β-glucan leads to an increased ability to resist viral, bacterial and fungal challenge in innate immunity [94]. Oral administration of mushroom and yeast β-glucan have shown effects *in vivo* [93]. These effects could be supported by *in vitro* studies like those from Olson *et al.* [95] demonstrating that yeast *S.cerevisiae* β-glucan increased *in vitro* TNF-α production by alveolar macrophages isolated from mice. Besides β-glucan from mushroom and yeast, also β-glucan from oat shows these immune modulating effects *in vitro*. Estrada *et al.* [96] showed an increase of IL-1α 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.

VitaminD3

VitaminD3, 1α, 25-dihydroxyvitamin D3, has been shown to promote antibacterial activity of monocytes through activation of several antibacterial proteins and enzymes [97, 98]. The suppressive effects of vitaminD3 on NF-κB signalling pathways has been observed in T cells, monocytes and macrophages [98]. VitaminD3-stimulated human monocytes are shown to inhibit the expression of innate receptors TLR-2, TLR-4 and TLR-9 and the production of IL-6 [99], in which TLR-2 and -4 were suppressed in both mRNA and protein expression in a time-dependent and dose-dependent way [100, 101]. Macrophage differentiation, dendritic cell maturation and T- and B-lymphocytes were also reported to be modulated by vitaminD3 [98, 102].

Polyphenols

Several thousands of polyphenols have been grouped into major classes, such as flavonoids, phenolic acids, stilbenes and lignans [103]. They can be found in many fruits, vegetables and spices [104]. Several studies showed anti-allergic effects of polyphenols. Crude apple polyphenol extract inhibited the release of mediators involved in allergic reactions including histamine or leukotriene in an *in vitro* system [105]. In an animal model, it was shown that a quercetin-enriched diet attenuated respiratory symptoms in sensitized guinea-pig [106]. Pilot human intervention studies indicated that consumption of apple-condensed tannins in a period of 8 weeks reduced symptoms of atopic dermatitis (eczema) [107]. Epidemiological studies in Finland [108], the Netherlands [109] and the United Kingdom [110] have demonstrated a protective effect of quercetin/apple consumption on asthma incidence.

Polyphenols not only show anti-allergic properties, but also anti-inflammatory effects. Antioxidative capacities of flavonoids have been described *in vivo* [111, 112] and *in vitro* [105, 113]. Recent studies reported that quercetin could suppress the production of various pro-inflammatory cytokine genes in the presence of LPS in THP-1 monocytes and macrophages [52] and pro-inflammatory cytokine production in RAW 264.7 [114]. It has been mentioned that quercetin suppresses inflammatory status through the MAP kinase and NF-κB pathway [114, 115] as well as does curcumin [116]. Other flavonoids such as epicatechin derivatives showed antioxidant activity, thus decrease production of IL-1β, IL6 and IL-8 cytokines [117, 118]. Similar findings were reported for the flavonoids apigenin, luteolin and chrysin [119].

Polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFAs) are fatty acids that contain more than one double bond in their backbone. They can be divided into omega-3, omega-6 and omega-9 fatty acids, in which omega-3 fatty acids (n-3 FAs) attract the most attention among researchers and consumers [120]. The main n-3 FAs are EPA and DHA and α-linolenic acid. EPA and DHA are mainly found in fish and seafood, while α-linolenic acid is in flaxseed, canola, soy and walnut oils. It has been shown that high intake of long chain n-3 polyunsaturated fatty acids (PUFAs) decreased the production of inflammatory mediators such as cytokines and reactive oxygen species (ROS) [121]. Several animal and human studies indicated that increasing consumption of n-3 FAs caused anti-inflammatory

effects [122-125]. Moreover, clinical data have been documented that n-3 FAs suppressed chronic inflammatory diseases, for instance, rheumatoid arthritis and IBDs [126]. Other highly inflammatory circumstances like in surgery and sepsis appeared to be beneficially affected by n-3 FAs [127].

Fungal Immunomodulatory Proteins (FIPs)

FIPs have a molecular weight approximately of 13 kDa protein. A number of FIPs have been found, for instance, FIP-*fve* from *Flammunila velutipes*, FIP-*vvo* from *Volvariella volvacea*, LZ-8 from *Ganoderma lucidum*, FIP-*gja* from *Ganoderma japonicum* and FIP-*gts* from *Ganoderma tsugae* [128]. Biological relevance of FIPs in allergy mitigation has been suggested by their attenuating ability in food-allergic and respiratory-allergic reactions in murine models. LZ-8 demonstrated the activation of human monocyte-derived dendritic cells by enhancing cell surface expression of CD80, CD86, CD83 and HLA-DR, as well as the enhanced production of IL-12p40, IL-10 and IL-23 [129]. FIP-*fve* increased IFN-γ-production though the p38 MAPK pathway in human PBMCs and showed a potent effect on T cells [130].

Pectin

Pectin is found in plant cell walls; it is most abundant in citrus fruit (lemon, orange, lime and grapefruit). It is composed of complex polysaccharides rich in galacturonic acid residues [131]. Pectin has been suggested to exhibit antimutagenic activity against nitroaromatic compounds [132]. Citrus pectin with different degree of esterification was shown to have different inhibitory effects on expression of iNOS and COX-2 genes in LPS-activated macrophages [133]. Mice fed with a diet supplemented with 20% apple pectin significantly decreased the number of colon tumours and reduced prostaglandin E2 levels in the distal colonic mucosa. Antioxidant activity and radical scavenging function have been indicated for apple pectin [134].

Others

Apart from the above mentioned food compounds, there is a vast number of compounds showing immunomodulating effects *in vivo*, *ex vivo* and *in vitro* studies. For example, chitin (β-(1-4)-poly-N-acetyl D-glucosamine) found in the cell walls of the exoskeleton of crustaceans like crabs, shrimp etc., and insects has been shown to activate the innate immune response by up-regulation of IL-12, TNF-α and IL-18 genes [135]. In addition, it has been also noted that chitin induced eosinophil and basophil recruitment and enhanced the generation of alternatively activated macrophages (M2) [136]. Milk and soy proteins, as well as probiotics, have been also reported for their immunomodulating properties.

Gene expression studies using RT-qPCR

Real-time quantitative PCR (RT-qPCR) technology has proven to be a powerful method to quantify gene expression [137, 138]. Its prime advantage is the ability to quantitatively compare gene expression over a range of 10^7 fold. In addition, RT-qPCR based assays are characterized to be sensitive and specific with very reliable reproducibility, relatively easy to apply in clinical setting and efficient in terms of biological material consumption [139]. In case marker genes are known or can be selected based on an educated hypothesis, RT-qPCR is relatively cost-effective compared to the more black box approach of using microarray, thus makes it suitable for every day laboratory routine work. Spending careful attention to experimental design, sample and assay quality control and selection of proper reference genes for normalization will significantly increase the reliability of results using RT-qPCR [140].

Correlations between RNA and protein expression profile

The central Dogma of molecular biology states that "DNA makes RNA makes proteins" suggesting there is a direct relationship between mRNA and protein levels [141].
This assumed relationship is based on the correlations between the significant general correlations between mRNA and protein expression which have been found in many studies [142-146]. However, recent genome wide correlation studies have shown that levels of transcripts and proteins correlate significantly for only about half of the genes tested and this correlations also depends on

the cellular location and biological function of the gene [147]. In inflammatory situation, immune responses require the trafficking of inflammatory leukocytes to appropriate anatomic locations. This process is regulated by chemoattractants, such as chemokines and cytokines, and by receptors through which they mediate their activities. This means that the secreted chemokines and cytokines will be recognized by receptors of other immune cells in paracrine and autocrine manner. An example for such process is the binding of IL-10 cytokine to their receptors on cell surface IL-10R-1 and IL-10R-2 which could decrease the available amount of IL-10 cytokine in culture supernatant [56, 148]. This relatively complex cell-cell signals in paracrine and autocrine manner makes correlation between mRNA and protein level in immune cells less straight forward.

Research aim and thesis outline

This thesis aims to investigate the utilization of the THP-1 cell line in either the monocyte or the macrophage state as a model to study immunomodulating properties of food-derived compounds. A variety food-derived stimuli, together with different read-outs have been applied to provide detailed knowledge on THP-1 cell responses for future use as a screening tool. In **Chapter 2**, various inflammation-related genes were analyzed for their expression kinetics in LPS-stimulated THP-1 monocytes and PMA-differentiated THP-1 macrophages. Time-dependent cytokine secretion was also determined and correlated. A number of food-derived compounds were tested in the LPS-stimulated THP-1 cell model to address a possible use as a tool to study inflammatory modulating effects. **Chapter 3** describes *in vitro* analyses of THP-1 cells upon β-glucan stimulation. The PMA-differentiated THP-1 macrophages were tested with β-glucans extracted from different sources. The application of this *in vitro* model is expected to set up strategies to characterize, compare and predict immunomodulating properties of β-glucans from diverse source.

Polarizing ability due to changes of microenvironment is one of the main characteristics of macrophages. **Chapter 4** focuses on polarization of resting PMA-differentiated THP-1 macrophages (M0) into either the M1 or M2 phenotype using stimuli applied *in vivo*. Examples of food compounds were also tested to see whether they are able to polarize M0 towards M1 or M2 state. **Chapter 5** consists of production and purification of different recombinant fungal immunomodulatory proteins (rFIPs). Transepithelial electrical resistance (TEER) values were measured in confluent differentiated Caco-2 monolayer after exposure to different kinds of purified

rFIPs. Inflammation-related gene expression was determined in THP-1 macrophage stimulated with different purified rFIPs in different concentration.

Finally, the results obtained in this research are discussed in **Chapter 6**. This chapter indicates the future perspectives using THP-1 cell line as a screening tool and its impact on further research towards development of this *in vitro* cell model.

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Chapter 2

Transcription profiles of LPS-stimulated THP-1 monocytes and macrophages : a tool to study inflammation modulating effects of food derived-compounds

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Abstract

An assay was developed to study inflammation-related immune responses of food compounds on monocytes and macrophages derived from THP-1 cell line. First strategy focussed on the effects after stimulation with either lipopolysaccharide (LPS) or Concanavalin A (ConA). Gene expression kinetics of inflammation-related cytokines (IL-1β, IL-6, IL-8, IL-10 and TNF-α), inflammation-related enzymes (iNOS and COX-2), and transcription factors (NF-κB, AP-1 and SP-1) were analyzed using RT-qPCR. Time dependent cytokine secretion was investigated to study the inflammation-related responses at protein level. LPS stimulation induced inflammation-related cytokine, COX-2 and NF-κB genes of THP-1 monocytes and THP-1 macrophages with the maximum up-regulation at 3 and 6 h, respectively. These time points, were subsequently selected to investigate inflammation modulating activity of three well known immuno-modulating food-derived compounds; quercetin, citrus pectin and barley glucan. Co-stimulation of LPS with either quercetin, citrus pectin, or barley glucan in THP-1 monocytes and macrophages showed different immuno-modulatory activity of these compounds. Therefore, we propose that simultaneous exposing THP-1 cells to LPS and food compounds, combined with gene expression response analysis are a promising *in vitro* screening tool to select, in a limited time frame, food compounds for inflammation modulating effects.

Introduction

Monocytes and macrophages are an important part of the innate arm of the immune system. These cells are involved in inflammatory processes, with a profound capacity to synthesize and secrete pro- and anti-inflammatory cytokines [1, 2]. THP-1 cells, a human leukemia monocytic cell line, have widely been used as a model to study the immune response capacity of monocytes and monocyte-derived macrophages, because of similarities in their responses when compared to the monocyte fraction present in peripheral blood mononuclear cells (PBMCs) [3, 4].

The innate immune system relies on pathogen recognition receptors (PRRs) such as Toll-like receptors (TLRs), to recognize conserved molecular structures of invading pathogens called PAMPs. PAMPs, like lipopolysacharide (LPS), play a pivotal role in the initiation of a variety of host responses caused by infection with Gram-negative bacteria. Such action leads to systemic inflammatory response, for instance up-regulation of pro-and anti-inflammatory cytokine genes, resulting in secretion of cytokine proteins into the blood stream [5, 6]. Some transcription factors have been shown to be directly or indirectly related to the receptor-mediated expression of inflammation related-cytokine and inflammation-related enzyme genes. The transcription factor Nuclear Factor (NF)-κB is involved in the transcriptional regulation of the IL-1β, IL-6, IL-8, TNF-α, iNOS and COX-2 genes [7, 8]. The transcription factor AP-1 is associated with the regulation of IL-8 and TNF-α genes [7, 9]. Activation of the AP-1 transcription factor occurs by an increased the production of c-Jun and c-Fos proteins which need to form a dimeric complex binding the promoter region of AP-1 [10]. Expression of IL-10 gene is regulated by SP-1 transcription factor [11, 12].

Immune responses are commonly determined by measuring the presence of cytokines in culture medium after challenging cells. However, these assays are often performed with relative long exposure time (generally hours) in order to obtain cytokine levels above the threshold which can lead to the initiation of further signalling pathways. Furthermore, cytokine protein secretion is only partly related to the expression of cytokine-related genes and their upstream transcription factors, because of extensive regulation of the transcription and translation processes [13, 14]. We, therefore, investigated the gene expression kinetics of inflammation-related cytokines, inflammation-related enzymes and relevant transcription factors, together with time-dependent cytokine protein secretion. Genetically identical THP-1 monocytes and macrophages, which were stimulated with LPS and ConA, were used as a model. Furthermore, the application of this assay for screening immuno-modulatory effects of food-derived components was tested and discussed.

Materials and methods

Chemicals and cell culture

The human monocytic leukemia cell line THP-1 (American Type Culture Collection, Rockville, Md.) was grown in RPMI 1640 culture medium (Lonza, Switzerland) supplemented with foetal bovine serum (FBS; Invitrogen, UK.) and penicillin/streptomycin (P/S) (Invitrogen) to respectively 10% and 1 %, at 37 ^{o}C in 5 % CO_2 in a humidified incubator. Cells were sub-cultured twice per week. THP-1 cells change their culture properties after prolonged periods in culture, cells were therefore discarded and replaced by frozen stocks after 25 passages. LPS (E.*coli* 0111:B4), Concanavalin A (ConA), quercetin, citrus pectin and barley glucan were purchased from Sigma (St. Louis, MO, USA), the latter three of the highest possible purity grade to respectively 99%, ≥74% galacturonic acid and >95%.

Macrophage differentiation and stimulation

The mature macrophage-like state was induced by treating THP-1 monocytes (10^6 cells/ml) for 48 h with 100 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma Chemical) in 12-wells cell culture plates (Greiner, Germany) with 1 ml cell suspension in each well. It has been demonstrated that this differentiation method of THP-1 cells resulted in the expression of macrophage specific surface markers CD11b and CD36 and also phagocytic activity [43]. Differentiated, plastic-adherent cells were washed once with sterile phosphate-buffered saline (PBS; Sigma Chemical, USA) and RPMI 1640 medium without PMA but containing 10% FBS and 1% P/S. THP-1 monocytes (undifferentiated cells) and THP-1 macrophages (differentiated cells) were treated with either 1 μg/ml LPS or 5 μg/ml ConA, which concentrations were chosen according to our preliminary optimization studies. RPMI 1640 medium containing 10% FBS and 1% P/S was used as a control. Both types of cells were harvested at different time points ranging from 0-30 h to investigate gene expression kinetics, while cell-free culture supernatants were collected and stored at -80 ˚C to measure time-dependent cytokine secretion. The experiments were performed by two independent biological replications, started from a new batch of cells.

Gene expression kinetics by Real-Time qPCR

Total RNA was isolated by using RNeasy mini kit (Qiagen, USA) with a RNase-free DNase (Qiagen) treatment for 15 min according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from isolated RNA with iScript cDNA systhesis kit (Bio-Rad, USA). Of the synthesized cDNA 200 ng was mixed with 10 μl of IQ^{TM} SYBR Green Supermix (Bio-rad) and primer pairs in a 20 μl reaction volume and preheated at 95 ^{o}C for 90 sec, followed by PCR for 40 cycles, denaturing temperature of 95 ^{o}C for 10 sec, annealing temperature of 58 ^{o}C for 10 sec, and elongation temperature of 72 ^{o}C for 15 sec, and finally elongation temperature of 72 ^{o}C for 2 min. Primer sets (see Table 1) were tested by dilution series of cDNA from LPS-stimulated THP-1 monocytes to analyze PCR efficiency. Amplified PCR products were also analyzed by ethidium bromide stained agarose gel to check for amplification of a single product. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen for normalisation because this gene was stably expressed in both THP-1 monocytes and macrophages, both in challenged and unchallenged cells (data not shown). The PCR products of all samples were subjected to a melting curve analysis to verify the single amplification product. The relative messenger RNA (mRNA) expression were exhibited in two ways; firstly, the expression relative to GAPDH, calculated as ΔCt [ΔCt =2^-(Ct_{GAPDH}-Ct_{sample})][44]. Secondly, the values expressed as fold change relative to the value at time point zero, calculated as $\Delta\Delta$Ct [$\Delta\Delta$Ct =2^-(ΔCt $_{GAPDH}$- ΔCt $_{sample}$)][44]. All experiments were performed with the same amount of cells (10^6 cells/ml) and the same quantity of RNA input. RT-qPCR was performed twice on each sample.

Table 1 Sequence of Real Time-PCR primers

Gene	Accession number	Primer working concentration (μM)	Sequence (5'→3')
IL-1β	NM_000576.2	0.1	F- GTGGCAATGAGGATGACTTGTTC
			R- TAGTGGTGGTCGGAGATTCGTA
IL-6	NM_000600.3	0.1	F- AGCCACTCACCTCTTCAGAAC
			R- GCCTCTTTGCTGCTTTCACAC
IL-8	NM_000584.2	0.1	F- CTGATTTCTGCAGCTCTGTG
			R- GGGTGGAAAGGTTTGGAGTATG
IL-10	NM_000572.2	0.4	F- GTGATGCCCCAAGCTGAGA
			R- CACGGCCTTGCTCTTGTTTT
TNF-α	NM_000594.2	0.1	F- CTGCTGCACTTTGGAGTGAT
			R- AGATGATCTGACTGCCTGGG
iNOS (1)	NM_000625.3	0.1	F- CATCCTCTTTGCGACAGAGAC
			R- GCAGCTCAGCCTGTACTTATC
iNOS (2)	NM_000625.4	0.1	F- GGCTGGAAGCCCAAGTACG
			R- CTCAGGGTCACGGCCATTG
COX-2	NM_000963.2	0.1	F- CAGCACTTCACGCATCAGTT
			R- CGCAGTTTACGCTGTCTAGC
NF-κB	NM_003998.2	0.4	F- TGAGTCCTGCTCCTTCCA
			R- GCTTCGGTGTAGCCCATT
SP-1	NM_138473.2	0.4	F- GGTGCCTTTTCACAGGCTC
			R- CATTGGGTGACTCAATTCTGCT
c-Jun	NM_002228.3	0.4	F- TGGAAACGACCTTCTATGACGA
			R- GTTGCTGGACTGGATTATCAGG
c-Fos	NM_005252.3	0.4	F- GGATAGCCTCTCTTACTACCAC
			R- TCCTGTCATGGTCTTCACAACG
GAPDH	NM_002046.3	0.1	F- TGCACCACCAACTGCTTAGC
			R- GGCATGGACTGTGGTCATGAG

Time-dependent cytokine secretion by cytometric bead array

Cytokine secretion (IL-1β, IL-6, IL-8, IL-10 and TNF-α) in cell-free culture supernatants of THP-1 monocytes and macrophages was measured by cytometric bead array (CBA) analysis (BD Biosciences, USA) according to the manufacturer's guidelines. Briefly, a mixture of 5 capture bead populations (50 μl) with distinct fluorescence intensities coated with capture antibody proteins for the above mentioned cytokines was first mixed with each sample or standard (50 μl) and incubated in the dark for 1 h on an orbital shaker. Additionally, PE-conjugated detection antibodies (50 μl)

were added to form sandwich complexes and then incubated in dark for 2 h on an orbital shaker. Subsequently, the samples were resuspended in 200 μl of wash buffer before acquisition on a FACScan cytometer (BD Bioscience). The sample results were analyzed using FCAP^{TM} Array analysis software. The measurement was performed twice from one of two biological replicates.

Food-derived compounds on LPS-stimulated cells

THP-1 cells were stimulated with either 50 μM quercetin, 0.75 mg/ml citrus pectin, or 100 μg/ml barley glucan, with and without the presence 700 ng/ml LPS. The LPS concentration was reduced from 1 μg/ml to 700 ng/ml to achieve a lower up-regulation of inflammation-related genes. Expression kinetics of inflammation-related genes with 700 ng/ml LPS were similar to 1 μg/ml LPS, but with a reduced amplitude (data not shown). The concentrations of food compounds were chosen according to preliminary optimization studies and literature search [39, 45, 46]. After stimulation, THP-1 monocytes and macrophages were harvested at 3 h and 6 h, respectively. Expression of inflammation-related genes was determined. The experiments were performed by two independent biological replications, started from a new batch of cells.

Results

Gene expression kinetics of THP-1 monocytes and macrophages stimulated with LPS and ConA

Expression of genes for inflammation-related cytokines

Pro- and anti-inflammatory cytokine gene expression was analysed in undifferentiated and differentiated THP-1 cells, designated as THP-1 monocytes and THP-1 macrophages, respectively. In this analysis, mRNA expression level of the pro-inflammatory cytokine genes IL-1β, IL-6, IL-8 and TNF-α, and of the anti-inflammatory cytokine gene IL-10 were determined by RT-qPCR. In both non-stimulated THP-1 monocytes and macrophages at time zero, IL-8 was the most abundant gene relative to GAPDH, followed by IL-1β and TNF-α genes, respectively (Fig 1). IL-6 and IL-10 exhibited low abundance in both cell types. All cytokine genes were higher expressed in non-stimulated THP-1 macrophages than in monocytes (Fig 1).
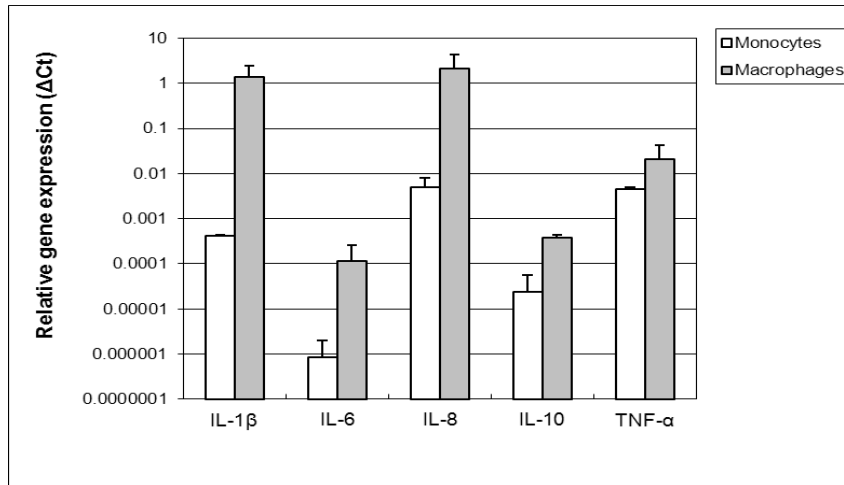
Figure 1 Inflammation-related cytokine gene expression relative to GAPDH (ΔCt) of THP-1 monocytes and macrophages before stimulation. Data shown are the means + standard deviation (SD bars) from two independent biological replications.

THP-1 cells, like primary monocytes and macrophages, expressed a variety of inflammation-related cytokine genes in response to LPS (Fig 2A, C). The IL-6 gene showed, among the analyzed cytokine genes, the highest fold change of expression in both THP-1 monocytes and macrophages. Exposure of THP-1 monocytes to LPS strongly induced IL-1β, IL-6, IL-8, IL-10 and TNF-α gene expression, with maximal expression after 3 h of stimulation, except for IL-6 expression which gradually increased throughout the incubation time (Fig 2A). All inflammation-related cytokine genes of THP-1 macrophages were also highly up-regulated by LPS-stimulation but less than those of monocytes, except IL-6, with a maximal expression after 6 h of stimulation (Fig 2C). The onset of up-regulation of all analyzed inflammation-related cytokine genes appeared to be earlier in THP-1 monocytes (within 1 h) compared to that of macrophages (within 2 h) (Fig 2A,C).
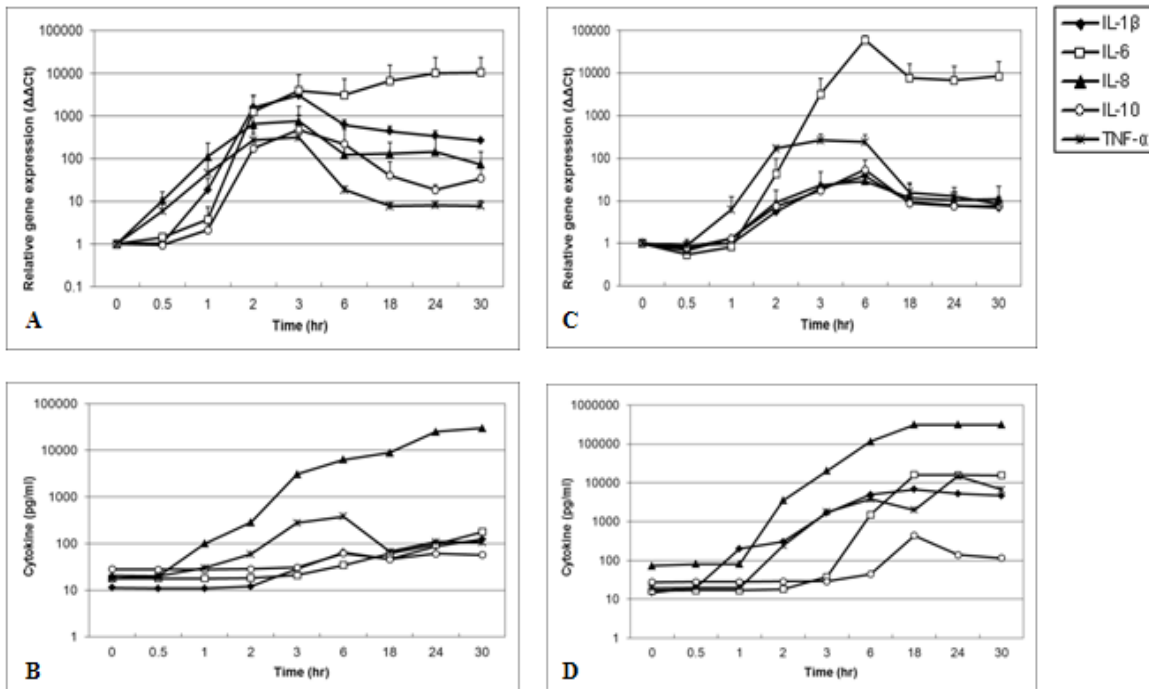
Figure 2 Inflammation-related cytokine gene expression kinetics and cytokine secretion kinetics of THP-1 monocytes (A-B) and THP-1 macrophages (C-D) stimulated with 1 µg/ml LPS. Gene expression was expressed as relative gene expression towards GAPDH-expression and non-stimulated cells at time zero ($\Delta\Delta$Ct). Data shown from RT-PCR are the means + standard deviation (SD bars) from two independent biological replications.

In all experiments, controls were performed using non-stimulated cells (medium). ConA was chosen as a negative or weak stimulus of the innate immunity. Non-stimulated THP-1 monocytes and macrophages and those-stimulated with ConA showed no or very low effects on gene expression of the inflammation-related cytokine genes (Fig 3A, C).
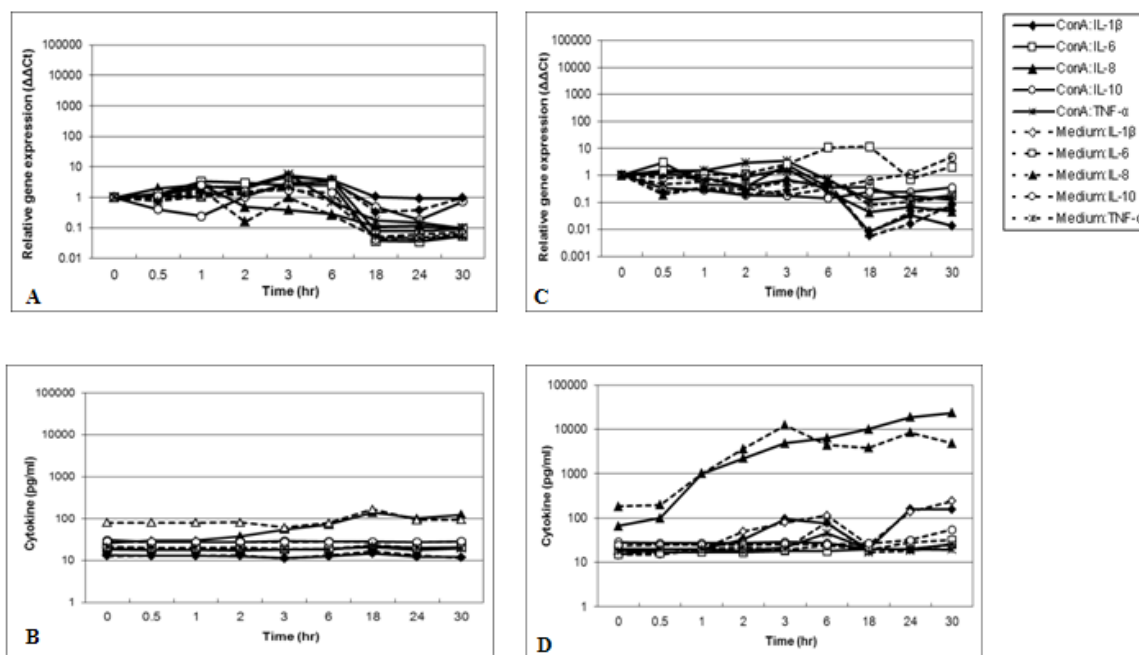
Figure 3 Inflammation-related cytokine gene expression kinetics and cytokine secretion kinetics of THP-1 monocytes (A-B) and THP-1 macrophages (C-D) stimulated with 5 μg/ml ConA and non-stimulated (medium). Gene expression was expressed as relative gene expression towards GAPDH-expression and non-stimulated cells at time zero (ΔΔCt). Data shown are the means from two technical measurements.

Expression of genes for inflammation-related enzymes

Apart from inflammation-related cytokine genes, two candidate inflammation-related enzyme genes, COX-2 and iNOS, were also investigated in THP-1 monocytes and macrophages stimulated with LPS and ConA. Incubation of THP-1 monocytes with LPS led to an induction of COX-2 gene expression within 0.5 h and reached the highest expression after 3 h of stimulation (Fig 4A). For THP-1 macrophages, the first up-regulation of the COX-2 gene was observed after 2 h and the highest expression was after 6 h of stimulation (Fig 4B). The overall COX-2 gene expression in LPS-stimulated THP-1 macrophages was approximately 10 times higher than that of monocytes. ConA only weakly induced COX-2 gene expression in THP-1 monocytes (data not shown). In contrast to the inflammation-related cytokine genes, the expression level of the COX-2 gene relative to GAPDH (ΔCt) in LPS-stimulated THP-1 monocytes and macrophages was similar (data not shown). The expression of the iNOS gene could not be detected in both LPS-and ConA-stimulated THP-1 monocytes and macrophages throughout the incubation time, although two different iNOS primer sets in Table 1 have been used (data not shown).

Expression of genes for inflammation-related transcription factors

LPS caused considerable up-regulation of NF-κB gene expression in both THP-1 monocytes and macrophages up to 30 h with the highest expression after 3 h and 6 h of stimulation, respectively (Fig 4). c-Jun and c-Fos gene expression in LPS-stimulated THP-1 macrophages was up-regulated for a rather short period 0.5-3 h after stimulation. A bi-phasic pattern was found in c-Fos gene expression of THP-1 macrophages treated with LPS (Fig 4B). SP-1 gene expression was not affected by LPS stimulation in both THP-1 monocytes and macrophages (Fig 4). No up-regulation from any of the studied transcription factor genes was observed in ConA-stimulated and non-stimulated THP-1 monocytes and macrophages (data not shown).
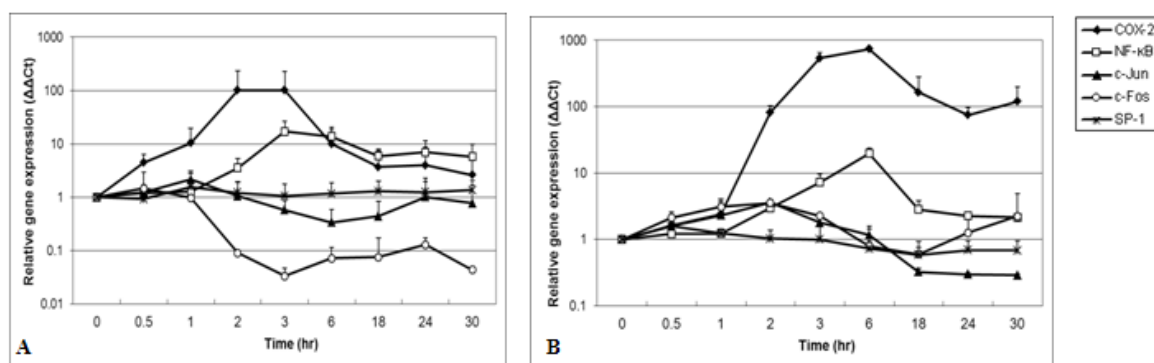

Figure 4 Inflammation-related enzyme and transcription factor gene expression kinetics of THP-1 monocytes (A) and THP-1 macrophages (B) stimulated with 1 μg/ml LPS. Gene expression was expressed as relative gene expression towards GAPDH-expression and non-stimulated cells at time zero (ΔΔCt). Data shown are the means + standard deviation (SD bars) from two independent biological replications.

Time-dependent cytokine secretion of THP-1 monocytes and macrophages stimulated with LPS and ConA

Stimulating THP-1 monocytes and macrophages with LPS resulted in a dramatic increase in the secretion of IL-1β, IL-6, IL-8, IL-10 and TNF-α (Fig 2B, 2D). At time zero of THP-1 monocytes and macrophages, all cytokines were present at a basal level of 20 to 30 pg/ml. Inflammatory cytokine produced by THP-1 macrophages was higher than those of monocytes, likely as a consequence of higher abundance of inflammation-related cytokine genes relative to GAPDH in non-stimulated THP-1 macrophages than in monocytes, as described above (Fig 1).

IL-8 was the most predominant cytokine in the supernatant of both THP-1 monocytes and macrophages (Fig 2B, D). IL-8 concentration in the supernatant increased overtime and only in THP-1 macrophages reached a plateau within the tested time frame after 18 h of stimulation. The relative order in abundance of cytokines secreted from THP-1 monocytes and macrophages was similar to the order of their responsive genes relative to GAPDH (Fig 1), except for IL-1β from THP-1 monocytes, of which the expression was relatively close to that of the IL-8 gene, but not at protein level. All analyzed cytokines, except for TNF-α from monocytes and IL-10 from macrophages, continued to accumulate over the incubation time (Fig 2B, D). Cytokines secreted from ConA-stimulated THP-1 monocytes and macrophages were detected in relatively low amounts (Fig 3B, D), except IL-8 from ConA-stimulated and non-stimulated THP-1 macrophages (Fig 3D).

These results suggest that the RNA expression and protein secretion are correlated to a large extent. The lag-phase in up-regulation of the mRNA level was approximately 1 h before cytokine proteins were secreted at a detectable concentration. Cytokine production demonstrated more variable kinetics than the expression of corresponding genes, which results in less uniform time points in quantification of effects.

Modulating effects of quercetin, citrus pectin and barley glucan on the expression of inflammation-related genes in LPS-stimulated THP-1 monocytes and macrophages

The effect of co-stimulation of LPS and purified food compounds was determined after stimulation at 3 h and 6 h of THP-1 monocytes and macrophages respectively, since the maximal gene expression response was observed at these time points (Fig 2A, C). Quercetin, citrus pectin and barley glucan, were chosen as food-derived compounds. Exposure of the cells to quercetin, citrus pectin and barley glucan or the solvent control (DMSO) and PBS did not show any altered expression of the measured genes (data not shown), indicating that these compounds do not possess direct inflammation-enhancing properties.

Data represented in Figure 5 show the effect of quercetin, citrus pectin and barley glucan on modulation of LPS-induced responses. Quercetin, citrus pectin and barley glucan lowered the LPS-induced expression of most inflammation-related genes expressed by THP-1 monocytes, except TNF-α and COX-2 (Fig 5A). Different effects were observed from THP-1 macrophages as compared to monocytes for specific food components. After 6 h of stimulation, quercetin reduced

expression of all inflammation-related genes of LPS-stimulated THP-1 macrophages, except COX-2 (Fig 5B). Less inflammatory reducing effects were found for citrus pectin compared to quercetin in THP-1 macrophages. Barley glucan appeared to enhance expression of inflammation-related genes of LPS-stimulated THP-1 macrophages (Fig 5B) whereas in monocytes had a reducing effect. Our results indicate that gene expression after simultaneous exposure of LPS with food components to THP-1 monocytes for 3 h and THP-1 macrophages for 6 h is an adequate model to examine inflammation-modulating activity of food compounds.
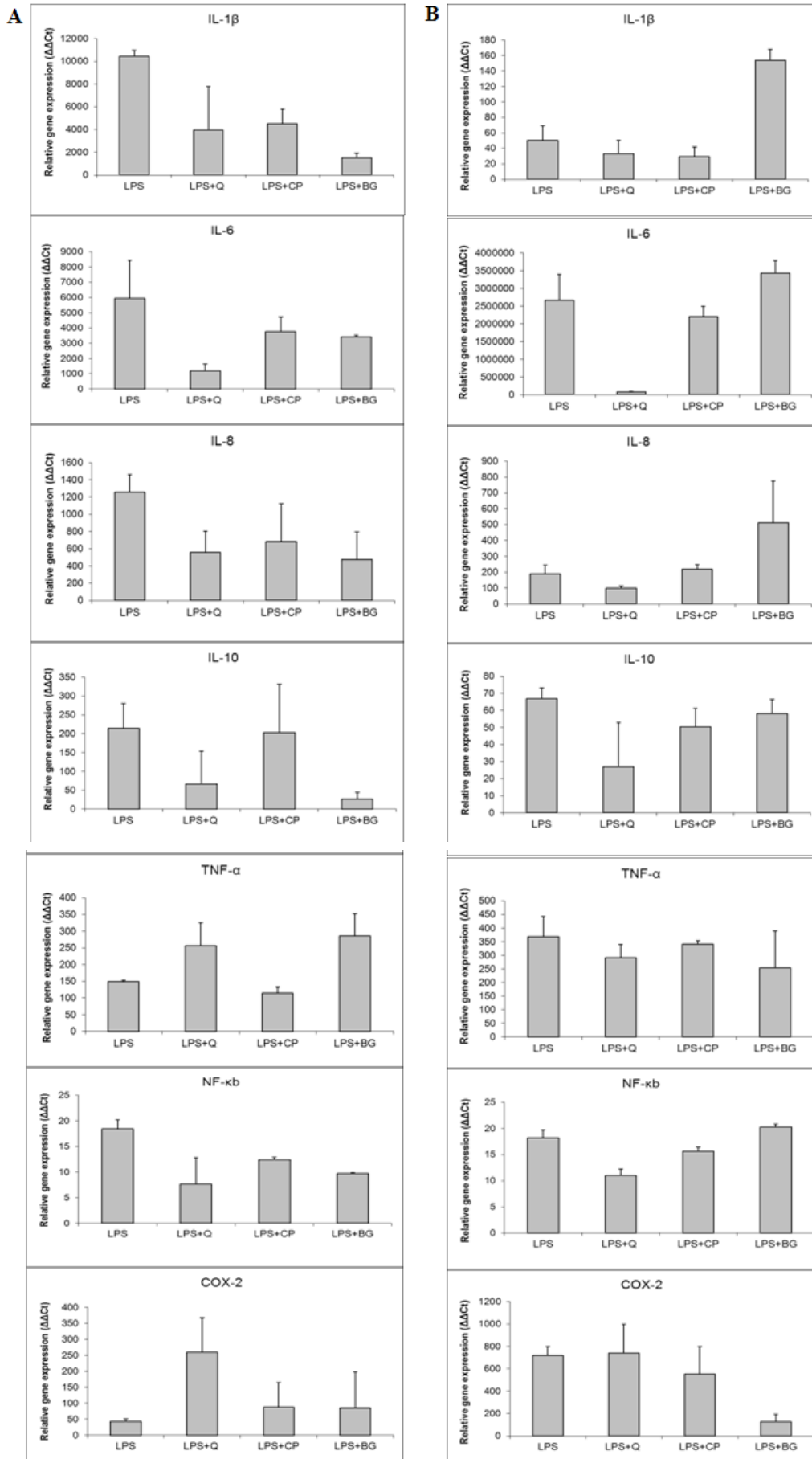
Figure 5 Inflammation-related cytokine gene expression of 700 ng/ml LPS-stimulated THP-1 monocytes (A) and macrophages (B) with 50 μM quercetin (LPS+Q), 0.75 mg/ml citrus pectin (LPS+CP) and 100 μg/ml barley glucan (LPS+BG). THP-1 monocytes were collected at 3 h and macrophages at 6 h after stimulation. Gene expression was expressed as relative gene expression towards GAPDH-expression and non-stimulated cells at time zero (ΔΔCt). Data shown are the means + standard deviation (SD bars) from independent biological replications.

Discussion

In this study, a new test method to investigate immuno-modulating effects of food-derived compounds was developed based on the THP-1 cell line. Two stimuli were chosen with different actions on the cellular responses. LPS is widely used as a potent and prototypical inducer of cytokine production in innate immunity which begins with the orchestration of monocytes [7, 15]. ConA, a lectin from jack-bean (*Canavalia ensiformis*), was reported to function as a T-lymphocyte mitogen in adaptive immunity [16], and to control some non-immune responses of THP-1 monocytes such as growth, proliferation, metabolism and survival processes [17].

Differences in RNA stability, protein translation kinetics, post-translational modification factors and proteolytic processing events, make the production of individual cytokines unique [18]. However, a general relation between mRNA and protein level in both LPS-stimulated THP-1 monocytes and macrophages was found in our studies. The higher mRNA expression level relative to GAPDH of inflammation-related cytokine genes and higher cytokine secretion level of LPS-stimulated THP-1 macrophages, as compared to monocytes, could be caused by higher expression of TLR4-mRNA (a PRR for LPS) [19].

LPS strongly up-regulated inflammation-related cytokine, COX-2 and NF-κB genes in THP-1 monocytes and macrophages, while no expression of the iNOS gene after LPS-stimulation was found. This last finding was in concordance with studies making use of PBMCs, indicating that human peripheral monocytes and their derived macrophages are not able to express the iNOS gene after LPS induction [20-23]. However, some PBMC-based studies indicated the ability of LPS to up-regulate the iNOS gene [24, 25]. No unequivocal explanation for this controversy has been presented yet. Based on literature, it seems that species differences, genetic background, and perhaps details of experimental procedure play a role [7, 20-25]. The relatively low and short expression of c-Jun and c-Fos in LPS-stimulated THP-1 monocytes and macrophages can be explained by the fact that these genes are typical early response genes with a very short mRNA

half-life of only 35-45 minutes [26, 27]. A bi-phasic pattern of c-Fos gene expression in LPS-stimulated THP-1 macrophages was also reported [26, 27]. The binding activity of nuclear proteins to SP-1 target genes was constitutive and unchanged by LPS stimulation in THP-1 monocytes and murine macrophages [11, 28] but it can be up-regulated during the PMA or vitamin D3 induced differentiation process of THP-1 monocytes to regulate the expression of CD14 [29, 30]. Therefore, it could be argued that SP-1 might not be an appropriate gene to serve as an indicator in LPS exposure of THP-1 monocytes and macrophages.

Accumulation of TNF-α in supernatants of both THP-1 monocytes and macrophages declined after 6 and 24 h of stimulation, respectively. The decline of TNF-α-accumulation in our studies was consistent with the findings in several studies which demonstrated that IL-10 can suppress TNF-α production in human monocytes and macrophages, or even cause diminished levels of TNF-α, IL-1β and IL-8 mRNA upon prolonged stimulation [31-33]. However, to drive such mechanisms, IL-10 needs to bind to IL-10R-1 and IL-10R-2 which should cause a decrease in the measurable (unbound) amount of IL-10 in culture supernatants [34, 35]. Similar to our results, IL-10 cytokine accumulation by LPS-stimulated THP-1 monocytes slightly dropped at 6 h and increased again at 18 h after stimulation, while it dramatically decreased in THP-1 macrophages at 18-30 h after stimulation.

The beneficial health effects of quercetin and citrus pectin have been attributed to their anti-inflammatory activity [36-39], while β-glucans shows their immunological effect by enhancing innate immunity through induction of cytokine production and phagocytosis [40]. Incubating LPS-stimulated THP-1 monocytes for 3 h and macrophages for 6 h with the food compounds revealed different inflammation-modulating effects at mRNA level with similarity as described in the mentioned literature.

Studying effects of PAMPs and other (food based) immuno-modulating compounds using monocytes and macrophages isolated from PBMCs might be a more realistic model for human immune functioning. However, large variation between blood samples, time and cost efficiency make it difficult to apply this in a high throughput fashion. It has been indicated that THP-1 cell line has shown to be accurate model for native and monocytes-derived macrophages for studying LPS responses [3, 4, 41, 42]. Our findings suggested that LPS-stimulated THP-1 monocytes and macrophages are a sensitive *in vitro* system to analyze potential immunomodulatory activity of food components by using a detailed insight into the kinetics of mRNA expression. Therefore, THP-1

monocytes and macrophages could thus be a suitable and reliable model for screening a variety of components prior to a more detailed analysis with human derived cells.

Conclusion

A rather short incubation time is required for gene expression analysis, this approach facilitates the use of less sterile samples and allows a more reliable read-out for the early triggering events in which responses by various effector molecules such as cytokines and post-translational events have not yet occurred. This makes it possible to differentiate between primary food-cell signals and secondary cell-cell signal. RT-PCR is a method to analyze gene expression, which is very straight forward, can be performed in almost every molecular lab, is cost-effective compared to microarray analysis, and can be very accurate and informative when using a key selection of functional indicator genes. Analysing cytokine secretion profiling has some major drawbacks compared to gene expression analysis as longer incubation is needed to be detectable resulting in more uncertainties due to occurrence of forward and backward responses from various secreted cytokines. Also, every run needs many extra samples for calibration curves. These remarks together with the findings described in this paper show that gene expression measurement can give reproducible results and even on a wider spectrum of responses than cytokine measurements.

Acknowledgement

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Chapter 3

β-Glucans are involved in immune-modulation of THP-1 macrophages

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Abstract

Scope: We aimed to examine different immunological aspects of β-glucans derived from different food sources (oat, barley and shiitake) on PMA-differentiated THP-1 macrophages. Commercially purified barley β-glucan (Commercial BG) and lentinan were included to compare β-glucans from the same origin but different degree of purity and processing.

Methods and results: Chemical composition and molecular weight distribution of β-glucan samples were determined. Inflammation-related gene expression kinetics (IL-1β, IL-8, NF-κB and IL-10) after 3, 6 and 24 h of stimulation with 100 µg/ml β-glucan were investigated. All tested β-glucans mildly up-regulated the observed inflammation-related genes with differential gene expression patterns. Similar gene expression kinetics, but different fold induction values, was found for the crude β-glucan extracts and their corresponding commercial forms. Pre-incubation of THP-1 macrophages with β-glucans prior to LPS exposure decreased the induction of inflammation-related genes compared to LPS treatment. No production of nitric oxide (NO) and hydrogen peroxide (H_2O_2) was detected in β-glucan stimulated THP-1 macrophages. Phagocytic activity was not different after stimulation by β-glucan samples.

Conclusion: Based on these *in vitro* analyses, it can be concluded that the analysed β-glucans have varying levels of immunomodulating properties, which are likely related to structure, molecular weight and compositional characteristic of β-glucan.

Introduction

β-(1→3)-(1→4)- and β-(1→3)-(1→6)-Glucans are found in cell walls of cereals, plants, fungi and bacteria [1]. Many β-glucans are considered as non-digestible carbohydrates and classified as Pathogen-Associated Molecular Patterns (PAMPs). These PAMPs can be recognized by Pattern Recognition Receptors (PRRs). Immunomodulatory effects of β-glucans on human and animals are considered to be mediated via PRRs like dectin-1, TLR-2, TLR-4, TLR-6, CR3, lactosylceramide and scavenger receptors which are located on innate immune cells like macrophages, dendritic cells and neutrophils [2-6]. The stimulation of these receptors results in up-regulation of cytokine gene expression and subsequent stimulation of the humoral and cell mediated immunity [4, 7].

β-Glucans found in cereals are composed of a β-(1→3) backbone, branched via β-(1→4) linkages, whereas those of yeast and mushroom are characterised by a similar backbone structure with β-(1→6) linked side chains [8]. Because vertebrates do not express β-glucanase, orally administered β-glucan is not digested in the gastro-intestinal tract. It thus reaches the small intestine intact where it comes in contact with enterocytes [9, 10]. It has been reported that β-glucan can be taken up and presented to immune cells in Payer's patches by either microfold cells (M cells) or dendrites of dendritic cells (DCs) [11, 12]. Heterogeneity of β-glucans, for instance, solubility, size, structure and degree of branching impacts on their bioavailability and on the interactions of β-glucans with immune cells [13].

Immunomodulating properties of β-glucans in innate and adaptive immunity have been widely investigated over the past years. β-Glucan induces NF-κB gene expression via binding to dectin-1 in association with TLR-2 and -4 in a MyD88 –dependent signaling cascade [10]. It has been indicated in *in vivo* and *in vitro* studies that stimulation of β-glucan increased phagocytosis activity, nitric oxide production and inflammatory responses [10, 14, 15]. Production of inflammatory cytokines and mediators enhances the ability to counteract viral and bacterial or fungal challenge in innate immunity [10]. Furthermore, anti-inflammatory activities [16], ability in activating naïve T lymphocytes [17] and even anticancer properties [18] of β-glucans have been also concluded from *in vitro* studies.

We examined different immunological aspects of β-glucans derived from different food sources (oat, barley and shiitake). Besides the different origins, we also used β-glucans from the

same source but prepared and purified differently to compare their immunomodulating properties after food processing. PMA-differentiated THP-1 macrophages were used as a model since this cell type shares many characteristics with *in vivo* human macrophages [19-21]. The application of these assays will help to set up strategies to characterize, compare and predict immunomodulating properties of β-glucans from diverse sources and prepared with wide range of food processing methods prior to *in vivo* interventions.

Materials and methods

Samples and chemicals

De-hulled barley (*Hordeum vulgare*), de-hulled oat (*Avena sativa*) and fresh shiitake mushroom (*Lentinula edodes*) were purchased from a local supermarket, Wageningen, The Netherlands. Purified lentinan was kindly provided from Ajinomoto Co., Inc, Japan. Lipopolysaccharide (LPS) (*E.coli* 0111:B4), commercial barley β-glucan (BG), α-amylase, β-glucanase, phenol red sodium salt and peroxidase from horse radish were purchased from Sigma (St. Louis, MO, USA).

Extraction of β-glucan

The β-glucan extraction condition was slightly modified after Burkus and Temelli (1998) and Ahmad *et al.* (2009). Fresh shiitake mushrooms were washed with water prior to freeze drying. Ground freeze-dried shiitake were mixed with hot water in the ratio 1:60 (w/v), while for ground barley and oat this was 1:10 (w/v). The suspensions were incubated at 80 °C in a shaking water bath for 8 h, then cooled down to 60 °C before adding 30U/ml α-amylase and incubated for 1 h in a shaking water bath. After centrifugation at 10,000 g for 15 min, to clear supernatants, two volumes of 96% ethanol were added and this mixture was kept at 4 °C overnight. The mixtures were subsequently centrifuged at 10,000 g for 15 min and the glucan pellets were freeze-dried.

Chemical analysis

In all starting materials and β-glucan extracts (except the lentinan solution), the β-(1,3)(1,4)-glucan and β-(1,3)(1,6)-glucan content were determined. The enzymatic kits used in the β-glucan assay were K-BGLU 04/06 and K-YBGL 09/2009 (Megazyme International Ireland Ltd, Wicklow, Ireland) for β-(1,3)(1,4)-glucan and β-(1,3)(1,6)-glucan determination, respectively. The β-glucan contents were reported on moisture-free basis.

Freeze-dried β-glucan extracts and commercial BG were ground and dissolved in PBS pH 7 to reach the working concentration of 4 mg/ml β-glucan (dry basis) followed by sterilization by autoclaving. The sterilised pure lentinan was provided by Ajinomoto in solution of 4 mg/ml β-glucan. Protein analysis (Bradford reagent), total phenolic analysis (Folin-Ciocalteau reagent) and the LPS contamination (L00350, GenScript, New Jersey, USA) were determined in autoclaved samples.

Size-Exclusion Chromatography

The molecular weight (MW) distribution of β-glucan samples and β-glucanase treated β-glucan samples were accessed using high pressure size-exclusion chromatography (HP-SEC) coupled with multi-angle laser light scattering (MALLS) and refractive index (RI) detectors. The Viscotek GPC system was used in the HP-SEC measurement. The measurement was performed using two ViscoGEL columns (300 x 7.8 mm), type GMHHR-M + Guard column running at 70 $^{\circ}$C. The mobile phase was N,N-dimethylacetamide with 0.5% LiCl at a rate of 0.5 ml/min. Samples were prepared at 3 mg β-glucan powder in 1 ml DMAc+0.5% LiCl and passed through 0.45 µm filter before injection (100 µl). The OmniSEC^{TM} (version 4.0) was used to calculate MW distribution. Shodex pullulan standard from 0.59×10^4 to 160×10^4 Da was used to prepare a calibration curve.

THP-1 cell culture and differentiation

The human monocytic leukemia cell line THP-1 (American Type Culture Collection, Rockville, Md.) was grown in RPMI 1640 culture medium (Lonza, Switzerland) supplemented with

foetal bovine serum (FBS; Invitrogen, UK.) and penicillin/streptomycin (P/S) (Invitrogen) to respectively 10% and 1 %, at 37 ^{o}C in 5 % CO_2 in a humidified incubator. Cells were sub-cultured twice a week. THP-1 cells were used between passage 12 and 25. The macrophage-like state was obtained by treating THP-1 monocytes for 48 hr with 100 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma) in 12-wells cell culture plates (Greiner, Germany) with 1 ml cell suspension (10^6 cells) in each well. Differentiated, plastic-adherent cells were washed twice with culture medium (RPMI 1640 medium without PMA but containing 10% FBS and 1% P/S) and rested for another 24 h in the culture medium. The culture medium was removed and replaced by the culture medium containing 100 µg/ml β-glucans from either barley, oat, shiitake, commercial BG or lentinan. The control used in all measurements was THP-1 macrophages exposed with the culture medium containing PBS in similar amount as other treatments.

Cytotoxicity assay

Cytotoxicity was determined by the MTT assay. The MTT assay determines the viability of cells by the reduction of yellow soluble MTT in the metabolically active cells. Briefly, THP-1 monocytes were induced for differentiation into macrophages in 96-wells cell culture plate ($8.2x10^5$ cells/well). THP-1 macrophages were exposed to 100 µg/ml β-glucan from either oat, barley, shiitake, commercial BG or lentinan and incubated for 3, 6 and 24 h at 37 ^{o}C in 5 % CO_2 in a humidified incubator. Co-stimulation by 700 ng/ml LPS and 100 µg/ml β-glucan for 6 h on THP-1 macrophages was also determined to assess potential cytotoxicity. Ten microliters of fresh medium containing 5.5 mg/ml MTT were added into each well and incubated for 2 h at 37 ^{o}C. After this incubation period, 10 µl DMSO:ethanol (1:1) was added into each well after which the plate was mildly shaken on a shaker for 5 min. The absorbance was measured at 570 nm using an ELISA plate reader. The results were expressed relative to the control (non-stimulated cells).

Release of reactive oxygen

Nitric Oxide

Nitric oxide production from non-, LPS- and β-glucan-stimulated THP-1 macrophages was measured by Griess reagent using 96-wells culture plate. THP-1 macrophages were incubated with LPS by ten-fold serial dilution from 10 µg/ml – 0.1 pg/ml or β-glucan by two-fold serial dilution from 200 µg/ml – 1.56 µg/ml in 100 µl volume and incubated for 3 and 6 h in an incubator at 37 ^{o}C in 5 % CO_2. One hundred microliter of the Griess reagent was added into each well and incubated at room temperature for 15 min, after which the absorbance at 540 nm was measured using an ELISA plate reader.

Hydrogen peroxide production

Hydrogen peroxide production from non-, LPS- and β-glucan-stimulated THP-1 macrophages was measured based on the horseradish peroxidise mediated oxidation of phenol red. THP-1 macrophages were incubated with LPS by ten-fold serial dilution from 10 µg/ml – 0.1 pg/ml or β-glucan by two-fold serial dilution from 200 µg/ml – 1.56 µg/ml in 100 µl volume which were diluted in phenol red solution. After incubation at 3 and 6 h in an incubator at 37 ^{o}C in 5 % CO_2, 10 µl of 1M NaOH was added and subsequently the absorbance at 600 nm measured using an ELISA plate reader.

Phagocytic activity

Phagocytic assay was basically performed according to Shiratsuchi and Basson (2004) with some small modifications. In summary, THP-1 macrophages were incubated with 100 µg/ml β-glucan for 6 h before exposure to serum-opsonized fluorescence-labelled beads. These fluorescence-labelled latex beads (Fluoresbrite® YG carboxylate microspheres 2.0 µm, Polysciences, Germany) were opsonized with 10% unheated FBS at 37 ^{o}C for 60 min prior the experiment. Opsonized beads were suspended in culture medium containing 10% FBS and 1% P/S in a working concentration. THP-1 macrophages (4.1 x 10^4 cells) in each well of 96-wells plate

were added with opsonized beads at the ratio of 1:5 (cells:beads) in the final volume of 164 µl and incubated for 2 h in an incubator.

After 2 h, macrophage monolayers were washed extensively with ice-cold PBS containing 1% paraformaldehyde. The uptake of fluorescent latex beads was counted under a fluorescence microscope. One hundred cells were counted in each well and three wells for each treatment. Three biological replications were performed. Phagocytic activity was calculated as [22, 23]:

- % Phagocytosis = number of cells with at least one intracellular bead x 100%
                              total number of cells counted

- Phagocytic index = 100 x [{number of cells with 1 bead + (3.5 x number of cells with 2-5 beads) + (8 x number of cells with 6-10 beads) + (20 x number of cells with over 10 beads)} / total number of cells counted]

Inflammation-related gene expression kinetics of β-glucan stimulated THP-1 macrophages

The mature THP-1 macrophages in 12 wells-cell culture plates were stimulated with 1 ml culture medium containing 100 µg/ml β-glucan from either oat, barley, shiitake, commercial BG or lentinan. Cells were harvested at different time points ranging from 0, 3, 6 and 24 h. RNA isolation, cDNA synthesis and RT-qPCR were performed according to Chanput *et al*. (2010). The RT-qPCR analysis was performed twice on each sample. The experiments were performed by two independent biological replications, started from a new batch of cells.

Inflammation-related gene expression of THP-1 macrophages pre-incubated with β-glucan prior to LPS exposure

Mature THP-1 macrophages in 12 wells-cell culture plates were pre-incubated with 1 ml culture medium containing 100 µg/ml β-glucan from either oat, barley, shiitake, commercial BG or lentinan for 3 h prior to 700 ng/ml LPS exposure for 6 h. Expression of inflammatory genes was determined using RT-qPCR. The experiments were performed by two independent biological replications, started from a new batch of cells. The incubation time and concentration were chosen according to our previous kinetic studies [24].

β-Glucanase treatment

Briefly, the β-glucanase digestion was performed at 50 ^{o}C in a heat block for 1.5 hr with every 15 min vortex for 10 sec. After that, the samples were heated for 90 min at 100 ^{o}C in a heat block with every 15 min vortex for 10 sec to inactivate the enzyme. Non-treated samples were also exposed to the same incubation conditions but omitting the β-glucanase enzyme. Both β-glucanase-treated and non-treated samples were analysed with HP-SEC analysis to check the breakdown of β-glucan and via exposing THP-1 cells (100 μg/ml β-glucan for 6 h) to determine changes in expression of inflammation-related genes using RT-qPCR.

Statistical analysis

Comparison between treatments was calculated using one-way ANOVA with Duncan post hoc comparison test. A value of $p<0.05$ was considered to be significant.

Results

Composition and yield of β-glucan extracts

The composition of β-glucan extracts was determined on moisture free basis as presented in Table 1. The β-glucan contents in the starting materials (barley and oat seeds and freeze-dried fresh shiitake) are also shown in parentheses in Table 1. Of the total mass of the barley β-glucan extract, 37.2% was found to be β-(1→3)(1→4) glucan while this value was 3.2% for barley seeds. The β-glucan extract obtained from oat contained 20.5% of β-(1→3)(1→4) glucan. As expected, β-(1→3)(1→4) glucan was not found in the shiitake β-glucan extract while no β-(1→3)(1→6) glucan was detected in the barley and oat β-glucan extract. Out of the three β-glucan extracts, shiitake contained the highest β-glucan content (43.1%). The percentage β-glucan yield from barley, oat and shiitake was 32.4, 28.0 and 3.9% (w/w, based on dry weight), respectively.

The starch content of the β-glucan extracts varied among sources. The highest amount of starch was observed in the oat β-glucan extract while lowest was found in shiitake. The protein content was very low in all β-glucan extracts (< 2%) and the total phenol content was below detection limit. Based on the approximation that 1 endotoxin unit (EU) is equivalent to 0.2 ng of

LPS [25], we calculated that the LPS contamination in all β-glucan extracts was less than 1 pg of LPS/100 μg of β-glucan (data not shown) which is 7×10^5 times less than the LPS concentration used in the LPS treatment (700 ng) and for which no cell responses were found when they were exposed to concentration of 1 pg/ml LPS (data not shown).

Table 1 Composition of β-glucan extracts

Percentage of composition as dry basis	β-Glucan extracts			Commercial BG
	Barley	Oat	Shiitake	
% β-Glucan				
• β-(1→3)(1→4)-glucan	37.2 (3.2)	20.5 (3.4)	Ud	90.4
• β-(1→3)(1→6)-glucan	Ud	Ud	43.1 (28.3)	Ud
% Starch	17.9	49.8	4.5	0.2
% Protein	1.6	0.8	2.1	0.5
% Total phenol	Ud	Ud	Ud	Ud
% Non-determined	43.3	28.9	50.3	8.9
% β-Glucan yield	32.4	28.0	3.9	-

Ud : Undetectable

Numbers in parentheses are percentages of β-glucan in starting materials

Size exclusion chromatography

The integrated peak area from β-glucanase non-digested and digested β-glucan extracts, as identified by HP-SEC are displayed in Table 2. As a representative example of typical HP-SEC profiles, those of non-digested and digested barley β-glucan extract are shown in Fig 1. As deduced from the main peak 1 (Table 2), similar MW for non-digested β-glucan from barley and oat were observed. However, this differed from non-digested commercial BG, which appeared to have a smaller MW and for which no peak 2 was detected (Table 2). The β-glucan extracted from shiitake showed higher MW than the purified lentinan. After digestion with β-glucanase, large shifts in MW was observed for the β-glucans from barley, oat and commercial BG, while β-glucan extracted from shiitake was not affected. Digestion of commercial lentinan was not performed for HP-SEC because of the limited amount we had available.

Table 2 Molecular weight distribution of non- and digested β-glucans

| Sample	Peak 1		Peak 2	
	Retention volume (ml)	MW (Dalton)	Retention volume (ml)	MW (Dalton)
Barley				
Non-digested	11.152	1.59×10^6	17.338	3,904
Digested	Ud	Ud	16.952	294
Oat				
Non-digested	10.728	1.55×10^6	14.668	8,299
Digested	Ud	Ud	16.872	749
Commerical BG				
Non-digested	11.698	4.87×10^5	Ud	Ud
Digested	16.742	46	Ud	Ud
Shiitake				
Non-digested	11.782	1.53×10^6	17.187	3,671
Digested	11.245	9.66×10^5	17.227	2,355
Lentinan				
Non-digested	12.412	6.87×10^5	Ud	Ud
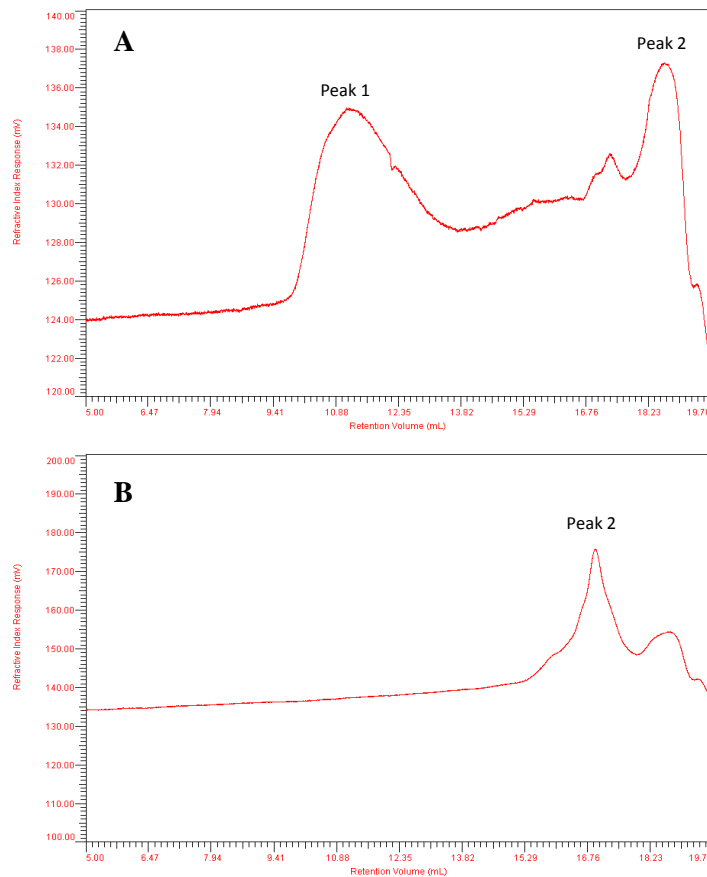
Figure 1 Molecular weight distribution of β-glucan from HP-SEC analysis; non-digested barley β-glucan extract (A) and β-glucanase-digested barley β-glucan extract (B).

Cytotoxicity

The 100 µg/ml β-glucan containing samples were investigated for potential cytotoxicity on THP-1 macrophages after three incubation periods: 3, 6 and 24 h. Cell viability was expressed relative to the non-stimulated cells which was set at 100%. Overall relative cell viability of only β-glucan stimulation was over 94% (data not shown). Co-stimulation of LPS and β-glucans for 6 h slightly affected cell viability (90-95%) (data not shown). At a β-glucan concentration of 200 µg/ml, cell viability decreased to *ca*. 85% (data not shown). From this we concluded that hot water extracted β-glucan, up to 100 µg/ml, was not toxic to THP-1 macrophages but simultaneous stimulation of β-glucan and LPS over a long incubation period slightly decreased cell viability.

Nitric oxide and hydrogen peroxide production

Two assays were performed in order to investigate the production of reactive oxygen species produced after exposure to either β-glucan, LPS or both simultaneously. Neither nitric oxide nor hydrogen peroxide could be detected in all tested compounds in the concentration range of β-glucan 1.56 μg/ml –200 μg/ml and LPS 0.1 pg/ml –10 μg /ml (data not shown).

Phagocytic activity

The effect of β-glucan on % phagocytosis and phagocytic index was examined in THP-1 macrophages using 2 μm fluorescence-labelled beads. Most β-glucan samples showed similar % phagocytosis and phagocytic index compared to the non-stimulated cells, except for lentinan, which significantly increased the phagocytic index (p<0.05) (Fig 2).
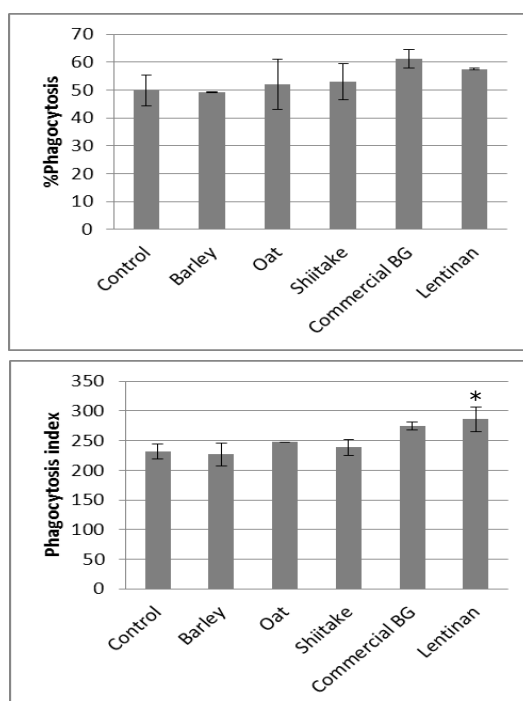

Figure 2 Phagocytic activity of 6 h pre-incubated THP-1 macrophages with 100 μg/ml β-glucan prior to 2 h incubation with serum-opsonized fluorescence-labeled latex beads (5 beads/cell). Cells were washed extensively with ice-cold PBS and fixed. Data were expressed as % phagocytosis and phagocytic index calculated as given in "Material and Methods" section. Data shown are the means from three independent biological replications. *P<0.05 compared with non-stimulated cells.

Inflammation-related gene expression kinetics of β-glucan stimulated THP-1 macrophages

Mature THP-1 macrophages, derived from PMA-differentiated THP-1 monocytes, were challenged with 100 µg/ml β-glucans from either barley, oat, shiitake, commercial BG or lentinan. In these exposed cells, expression levels of the inflammation-related genes IL-1β, IL-8, NF-κB and IL-10 were analysed relative to the non-stimulated cells. Exposure of THP-1 macrophages to all tested β-glucans slightly increased the expression of the analysed genes with a maximal expression of IL-1β, IL-8 and NF-κB genes at either 3 or 6 h after stimulation (Fig 3). The IL-10 gene expression was highest up-regulated with a unique late up-regulation profile after 24 h of incubation in all β-glucan treatments. The inflammation-related gene expression patterns of THP-1 macrophages to β-glucans derived from the same origin were found to be similar. For instance, commercial BG (a purified form of barley β-glucan) with a purity of 90%, showed relatively similar inflammatory gene expression profiles compared to those of barley β-glucan extract, with only a difference in the relative fold induction values. Also the gene expression patterns observed for exposure to shiitake β-glucan extract and its purified form, lentinan, were comparable. β-Glucan samples obtained from different origin showed different gene expression profile, although equal β-glucan concentrations were applied.

To compare pro- and anti-inflammatory properties of these five β-glucans, the expression of IL-1β, IL-8 and NF-κB genes at 3 h and IL-10 gene at 24 h after stimulation were statistically analysed (data not shown). Among the tested β-glucans, β-glucan from oat showed the highest up-regulation of IL-1β, NF-κB and IL-8 expression although there was no significantly difference between the five glucans tested (after 3 h) ($p \geq 0.05$) while IL-10 expression of lentinan was significantly higher than other tested β-glucan ($p < 0.05$).
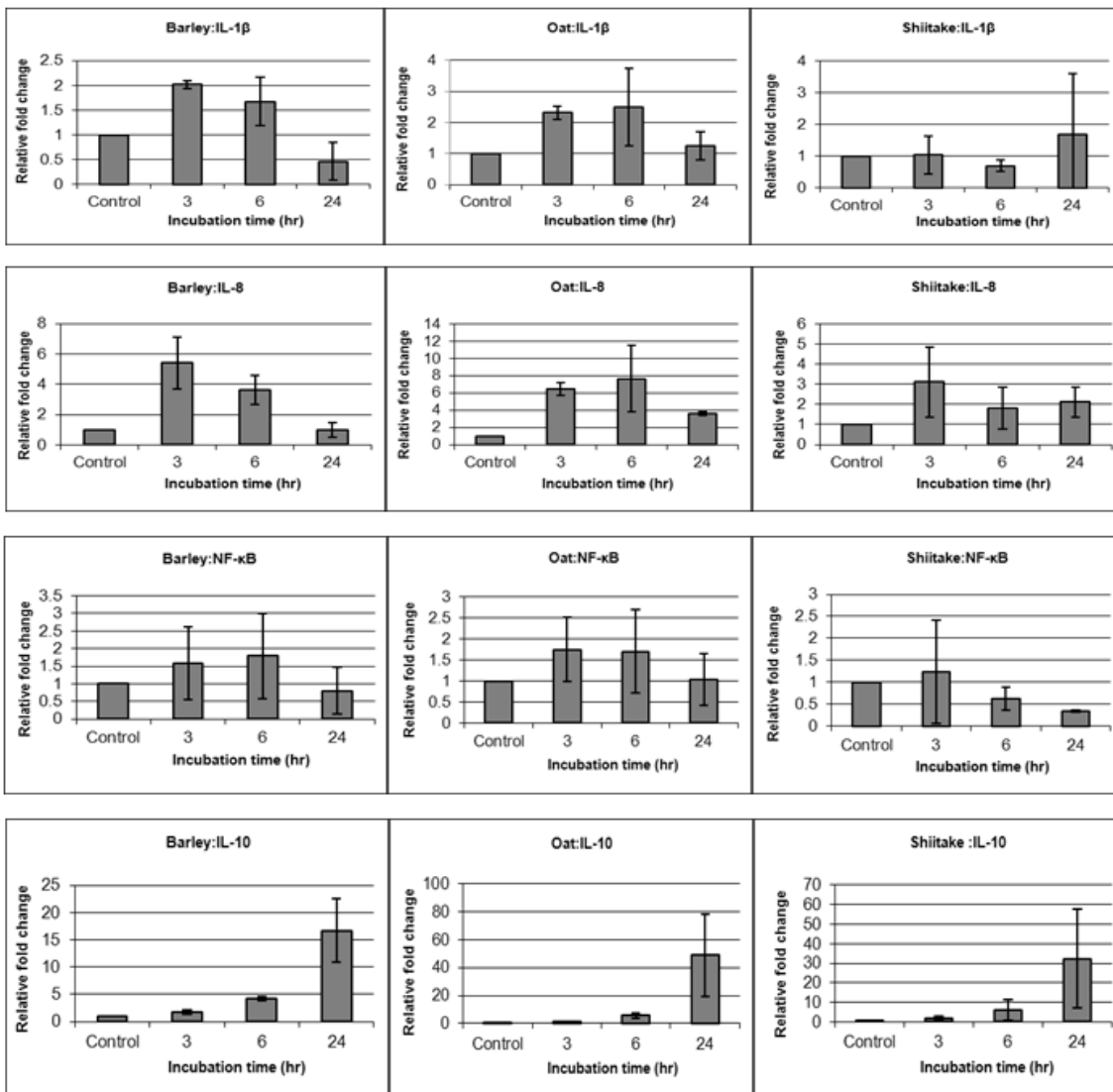
Figure 3 Differential inflammation-related gene expression kinetics of 100 μg/ml β-glucan stimulated THP-1 macrophages. Gene expression was expressed relative to GAPDH-expression and the control (non-stimulated cells) (ΔΔCt). Data shown from RT-qPCR are the means ± standard deviation (SD bars) from two biological and two technical replications.
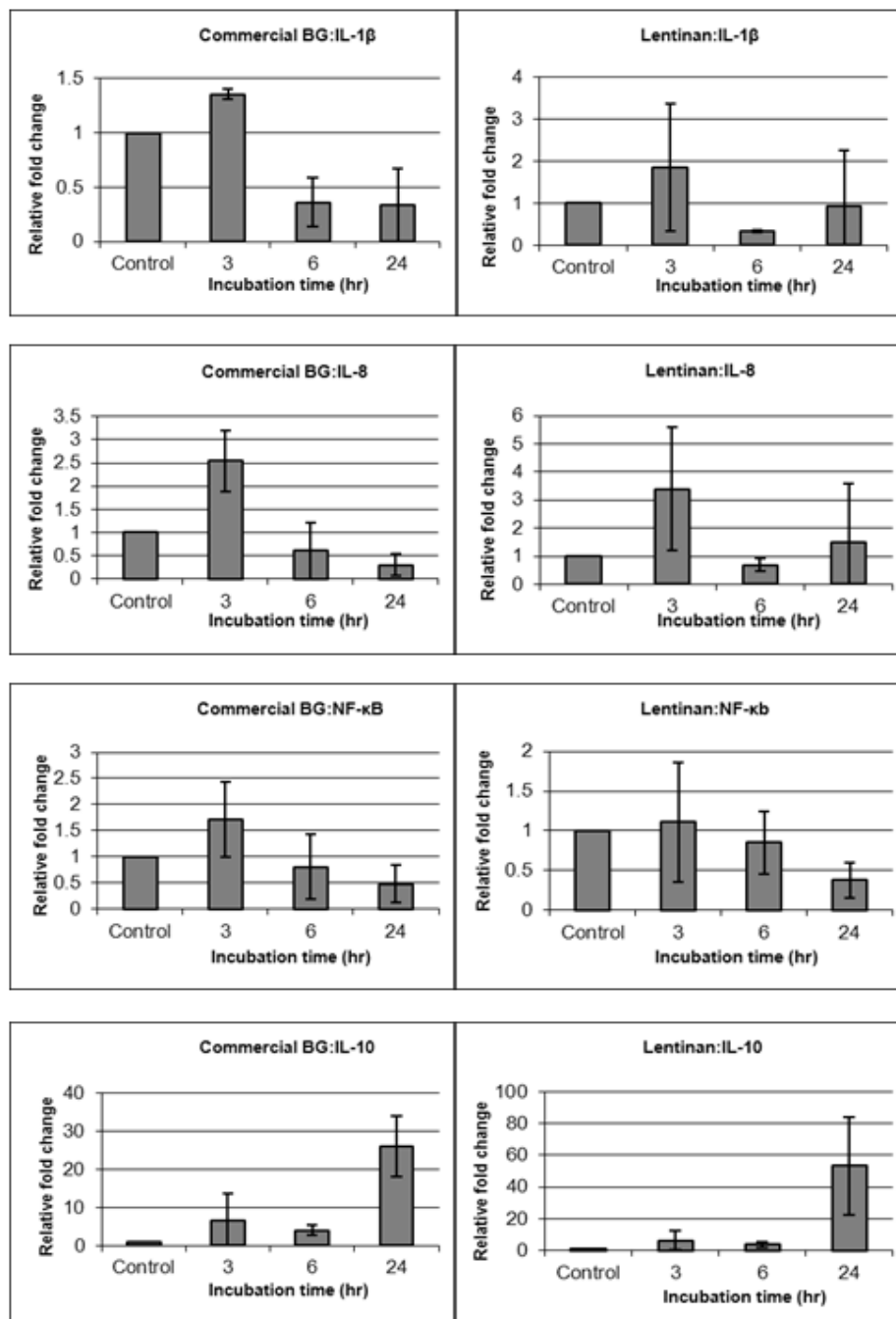
Figure 3 Continued

Inflammation-related gene expression of THP-1 macrophages pre-incubated with β-glucan prior to LPS exposure

THP-1 macrophages were pre-incubated with β-glucan for 3 h prior to 6 h exposure to LPS. The pre-incubation time point was selected from our preliminary study (data not shown). Relative fold induction of inflammation-related genes from LPS stimulation was set as a reference for inflammatory status; thus, the expression of genes above and below this LPS reference were designated as inductive and suppressive inflammatory effects, respectively.

In general, pre-incubation showed suppressive effects on LPS exposure as shown in Fig 4. Among β-glucan samples, barley β-glucan and commercial BG significantly suppressed LPS-induced IL-1β and IL-10 gene expression compared with solely LPS stimulation (p<0.05). Pre-incubation with oat and shiitake β-glucan significantly decreased only IL-1β expression (p<0.05).

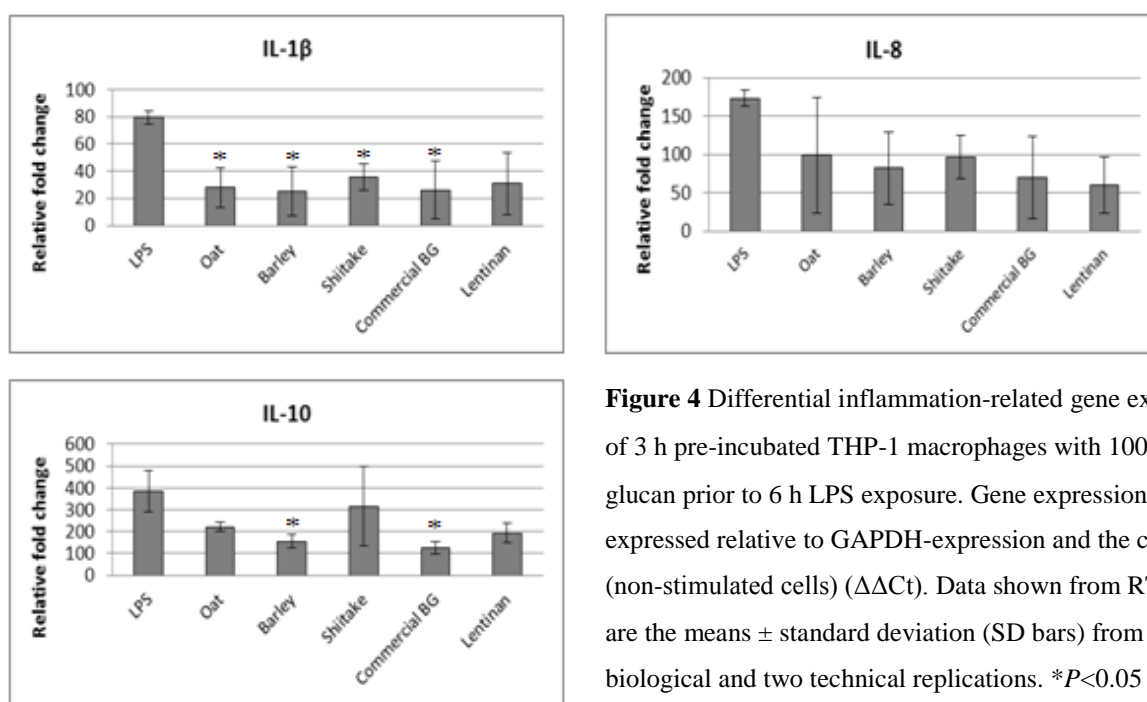**Figure 4** Differential inflammation-related gene expression of 3 h pre-incubated THP-1 macrophages with 100 µg/ml β-glucan prior to 6 h LPS exposure. Gene expression was expressed relative to GAPDH-expression and the control (non-stimulated cells) (ΔΔCt). Data shown from RT-qPCR are the means ± standard deviation (SD bars) from two biological and two technical replications. *$P<0.05$ compared with LPS treatment.

β-glucanase treatment

Water with and without β-glucanase added were used as controls for digested and non-digested treatments, respectively. Gene expression was expressed as relative value towards GAPDH-expression and corresponding controls (ΔΔCt). The expression of IL-1β, IL-8 and IL-10 genes after 6 h of stimulation of THP-1 macrophages with digested or non- digested β-glucan, were investigated. Figure 5 demonstrates that a β-glucanase treatment of the samples dramatically reduced expression of the observed inflammation-related genes ($p<0.05$). This corroborates the HP-SEC analysis that confirmed the breakdown of the β-glucan molecules by the treatment.

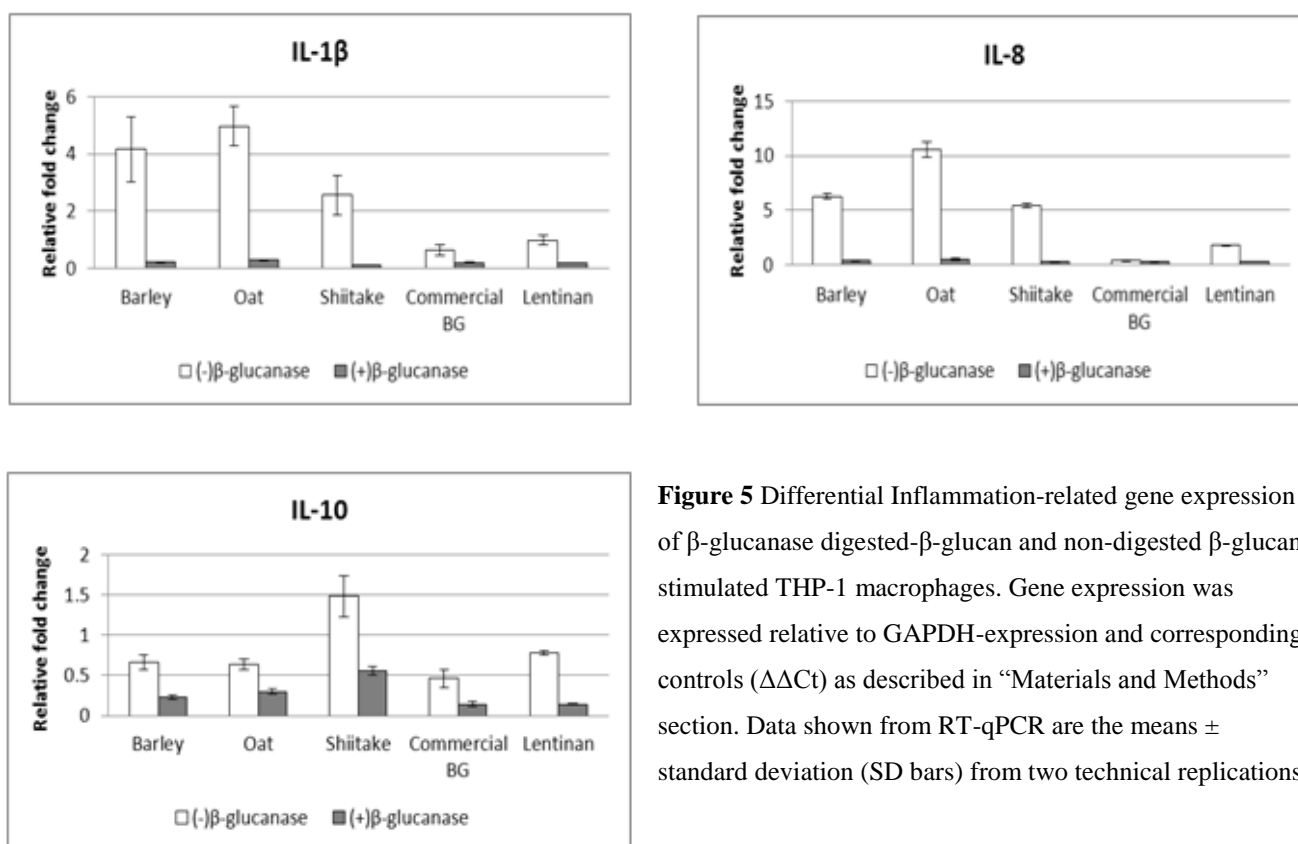**Figure 5** Differential Inflammation-related gene expression of β-glucanase digested-β-glucan and non-digested β-glucan stimulated THP-1 macrophages. Gene expression was expressed relative to GAPDH-expression and corresponding controls (ΔΔCt) as described in "Materials and Methods" section. Data shown from RT-qPCR are the means ± standard deviation (SD bars) from two technical replications.

Discussion

It is widely known that structures of β-glucans from various origins differ, which consequently has direct effects on their bioactive properties [3, 6, 8]. In our study, the immunomodulating properties of hot water extracted β-glucans from different sources (oat, barley and shiitake) were investigated. Cytotoxicity of β-glucan extracts on THP-1 cells, production of reactive oxygen species, phagocytic activity, inflammation-related gene expression kinetics and effects of pre-incubation with β-glucan on the alteration of endotoxin-induced cytokines responses were analysed. Moreover, commercially purified β-glucans from barley and shiitake mushroom were also included in our study in order to analyse impact of degree of purity and method of preparation on the immune responses.

A hot water extraction, as the mildest β-glucan extraction process, was used in the study to avoid effects of remaining salts and chemical traces in the extract which might influence cell responses. It has been reported that hot water extraction results in β-glucan yields of approximately 5-6% [26, 27]. Other extraction procedures, such as use of alkaline conditions, resulted in a higher protein content in β-glucan extract [27]. As a consequence of the mild extraction conditions used, without application of post-extraction techniques, other soluble dietary fibres than β-glucan (arabinoxylan, arabinogalactan, mannogalactan, pectin and other oligosaccharides) and also insoluble dietary fibres (cellulose, lignin, hemicellulose and waxes) can be present in the hot water extracted β-glucan preparations [27, 28]. Thus, we estimated that approximately 50% of the total composition in our β-glucan extracts, which was designated as non-determined, may be accounted for by the above mentioned compound types.

Neither H_2O_2 nor NO production was detected in THP-1 macrophages in response to either β-glucans or LPS. The latter observation is in concordance with the previous study indicating that iNOS gene was not expressed in THP-1 macrophages after LPS induction [24]. It has been shown that THP-1 macrophages produced H_2O_2 after stimulation by particulate β-glucan from *Saccharomyces cerevisiae* cell wall, while soluble β-glucan from edible mushroom such as *Grifola frondosa* and *Lentinus edodes* did not trigger H_2O_2 production [25]. Since in our β-glucan solutions, after autoclaving, no insoluble residues were visible. It can be assumed that these contained mostly soluble β-glucan which has less H_2O_2 inducing ability. Apparently, solubility, i.e. particulate or soluble, and structure of β-glucan are involved in regulating H_2O_2 production.

In our study, all β-glucan samples showed the ability to modulate innate immune functions determined by altered expression of inflammation-associated genes, which corroborates other literature reports [8, 10, 29]. Inflammation-related gene expression patterns and induction values were found to be different for β-glucans from different sources which may be attributed to structure, degree and type of branching and chain length of β-glucan [7, 30, 31]. Similar gene expression patterns but different fold induction values were found for β-glucans derived from the same origin but obtained after different extraction procedure, for instance, barley β-glucan and commercial BG, and for shiitake β-glucan and lentinan. A similar finding was also observed in an *in vivo* study which found that IFN-γ, TNF-α, IL-1α and IL-2 cytokine levels were higher in blood from mice fed with pure lentinan as compared to those fed with crude shiitake extract, while the secretion kinetics remained the same [32]. Slightly different gene expression patterns were found between barley and oat β-glucan extracts, although they possess, basically, a similar β-glucan structure. It can be concluded that different structure, due to different sources, of β-glucan has more impact on gene expression kinetics than different degree of purity or a different purification technique. However, impurities such as starch and soluble/insoluble dietary fibre which are possibly present in these extracts, may affect cell responses. [33, 34].

The anti-inflammatory cytokine gene, IL-10, was highest expressed after 24 h of β-glucan stimulation. The expression was even higher than observed for exposure of the same macrophage cell line at the same time point to 700 ng/ml LPS [24]. Due to its autocrine growth activity, the expression of the IL-10 gene might be an indication of the skewing ability of β-glucan to differentiate naïve T cells (Th_0) towards regulatory T cells (T_{reg}), a T cell subpopulation that suppresses and regulates activity of the immune system [35]. Other T helper cells (Th) skewing cytokine genes, for instance, IL-12 (reflecting Th_1) and IL-4 (reflecting Th_2) were analysed in the same experiment but no expression was found (data not shown). IL-6 (associated with Th_{17}) gene expression was declined to the base line after 6 h (data not shown). Therefore, we hypothesize that the expression of IL-10 gene caused by β-glucan might skew Th_0 towards T_{reg}. Our qPCR analysis also demonstrated that dectin-1 mRNA transcripts were predominantly present in β-glucan stimulated THP-1 macrophages (data not shown) leading to the assumption that the recognition of β-glucans by THP-1 macrophages involves dectin-1 as has been described before [36].

β-Glucanase (EC 3.2.1.6) catalyses the hydrolysis of endo β-(1→3)- or β-(1→4)-glycosidic linkages in cereal β-glucans. HP-SEC was performed after β-glucanase treatment to verify the

digestion of β-glucans by the enzyme. Most β-glucans in the extracts from barley, oat and commercial BG disappeared after the digestion. The β-glucan in the shiitake extract was not digestible by β-glucanase due to the presence of β-(1→6) glycosidic side chain linkages [8]. Expression of inflammation-related genes by THP-1 macrophages stimulated with digested β-glucan were analysed to ascertain that β-glucans were indeed involved in the immune-modulating properties of the β-glucan extracts. Large reductions of gene expression were observed in THP-1 macrophages exposed to the digested β-glucans, although other putative immuno-stimulants such as protein and total phenol have been found to be present in a very low amount. This observation implies that β-glucans are very likely responsible for the stimulatory effects of the extracts on THP-1 macrophages.

Our THP-1 macrophage *in vitro* studies suggest that all tested β-glucans mildly induced pro-inflammatory genes (IL-1β, IL-8 and NF-κB) in the short period of time, approximately 3 h after stimulation. Similar findings were observed in *ex vivo* and *in vivo* studies, which reported that the expression of inflammatory cytokine secretion peaked between 4-5 h after β-glucan challenge [32, 37]. This is in concordance to general observations that cytokine secretion is delayed approximately an hour after expression of their corresponding genes [24]. As indicated in many *in vivo* studies, oral intake of crude β-glucan extracts derived from yeast and mushroom, as well as purified lentinan and curdlan, enhanced the protection against acute bacterial shock and injury in mice [37-41]. This may be explained by the ability of β-glucan to be present in plasma after 24 h in mice that were orally fed a β-glucan [13] or after 14 days of mice that were daily fed a yeast derived β-glucan [40]. We thus analysed the effect of pre-incubating THP-1 macrophages with β-glucan *in vitro* before endotoxin challenge. As found in our study, pre-incubation showed suppressive effects of β-glucan on endotoxin-induced response.

It should be realized that *in vitro* test systems have their limitations with respect to predictive power for *in vivo* situations. However, fast screening of various β-glucan-containing preparations and differences in structure and source for their biological effects is not always feasible, neither financially nor ethically, in *in vivo* experiments. Yet such analysis is desirable for the development of specific applications, considering e.g. variability in food products that result from raw material differences, processing and matrix effects. In particular for immune functioning studies have been performed acceptable correlation between *in vitro* and *in vivo* readings [42-46].

Moreover, the resistance of β-glucans to digestion may increase the relevance of *in vitro* read-out systems to estimate their bioactivity.

In our experiments, we have shown that β-glucans differing in structure, molecular weight, origin and purification degree have a varying effect on THP-1 macrophages responses. Therefore, it might be interesting to further develop THP-1 macrophages based assays, combined with computational and statistical analysis, as a testing tool to obtain insight in the structure-bioactivity relationships of β-glucan. This will help to develop a strategy that can predict possible *in vivo* effects after oral consumption of β-glucan containing extracts or products.

Acknowledgement

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We are shaped by our thoughts, we become what we think.

When the mind is pure, joy follows like a shadow that never leaves.

Buddha

Chapter 4

Characterization of polarized THP-1 macrophages and polarizing ability of LPS and food compounds

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Abstract

Little is known about the polarizing potential of currently used human macrophage cell lines, while a better understanding phenomena can support the prediction of effects *in vivo* based on *in vitro* analysis. To test the polarization capability of PMA differentiated-THP-1 macrophages (M0), cells were stimulated with 20 ng/ml IFNγ+1μg/ml LPS and 20 ng/ml IL-4, which are known to influence macrophage polarization *in vivo* and *ex vivo* into the M1 and M2 state, respectively. Apart from several well-known M1 and M2 markers, also new possible markers for M1 and M2 polarization were analysed in this study. The expression of M1 marker genes was up-regulated in IFNγ+LPS stimulated-M0 THP-1 macrophages. The IL-4 stimulated-M0 THP-1 macrophages expressed M2 cell membrane receptor genes. However, M2 chemokine and their receptor genes were only slightly up-regulated which might be due to complexity of the secondary cell-cell interaction of the chemokine system. Lipopolysaccharide from *E.coli* (LPS) and food compounds (lentinan, vitamin D3 (vD3) and the combination of lentinan+vitaminD3 (Len+vD3) were investigated for their polarizing ability on M0 THP-1 macrophages towards either the M1 or M2 state. LPS (700 ng/ml) was able to skew M0 THP-1 macrophages towards the M1 direction since all analysed M1 marker genes were strongly expressed. Lentinan, vD3 and Len+vD3 did not induce expression of either M1 or M2 markers indicating no polarizing ability of these compounds. Based on the expression of M1 and M2 marker genes we concluded that THP-1 macrophages could be successfully polarized into either the M1or M2 state. Therefore, they can be used as a new macrophage polarizing model to estimate the polarizing/switching ability of test food compounds.

Introduction

Human macrophages *in vivo* can be classified into two macrophage activation states, based on their reactions to different stimuli [1]. M1 macrophages, or classically activated macrophages, are involved in the defence against bacterial and viral infection and in tumour regression [2]. The M1 phenotype is initiated from resting macrophages, responding to pathogen associated molecular patterns (PAMPs) or to Th1 cytokines such as IFNγ and TNF-α [1, 3]. The M1 phenotype is characterised by a high production of pro-inflammatory cytokines, such as TNFα, IL-1β, IL-6, IL-8 and IL-12, and by expression of pattern recognition receptors (PRRs), such as Toll like receptors (TLRs) and NOD-like receptors (NLRs) [4, 5]. M2 macrophages, or alternatively activated macrophages, play a role in parasite infection, tissue modelling, immunoregulation, allergy and tumour progression [6, 7]. M2 macrophages are derived from resting macrophages by exposure to Th2 cytokines, such as IL-4 or IL-13 [4]. Three subsets of M2 macrophages have been described: M2a induced by IL-4 or IL-13, M2b induced by exposure to IC/TLRs agonist or IL-1R and M2c induced by IL-10 [8]. These subclasses can be identified on the basis of chemokine production [8-10]. The M2 type is characterised by expression of arginase-1, Ym-1, Fizz-1, mannose receptor, scavenger receptors and chemokine genes such as CCL1, CCL17, CCL22, CCL16, CCL18 and CCL24 [8]. Recently, a new macrophage phenotype, M4, has been described showing a unique transcriptional profile upon CXCL4 induction, characterised by a reduction of CD163 and other scavenger receptor expression, as well as phagocytic capacity [11, 12].

Macrophages can, therefore, be described as unique cells which are very heterogeneous, very versatile and highly plastic, in which plasticity is controlled by small changes in micro-environmental signals. Lopez-Castejón *et al.*[6] described several plasticity levels in mouse macrophages, which were shown to be highly reversible and dynamic by being able to switch from one activation state to the other. It has been hypothesized that imbalances in M1/M2 are causally related to atherosclerosis [13], cancer [14, 15] and insulin resistance [16]. Low-grade chronic inflammation in obesity is also associated with an increased number of M1-type adipose tissue macrophages [17]. Furthermore, relative strong differentiation to a M2 phenotype is related to the development of allergic symptoms [18, 19]. There are quite a few reports on mouse *in vivo* and *ex vivo* and human *ex vivo* studies on the characterisation of polarized macrophages [6, 17, 20]. A number of compounds, such as C-reactive protein [21] , adiponectin [20] and methionine encephalin [22], have been investigated in *in vivo* and *ex vivo* mouse models for their macrophage

polarizing ability. It has been suggested that food-derived compounds which exert anti-inflammatory effects might contribute to prevent skewing of M1 polarization which may be involved in the development of chronic disease [23]. However, no experimental data have been provided yet. Spencer *et al.*[24] and Caras *et al.*[25] recently reported the polarizing ability of a human monocytic cell line, THP-1, into the M1 and M2 phenotypes. Different stimulating conditions were observed in these two studies according to different research aims, for instance, Spencer *et al.*[24] intended to understand adipose tissue inflammation and the development of fibrosis, while Caras *et al.*[25] aimed to study the effect of tumour-secreted soluble factors on the macrophage phenotypes.

In this study, we analysed the polarizing ability of THP-1 macrophages exposed to stimuli, which are known to influence polarization *in vivo* and *ex vivo*. We expected that our study will contribute to better understand this balance *in vitro* and to aid in predicting effects *in vivo*. The expression of M1 and M2 marker genes, like cytokines, cell membrane receptors and chemokines and their receptors have been analysed in polarized THP-1 macrophages. Furthermore, the effects of LPS and food compounds have been investigated on their polarizing ability towards resting THP-1 macrophages (M0).

Materials and methods

Samples and chemicals

Interferon gamma (IFNγ), interleukine-4 (IL-4) and interleukine-10 (IL-10) were purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). Purified lentinan was kindly provided from Ajinomoto Co., Inc, Japan. Lipopolysaccharide (LPS) (*E.coli* 0111:B4) and 1α, 25-dihydroxyvitamin D3 (≥ 99% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

THP-1 cell culture and macrophage differentiation

The culture conditions for the human monocytic leukemia cell line, THP-1 (American Type Culture Collection, Rockville, Md.), were as described earlier [26]. The macrophage-like state was obtained by treating THP-1 monocytes for 48 h with 100 ng/ml phorbol 12-myristate 13-acetate

(PMA; Sigma) in 24-wells cell culture plates (Greiner, Germany) with 0.5 ml cell suspension ($5x10^5$ cells) in each well. Differentiated, adherent cells were washed twice with culture medium (RPMI 1640 medium without PMA but containing 10% FBS and 1% penicillin/streptomycin (P/S)) and rested for another 24 h in the culture medium to obtain the resting state of macrophages (M0).

THP-1 macrophage polarization and effects of LPS and food compounds

The resting macrophages (M0) were primed with fresh medium supplemented with 20 ng/ml IFNγ +1 µg/ml LPS to differentiate into the M1 phenotype and with 20 ng/ml IL-4 to the M2 phenotype [6]. The incubation time was 6 and 24 h in both stimulating conditions. These polarization conditions were, in addition to being based on literature, also derived from preliminary results (data not shown).

When analysing polarizing effects of LPS and food compounds, M0 THP-1 macrophages were stimulated with either 700 ng/ml LPS, 100 µg/ml lentinan, 100 nM vitamin D3 (vD3), or the combination of 100 µg/ml lentinan with 100 nM vitamin D3 (Len+vD3) and incubated for 6 or 24 h. The concentrations for these food compounds were optimized based on literature and preliminary experiments (data not shown). The controls used in all experiments were M0 THP-1 macrophages exposed to the culture medium (RPMI 1640 supplemented with 10% FBS and 1% P/S) at either 6 ($M0_{6h}$) or 24 h ($M0_{24h}$).

Gene expression of M1 and M2 markers in THP-1 macrophages by Real-Time qPCR

The polarized M1 and M2 cells, LPS-stimulated M0 and food compound stimulated-M0 cells were harvested for RNA isolation, cDNA synthesis and RT-qPCR as previously published [27]. Primer sets (see Table 1) were quantified as described before [27]. M1 and M2 marker genes that represent inflammation-related cytokines and cell membrane receptors were selected from literature and analysed for their expression. Relative fold change was calculated using $2^{-\Delta\Delta Ct}$ method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and M0 cells exposed with culture medium at each responsive time point, 6 or 24 h of incubation, were used for normalization. The RT-qPCR analysis was analysed twice on each sample. Two biological replicates were performed.

Table 1 Sequence of Real Time-PCR primers

Gene	Accession number	Primer working concentration/ μM	Sequence (5'-3')
IL-12p40	NM_002187	0.1	F-CTCTGGCAAAACCCTGACC
			R-GCTTAGAACCTCGCCTCCTT
MRC-1	NM_002438	0.1	F-CAGCGCTTGTGATCTTCATT
			R-TACCCCTGCTCCTGGTTTTT
DC-SIGN	AY042222	0.1	F-TCAAGCAGTATTGGAACAGAGGA
			R-CAGGAGGCTGCGGACTTTTT
Dectin-1	AF00599	0.4	F-AACCACAGCTACCCAAGAAAAC
			R-GGGCACACTACACAGTTGGTC
LOX-1	NM_02543	0.1	F-GCGACTCTAGGGGTCCTTTG
			R-GTGAGTTAGGTTTGCTTGCTCT
MARCO	NM_006770	0.1	F-CAGCGGGTAGACAACTTCACT
			R-TTGCTCCATCTCGTCCCATAG
Arginase-1	NM_000045	0.4	F-GTGGAAACTTGCATGGACAAC
			R-CCTGGCACATCGGGAATCTTT
Yim-1	NM_009892	0.4	F-CATGAGCAAGACTTGCGTGAC
			R-GGTCCAAACTTCCATCCTCCA
Fizz-1	NM_032579	0.4	F-AGTGGTCCAGTCCACCACAC
			R-AGTGTCAAAAGCCAAGGCAG

Chemokines and their receptors gene expression in THP-1 macrophages by RT^2 Profiler^{TM} PCR array

The human chemokines and receptors PCR array (PAHS-022) was purchased from SABiosciences (Qiagen, USA). RNA from the polarized M1 or M2 cells, LPS- or food compound-stimulated M0 was isolated using the RNeasy mini kit (Qiagen) and treated with an RNase-free DNase (Qiagen) following the recommended RNA preparation of manufacturer's protocol. The purity and quality of the isolated RNA were determined using the nanodrop and agarose gel electrophoresis under UV detection as described in the RNA quality control of manufacturer's protocol. One microgram of isolated RNA was used to make complementary DNA (cDNA) using RT^2 First strand cDNA kit (Qiagen) according to the manufacture's protocol. The synthesized cDNA was mixed with RT^2 qPCR master mix (Qiagen) and loaded in a volume of 25 μl to each well of the 96-well custom PCR array. The two-step cycling program of Bio-Rad CFX96 was

adjusted according to the manufacturer's protocol with pre-heating at 95 ^{o}C for 10 mins, followed by 40 PCR cycles with a denaturing temperature of 95 ^{o}C for 15 s, an annealing temperature of 60 ^{o}C for 1 min and an elongation temperature of 72 ^{o}C for 2 min, followed by 95 ^{o}C 30 s. The ramp rate was adjusted to 1 ^{o}C/s. Five housekeeping genes (B2M, HPRT1, RPL13A, GAPDH and ACTB) were aligned in the PCR array plate. After checking no significant changes observed among five different housekeeping genes upon treatments, the average of their Ct values was used for normalization as recommended in manufacture's protocol. Three PCR controls (genomic DNA control, reverse transcriptase control and positive PCR control) were included in the PCR array plate. All data obtained from PCR-based array were carefully checked to meet the data criteria according to manufacturer's protocol. The analysis was analysed once on each sample. Two biological replicates were performed.

Cytotoxicity assay

Differentiated THP-1 macrophages in 96-wells cell culture plates (8.2×10^5 cells/well) were exposed to either 20 ng/ml IFNγ +1 µg/ml LPS or 20 ng/ml IL-4 and incubated at 37 ^{o}C and 5 % CO_2 in a humidified incubator. Cytotoxicity was determined after 6 and 24 h of incubation by the MTT assay as described before [26]. The M0 THP-1 macrophages, stimulated with either LPS or with food compounds as described previously were also analysed for their potential cytotoxicity after 6 and 24 h of incubation.

PCA analysis

To perform PCA analysis, Perseus-Post MaxQuant analysis software version 1.1.1.34, Germany, was used.

Statistical analysis

Comparison between treatments was calculated with one-way ANOVA with Multiple Comparisons and Duncan post-hoc test using IBM SPSS 19 (PASW statistics, IBM, Amsterdam, The Netherlands). A value of $p < 0.05$ was considered to be significant.

Results

Characterization of polarized M1 and M2 THP-1 macrophages

Expression of M1-M2 marker genes

Human and mouse PBMC macrophages appear to be polarized by IFNγ+LPS into the M1 state and by IL-4 into the M2 state [6, 28]. Based on the literature, we tested whether M0 THP-1 macrophages could also be polarized with 20 ng/ml IFNγ +1 µg/ml LPS or 20 ng/ml IL-4 to obtain the M1 or M2 state, respectively. Polarization was analysed using gene expression of well-known M1 marker genes (TNFα, IL-1β, IL-12p40, IL-6, IL-8) and M2 marker genes (MRC-1, dectin-1, arginase-1, Fizz-1 and Ym-1) as suggested in several studies [6, 8, 17]. It has been mentioned that M2 macrophages express scavenger receptors [8, 29, 30], of which MARCO, CD36 and LOX-1 belong to scavenger receptor class A (SR-A), class B (SR-B) and class E (SR-E), respectively [31]. Therefore, these three genes were also included in the analysis as new possible M2 marker genes. DC-SIGN is a C-type lectin receptor which tends to be expressed in dendritic cells and macrophages in anti-inflammatory situation (M2 state) [32, 33]. Thus, this gene was analysed as another possible M2 marker gene.

The IFNγ+LPS primed-THP-1 macrophages strongly expressed M1 marker genes TNFα, IL-1β, IL-12p40, IL-6 and IL-8 after 6 h, which dramatically declined after 24 h of incubation ($p < 0.05$) (Fig 1A). The same set of genes could not be detected in IL-4 polarized- cells at both time points. The inverse gene expression was found for the M2 markers, MRC-1 and dectin-1 (Fig 1B). Activation with IFNγ+LPS (M1 activation) did not induce these M2 marker genes, apart from a low induction of dectin-1 at 6 h of incubation (Fig 1B). After activation with IL-4 (M2 activation), the MRC-1 gene was significantly higher expressed at 24 h than at 6 h ($p < 0.05$), while dectin-1 expression behaved oppositely ($p < 0.05$). The newly hypothesized M2 marker LOX-1, was

unexpectedly expressed in IFNγ+LPS exposed macrophages (Fig 1C). Expression of this gene was also found in IL-4 treated cells, however, the expression was much lower than for IFNγ+LPS induced M1 activation (p<0.05). MARCO and CD36 genes showed no expression after both IFNγ+LPS and IL-4 stimulation (data not shown). Another possible M2 marker gene, DC-SIGN, was induced in IL-4 stimulated macrophages (Fig 1D). The expression of the earlier described M2 marker genes in mouse *in vivo*; arginase-1, Fizz-1 and Ym-1 (although the primer sets were designed based on human genome, except Ym-1), could not be detected after both IFNγ+LPS and IL-4 activation (data not shown). It can be concluded that LOX-1 and DC-SIGN can be hypothesized as a new M1 an M2 marker gene, respectively.
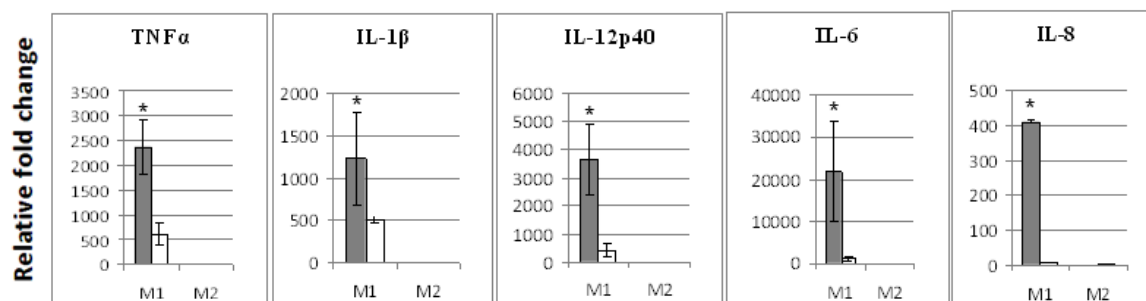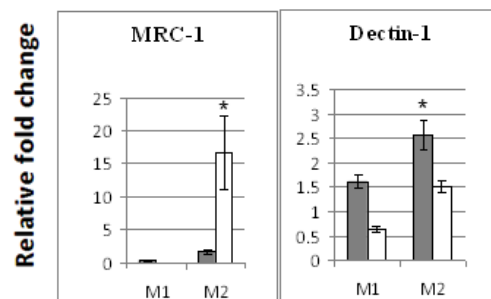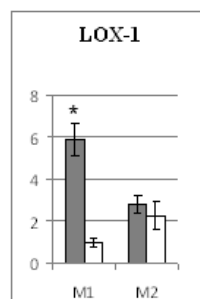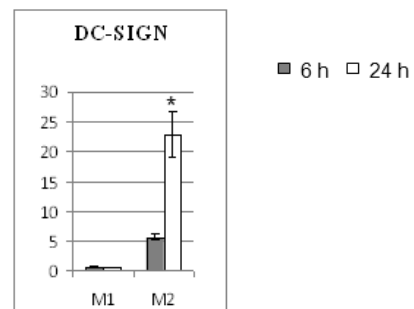

Figure 1   ■ 6 h   □ 24 h

Based on these analyses we can summarize that M0 THP-1 macrophages can indeed be polarized similar to mouse and human PBMC macrophages. IFNγ+LPS activates them into a M1-

like state and IL-4 into a M2-like state. Therefore, we will use the term "polarized M1 THP-1 macrophages" and "polarized M2 THP-1 macrophages" in the rest of the article.

Expression of human chemokine and their receptor genes

Apart from M1 and M2 cytokine and cell membrane receptor genes, the expression of a full range human chemokine and their receptor genes were determined in polarized M1 THP-1 macrophages after 6 h of incubation with IFNγ+LPS and in polarized M2 THP-1 macrophages after 24 h of incubation with IL-4 using RT^2 Profiler^{TM} PCR array. These two incubation times were selected according to the highest expression of M1-M2 marker genes as previously described. Chemokines and their receptors gene expression was expressed as fold changes relative to the average of five aligned housekeeping genes as described above and to M0 cells incubated with standard medium for the same time period of 6 or 24 h ($2^{\wedge -\Delta\Delta Ct}$). Polarization of M0 cells to the M1 state activated a variety of chemokine and their receptor genes. Out of the analysed 84 genes, 53 genes were up-regulated by more than 1.5 fold (supplementary Table 1). Figure 2 shows the fold change ratio (M1 : M2) of chemokine and their receptor gene expression, in which a ratio of more than 1 implies higher gene expression in polarized M1 than in polarized M2 THP-1 macrophages and oppositely for a ratio below 1.

The PCR array contained 16 cytokine and receptor genes known to be markers of M1 (symbolically marked as *) and 5 cytokine and receptor genes of M2 (symbolically marked as **§**) as described by Mantovani *et al.* [8]. The IFNγ+LPS treated macrophages abundantly expressed M1 chemokine and receptor genes as shown on the left side of Figure 2 (ratio > 1). This set of M1 genes was not found in the primed IL-4 THP-1 macrophages. The M2 cytokine and receptor genes were less clear expressed in the polarized IL-4 THP-1 macrophages (right side of Figure 2), and instead, most of them were slightly expressed in the polarized IFNγ+LPS THP-1 macrophages. The only exceptional gene was CCR2. However, the relative fold change of CCR2 gene expression in polarized M2 THP-1 macrophages was only 0.51 while in M1 was 0.08 (supplementary Table 1), indicating that this gene was down-regulated during M1 polarization. As shown in supplementary Table 1, the highest expressed chemokine gene in the polarized M2 THP-1 macrophages was CCL13 with *ca.*1610 fold induction, whereas in polarized M1 THP-1 macrophages was *ca.* 11 fold induction. This result leads to the assumption that CCL13 might be a new M2 marker gene, which

should be further evaluated in *ex vivo* cultured macrophages. The next two highest gene expressions in polarized M2 THP-1 macrophages were CCL8 and CCL7 with *ca*. fold change of 52 and 11, respectively. However, these two genes were also highly expressed in polarized M1 THP-1 macrophages (98102 and 91.3, respectively). Most of chemokine and receptor genes which exhibited an expression ratio below 1 (expressed in M2 more than in M1 cells) were indeed either slightly expressed or down-regulated in both M1 and M2 cells but more down regulated in M1 cells. Chemokine genes (CXCL1, 2, 3, CCL8, CCL19 and CSF3) were highly expressed in polarized M1 THP-1 macrophages. Therefore, these genes are possibly new M1 marker genes. However, as mentioned above, they should be further tested in *ex vivo* cultured macrophages.
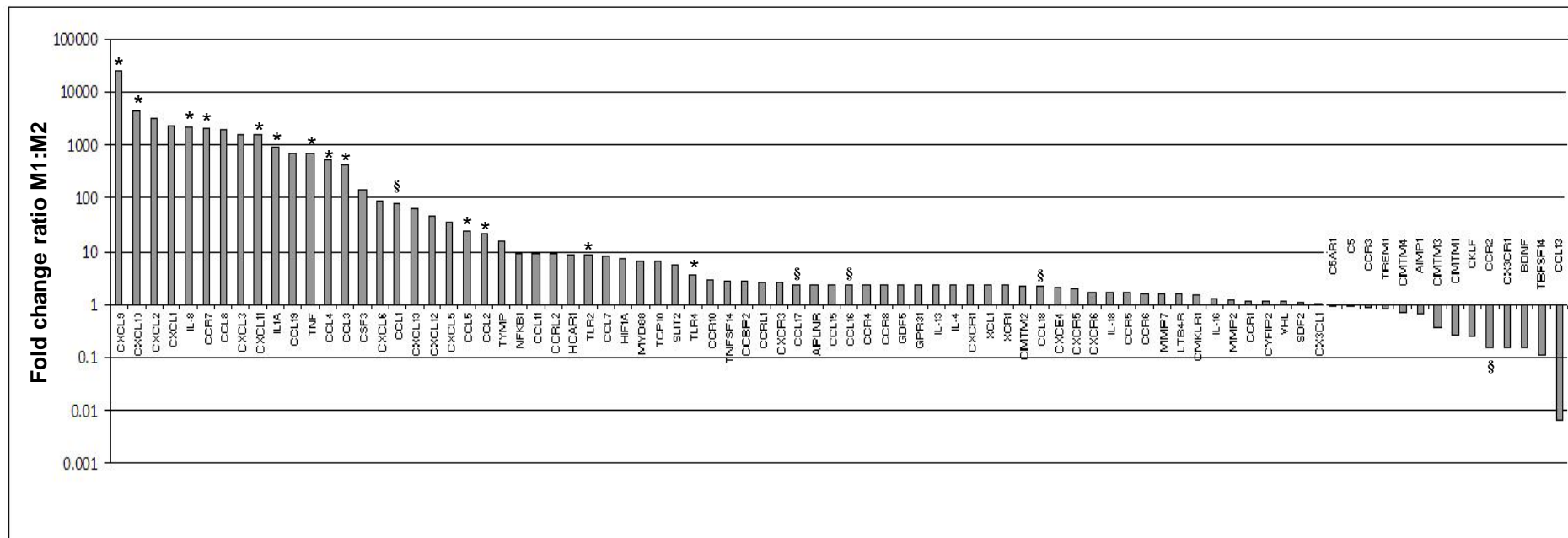
Figure 2 Fold change ratio M1:M2 chemokine and receptor gene expression from RT[2] Profiler[TM] PCR array. The ratio was calculated based on gene expression (ΔΔCt) relative to the average of five constitutively expressed housekeeping gene and to resting M0 macrophages incubated with culture medium for the same time period of either 6h for M1 state and 24 h for M2 state. Symbolically mark (*) and (§) represent M1 and M2 genes, respectively. The experiment was performed in two biological and one technical replicate.

Effects of LPS stimulation on THP-1 macrophage polarization

LPS is an often used pro-inflammatory stimulus [34, 35]. We, therefore, examined whether a single exposure of LPS also causes THP-1 polarization based on the above examined M1-M2 marker genes. With this aim, M0 THP-1 macrophages were stimulated with 700 ng/ml LPS for 6 and 24 h. Gene expression ($2^{\wedge-\Delta\Delta Ct}$) of M1 and M2 markers were analysed using RT-qPCR. The ΔCt values from RT^2 Profiler^{TM} PCR array (normalized with the average of five constitutively expressed housekeeping genes) was used in PCA analysis to compare gene expression profiles of chemokine and their receptor genes with those of resting THP-1 macrophages at time 0 h (M0), at 6 h (M0$_{6h}$), at 24 h (M0$_{24h}$) and together with polarized M1-M2 THP-1 macrophages.

Expression of M1-M2 marker genes

As shown in Figure 3, M1 cytokine marker genes; TNFα, IL-1β and IL-12p40, were highly up-regulated upon LPS stimulation after 6 h of incubation, after which expression dramatically declined at 24 h of incubation (p<0.05). LOX-1, a possible new M1 marker, was also expressed after LPS stimulation with *ca*. 87 fold up-regulation after 6 h of incubation. In general, the gene expression pattern of the known M1 cytokine markers and of the LOX-1 gene after LPS stimulation were found to be very similar to those of polarized M1 THP-1 macrophages as described earlier. The M2 marker genes, MRC-1, DC-SIGN and dectin-1, were slightly up-regulated (1.3-2.8 fold induction) upon LPS stimulation (Fig 3). Expression of arginase-1, Fizz-1 and Ym-1 could not be found after LPS stimulation (data not shown). A mild expression of the MARCO gene was unexpectedly observed since the expression of this gene could not be detected in either M1 or M2 polarized macrophages.
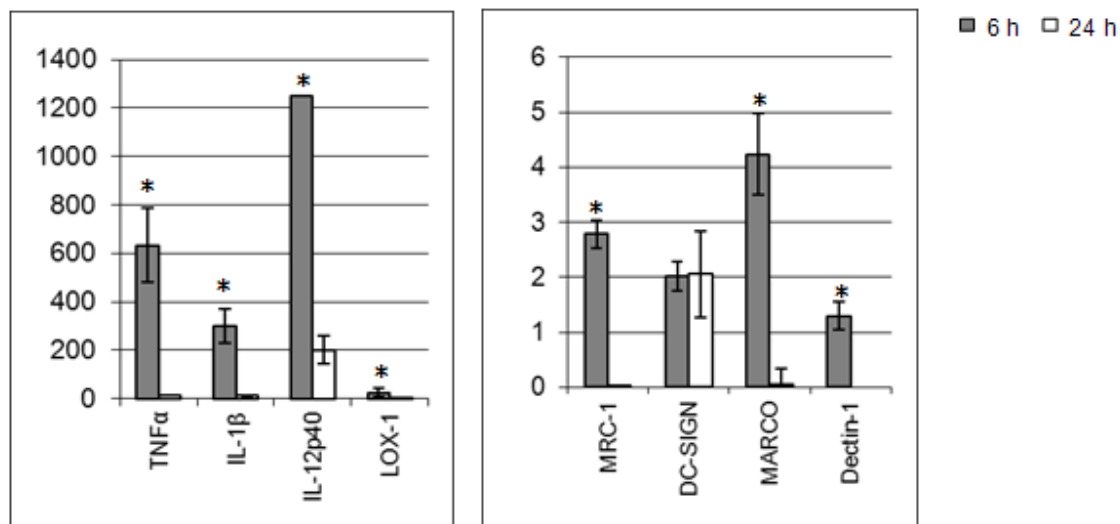
Figure 3 Expression of representative M1 and M2 cytokine and cell surface receptor marker genes of 700 ng/ml LPS stimulated-M0 THP-1 macrophages after 6 h and 24 h of incubation. Gene expression was expressed relative to GAPDH-expression and to resting M0 macrophages incubated with culture medium for the same time period of 6 or 24 h ($\Delta\Delta$Ct). Data shown from RT-qPCR are the means ± standard deviation (SD bars) from two biological and two technical replications. *P<0.05 compared with 24 h of LPS stimulation.

Expression of chemokine and their receptor genes

The M0 THP-1 macrophages stimulated with 700 ng/ml LPS after 6 h of incubation were chosen to examine expression of chemokine and receptor genes using RT^2 Profiler^{TM} PCR array since M1 markers genes were highly expressed at this time point. ΔCt values for chemokine and their receptor genes in LPS-stimulated M0 THP-1 macrophages at 6 h, M0, M0$_{6h}$ and M0$_{24h}$ and polarized M1-M2 THP-1 macrophages were calculated and analysed with PCA. As shown in Figure 4A-D, total variance of all four scattergrams was between 91-96.6% for the first two components. Results from PCA of the complete chemokine and their receptor genes showed that LPS-stimulated THP-1 macrophages at 6 h were clearly separated from resting M0 cells and from the polarized M2 state, but were relatively similar to the polarized M1 state (Fig 4A). A sub-classification into three groups can be made based on the structures and functions of chemokine and receptor genes as included in the PCR array system (Table 2), for instance, based on chemokine C-C motif ligands and receptors, chemokine C-X-C motif ligands and receptors and inflammation-related genes. To establish which gene group leads to the best discrimination for M1-M2 classification, each of the above mentioned gene group was analysed by PCA. Similar scattergrams were obtained when

groups were categorized based on structures and functions of chemokine and receptor genes (Fig 4B-C) or whether a complete data set was used (Fig 4A). When all analysed genes which have been measured by both RT-qPCR (cytokines and cell membrane receptors) and RT^2 Profiler^{TM} PCR array (chemokine and their receptors) was used for PCA analysis, a very similar scattergram was observed as Fig 4A-D (data not shown). Therefore, it can be concluded that stimulation of M0 cells with 700 ng/ml LPS after 6 h of incubation resulted in polarization from the M0 state towards the M1 state but less than the combined treatment of IFNγ+LPS.

A *Complete chemokine and receptor genes*

B *Chemokine (C-C motif) ligands and receptors*

C *Chemokine (C-X-C motif) ligands and receptors*

D *Inflammation-related genes*
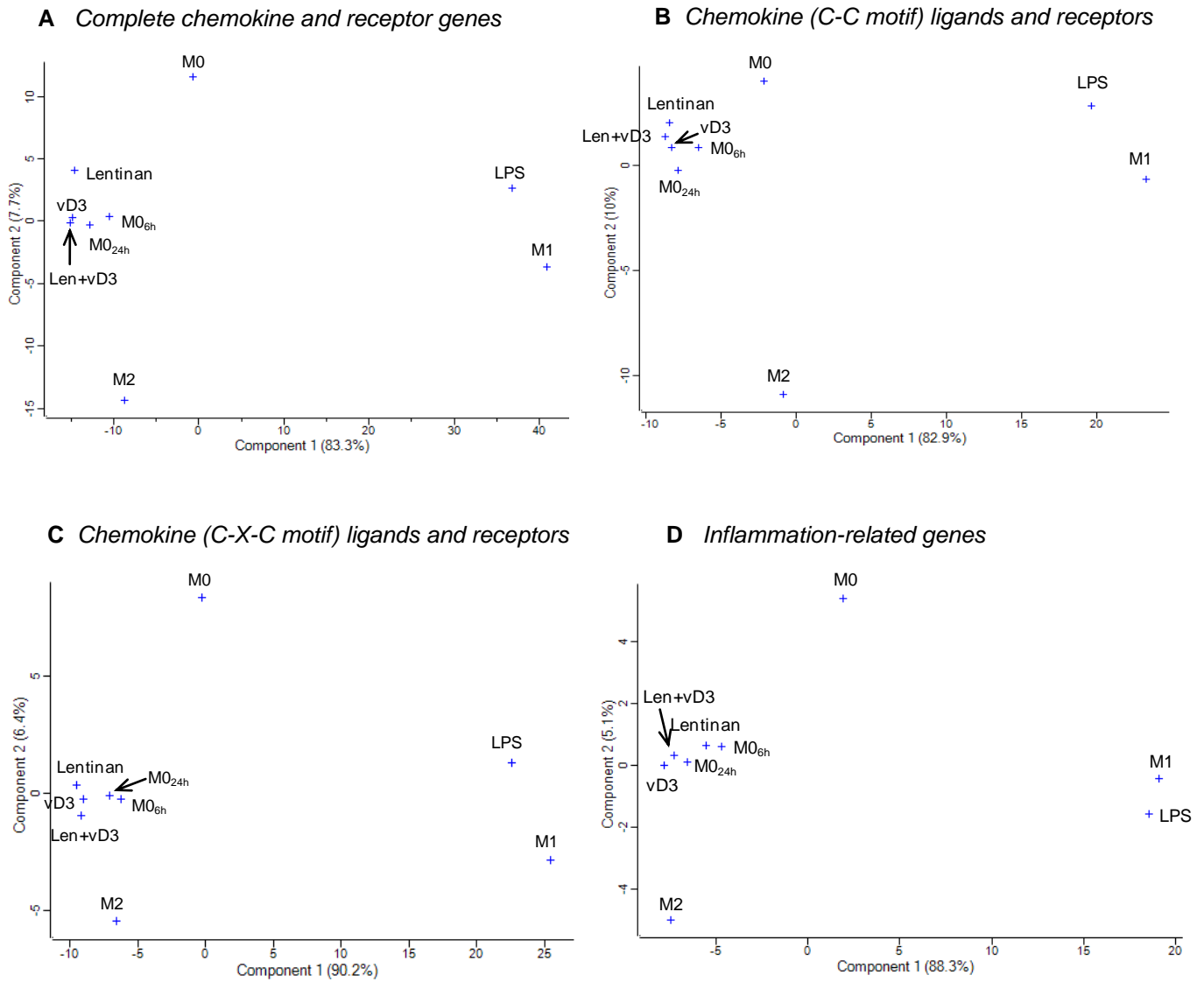
Figure 4 PCA analysis from ΔCt values of chemokine and their receptor genes from 700 ng/ml LPS stimulated M0 THP-1 after 6 h (LPS), 100 µg/ml lentinan, 100 nM vitaminD3 (vD3) and combination of 100 µg/ml lentinan+100 nM vitaminD3 (Len+vD3) stimulated M0 THP-1 macrophages after 24 h of incubation. ΔCt values from M1 and M2 THP-1 macrophages, as well as from resting M0 macrophages incubated with culture medium for the same time period of 6 (M0$_{6h}$) or 24 h (M0$_{24h}$) were also included in this PCA analysis.

Table 2 Sub-classification of chemokines and receptors genes used in PCA analysis

Group 1 (Fig 4B) Chemokine (C-C motif) ligands and receptors		Group 2 (Fig 4C) Chemokine (C-X-C motif) ligands and receptors		Group 3 (Fig 4D) Inflammation-related genes	
Ligands	CCL1	Ligands	CX3CL1	PCR array	MYD88
	CCL11		CXCL1		NF-κB1
	CCL13		CXCL10		TLR-2
	CCL15		CXCL11		TLR-4
	CCL16		CXCL12		TNF
	CCL17		CXCL13		TNFSF14
	CCL18		CXCL2		TBFSF14
	CCL19		CXCL3		IL-8
	CCL2		CXCL5		IL-1A
	CCL3		CXCL6		
	CCL4		CXCL9		
	CCL5				
	CCL7	Receptors	CX3CR1		
	CCL8		CXCR3		
			CXCE4		
Receptors	CCR1		CXCR6		
	CCR10		CXCR1		
	CCR2		CXCR5		
	CCR3		CXCR6		
	CCR4				
	CCR5				
	CCR6				
	CCR7				
	CCR8				
	CCRL1				
	CCRL2				

Effects of food compounds on THP-1 macrophage polarization

The β-glucan lentinan (from shiitake mushroom) and vitamin D3 are generally known as immune-modulating compounds which exert activity towards macrophages [26, 36-38]. It is still unknown whether polarization to the M1 or M2 state is part of the bioactivity of these compounds. Therefore, their effect on M0 THP-1 macrophages polarization was examined. M0 THP-1 macrophages were stimulated with either 100 μg/ml lentinan, 100 nM vitamin D3 (vD3) or the

combination of 100 μg/ml lentinan and 100 nM vitamin D3 (Len+vD3) for 6 and 24 h. After that, gene expression ($2^{\wedge -\Delta\Delta Ct}$) of M1 and M2 marker genes were analysed using RT-qPCR and ΔCt values using RT^2 Profiler^{TM} PCR array (normalized with the average of five constitutively expressed housekeeping genes) were used in the PCA analysis to compare gene expression profiles of chemokine and receptor genes with those of M0, M0$_{6h}$, M0$_{24h}$ and polarized M1-M2 THP-1 macrophages (Fig. 4).

Expression of M1-M2 marker genes

Incubation of resting M0 THP-1 macrophages with lentinan showed a small, but statistically significant up-regulation of the M1 marker genes (TNFα, IL-1β and IL-12p40) from M0$_{24h}$ ($p<0.05$) after 24 h of incubation (Fig 5A). Len+vD3 also mildly up-regulated TNFα and IL-12p40 genes at 24 h after stimulation, but only the IL-12p40 gene was statistically different from M0$_{24h}$ ($p<0.05$). The IL-6 and IL-8 genes did not express in lentinan, vD3 and Len+vD3 treated macrophages (data not shown). The M2 marker genes DC-SIGN and dectin-1 were down-regulated upon exposures to all tested food compound, whereas the MRC-1 gene was slightly up-regulated by vD3 after 24 h of incubation (Fig 5B), but this was not statistically different from M0$_{24h}$ ($p\geq0.05$). Expression of the LOX-1 gene seems to be down-regulated by food compound exposure, in which vD3 and Len+VD3 was the most effective in down regulation of this gene (Fig 5C). Both treatments also suppressed the expression of the DC-SIGN and dectin-1 genes ($p\geq0.05$). MARCO, arginase-1, Fizz-1 and Ym-1 gene expression could not be detected in all food compound exposures (data not shown).
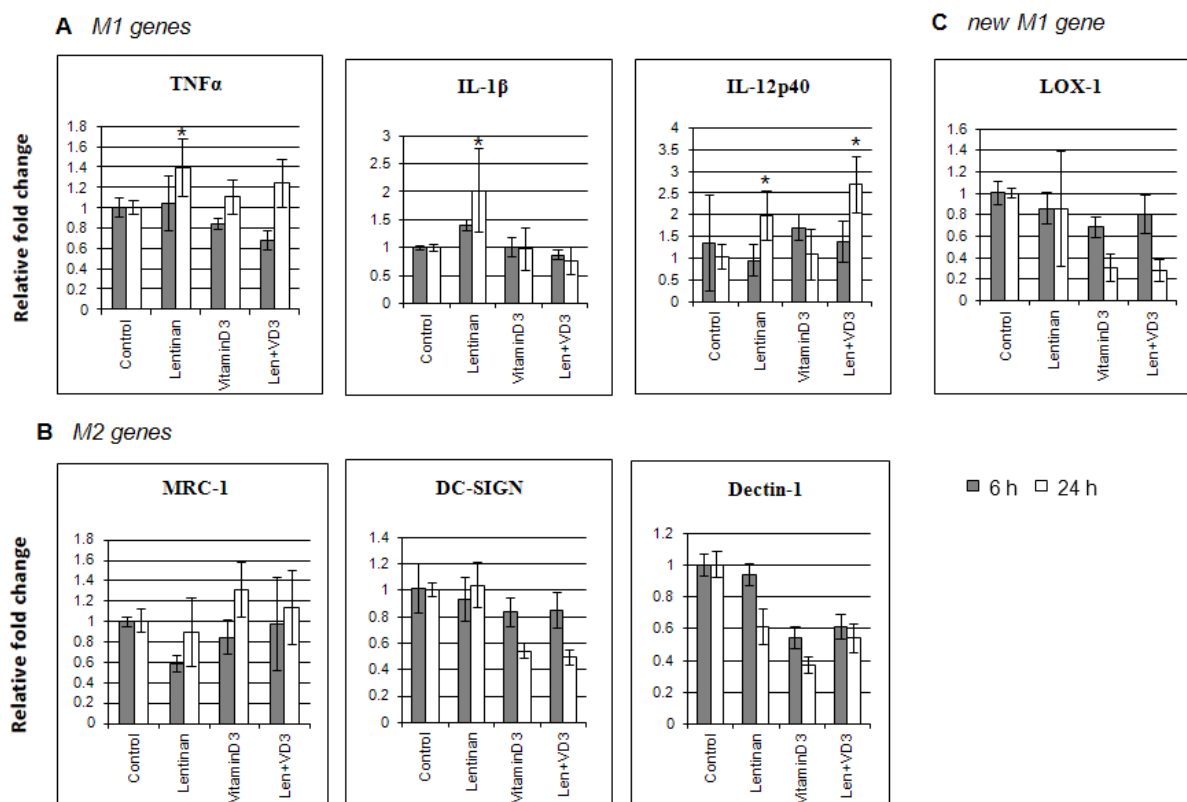
Figure 5 Expression of representative M1 and M2 cytokine and cell surface receptor maker genes of 100 µg/ml lentinan, 100 nM vitaminD3 and combination of 100 µg/ml lentinan+100 nM vitaminD3 stimulated M0 THP-1 macrophages after 6 h and 24 h of incubation. Gene expression was expressed relative to GAPDH-expression and to resting M0 macrophages incubated with culture medium for the same time period of 6 or 24 h (ΔΔCt). Data shown from RT-qPCR are the means ± standard deviation (SD bars) from two biological and two technical replications. *$P<0.05$ compared with resting M0 incubated with culture medium at 24 h.

Expression of chemokines and their receptor genes

ΔCt values of chemokine and their receptor genes of food compound stimulated-M0 THP-1 macrophages after 24 h of incubation were analysed by PCA and compared with those of M0, $M0_{24h}$ and polarized M1-M2 THP-1 macrophages. PCA results, calculated as either a complete chemokine and receptor data set (Fig 4A) or as structures and functions of chemokine and receptor gene groups (Fig 4B-D), showed similar scattergrams. Expression profiles of M0 stimulated lentinan, vD3 and Len+vD3 were clustered very close to each other and all displayed clear separation from those of LPS stimulation and from the polarized M1 state. Although they may have

a small tendency to move towards polarized M2 direction, it has to be concluded that macrophages exposed to these food compounds clustered closely to the gene expression profile of $M0_{24h}$ macrophages indicating that these food compounds have no or a very mild effect on polarization of macrophages.

Cytotoxicity

THP-1 macrophages stimulated with either 20 ng/ml IFNγ +1 μg/ml LPS, 20 ng/ml IL-4, 700 ng/ml LPS or food compounds; 100 nM vitamin D3, 100 μg/ml lentinan, the combination of lentinan and vitaminD3, showed overall relative cell viability over 95% after both 6 and 24 h after incubation (data not shown). From this we concluded that all stimulating treatments were not toxic to THP-1 macrophages.

Discussion

Several studies have examined gene expression or signalling molecules that control macrophage plasticity in human or mouse *ex vivo* [6, 28, 39]. Our study was designed to characterize whether THP-1, a human monocytic cell line, is able to polarize from resting macrophages (M0) to the M1- and M2-like state as described for *in vivo* and *ex vivo* systems. The gene expression profile of several known M1-M2 marker genes *ex vivo* were tested in the polarized M1-M2 THP1 macrophages, using the same stimuli with similar concentration used for *ex vivo* analyses [6, 28].

As previously mentioned, a hallmark for M1 polarization is a high production of pro-inflammatory factors and expression of TLRs and NLRs, while M2 polarization governs anti-inflammatory factors and expression of SRs. For murine *in vivo* and *ex vivo*, and for human *ex vivo* systems, it has been reported that IL-1β, TNFα, IL-12, IL-6, IL-8 genes were expressed upon M1 polarization [6, 20, 28]. Our study revealed a similar set of up-regulated genes in polarized M1 THP-1 macrophages, indicating successful M1-like differentiation in THP-1 macrophages. MRC-1 and dectin-1 genes were highly recommended to be used as M2 markers in animal and human *ex vivo* systems [28, 40] and also DC-SIGN (CD209) gene was hypothesised as it was expressed in an

anti-inflammatory situation [28, 41]. These three genes were also found to be up-regulated by polarized M2 THP-1 macrophages. Although it has been described in many reviews that SRs were expressed in the M2 state in *in vivo* murine models [8, 29, 30], different observations were found in our study in which the MARCO gene (SR-A) and CD36 (SR-B) were not expressed in polarized M2 THP-1 macrophages, while LOX-1 (SR-E) was up-regulated in polarized M1 THP-1 macrophages. Our study was, however, the first detailed gene expression analysis for each of the scavenger receptor sub-classes in M2 polarization. Based on murine macrophage polarization studies arginase-1, Fizz-1 and Ym-1 genes were identified as M2 markers, however, no expression of these genes has been observed in the M2 polarization of the human THP-1 macrophage in our study. This is in line with recent studies based on human PBMCs that did not find these genes as useful M2 markers [39, 42, 43] indicating that human polarization and murine polarization involves somewhat different mechanisms.

Phenotypic M1 and M2 cells have been shown to be discriminated by different sets of chemokines and their receptors [8, 28]. The chemokines CXCL 8, 9, 10, 11, 16 and CCL2, 3, 4, 5 and chemokine receptor CCR7, have been indicated to be specific for the M1 state [8]. The chemokines CCL1, 16, 17, 18, 22, 24 and chemokine receptors CCR2, CXCR1 and 2 are classified to characterize the M2 state [8]. Our finding shows that polarized M1 THP-1 macrophages expressed all of the above mentioned M1 chemokines and receptors, whereas the polarized M2 THP-1 macrophages showed relatively minor expression of the M2 chemokine and receptor markers. However, our study shows that a specific M1 chemokine gene cluster is actually down-regulated or very slightly expressed in M2 THP-1 macrophages, indicating that the pro-inflammatory mediators are not induced or even down-regulated. It has been reported that macrophages frequently respond to the early cytokines that they secrete in an autocrine and paracrine fashion [44], and by consequence such secreted cytokines may induce differentially expressed chemokine secretion pattern [45]. This may make the M2 state less sharply defined or consistent because chemokine genes are actually influenced by secondary cell-cell signals. Long stimulation, which is necessary to drive to the M2 activation state [28], and also several intermediate M2 states occurring during polarization [6] might be factors that cause variations of chemokine gene expression. Although we tested IL-4 concentrations of 20, 40 and 80 ng/ml with M0 THP-1 macrophages for 24 h of incubation, the expression of the M2 makers MRC-1, dectin-1, DC-SIGN, CD36 and MARCO did not differ (data not shown). Thus, the THP-1 macrophage

system using chemokines and receptors as marker genes might not be suitable to investigate polarizing effects towards the M2 state. The CCL13 gene was the only cytokine gene, which was expressed strongly in M2 polarization (*ca*. 1610 fold). This gene has been reported to be involved in chemotaxis of monocytes, basophils, T-lymphocytes by binding to CCR2, CCR3 and CCR5 receptors [46]. Besides that, CCL13 chemokine has been implicated in asthma, a disease in which M2 polarization is involved [47, 48]. Therefore, CCL13 might be a new exceptional chemokine gene expressed in polarized M2 THP-1 macrophages. Although our studies proposed possible new M1 and M2 marker genes, they should be confirmed with higher number of *in vitro* biological replications, as well as should be further evaluated in *ex vivo* cultured macrophages before establishing them as reliable biomarkers.

LPS has been shown to activate macrophages to produce series of pro-inflammatory mediators, TLRs and NLRs [27, 34, 49], suggesting LPS to be a potent M1 inducer. We previously showed up-regulation of several pro-inflammatory genes in THP-1 macrophages stimulated with 700 ng/ml LPS [27]. Thus, in this study, we were interested to investigate expression of the M2 markers upon LPS stimulation. The M1 marker genes were up-regulated upon LPS-stimulated THP-1 macrophages but less than THP-1 macrophages stimulated with IFNγ+LPS. This data, together with our previous study, demonstrates that 700 ng/ml LPS can activate M0 THP-1 into an M1-like state within 6 h.

Macrophages have been reported to react to lentinan via dectin-1, TLR-2 and -4 which induces the MyD88-dependent signalling cascade and enhances the ability to counteract viral and bacterial invasion [50]. VitaminD3 regulates the differentiation process of monocytes into macrophages [51] and the growth and function of a broad range of immune cells [52]. Our study intends to investigate whether these two compounds have an influence on M1-M2 THP-1 macrophage polarization, starting from resting macrophages (M0). Based on gene expression of cytokines genes, cell membrane receptors and chemokine and their receptors with the aid of PCA analysis, we found that food compound stimulated-M0 THP-1 macrophages for 24 h clustered very closely to $M0_{6h}$ and $M0_{24h}$ indicating that there is a relatively mild polarizing effect caused by lentinan, vD3 and Len+vD3 on the macrophage polarizing process. Another possibility to study polarizing activity of food compounds on macrophages might be to switch between activated M1 and M2 cells. Quantitative changes in these markers could be used as an indicator for macrophages switching from M1 to M2 state, or vice versa. These may also be applied to study in more detail the

effect of food on modification of the strong M1 and M2 signals like also performed previously [26, 27].

Conclusions

Our studies have shown that PMA-differentiated THP-1 macrophages are able to polarize into either M1 or M2-like phenotypes utilizing the same stimuli as applied in mouse and human culture systems *ex vivo*. Several known M1 and M2 marker genes have shown to be relevant in THP-1 macrophage polarization. Newly established M1 marker genes (LOX-1, CXCL1, 2, 3, CCL8, CCL19 and CSF3) and M2 marker genes (DC-SIGN and CCL13), in this THP-1 system should be further analysed in freshly isolated PBMC-derived macrophages in order to evaluate the potency of these markers for functionally categorizing effects of food towards the immune system.

Acknowledgement

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Supplementary Table 1 Fold induction of chemokines and their receptor genes in polarized

M1-M2 THP-1 macrophages

Gene name	GenBankID	M1	M2	Ratio M1:M2
CXCL9*	NM_002416	179298.02	7.12	25180.16
CCL8	NM_005623	98102.34	**52.06**	1884.54
CXCL10*	NM_001565	9165.51	2.00	4576.41
CXCL11*	NM_005409	2339.53	1.42	1652.00
CCR7*	NM_001838	2152.80	1.07	2019.80
CXCL2	NM_002089	1522.26	0.47	3236.01
CCL4*	NM_002984	1343.70	2.55	526.39
TNF*	NM_000594	1076.40	1.62	666.29
CXCL1	NM_001511	930.59	0.42	2210.26
CXCL3	NM_002090	917.78	0.56	1652.00
IL1A	NM_000575	626.86	0.68	916.51
IL-8	NM_000584	502.16	0.24	2091.03
CCL3*	NM_005408	407.88	0.94	433.53
CCL19	NM_006274	269.10	0.40	680.29
CCL1§	NM_002981	232.65	2.85	81.57
CSF3	NM_000759	157.81	1.10	144.01
CCL2*	NM_002982	144.21	6.69	21.56
CCL7	NM_006273	91.27	**10.87**	8.40
CCL5*	NM_002985	53.15	2.15	24.76
CXCL6	NM_002993	40.00	0.46	86.82
CXCL13	NM_006419	38.91	0.61	64.00
CXCL12	NM_000609	38.64	0.86	44.94
NFKB1	NM_003998	12.57	1.34	9.38
CCL18§	NM_002988	12.06	5.55	2.17
CCL13	NM_005408	11.02	**1609.06**	0.01
CCRL2	NM_003965	9.01	1.01	8.94
HIF1A	NM_001530	6.78	0.94	7.21
TYMP	NM_001953	5.59	0.36	15.45
SLIT2	NM_004787	5.14	0.96	5.35
HCAR1	NM_0032554	4.93	0.57	8.69
CCL11	NM_002986	3.19	0.35	9.19
TLR4*	NM_138554	2.99	0.81	3.71
MYD88	NM_002468	2.87	0.43	6.68
CXCL5	NM_002994	2.81	0.08	34.78

CCL17§	NM_002987	2.77	1.19	2.33
MMP7	NM_002423	2.74	1.74	1.57
TLR2*	NM_003264	2.59	0.30	8.51
CCR4	NM_005508	2.47	1.10	2.25
GPR31	NM_005299	2.47	1.10	2.25
APLNR	NM_005161	2.47	1.10	2.25
CCL15	NM_032965	2.47	0.84	2.93
CCR10	NM_016602	2.47	1.10	2.25
CCR6	NM_004367	2.47	1.55	1.59
CCR8	NM_005201	2.47	1.10	2.25
GDF5	NM_000557	2.47	1.10	2.25
IL-13	NM_002188	2.47	1.10	2.25
IL-4	NM_000589	2.47	1.10	2.25
CXCR1	NM_001337	2.47	1.10	2.25
XCL1	NM_002995	2.47	1.10	2.25
XCR1	NM_005283	2.47	1.10	2.25
CCL16§	NM_004590	2.47	1.10	2.25
CX3CL1	NM_002996	2.00	1.88	1.06
CMTM2	NM_144673	1.72	0.79	2.19
CYFIP2	NM_014376	1.44	1.24	1.16
CXCR3	NM_001504	1.35	0.51	2.62
TNFSF14	NM_003807	1.30	0.46	2.81
CCR5	NM_000579	1.19	0.73	1.62
VHL	NM_000551	1.13	1.00	1.13
CCR1	NM_001295	1.04	0.87	1.19
TCP10	NM_004610	1.01	0.15	6.54
CXCR4	NM_003467	0.95	0.45	2.08
LTB4R	NM_181657	0.93	0.61	1.54
AIMP1	NM_005161	0.88	1.32	0.67
SDF2	NM_006923	0.84	0.75	1.12
TREM1	NM_018643	0.82	0.96	0.86
C5	NM_001735	0.68	0.74	0.93
CMTM1	NM_181269	0.60	2.21	0.27
IL-18	NM_001562	0.54	0.33	1.64
CCBP2	NM_001296	0.51	0.19	2.69
CCRL1	NM_016557	0.50	0.19	2.66
CXCR6	NM_006564	0.48	0.29	1.68
CXCR5	NM_001716	0.44	0.23	1.93

CMKLR1	NM_004072	0.39	0.27	1.45
MMP2	NM_004530	0.37	0.31	1.20
CKLF	NM_181641	0.36	1.48	0.24
CCR3	NM_001837	0.31	0.34	0.91
CMTM4	NM_178818	0.23	0.31	0.73
CMTM3	NM_144601	0.20	0.53	0.37
BDNF	NM_001709	0.19	1.23	0.15
C5AR1	NM_001736	0.18	0.20	0.93
TNFRSF1A	NM_001065	0.16	1.41	0.11
IL-16	NM_004513	0.10	0.08	1.34
CCR2§	NM_001123396	0.08	0.51	**0.16**
CX3CR1	NM001337	0.05	0.30	0.16

Chapter 5

Biochemical and functional characterization of recombinant fungal immunomodulatory proteins (rFIPs)

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Abstract

In this study two novel FIPs have been identified and characterized. The first is FIP-*nha*, identified in the ascomycete *Nectria haematococca*, and as such, FIP-*nha* would be the first FIP to be identified outside the order of Basidiomycota. The second is LZ-9, an LZ-8 like protein identified in *Ganoderma lucidum*. Recombinant FIP proteins were produced in *Pichia pastoris* and purified using His-affinity magnetic beads. The bioactive characteristics of FIP-*nha* and LZ-9 was compared to two other well-known FIP proteins, LZ-8 from *Ganoderma lucidum* and FIP-*fve* from *Flammulina velutipes,* which were produced and purified using the same method. The produced recombinant FIPs (rFIPs); rLZ-8, rLZ-9, rFIP-*fve* and rFIP-*nha* were investigated for their hemagglutinating activity which revealed that rLZ-8, rLZ-9 and rFIP-*nha* were able to agglutinate rabbit, mouse and sheep red blood cells while rFIP-*fve* only agglutinated rabbit red blood cells. None of the rFIPs were able to agglutinate human red blood cells unless the cells were trypsinized. In addition, all rFIPs were studied and compared to several lectins for their effect on Caco-2 intestinal cell layer integrity using transepithelial electrical resistance (TEER) measurement. rLZ-9 appeared to have the highest effect in lowering TEER, similar to one of the tested lectins. Testing of rFIPs for their activation of inflammation-related genes of THP-1 macrophages showed rFIP-*fve* to be the strongest inducer of pro-inflammatory cytokine transcription. These results indicate that each rFIP has a unique bioactive profile as well as each lectin, creating the basis for further studies to relate structure to biological activity.

Introduction

Fungal immunomodulatory proteins (FIPs) are a class of small proteins extensively studied for their immunomodulating activities [1-3]. They have shown to potentially exert, for instance, anti-cancer, anti-tumour, anti-allergy and anti-anaphylaxis activity and stimulation of immune cells to produce cytokines [1, 4, 5]. Moreover, it has been described that FIPs can be applied in pharmaceutical preparations or vaccines in order to enhance the level of immune regulation and suppress tumour and autoimmune diseases [6, 7]. Several FIPs belonging to the fungal order of Basidiomycota were identified: LZ-8 from *Ganoderma lucidum* [8], FIP-*gts* from *G. tsugae* [9], FIP-*gja* from *G. japonicum* (GeneBank : AAX98241), FIP-*gsi* from *G. sinense* [10], FIP-*gmi* from *G. microsporum* [11], FIP-*gap* from *G. applanatum* (GeneBank : AEP68179), FIP-*fve* from *Flammulina velutipes* [12], FIP-*vvo* from *Volvariella volvacea* [13] and FIP-*tvc* from the mushroom *Trametes versicolor* [14]. Based on BLAST analysis, we identified a novel fungal immunomodulatory protein gene in the *Nectria haematococca* genome sequence (DOE Joint Genome Institute, http://www.jgi.doe.gov/) and designated it as FIP-*nha* (GeneBank : XP3043654). This pathogenic fungus, also commonly referred as *Fusarium solani*, is a member of the Sordariomycetes family belonging to the order of Ascomycota which makes FIP-*nha* the first FIP member identified outside the Basidiomycota order. Besides this complete new FIP, we previously identified a slightly different FIP as currently published for *Ganoderma lucidum* (in preparation). This FIP has a high similarity to LZ-8 and was therefore named LZ-9.

The resemblance in the bio-functional properties of FIPs to plant lectins has been well recognized in their ability to agglutinate red blood cells [1, 14, 15]. Both types of proteins are able to interact and bind to cell surface sugar moieties [16, 17]. Some lectins are known for their anti-nutritional effect as they can bind to membrane glycosyl groups of the cells lining the digestive tract. As a result of that, they can damage luminal membranes of the epithelium and interfere with nutrient digestion and absorption (noticeable as causing diarrhoea) as also shown by decreasing transepithelial electrical resistance (TEER) values in *in vitro* cell model studies, using Caco-2 cells [18, 19]. However, it still remains unknown whether FIPs also have an effect on the intestinal cell layer integrity, which is important when developing novel functional food or feed products based on FIPs. Besides that, there is no assay available for comparing lectins and FIPs to study their specific bioactivity towards immune cells systematicly, while such method could support risk-benefit

analysis. Therefore, the human macrophage cell line model, THP-1, was explored as a model to analyse the bioactivity of this class of proteins.

In this study, we expressed, isolated and purified four FIPs: LZ-8 and LZ-9 from *G. lucidum*, FIP-*fve* from *F. velutipes* and FIP-*nha* from *N. haematococca* using the *Pichia pastoris* expression system. Bioactivity characteristics of the purified recombinant FIPs (rFIPs) were investigated and compared to some lectins in terms of hemagglutinating activity, kinetic of transepithelial electrical resistance (TEER) values of confluent differentiated Caco-2 monolayers and expression of inflammation-related genes in THP-1 macrophages. The characterizations of rFIPs provided in this research was aimed to support risk-benefit analysis and raise some issues which should be taken into consideration when designing human and animal intervention trials in the future.

Materials and methods

Samples and chemicals

Yeast extract peptone dextrose (YPD) broth, low salt Luria-Bertani (LB) broth, buffered glycerol-complex medium (BMGY), glycerol, peptone, micro agar, yeast extract and yeast nitrogen base (YNB) with ammonium sulphate without amino acids were purchased from Duchefa (The Netherlands). The methanol was obtained from Merck (Germany). All tested lectins were purchased from Sigma-Aldrich (St. Louis, MO, USA): BSI from *Bandeirae simplicifolia* (*Griffonia simplicifolia*), Concavalin A type IV from *Canavalia ensiformis* (Jack bean), SBA from *Glycine max* (soy bean), WGA from *Triticum vulgaris* (wheat germ) and LE from *Solanum esculentum* (tomato) with protein content according to manufacturer's datasheet >90%, non-determined, >80%, 100% and >40%, respectively. All PCR and RT-qPCR primers used were synthesised by Biologio (The Netherlands).

Recombinant FIPs (rFIPs) cloning and construction of expression vector

The nucleotide sequence of LZ-8 and FIP-*nha* were synthesized by BaseClear B.V. (Leiden, the Netherlands) based on the sequence identified in *Ganoderma lucidum* (GeneBank : ACD44335) and *Nectria haematococca* (GeneBank : XM3043608), respectively. The FIP-*fve* and LZ-9 genes were obtained by PCR based cloning from *Flammulina velutipes* (strain M4600) and *Ganoderma lucidum* (strain M9720), respectively, in which both strains were obtained from Mycelia BVBA, Belgium. To facilitate sub-cloning of the FIP genes into Pichia expression vector pPICZα-A (Life Technologies Ltd, Paisley, UK), the following forward and reverse primers were used:

5'-GGG<u>GAATTC</u>TCCGACACTGCCTTGATCT-'3 (Forward LZ-8 and LZ-9),

5'-GGG<u>GAATTC</u>GCTACCACCAATGACTC-3' (Forward FIP-*nha*),

5'-GGG<u>GAATTC</u>TCCGCCACGTCGCTCAC-3' (Forward FIP-*fve*),

5'-GGG<u>TCTAGA</u>**CCTCCCCCGCCTCCCCC**GTTCCACTGGGCGATGATG-3' (Reverse LZ-8 and LZ-9),

5'-GGG<u>TCTAGA</u>**CCTCCCCCGCCTCCCCC**TTCCACTGGGCAACAAGAT-3' (Reverse FIP-*nha*) and

5'-GGG<u>TCTAGA</u>**CCTCCCCCGCCTCCCCC**AGTCTTCTTCCACTCAGCG-3' (Reverse FIP-*fve*).

An *EcoR*I and *Xba*I restriction site, as underlined above, were added to the forward and reverse primers, respectively. In addition, all reverse primers contained an additional flexible linker consisting of six glycine amino acids (6x Gly, indicated in bold) to stimulate proper structure formation and free rotation in between the rFIP and c-myc epitope /6x His tag fusion protein [20]. All FIP fragments were amplified using the AccuPrime™ *pfx* polymerase of Life Technologies Ltd. PCR amplification conditions were as follows: a 2 min denaturing step at 94˚C followed by 35 cycles of amplification, 15 sec at 94˚C for denaturation, 30 sec at 63˚C for annealing and 1 min at 68˚C for extension. Amplified fragments were run on a 2% agarose gel. DNA bands were purified using the agarose gel purification kit (QIAGEN, Valancia, USA) and sub-cloned into pGEM-T vector (Promega Corporation, Wisconsin, USA). The ligated plasmids were used to transform *E.coli* XL1-Blue heat-shock competent cells (Stratagene, Santa Clara, USA). The transformants were checked by restriction endonuclease digestion and verified by full length sequencing (BaseClear B.V., Leiden, The Netherlands). Finally, the verified FIP genes were transferred into the pPICZα-A plasmid using the *EcoR*I/*Xba*I restriction sites to transform *E.coli* Top10 heat-shock competent cells (Life Technologies Ltd, Paisley, UK). The transitions between the FIP gene and the alpha signal and in between the c-myc epitope/polyhistidine (6x His) was checked by sequencing using the sequencing primers 5'-TCATCGGTTACTCAGATTTAGAAG-'3

and 5'-ACGGCGCTATTCAGATCCTC-'3, respectively.

Transformation of *P. pastoris* and selection of transformants

The pPICZα-A-FIP plasmids were introduced into *Pichia pastoris* strain X-33 according to the EasyComp™ *Pichia* Transformation kit (Life Technologies Ltd, Paisley, UK). Transformed cells were spread onto YPD plates with different Zeocin concentrations (100, 250, 500 and 1000 µg/ml) for direct selection of multi-copy insertion transformants. Six integrant strains, chosen from different Zeocin selection plates were checked by colony PCR using FIP forward and 3'AOX reverse primers. Of these, three integrant strains, preferably single-copy as well as multi-copy strains, were evaluated for protein expression.

Expression and purification of rFIPs from *Pichia pastoris*

A single colony, picked from a fresh plate, was grown in 25 ml of BMGY medium at 30˚C for 18-24 h to generate cell biomass. The yeast cells were collected by centrifugation at 1,500xg for 5 min and resuspended in BMMY medium (baffled flasks, 1:10 v/v medium:air) to a final OD600 of 1.0. Protein expression was induced by adding 100% methanol to a final concentration of 1.5% every 24 h for 3 days. The rFIP proteins secreted into the growth medium were purified using his-affinity electromagnetic beads (Dynabeads; Life Technology Ltd, Paisley, UK) according to the manufacturer's protocol, dialyzed twice against PBS ([-]$MgCl_2$ [-]$CaCl_2$) in the total period of 16 h and quality checked by SDS-PAGE gel and western blot. The contamination of LPS was determined in all test samples using LAL endotoxin test (L00350, GenScript, NJ).

As a negative control for protein purification, the *P. pastoris* wild type strain X33 was grown, treated with methanol and processed in exactly the same way as was done for the FIP expressing strains. The obtained control yeast protein sample, designated as X33nc, functioned as a control in all cell-based assays.

Biochemical characterization of purified FIPs

- *Protein yield*

Protein concentration was determined by Bradford Assay using Coomassie Protein Assay Reagent (Thermo Fisher Scientific Inc, MA, USA). The protein yield per liter of growth medium in all purified rFIPs was calculated.

- *SDS-PAGE of purified FIPs*

Purified rFIPs were visually analyzed by SDS-PAGE gel using precast NUPAGE 10% Bis-Tris gels in 1x NuPAGE® MES SDS Running Buffer, stained by SimplyBlue™ SafeStain (Life Technologies Ltd, Paisley, UK). Protein sample (7.8 µl) containing 3 µl 4xNuPAGE® LDS buffer (Life Technologies Ltd, Paisley, UK) and 1.2µl of β-mercaptoethanol was boiled for 5 min before loading on gel. The unstained protein molecular weight marker SM0431 (Fermentas Molecular Biology, St. Leon-Rot, Germany) was used as a size indicating marker.

- *Western blotting*

Western Blot was performed according to the Criterion™ Blotter Instruction Manual (Bio-Rad Laboratories, California, USA). Proteins were transferred to a 0.20 µm nitrocellulose membrane (LKB Bromma), blocked and washed with TBST (50 mM Tris, 150 mM NaCl, 1% Tween-20, pH 7.5) with and without 5% w/v bovine albumin, respectively. The washed membrane was incubated overnight with 1:5,000 Mouse Anti-*myc* IgG (Life Technologies Ltd, Paisley, UK) under continuous agitation, washed 2 times with TBST prior to incubation with 1:40,000 diluted AP-conjugated Goat Anti-Mouse IgG (Sigma-Aldrich, MO, USA) for 1h and subsequently stained with 1-Step™ NTB/BCIP (Thermo Fisher Scientific Inc, MA, USA)

- *Glycosylation staining*

Glycoprotein detection on gel was performed according to the Pierce Glycoprotein Staining Kit instruction Manual (Thermo Fisher Scientific Inc, MA, USA). For the SDS-page protein separation step, 8 µg of each rFIPs was used, while for the positive and negative control provided in the kit, 10 µg was loaded on gel.

- *FIPs protein sequencing*

Purified FIPs, 7.8 µl to which 3 µl 4xNuPAGE® LDS buffer (Life Technologies Ltd, Paisley, UK) and 1.2 µl of β-mercaptoethanol were added, were boiled for 5 min prior to loading on a 12% SDS-PAGE gel (Biorad Mini-PROTEAN Precast Gels) and stained with Colloidal Blue Staining (Life Technologies Novex, USA). The protein bands of interest were cut out of the gel and sliced in 1 mm^3 pieces, destained by washing three times with 0.2M NH_4HCO_3: acetonitrile (1:1). After drying (Speedvac), the gel slices were soaked in 10mM DTT in 0.1M NH_4HCO_3 for 45 min at 56°C. The gel slices were chilled to room temperature and incubated with 55mM iodoacetamide in 0.1M NH_4HCO_3 for 30 min in the dark. The gel slices were washed once more with 0.2M NH_4HCO_3: acetonitrile (1:1), dried and soaked into trypsin solution (12.5 ng/µl trypsin in 0.1M NH_4HCO_3 and 5mM $CaCl_2$) overnight at 37°C. After centrifugation, the digest was transferred to a new tube and the gel slices were washed with 20 µl 5% formic acid : acetonitrile (1:1). The collected supernatant was dried by vacuum centrifugation and the peptides were re-dissolved in 20µl 0.1M ammonium formate. Peptide samples were analysed for peptide composition by LC-MS injection as described in [21]. ProteinLynx Global Server 2.4 (Waters, Milford, USA) was used for protein identification and Progenesis (Nonlinear dynamics Ltd, USA) for peptide quantification [22].

Functional characterization

- *Hemagglutination assay*

Mouse, rabbit and sheep whole blood was ordered from Harlan Laboratories (Horst, The Netherlands). Human whole blood was provided by Sanquin Bloedbank Souh-East Region (Nijmegen, The Netherlands). All blood samples were used within one week after drawing. The animal red blood cells were washed in PBS repeatedly until the wash buffer remained clear. The cells were resuspended to a 1.5% suspension of red blood cells in PBS (v/v). Human red blood cells were washed similar to animal blood, but resuspended to 10% of cells in PBS with 1% (w/v) trypsin from hog pancreas (Sigma-Aldrich, MO, USA). After 1 h incubation at 37°C, the cells were washed twice with PBS and also resuspended to a final concentration of 1.5% red blood cells in PBS. A serial dilution series (40 µg/ml to 0.625 µg/ml) of purified rFIPs was added to each red blood cell type in 96-well U-bottom plates (Greiner Bio-one, The Netherlands). The assay was performed in

duplicate and the hemagglutination activity was recorded after 2 and 4 h of incubation at room temperature. ConA type IV and PBS were used as a positive and negative control, respectively.

- *TEER measurement*

Caco-2 cells (American Type Culture Collection, Rockville, MD, USA) were grown in 24 suspension culture plates on transwell inserts of 33.6 mm^2, 0.4 µm pore size, 1 x 10^8 pores/cm^2 (Greiner Bio-one, The Netherlands). Cells were seeded with 2 x 10^5 cells (150 µl) per insert and grown for 21 days in an incubator humidified with 5% CO_2 at 37 ^{o}C. The culture medium DMEM 42430 (Life Technologies Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone PerBio, Etten-Leur, The Netherlands) was used to maintain Caco-2 germ line once a week and to replace medium of cells growing on the transwells three times a week. Caco-2 cells used were at a passage number between 30-40. TEER was measured using a MilliCell-ERS Ω-meter (Millipore, Molsheim, France) on 21 days-old cells grown on transwells before adding test samples. In all experiments, transwells with TEER values outside the boundary of 850-1050 Ω.cm^2 were excluded from the experiment. From the selected transwells, medium was removed from the apical and basal compartment prior to adding culture medium containing treatment samples in the volume of 150 µl in the apical compartment and 700 µl culture medium without samples in the basal compartment. TEER was determined after 1, 3, 6, 9 and 24 h of sample exposure. As all test samples were either dissolved in or dialysed against PBS pH 7.4 ([-]MgCl$_2$ [-]CaCl$_2$) in case of lectins and rFIPs, respectively, thus an equal amount of PBS was equally adjusted in all stimulating conditions prior to adding to cells. TEER values from cells exposed with culture medium containing PBS and those of before sample addition were used for TEER normalization to obtain relative TEER as later designated as "TEER". The measurement was performed by three replicate transwells.

- *Activation of THP-1 macrophages*

The culture condition of the human monocytic leukemia cell line, THP-1 (American Type Culture Collection, Rockville, Md.), was as described before [23]. The macrophage-like state was obtained by treating THP-1 monocytes for 48 h with 100 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma Aldrich, MO, USA) in 24-wells cell culture plates (Greiner Bio-one, Germany) with 0.5 ml cell suspension (5x10^5 cells) in each well. Differentiated, adherent cells were washed twice with culture medium (RPMI 1640 medium without PMA but containing 10% FBS and 1% Penicillin/Streptomycin (P/S)) and rested for another 24 h prior the exposure.

Rested THP-1 macrophages were exposed to culture medium (RPMI 1640) containing either isolated rFIPs; rLZ-8, rLZ-9, rFIP-*nha*, -*fve* and X33nc, or lectins (Table 1); BSI, ConA-IV, SBA, WGA and LE, in the concentration of 1, 50 and 100 µg/ml. Cells were harvested after 6 h of exposure. RNA isolation, cDNA synthesis and RT-qPCR were performed as reported previously [23]. Inflammation-related genes, as well as TLR-2 and -4, were analysed for their expression. Primer sets of inflammation-related genes were described in [23]. Primer pair sequences of TLR-2 were 5'-ATCCTCCAATCAGGCTTCTCT-'3 (forward)

and 5'-ACACCTCTGTAGGTCACTGTTG-'3 (reverse)

and of TLR-4 are 5'-TACAAAATCCCCGACAACCTCC-'3 (forward) and

5'-GCTGCCTAAATGCCTCAGGG-'3 (reverse). Relative fold change was calculated using the $2^{-\Delta\Delta Ct}$ method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and THP-1 macrophages exposed with culture medium containing similar amount of PBS as other treatments at 6 h after exposure were used for normalization. The incubation time was selected based on our previous investigation [23]. The RT-qPCR analysis was performed twice on each sample. The experiments were performed by two biological replications.

Table 1 Sugar specificity of lectins (adapted from [15])

Lectin	Abbreviation	Carbohydrate specificity
Bandeirae simplicifolia	BSI	α-Gal, α-GalNAc
Canavalia ensiformis	ConA	α-D-Man, α-D-Glc
Glycine max	SBA	$(D\text{-GalNAc})_2$
Triticum vulgaris	WGA	$(D\text{-GalNAc})_2$, NeuNAc
Solanum esculentum	LE	$(D\text{-GlcNAc})_3$

Cytotoxicity assay

THP-1 monocytes were induced for differentiation into macrophages in 96-wells cell culture plate (8.2×10^5 cells/well). Differentiated THP-1 macrophages were washed twice with culture medium (RPMI 1640 medium without PMA but containing 10% FBS and 1% P/S) and rested for another 24 h before exposure to samples for 6 h at 37 °C at 5 % CO_2 in a humidified incubator. MTT assay was used to determine cytotoxicity of test samples as described by [24].

Hierarchical clustering

GeneMaths XT software analysis version 2.12 (Applied Maths, Belgium) was used to hierarchical cluster rFIPs and lectins regarding to hemagglutinating activity (depicted as the reciprocal of the highest dilution giving visible agglutination [25]), reduction of Caco-2 cell layer (100-X) and activation of THP-1 macrophages. All analysed data were obtained from 100 μg/ml stimulating concentration, except for the hemagglutination data. The values were expressed as [(X-X_{mean})/SD]. The green color indicates low bioactivity of the three assays, while the red color represents high bioactivity.

Statistical analysis

Comparison between treatments were calculated with one-way ANOVA with Multiple Comparisons and Duncan post-hoc test using SPSS 19 (PASW statistics, IBM, Amsterdam, The Netherlands). A value of $p<0.05$ was considered to be significant.

Results

Two novel FIPs : FIP-*nha* and LZ-9

FIP-*nha* was identified by a homology-based BLAST search using the amino acid sequence of FIP-*fve* on the *Nectria heamatococca* genome after it was released by the DOE Joint Genome Institute (http://www.jgi.doe.gov/). The nucleotide sequence of 785 base pairs (bp), including an intron sequence of 440 bp long, encodes for a protein of 114 amino acids with a calculated molecular weight of 12.84 kDa and a *p*I of 9.25. The 5-UTR-region contains a TATA-box at position -177 till -171 from the start codon. According to the amino acid PROSITE database (http://www.expasy.org/prosite/) intra-domain feature scan, FIP-*nha* contains six putative phosphorylation sites, of which only Thr[47]-Asn-Lys is conserved among the four FIPs tested (Fig 2). In addition, the scan predicted two putative N-glycosylation sites, as well as four putative N-myristoylation sites, of which only the sequence at position sites Gly[31]-Ser-Pro-Asn-Ser-Tyr and Gly[63]-Val-Arg-Asp-Ser-Tyr appeared to be conserved (Fig 1, 2) [26].

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   1 CACGTTTACTTGGCGCTTTCAGGTATATAAGAACCTCTTTATTCCTCTCAATCTCTGTTC
  61 TCAACTCAACCCTTCAATCTACACTTCCCTCTACCCAAGACAACATTTCAAAGGTCCGCA
 121 CCATCCTTGGGACTTGGTCTCGCTTGAACAAGACCTTGACTGACCCACCCCGTCACAGTT
 181 CAACCACCACACACTACACCATGGCTACCACCAATGACTCCGCCGTTCTCTTCTACATTG
                           M  A  T  T  N  D  S  A  V  L  F  Y  I
 241 TCGCGAGCCAGAAGAAGCTCAGCTTCGACTACACTCCCAACTGGGGTCGCGGAAGCCCCA
        V  A  S  Q  K  K  L  S  F  D  Y  T  P  N  W  G  R  G  S  P
 301 ACTCCTACATCGACAACCTCACCTTCCCCCGTGTCCTGACCAACAAGCCCTATAAGTACC
        N  S  Y  I  D  N  L  T  F  P  R  V  L  T  N  K  P  Y  K  Y
 361 GTGTCGTCAAGGCTGGTCAGGATCTGGGAGTTCGTGACTCCTACTCTGTGCAGTCTGACG
        R  V  V  K  A  G  Q  D  L  G  V  R  D  S  Y  S  V  Q  S  D
 421 GCTCGCAGAAGGTCAACTTCCTTGAGTACAACGCTGGTCGTGGCATCGCCGACACCCAGA
        G  S  Q  K  V  N  F  L  E  Y  N  A  G  R  G  I  A  D  T  Q
 481 CCATCCAGGTCTATGTCGTCGACCCTGACAATGGCAACCAATATCTTGTTGCCCAGTGGA
        T  I  Q  V  Y  V  V  D  P  D  N  G  N  Q  Y  L  V  A  Q  W
 541 Agtaagatgggcgcagcgtgatgagtacgatctctgtaactagccgagcgccttggctcg
        K
 601 ttgaatggaatggcggtggataggatggaatcaggcaatgttaggtagctcagccacatc
 661 aatcacattcttgactaccaaaagccgaagaatatcatctgcacagtgaagagtcgctca
 721 ttgatctcatctcatatcaaagtgttgacccgccatgtgatgctatgaaactaatgtact
 781 acactgatgtggtcttgctctggcccttcgctgctctaacaaaaccactcagcagttcca
 841 gcagtgagaagtattgtagttcaagatgtgacccgaatcttggagtgggttcatagcgca
 901 agagagtgacctgtcactcactttctcgctatttggatctatttaatactagcgttaggt
 961 atgctgatataactataacagGTGATGAGTACTTTTTATTAAAAGCCATGCATTTTTTTT
1021 TTTTTTTTAATTTTTTTTTCTTTTATTATTTTTAGGCATATATACTGAGTCATAAAAAAA
1081 TCCAGTTTATAGGCAAACCCAAAAGAGCAGTCATGGCCATTAACCGCACTTGATCGCATG
1141 TCCTTTTTGCAATGCAGGTAAAAGCTACATACTCTACAAAGCTTA
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Figure 1 The genomic DNA sequence and protein sequence of FIP-*nha* as predicted by the DOE Joint Genome Institute. The putative TATA-box is underlined. The start and stop codon are indicated in bold italic. The predicted intron is marked in grey and its splice sites are underlined. Putative serine and threonine phosphorylation sites as predicted by PROSITE are underlined in the amino acid sequence and putative N-glycosylation sites are marked in bold.
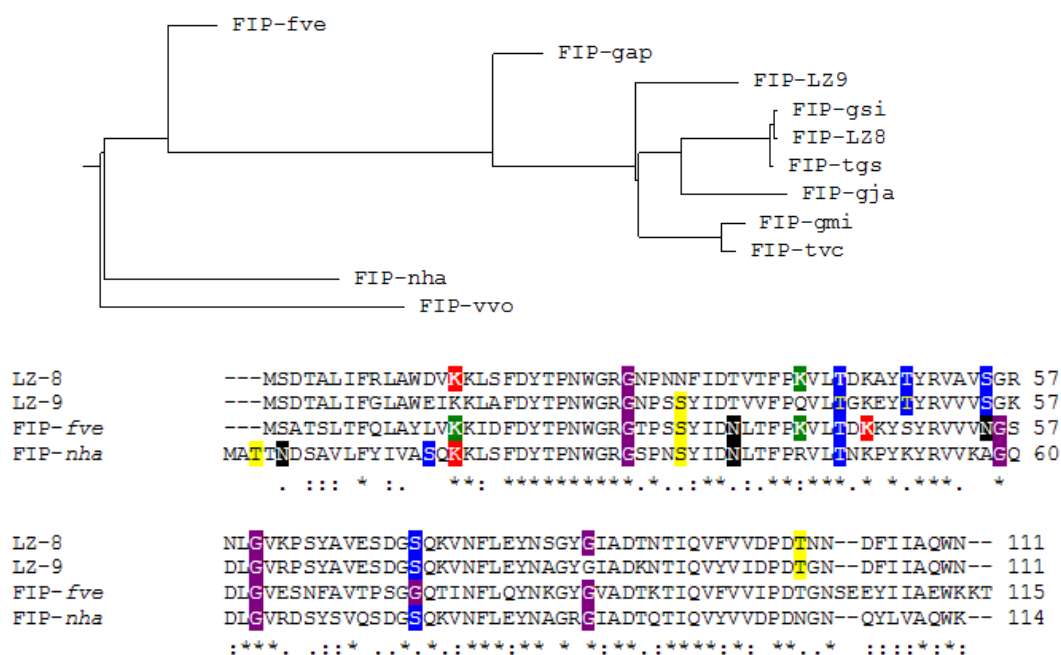
Figure 2 The Phylogenetic tree generated by Clone Manager 9.0 software using the BLOSUM62 alignment scoring matrix (upper segment) and sequence alignment generated by ClustalW2 (lower segment) based on the amino acid sequence of FIPs used in this study. Specific posttranslational modification sites are indicated as colored amino acids; casein kinase II phosphorylation (yellow), protein kinase C phosphorylation (blue), cAMP- and cGMP-dependent protein kinase phosphorylation (red), tyrosine kinase phosphorylation (green), N-glycosylation (black) and N-myristoylation (purple).

The LZ-9 gene was identified by homology based cloning using a *Ganoderma lucidum* strain while attempting to clone the LZ-8 gene. Sequence alignment analysis revealed a somewhat homologous FIP gene which shares the same N-terminal and C-terminal sequence. At the protein level, LZ-9 has 81% homology to LZ-8 and therefore it is unlikely that this gene is an analog of LZ-8. The 111 amino acid sequence, with a calculated molecular weight of 12.40 kDa and a *p*I of 4.33 lacks any putative N-glycolysation, however does possess eight predicted phosphorylation sites and two putative N-myristoylation sites.

Homology analysis using all FIPs that are currently published and that belong to this class of FIPs reveals that both FIP-*nha* and LZ-9 formed a unique separate lineage as also found for FIP-*vvo*, FIP-*fve* and FIP-*gap* indicating a substantial phylogenetic difference with the other FIPs (Figure 2). In terms of homology, FIP-*nha* shares between 57-66% amino acid sequence similarity

with other identified FIPs : the highest homology (66%) to LZ-9 and FIP-*gap*, 65% to FIP-*gmi*, 64% to Fip-*tvc*, 63% to FIP-*vvo*, FIP-*gsi* and LZ-8, 62% to FIP-*gja*, and 57% to FIP-*fve*. LZ-9 showed relatively high similarity to FIPs isolated from other Ganoderma species, 86% homology to FIP-*gmi*, 81% to LZ-8, FIP-*gja, -tgs,* and −*gsi*, 77% to FIP-*gap*, however, exhibited the highest similarity (87%) with FIP-*tvc*. With FIP-*fve, -vvo* and -*nha,* LZ-9 shares 60%, 62% and 64% of homologous amino acids, respectively. Sequence alignment by ClustalW2 indicates the existence of several conserved domain stretches among all identified FIPs as shown in Figure 2, of which functions need yet to be elucidated.

rFIPs production and purification

Recombinant proteins of LZ-8, LZ-9, FIP-*fve*, and −*nha* were produced in *P. pastoris* by means of extracellular secretion of the protein into the growth medium using the vector pPICZα-A. The protein expression of each rFIP was determined and analysed. The highest yield, of approximately 34.4 mg/litre of growth medium, was obtained for rLZ-8. rFIP-*nha* and rFIP-*fve* yielded an equal production of 18.9 mg/L, while for rLZ-9 a yield of 6.7 mg/L was reached. SDS-PAGE of the rFIPs after His-affinity based purification showed that rLZ-8 and rLZ-9 consisted of one single band of around 17 and 15 kDa, respectively, whereas rFIP-*nha* and -*fve* consisted of multiple bands (Figure 3). Protein sequencing confirmed that major protein bands in all rFIPs contained the correct FIP-amino acid sequence (data not shown). Western blot analyses indicated the presence of a c-myc-tag attached to all major bands visualized on SDS-PAGE gel (data not shown). Glycosylation staining showed that the two upper bands of rFIP-*nha* and −*fve,* indicated by arrows in Figure 3, are glycosylated proteins while the lower bands of rFIP-*nha* and −*fve*, as well as the protein bands of rLZ-8 and rLZ-9 are not glycosylated (data not shown). These results are consistent with the PROSITE N-glycosylation feature prediction showing no N-glycosylation on rLZ-8 and rLZ-9 and two N-glycosylation sites on rFIP-*nha* and −*fve*. According to the NetOGlyc 3.1 server prediction, only rFIP-*fve* contains 1 putative predicted O-glycosylation site (Thr[115]). However, as none of the rFIPs contain a signal peptide for O-glycosylation, therefore, such a glycosylation is not to be expected.
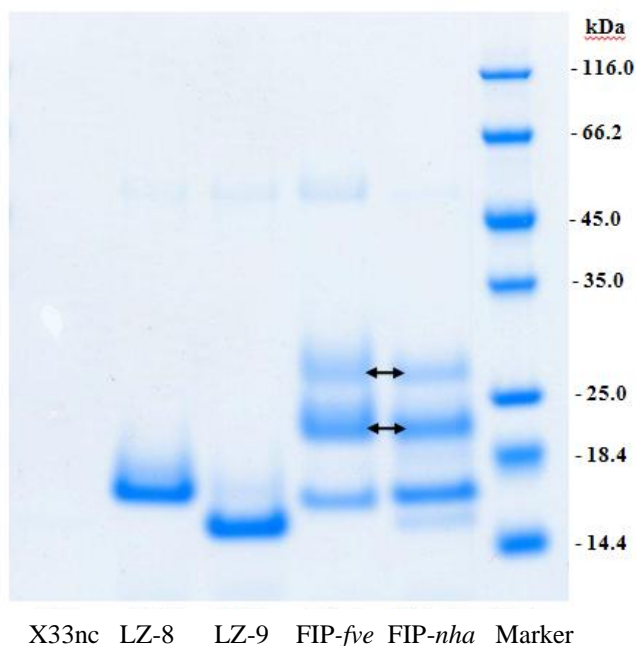
Figure 3 SDS-PAGE gel of purified rFIPs. Arrows indicate glycosylated rFIPs

LPS determination

In cell-based assays, the presence of LPS may cause biased interpretation of results. Therefore, the LPS content was analysed in the rFIPs and commercially purchased lectins using the LAL endotoxin assay kit. According to [27], one endotoxin unit (EU) is equivalent to 0.2 ng of LPS. We calculated that rLZ-8, rLZ-9, rFIP–*fve*, rFIP–*nha* were free from LPS (data not shown). Unexpectedly, *P. pastoris* wild type control (X33nc), which was produced and purified in the same way and at the same time as the other rFIPs, contained *ca*. 0.15 pg LPS/μg isolated protein. Commercial lectins LE and BSI were contaminated with 0.7 and 0.64 pg LPS/μg material, respectively, whereas ConA contained approximately 0.002 pg LPS/μg. SBA showed the highest LPS contamination with more than 550 pg LPS/μg. WGA was the only tested commercial lectin in which no LPS contamination was detected.

Hemagglutinating activity

FIPs are known for their hemagglutinating activity towards red blood cells in which different FIPs appear to have different specificity and activity towards blood from different species. Therefore, the hemagglutinating activity of all rFIPs was determined for four different blood origins: sheep, rabbit, mouse and human type O. No activity was observed on human red blood cells unless they were trypsinized before exposure to the rFIPs (Table 2). rLZ-8 and rLZ-9 showed comparable activity in all blood origins. rFIP-*fve* was only capable of agglutinating rabbit red blood cells, at a concentration of 5 µg/ml, while rFIP-*nha* exhibited agglutinating activity towards all blood origins, particularly in sheep and mouse red blood cells requiring a minimal concentration of only 0.625 µg/ml to agglutinate. Both negative controls, X33nc and PBS, showed no hemagglutinating effects on all tested blood origins. ConA-IV, used as a positive control, showed a lower hemagglutinating activity than all rFIPs, except for rLZ-8 and rLZ-9 tested in sheep red blood cells.

Table 2 The rFIP concentration required [µg/ml] to hemagglutinate different origins of red blood cells.

rFIP	Origin of red blood cells			
	Sheep	Rabbit	Mouse	Trypsinized human
LZ-8	≥20	≥2.5	≥1.25	≥0.156
LZ-9	≥5	≥2.5	≥1.25	≥0.156
FIP-*fve*	-	≥5	-	-
FIP-*nha*	≥0.625	≥5	≥0.625	≥0.156
X33nc	-	-	-	-
PBS	-	-	-	-
ConA-IV	≥5	≥20	≥5	≥2.5

Note : (-) means no agglutination observed for the highest concentration tested. PBS was tested in the same amount as present in the other treatments.

Effects of purified rFIPs and lectins on Caco-2 cell layer integrity

In order to test the effect of rFIPs and lectins on the intestinal cell integrity, 21 day-old Caco-2 cells grown in transwells were exposed to either purified rFIPs or commercial lectins in concentrations of 10 and 100 μg/ml.

LPS towards Caco-2 cell layer

Since a remarkable amount of LPS contamination was observed in most tested commercial lectins (except for WGA) and slightly for the X33nc sample, we performed an experiment to investigate whether LPS itself has an effect on TEER values of the Caco-2 cell layer. We found that exposure to an LPS concentration up to 1 μg/ml for 6 h resulted in a 95% TEER of the LPS-free condition and this value remained stable for up to 30 h (data not shown). From this we conclude that, under the chosen experimental conditions, the applied doses of LPS do not have an effect on TEER values of the Caco-2 cell layer. Therefore, all five commercial lectins and all four rFIPs including X33nc and PBS were included in this test.

Commercial lectins towards Caco-2 cell layer

Among the five tested plant lectins, only WGA showed TEER lowering effects towards the Caco-2 cell layer with approximately 17% and 25% reduction after stimulation by 10 and 100 μg/ml after 24 h of exposure, respectively. Only the latter one was statistically different from other treatments ($p < 0.05$) (Figure 4B). SBA, ConA, BSI and LE slightly lowered TEER values over the incubation in both stimulating concentrations with non-statistically difference from the un-stimulated control ($p \leq 0.05$) (data not shown). The result indicates that TEER values of these 21 days old Caco-2 cell layers can be lowered by a specific lectin type, e.g. WGA, which can be further used as a reference to assess the activity of other proteins, including FIPs, towards TEER values of Caco-2 cell layers.
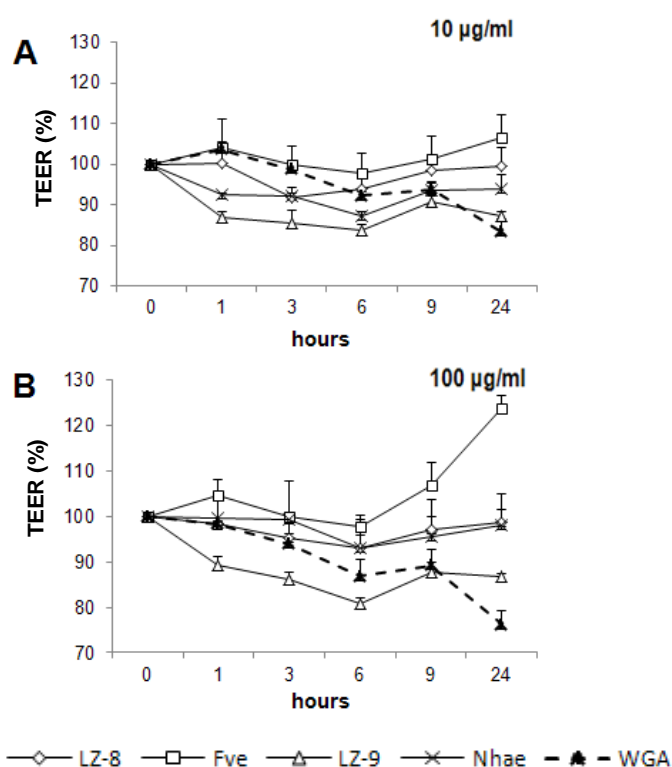
Figure 4 Kinetics of relative TEER (TEER) values of confluent Caco-2 monolayer after exposure to rFIPs and WGA at the concentration of 10 µg/ml (A) and 100 µg/ml (B). TEER values from cells exposed with culture medium containing same amount of PBS as other treatments and those of before sample addition were used for TEER normalization. Data shown are the mean plus SD from three transwells.

Purified rFIPs towards Caco-2 cell layer

A similar experimental setup as used for the plant lectins was applied for the rFIPs and X33nc in order to test their bioactivity towards changing TEER values. At the exposure concentration of 10 and 100 µg/ml, rLZ-9 appeared to significantly decrease the TEER values throughout the incubation time for both concentrations ($p<0.05$), while rFIP-*nha* exhibited a slight decrease of TEER only at 10 µg/ml concentration (Figure 4). There was no obvious TEER value difference between two stimulating concentrations of all rFIPs, as also observed for the tested lectins (data not shown), except WGA. rFIP-*fve* did not reduce TEER values at any time point. Instead, after 24h of incubation with 100 µg/ml, the TEER value statistically increased to 122%

(p<0.05). The X33nc caused no reduction of TEER in both concentrations during the complete incubation time (data not shown).

Cytotoxicity

FIPs and lectins are known to possess bioactivity towards immune cells. We, therefore, aimed to analyse the effects of rFIPs and of commercial lectins towards PMA- differentiated THP-1 cells, resembling macrophage-like cell types. However, the exposure concentration should not induce cytotoxicity, therefore purified rFIPs and commercial lectins at 50 and 100 µg/ml were firstly investigated for their cytotoxicity towards THP-1 macrophages after 6 h exposure. Overall cell viability in rFIPs treatments relative to the non-stimulated cells was shown to be over 90% (data not shown) indicating that none of the purified rFIPs and lectins were toxic to THP-1 macrophages up to 100 µg/ml concentration after 6 h of exposure. The WGA exposure in both concentrations at 6 h caused slightly decreased cell viability to 85% relative to the non-stimulated cells.

Expression of inflammation-related genes of THP-1 macrophages stimulated with purified rFIPs and lectins

As mentioned above, commercial lectins LE, BSI and SBA were contaminated with LPS with 0.7, 0.64 and >550 pg LPS/µg material, respectively. Thus, to prepare a stimulating concentration of 10, 50 and 100 µg/ml, a concentration higher than 1 pg LPS/ml exposure medium would be reached in all three concentrations for all mentioned lectins. This LPS concentration is known to activate expression of pro-inflammatory genes in THP-1 macrophages [24, 28]. Therefore, these three lectins were excluded from the THP-1 macrophage stimulation assay to avoid biased effects obtained from LPS over those of lectins. Consequently, THP-1 macrophages were stimulated with the two lectins, WGA and ConA-IV, and with the four rFIPs and its controls at a concentration of 10, 50 and 100 µg/ml to analyze their effects towards responses of immune related gene expression of macrophage after 6h of incubation.

Commercial lectins towards THP-1 macrophages

After stimulating THP-1 macrophages for 6 h with wheat germ lectin (WGA) at concentration of 10, 50 and 100 µg/ml, a high induction of pro-inflammatory genes IL-1β, IL-6, TNF-α, IL-10 and IL-8 was observed in a dose-dependent manner (Figure 5). Expression of this gene set, except for IL-6 and IL-10 which showed no expression, were only up-regulated moderately by Jack bean lectin (ConA-IV) at a exposure concentration of 100 µg/ml. The TLR-2 gene was up-regulated upon both WGA and ConA-IV exposure and TLR-4 gene was slightly induced after exposure to WGA. The highest induction of TLR-2 gene was obtained from 50 µg/ml WGA stimulation ($p<0.05$).
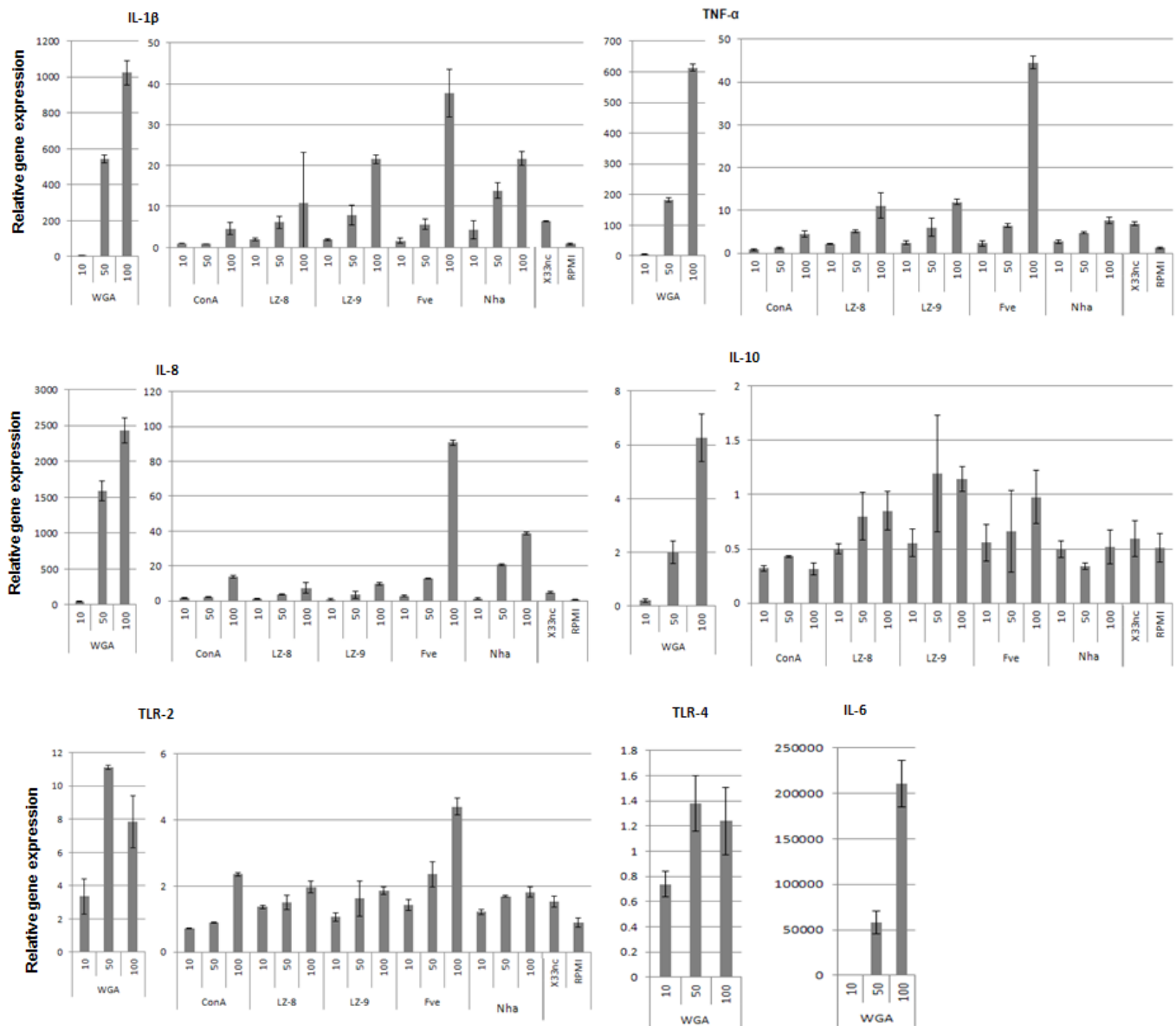
Figure 5 Expression of inflammation-related genes of THP-1 macrophages after exposure with lectin from wheat (WGA) and jack bean (ConA) at 10, 50 and 100 μg/ml after 6 h. Gene expression was expressed relative to GAPDH expression and those of cells exposed with culture medium containing same amount of PBS as other treatments (ΔΔCt). Data shown from RT-qPCR are the means ± SD from two biological and two technical replications.

Purified rFIPs towards THP-1 macrophages

As shown in Figure 5, all rFIPs activated the expression of pro-inflammatory cytokine genes IL-1β, IL-8 and TNF-α in a dose-dependent manner, while no expression was observed for the IL-6 gene (data not shown). Transcription of the IL-10 gene was down-regulated by exposure to rFIPs, for which a reverse dose-dependent effect could be observed, except for rFIP-*nha* stimulation. Among the four tested rFIPs, rFIP-*fve* was the most potent inducer of pro-inflammatory gene expression ($p<0.05$). The *P. pastoris* wild type control (X33nc) was used at a dose reflecting the highest volume addition of 100 μg/ml to stimulate THP-1 macrophages which was calculated to contain 1.31 pg LPS/ml medium. The changes in gene expression of the X33 exposure is very likely caused by the LPS contamination and possibly also the common *P. pastoris* protein background. The TLR-2 gene was slightly up-regulated upon rFIP-*fve* stimulation ($p<0.05$), for which a dose effect could be observed (Figure 5). No expression of the TLR-4 gene was found in THP-1 macrophages exposed to any rFIPs in all tested concentrations (data not shown). This indicates that both lectins and FIPs can induce the expression of inflammation-related genes in THP-1 macrophages but at the same time also demonstrate that these proteins induce unique expression patterns.

Comparison all observed bioactivities of the rFIPs and lectins

Figure 6 summarizes all results of the rFIPs and lectins in order to compare different types of *in vitro* bioactivity. The results from hierarchical clustering shows that the hemagglutinating activity clustered close to the reduction of Caco-2 cell layer integrity, while activation of THP-1 macrophages was separated in another lineage. However, the missing hemagglutinating activity of WGA, as this has not been determined, could have influenced clustering pattern. FIP-*fve* clustered closer to the control treatments than to the other three rFIPs, rLZ-8, rLZ-9 and rFIP-*nha*, indicating its unique bioactivities. LZ-8 and LZ-9 showed the closest relation in terms of bioactivities. ConA and WGA bioactivity clustered out of the rFIP protein group indicating minor relation between these two protein groups regarding to the three investigated bioactivities.
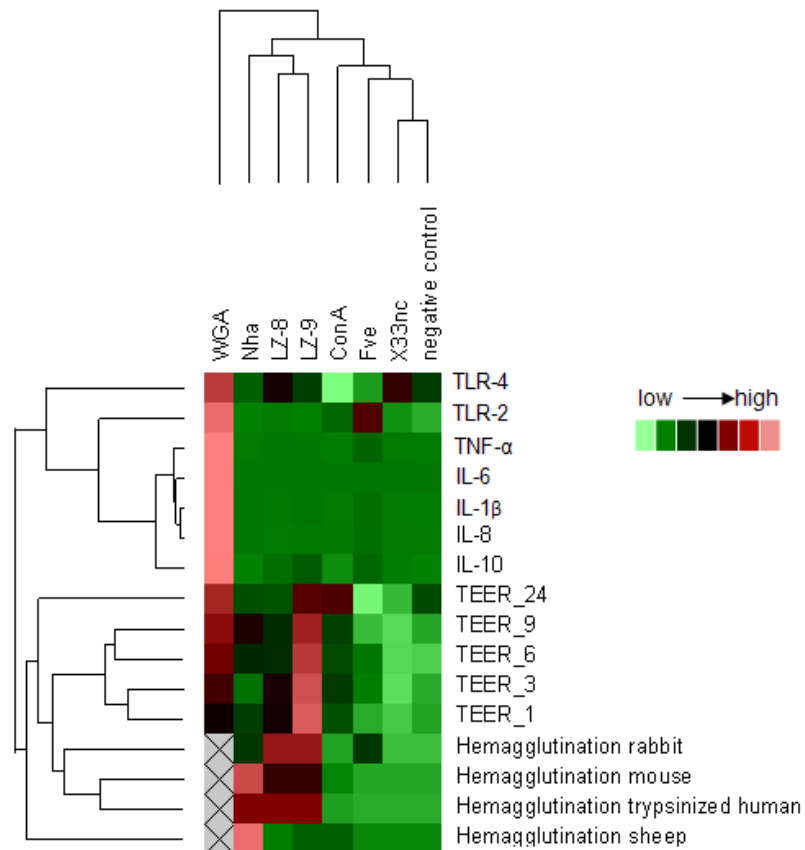
Figure 6 Hierarchical clustering of rFIPs, ConA and WGA regarding to hemagglutinating activity, interference with Caco-2 cell layer and activation of THP-1 macrophages, using GeneMaths XT v.2.12. All data were obtained from 100 µg/ml stimulating concentration, except for the hemagglutination data. The values were expressed as $[(X-X_{mean})/SD]$. The colour bar refers to low versus high bioactivity in all three assays.

DISCUSSION

Two new FIPs, FIP-*nha* and LZ-9, and two well-known FIPs, LZ-8 and FIP-*fve*, were cloned, recombinantly expressed and examined for their hemagglutinating activity, interference of Caco-2 cell layer integrity and activation of inflammation-related genes on THP-1 macrophages. To our knowledge, this study reflects the first time that four different rFIPs have been investigated for different bioactivities and in combination with comparison to certain lectins.

We were able to produce 34.4 mg of rLZ-8 per litre of growth medium using the *P. pastoris* extracellular expression system. Xue *et al.* [5] reached a yield of up to 270 mg/L rLZ-8 using a similar type of expression system. However, it was unclear whether these authors corrected for

possible presence of native yeast protein in the growth medium. Our findings showed that the control sample X33nc, representing the native protein content from *P. pastoris* wild type strain without the expression vector, contained up to 304 mg protein/L. If we do not correct for this, the protein yield of our rFIPs would be 338.4, 310.6, 322.8 and 322.8 mg/L for respectively, rLZ-8, rLZ-9, rFIP-*nha* and –*fve*. After affinity purification, the rFIP samples were examined for their purity using SDS-PAGE, western blot analysis, glycosylation staining and protein sequencing. Both rLZ-8 and rLZ-9 samples appeared as a single band of approximately 17kDa and 15kDa, respectively. For rLZ-8, this is consistent with results reported previously [5]. The deduced calculated molecular weight for both proteins including the extra c-Myc epitope and 6xHis-tag are shown to be respectively, 15.3 and 15.2 kDa for rLZ-8 and rLZ-9. The difference in protein size on SDS-PAGE from the calculation could be explained by difference in SDS-binding capacity and in splicing of the alpha signal N-terminally fused to the rFIPs sequences, which allows for extracellular protein transportation into the growth medium. Purified rFIP-*nha* and –*fve* appeared as multiple bands on SDS-PAGE gel, of which all contained a c-myc-tag and FIP amino acid sequence as confirmed by western blot and protein sequencing, respectively. In addition, our result indicates that during production of certain rFIPs, for instance, rFIP-*nha* and rFIP-*fve*, posttranslational modification like glycosylation plays a role. It is tempting to further investigate the types of glycosylation involved and whether bioactivities can be affected by such modifications.

Among the four purified rFIPs, rFIP-*nha* demonstrated the strongest hemagglutinating activity with a minimum required concentration of 0.625 µg/ml to aggregate sheep and mouse red blood cells. The recently identified FIP-*tvc* from *Trametes versicolor* exhibited hemagglutinating activity with a minimal required protein concentration of 1 µg/ml for mouse red blood cells [14]. The hemagglutinating activity of native and rLZ-8 produced in *E.coli* and *P. pastoris* was found to be 10 µg/ml for mouse red blood cells as examined by Xue *et al* [5]. None of the rFIPs in our study could agglutinate human red blood cell which corroborates previous reports [5, 14, 29].

The bioactivity of the rFIPs and lectins was evaluated towards changing the TEER of the Caco-2 cell layer to mimic the stability of intestinal epithelia. We found maximally decreased TEER values after 6 h of exposure to 10 and 100 µg/ml wheat germ lectin (WGA) which is similar to the finding of Ohno *et al*.[19]. In this latter study, 90 µg/ml of WGA exposure reduced the TEER values on Caco-2 monolayer for up to 15% but already after 4 h of exposure. This effect was not found for soybean lectin (SBA) stimulation which is identical to our experiment. Among the four

tested rFIPs, only rLZ-9 showed a pronounced decreasing effect on the TEER values of Caco-2 cell layer. At the same stimulating concentration of 10 and 100 μg/ml, rLZ-9 decreased TEER values starting after 1 h of incubation, while the effect of WGA was observable from 6 h of incubation onwards. The TEER value was found to be lowest (a 25% reduction) after 24 h of WGA incubation, while a minimum for rLZ-9 was reached at 6 h with 15% reduction, after which a slight recovery of TEER could be observed. Our report is the first to describe the influence of rFIPs on intestinal cell layer integrity, as reflected by TEER-readings. Therefore, it is difficult to speculate on a mechanism for the effect of FIPs on Caco-2 cells although the interaction between lectins and receptors at the tight junctions was hypothesized to play a role in affecting the intestinal cell layer [30].

Both lectins and FIPs have been claimed to be involved in regulation of immune functions, for instance, on antitumor activities and macrophage functions such as enhancing phagocytosis and the secretion of mediators like IFN-γ, TNF-α and nitric oxide [31-33]. Our study indicated that each type of lectin and of rFIP has a unique effect on THP-1 macrophage activating activity. Ou *et al.* [2] also showed different activity of FIP-*gts* and FIP-*fve* when human PBMCs were exposed to these preparations. The differences of FIP specific responses might relate to small changes in protein structure and folding, to the formation of homo-, di-, tri- and tetra-mers of rFIPs [2, 9], or to post-translational modification (this report) which all are likely to affect receptor binding [9]. Possible structural differences at the N-terminal α-helix region was also considered to be important for cell surface recognition [9, 34]. Although our purified rFIPs have not been investigated for their 3D-structure, the different protein band patterns visualized on SDS-PAGE are indicative for structural or posttranslational modification differences such as phosphorylation and glycosylation which might affect their bioactivities.

Considering that there was a slight correlation between the hemagglutinating activity and interference with Caco-2 cell layer but not for activation of THP-1 macrophages, we hypothesize that different regions of FIPs or lectins are required to drive the specific bioactivity towards the three different cell types used. For example, hemagglutination requires the ability to recognize and bind to cell surface carbohydrate moieties in the case of lectins [16]. However, the exact mechanism for FIPs is not known yet and needs further investigation. Based on our observations, the effects of FIPs on macrophages do not necessarily seem to be related to lectin-like carbohydrate-binding properties, but rather are based on involvement of receptors like TLRs. It has been reported that TLR-2 is necessary for the effect of an immunomodulatory protein from *Antrodia camphorate*

(bitter mushroom) on a mouse macrophage cell line to mediate NF-κB activation [4]. This could explain the up-regulation of the TLR-2 gene in our study in which rFIP-*fve* showed the strongest upregulation of TLR-2 expression as well as of the (NF-κB orchestrated) genes IL-1β, IL-8 and TNF-α. In contrast, a lectin purified from pupa enhanced translocation of NF-κB via TLR-4 in macrophages [35] which correlates to our result showing a mild up-regulation of the TLR-4 gene after stimulation by WGA, but not by rFIPs. rFIP-*fve* did not show any obvious effect on both hemagglutination and Caco-2 cell layer, but massively up-regulated inflammation-related genes IL-1β, IL-8, TNF-α and mildly the TLR-2 gene.

Taken all data from these three investigated bioactivities, we conclude that lectins and FIPs strongly share hemagglutinating activity which might be due to the presence of required active regions in both protein groups. Although hemagglutination can be a useful assay for primary bioactivity analysis of newly identified FIPs, testing for other *in vitro* biological activities, for instance, intestinal layer integrity or immune-related responses etc., should also be performed. Our observations suggest that each FIP has its own unique bioactivity profile. Since there is no clear difference among rFIPs on Caco-2 layer integrity, other activities such as effects on THP-1 macrophages are more suitable to characterize and classify FIPs. *In vivo* analysis will be necessary to develop this risk-benefit classification system for bioactive lectin-like proteins further and progress it to a practical tool in the future.

ACKNOWLEDGEMENT

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If you can't explain it simply,

you don't understand it well enough.

Albert Einstein

Chapter **6**

General Discussion

THP-1 cell line: a reliable cell model?

Due to either financial or ethical constraints related to animal and human *in vivo* studies, *ex vivo* or *in vitro* experiments become more relevant for the development of specific applications. *Ex vivo* systems have the advantage of their natural origin, however donor viability and high individual variation can make analyses and interpretation of results more complex. Mouse or human cell lines, originating from cancerous cells, are important *in vitro* tools to study cellular functions, mechanisms and responses, as well as signalling pathways, nutrient and drug transport/absorption. A drawback for the use of cell lines is that the malignant background and the cultivation of cells under controlled condition (outside their natural environment) might possibly result in different sensitivity and responses compared with normal cells in the body. Also, possibly relevant interactions between the target cells and surrounding cells, as in natural tissues, cannot be easily mimicked. Using immunological read-outs, based on decades of experimentation, a correlation between *in vitro* results and predicted *in vivo* responses has been described e.g. to evaluate probiotics [1]. An additional advantage of using human cell lines for the *in vitro* studies is that the results can be used to study cell metabolisms which can support the definite *in vivo* proven health promoting efficacy of selected compounds for human consumption.

The THP-1 cell line is isolated from the peripheral blood of a 1-year old male patient suffering from acute monocytic leukemia [2]. THP-1 cells are non-adhesive cells that express Fc and C3b receptors, but lack surface and cytoplasmic immunoglobulins. The average doubling time is 35 to 50 hours and cultures should be maintained between $2\text{-}9\text{x}10^5$ cells/ml. The THP-1 cell line has been widely used to study immune responses not only while cells are in the monocyte state but also in the macrophage-like state because of their ability to differentiate into a macrophage-like phenotype after exposure to phorbol 12-myristate 13-acetate (PMA) or to 1α, 25-dihydroxyvitamin D3 (vitaminD3) [3, 4]. There is no evidence for the presence of infectious viruses or toxic products in THP-1 cells, making the cell line relatively safe to use. Technically, THP-1 cells have some advantages over freshly isolated peripheral blood mononuclear cells (PBMCs) from both animal and human (*ex vivo*). Their homogeneous genetic background minimizes the degree of variability in the cell phenotype, which facilitates reproducibility of findings [5, 6]. In addition, THP-1 cells can be stored indefinitely in liquid nitrogen. By following an appropriate procedure, this cell line can be recovered without any obvious effect on monocyte-macrophage features and cell viability. In contrast, the availability of human PBMCs is limited, and only 3-9% of all leukocytes are

monocytes. Additional challenges for the use of PBMCs are donor variability and accessibility, contamination with other blood components (such as platelets) and a limited survival period in culture [5, 6]. However, it is important to realize that THP-1 monocytes and differentiated THP-1 macrophages can differ significantly from those derived from PBMCs, such as in the regulation of apoptosis. THP-1 is an immortalized cell line that can grow and divide indefinitely *in vitro* under proper culture conditions, while human PBMC monocytes require inflammatory mediators, for instance, IL-1β, TNF-α or LPS, to function as survival factors to prevent apoptosis [7, 8]. Under certain defined conditions, THP-1 cells cannot resemble the PBMC monocytes or tissue macrophages isolated from subjects with diseases, for instance, diabetes [9], obesity and chronic inflammation [10]. A number of publications have compared similarities and dissimilarities between the THP-1 cell line and human PBMCs with respect to various aspects as summarized in Table 1. The comparison indicates less similarity between THP-1 cells and PBMCs in the monocyte state than in macrophage state.

Table 1 Differences between THP-1 cells and human PBMCs under un-stimulated conditions

Primary cells	THP-1 cell	Human PBMCs	References
Monocyte state			
Resistin **:** a cytokine involves in inflammation, obesity and type II diabetes [11-13]	X	√	[14]
IRF5 isoforms 5-9 : proteins which regulate transcription of interferons in JAK-STAT signalling pathway [15]	X	√ (only IRF 5 and 6)	[16]
LILR-B1, -B2 and –B3 : receptors expressed by immune cell types of both myeoloid and lymphoid [17]	X	√	[18]
TLRs	Only TLR-1,-2 and -4	TLR-1 to -5	[19]
Macrophage state			
Increase of cytoplasm, mitochondria and ribosome numbers	√	√	[3]
Expression macrophage receptors : CD11b, CD 14 and CD 36	√	√	[4, 20-22]
Polarizing ability (plasticity)	√	√	Chapter 4
Macrophage morphology and adherence	√	√	[23]
Macrophage function : phagocytosis, accumulate lipids and present antigens	√	√	[24]
Resistance to apoptosis	√	√	[3]

Note : "x" and "√"means absence and presence of the indicated cell characters/functions, respectively.

Apart from comparisons between THP-1 cells and PBMCs under non-stimulated conditions, there are several studies comparing THP-1 cells with PBMCs, for instance, for cytotoxicity and expression of certain genes upon stimulated conditions. Cell sensitivity in terms of cytotoxicity was also observed to be similar for THP-1 cells and PBMCs [25]. THP-1 macrophages under LPS stimulating conditions expressed MD2, CD14 and MyD88 genes, which are also required for LPS signalling *in vivo* [26]. Sharif *et al.* [27] concluded that THP-1 cells provide an accurate and valid cell model system for evaluating the LPS response in macrophages. Other reports indicated that THP-1 cells in both the monocyte or the macrophage state are useful tools for drug and compound screening purposes [28-31] or can even be an alternative to replace the human monocytes-derived PBMCs [30]. Since PBMC-derived monocytes and macrophages have been known to express inflammation-related genes upon LPS stimulation [27, 32, 33], thus one of our preliminary experiments in Chapter 2 was aimed to investigate a dose response effect upon LPS stimulation in THP-1 monocytes in order to select a proper LPS concentration, in which cells can either be further up-regulated or be down-regulated. The THP-1 monocytes showed a LPS dose-dependent response in terms of inflammatory gene expression as shown in Figure 1. From this result, the 700 ng/ml LPS was selected to be used in the experimental chapter 2 and 4.
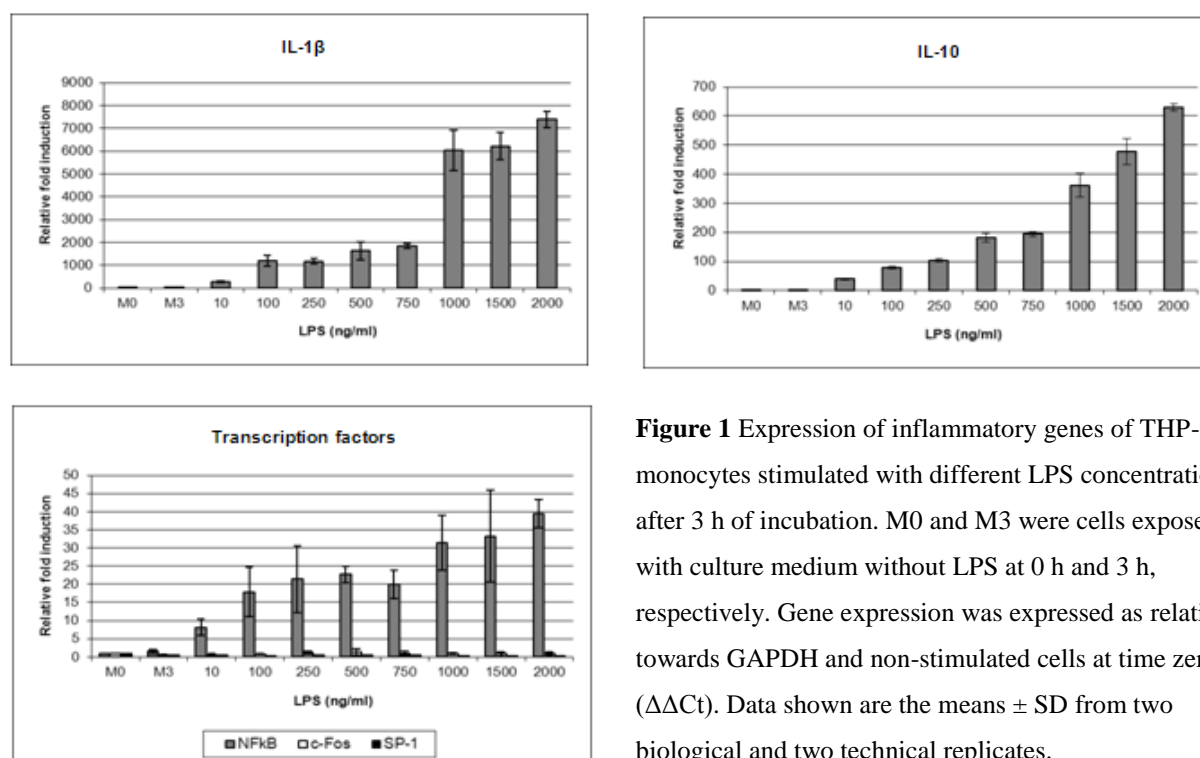

Figure 1 Expression of inflammatory genes of THP-1 monocytes stimulated with different LPS concentrations after 3 h of incubation. M0 and M3 were cells exposed with culture medium without LPS at 0 h and 3 h, respectively. Gene expression was expressed as relative towards GAPDH and non-stimulated cells at time zero ($\Delta\Delta$Ct). Data shown are the means ± SD from two biological and two technical replicates.

In addition to THP-1 cells, there are other immortalized human monocyte-macrophage cell lines such as U937, ML-2 and Mono Mac 6 cells, which have been used in biomedical research. The human monocytic U937 cell line has been widely used to study many aspects of responses of monocytes and macrophages [34-37]. By exposure to PMA or vitamin D3, U937 in the monocyte state can be differentiated to macrophage-like state, in which they showed the expression of the CD14 macrophage gene [38, 39]. Although this cell line has been also shown to express TLR-4, irrespective of differentiation status [40], and also a frequent used to study LPS response [29, 41, 42], Sharif *et al.* [27] showed that the LPS response of U937 monocytes were not responsive to LPS or were induced at very low levels which was dramatically different from THP-1 monocytes and PBMC derived macrophages. ML-2, a human myeoloblastic sub-line from ML-1 cell line, displayed morphological changes during cultivation in the presence of PMA to attach firmly to the surface of the culture flask and showed elongated and amoeboid morphology. This cell line was established in 1978, while the THP-1 cell line was developed in 1980, but the number of studies that used THP-1 related to LPS stimulation or inflammation is much higher than for ML-2 [43]. The Mono Mac 6 human cell line was established from peripheral blood of patient with a monoblastic leukemia, which showed monocyte morphological, cytochemical and immunological characteristics. This cell line can be differentiated into a macrophage-like state with the use of PGE_2, PMA or LPS [44]. However, this cell line is known to be more suitable for the study of phenotypic and functional features of *in vivo* mature monocytes due to its ability to constitutively phagocytose antibody-coated erythrocytes [45] and mycobacteria [46, 47]. In addition, it expresses the mature monocyte markers M42, LeuM3, 63D3, Mo2 and UCHMI, which could not be found on the THP-1 and U937 cell lines.

Taken together from the results of Chapter 2 and from literature studies referred to in this section, it can be concluded that THP-1 cells appear to represent a simplified, suitable and reliable model to study monocyte and macrophage functions/responses, macrophage differentiation and possible effects from external stimuli in the surrounding environment.

PMA as a differentiating stimulus

Phorbol esters are known to activate protein kinase C (PKC) (EC 2.7.11.13), which comprises a family of 12 related serine/threonine kinases implicated in the regulation of cell proliferation, differentiation and other cellular functions. The most common phorbol ester is 12-O-tetradecanoylphorbol-13-acetate (TPA), also called phorbol-12-myristate-13-acetate (PMA). PMA particularly stimulates strong expression of the iso-enzymes PKC-δ, -ε and –ζ [48]. PMA is not a naturally occurring compound, and therefore some side effects from the use of PMA as a differentiating stimulus are unavoidable. For instance, up-regulation of specific genes during the differentiation process might overwhelm the effect of stimuli on differentiated macrophages [21] or an increased sensitivity to LPS due to accumulation of NF-κB during differentiation might over-estimate effects from stimuli applied [26, 49]. Another, possibly naturally present, differentiating compound such as, 1α, 25-dihydroxyvitamin D3 is known to induce the differentiation of monocytes and the leukemia cell line THP-1, and has been shown to up-regulate transcription and strong expression of PKC-β during differentiation. However, it was suggested that THP-1 cell differentiation using vitamin D3 showed less similar to PBMC monocyte-derived macrophages than does PMA differentiation [3, 50]. As mentioned in several studies [3, 4, 50], PMA is a widely used as an acceptable stimulus to induce macrophage differentiation and in the study of macrophage functions, but possible deviations from the effects of other stimuli should not be ignored.

Comparison of responses between monocytes and macrophages

As mentioned earlier, monocytes are blood circulating cells, whereas macrophages can be found only at the site of infection/inflammation (so called inflammatory-monocyte derived macrophages) or at the tissue/lymph nodes (so called tissue-resident macrophages) [51]. Due to the circulation of monocytes throughout the body, these cells have easy access to the site of infection and respond faster in recognition of invaders or pathogen-derived substances than macrophages. Monocytes are subdivided into subsets that differ in size, trafficking and innate immune receptor expression. Their heterogeneity is typically based on their differential expression of CD14 and CD16. These monocytes are poised to rapidly extravagate into inflamed tissues at the sites of infection based on chemokine signalling as well as they can also be recruited to tumour sites and

inhibit tumour-specific immune defence mechanisms [51]. Monocytes exhibit differential gene expression profiles, including many genes involved in cell trafficking, tissue repair and destroying of ingested infectious particles, between the monocyte subsets [52]. It has been shown that inflammatory monocytes were recruited to the skin after injection of fluorescent latex beads, which are then also ingested. Most of the recruited monocytes remained in the tissue and differentiated into macrophages. In the meantime, some of the latex-bead-carrying monocytes did migrate from the skin to the lymph nodes where they differentiated into either macrophages or DCs [53, 54]. The results in Chapter 2 demonstrated that THP-1 monocytes respond faster to LPS than PMA-differentiated THP-1 macrophages do. THP-1 monocytes maximally expressed IL-1β, IL-6, IL-8, IL-10 and TNF-α genes after 3 h of LPS stimulation, while those of THP-1 macrophages appeared to be maximal after 6 h. This result illustrates the spontaneous responses of monocytes towards invasion by foreign substances.

Although slower and relatively similar fold induction in gene expression, secretion of cytokines was observed to be higher in THP-1 macrophages than in monocytes. Our results were in line with the finding of Takashiba *et al.* [49] and Schwende *et al.* [4], who observed higher TNF-α secretion in PMA-differentiated THP-1 macrophages than in monocytes as a result of higher TNF-α mRNA in macrophages under LPS stimulating conditions. This might be explained by TLRs being expressed more abundantly in THP-1 macrophages than in THP-1 monocytes under both un-stimulated and LPS-stimulated conditions [26]. Some external stimuli such as to β-amyloid fibrils could activate very rapidly THP-1 monocytes resulting in an increase of protein tyrosine phosphorylation and ERKs maximally within 1 min after stimulation [55]. These findings from both *in vivo* and *in vitro* studies lead to the conclusion that monocytes are faster in response to external stimuli such as LPS than macrophages. However, once macrophages start to react to such stimuli, they give much stronger responses in terms of mRNA expression, cytokine production, phagocytic activity and release of prostaglandin E_2 (PGE_2) than do monocytes [4]. These activities of macrophages reflect biological meaning in controlling an inflammatory situation, such as clearance of invaders and infected cells, oxidative burst, tissue homeostasis, remodelling and repair of tissues after inflammation and activation of the adaptive immune system [56, 57].

Inflammatory monocyte-derived macrophages

Monocytes are generated from committed stem cells in the bone marrow before they are released into peripheral blood, where they circulate for several days before entering tissues and replenishing the tissue macrophage population [58]. Tissue macrophages have a broad role in the maintenance of tissue homeostasis, through the clearance of senescent cells, remodelling and repair of tissues after inflammation [56, 57]. They show a high degree of heterogeneity, which reflects a specialization of function that is adopted by macrophages in different anatomical locations, for instance, alveolar macrophages in the lungs, macrophages from lamina propria in the gut, macrophages in the central nervous system, splenic macrophages, Kupfer cells in the liver, Langerhans cells in the skin and lastly inflammatory monocyte-derived macrophages [51].

It has long been believed that inflammatory monocytes are recruited and differentiated into macrophages at the site of infection [59]. In *in vitro* experiments, these macrophages are often described as being in an activation state [60-63], which can be achieved by stimulating resting macrophages with inflammation activators, for instance, LPS or pro-inflammatory cytokines. At the inflammatory lesion, macrophages are functionally and numerically dominant and many events take place, in which macrophages are involved (Figure 2). For instance, (i) the classical activation of macrophages (M1) with high microbicidal activity and pro-inflammatory cytokine production is known to take place at the site of infection, (ii) alternative activation of macrophages (M2) which associates with tissue repair and activation of humoral immunity, (iii) activation of innate immunity which is mediated by expression of TLRs, pro-inflammatory cytokine production and (iv) deactivation which is induced by the presence of IL-10 and TGF-β and reduction of MHC class II expression. In addition, also (v) macrophage plasticity, in which cells were able to easily switch from one activation state to the other, is an infection site located process [51], for which the mechanisms still have not been elucidated.
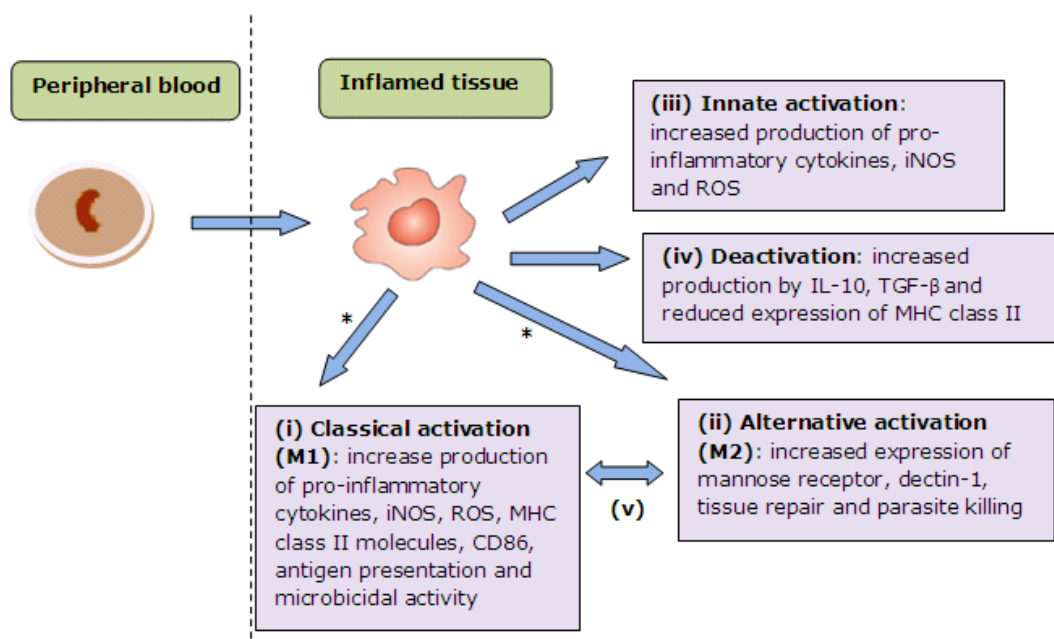
Figure 2 Macrophage heterogeneity during inflammation (adapted from [51])

In Chapter 4, we investigated whether THP-1 macrophages can be polarized into the M1 or M2 state, using stimuli applied in *ex vivo* polarization of mouse and human macrophages. The results successfully showed polarizing ability from resting THP-1 macrophage (M0) towards either the M1 or M2 state. In addition, we also examined whether an external stimulus like LPS could actually polarize the M0 state to become M1 in the THP-1 cell model. The results showed that LPS indeed up-regulated many M1 cytokine marker genes after 6 h, whose expression dramatically declined after 24 h of incubation as shown in Chapter 4. However, it is not clear whether similar profiles as found for cytokine gene expression can also be found in M1 cells for chemokine marker genes. The results showed that chemokine and their receptor M1 marker genes were up-regulated after 6 h which sustained up to 24 h of incubation (Figure 3). This finding correlates to the study of Martinez *et al.* [64] who found up-regulation of a cytokine gene, IL-6, remaining after 18 h of incubation of PBMC-derived macrophages with IFNγ+LPS, while also many M1 chemokine genes such as CXCL11, CCL19, CXCL9, CXCL10, CCL5 and CCL15 were still abundantly expressed at the same time point. It seems that when gene expression of pro-inflammatory cytokines is considered, then the M1 activation state is completed after 6 h of stimulation, but this does not apply to chemokines and their receptor genes. Further discussion can be found in Chapter 4.
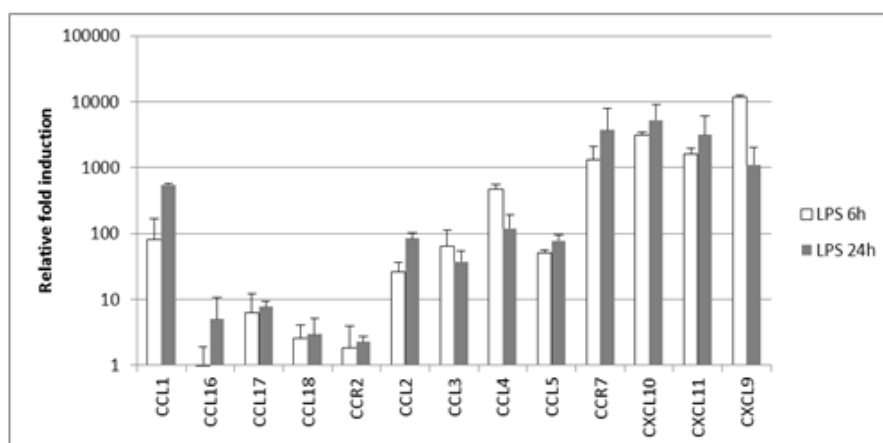
Figure 3 Chemokine and their receptor gene expression after PMA-differentiated THP-1 macrophages were stimulated with 700 ng/ml LPS for 6 and 24 h. All of these genes are described to be M1 markers according to [65]. Calculation of gene expression ($\Delta\Delta Ct$) was described in Materials and Methods of Chapter 4. Data shown + SD are from two biological and two technical replications.

THP-1 cell line as bioactive food compound screening tool

In all four experimental chapters, we showed immunomodulating properties of various food-derived compounds in the THP-1 cell model. Here, in this section, we will compare our findings on the THP-1 cell line with recent publications on *ex vivo* and *in vivo* studies, or with other immortal monocytic cell lines, to give an overview of the similarity and relevance of the data obtained.

Polyphenols and Pectin (Chapter 2)

Quercetin and citrus pectin were able to suppress expression of inflammation-related genes up-regulated during LPS stimulation in THP-1 monocytes and macrophages as described in Chapter 2. Although these two compounds share similar immunomodulating ability, different mechanisms could be expected. It has been addressed that both quercetin and kaempferol decrease IκBα degradation by inhibiting up-regulation of members of the IKK complex, thus they inhibit NF-κB activation [66]. This finding is in line with reports indicating that quercetin prevents LPS-IκB phosphorylation in bone marrow derived macrophages [67], decreases Rel-A/p65 without

modifying upstream signaling [68], and suppresses pro-inflammatory cytokine production through MAP kinase and the NF-κB pathway in LPS stimulated RAW 264.7 cells [69]. A contrary result stems from a mouse study indicated that dietary quercetin did not reduce NF-κB activation in mice with chronic diseases [70].

Pectin has been found to exhibit antimutagenic activity against nitroaromatic compounds [71]. A diet supplemented with 20% apple pectin significantly decreased a number of colon tumours in rats and reduced PGE_2 levels in the distal colonic mucosa [72]. Chen *et al.* [73] investigated citrus pectin with different degrees of esterification (DEs; DE30, DE60 and DE90) on phosphorylation of IκB in peritoneal macrophages *ex vivo* and in the mouse macrophage cell line RAW 264.7. After 30 min of LPS treatment, IκB degradation was found but DE90 pectin was able to significantly prevent such degradation, thus blocking the translocation of NF-κB p65 into the nucleus. Another suppressive mechanism of citrus pectin was its ability to bind to LPS, which might interfere with the binding of LPS to LBP and/or TLR-4 (see Fig 5).

β-glucan (Chapters 2, 3 and 4)

After oral administration, β-glucan comes in contact with the mucosal immune system, where intestinal epithelial cells together with immune cells of the Peyer's patches play a role in regulating immune responses [74, 75]. It has been suggested that orally administered barley and yeast β-glucans are taken up via Microfold (M)-cells [75], in which macromolecules of more than 2 MDa may be taken up from the intestinal lumen to the underlying lymphoid tissue [74] and via dendrites of DCs that sense and take up certain compounds across the intestinal cell layer [74, 76]. A number of receptors have been reported to recognize β-glucan, for example, TLR-2 and -4, dectin-1, complement receptor 3, lactosylceramide and some scavenger receptors, which are located on the cell membrane of macrophages, DCs and neutrophils [77-81].

After β-glucan binds to dectin-1, a cascade of intracellular signals is initiated which will ultimately lead to NF-κB activation and regulation of down-stream genes [82-84]. As mentioned in Chapter 2, NF-κB is a transcription factor regulating the expression of IL-1β and TNF-α. In Chapter 3, we also found up-regulation of these three genes in THP-1 macrophages stimulated with different β-glucans. Thus, we were interested whether the expression kinetics of these three genes were also

correlated. As shown in Figure 4, consistent expression of these three genes from THP-1 macrophages stimulated with β-glucan extracts, as well as commercial barley β-glucan and lentinan was found. This finding leads to the conclusion that PMA-differentiated THP-1 macrophages represent a reliable cell line which might be applied as a tool to further develop and examine bioactivity of β-glucans prior to investigating *ex vivo* or *in vivo* effects.
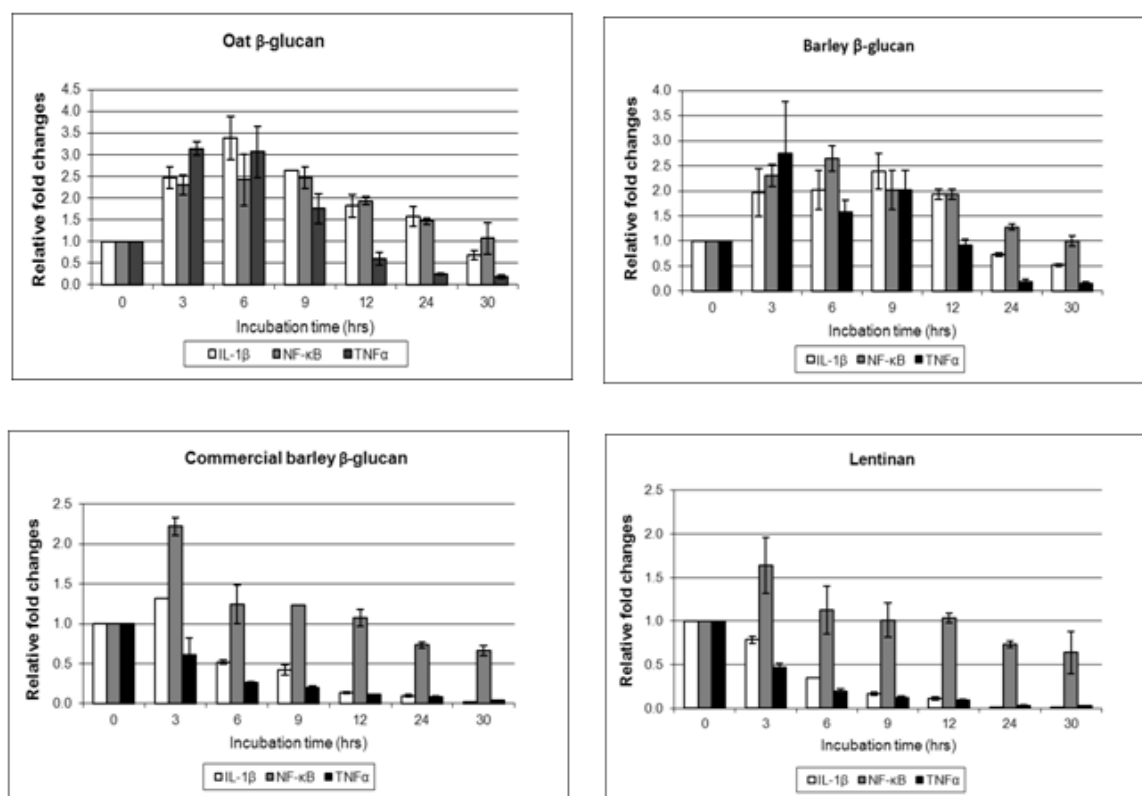

Figure 4 Expression of pro-inflammatory genes of THP-1 macrophages after stimulation with 100 µg/ml β-glucan from different sources up to 30 h of incubation. Gene expression was expressed as relative towards GAPDH and non-stimulated cells at time zero (ΔΔCt). Data shown are the means ± SD from two biological and two technical replicates.

Fungal immunomodulatory proteins (FIPs) (Chapter 5)

FIPs are immunomodulating proteins which are known in their ability as an immune modulator [85-87]. It has been shown that immunomodulatory proteins isolated from *Antrodia camphorata* (Bitter mushroom) will not exhibit bioactivities if TLR-2 is blocked. Its signalling, driven from activated TLR-2, is believed to be mediated by MyD88 which later switches on the NF-

κB [88]. Yeh *et al.* [87] demonstrated that recombinant LZ-8 (rLZ-8) protein primes macrophages via a TLR-4 independent route. Similar to our finding in Chapter 5, up-regulation of TLR-2 but not TLR-4 genes was found upon rFIPs stimulation resulted in expression of various NF-κB-regulated cytokine genes. In monocyte-derived dendritic cells, the finding of Lin *et al.* [89] suggested the immunomodulating activity of LZ-8 to proceed via TLR-4/MD2 signalling pathways. Wang *et al.* [90] demonstrated that FIP-*fve* induced the production of IL-4 and IFN-γ in T cells isolated from PBMCs via the p38 mitogen-activated protein kinase (p38 MAPK). Thus, allergy-associated disorders in which Th2 is up-regulated can be mitigated *via* the ability of FIP-*fve* to enhance activation of putative Th1 cells.

The overview of each experimental chapter in this thesis, except Chapter 4, has been simplified regarding to possible signalling pathways, participated cell surface receptors and cell responses as shown in Figure 5.

Why gene expression analysis?

In this thesis, we work with THP-1 cells in both monocyte and macrophage differentiation states. These two cell types are basically loaded with numerous receptors with various specificities. Such receptors do not function only to recognize pathogens and their fragments, but also can bind to hormones, cytokines or chemokines which are produced by the cells themselves or by neighbouring cells in an autocrine or paracrine way, respectively. The orchestration of monocytes and macrophages to react to infection or inflammation starts from cell receptor signalling resulting in up- or down-regulation of cytokine and chemokine downstream genes. Consequently, secretion of cytokines and chemokines, production of reactive oxygen species (ROS), cell migration, cell differentiation, phagocytosis and activation of adaptive immune cells etc. occurs. This allows to determine monocyte and macrophage cell responses on a scale of minutes for gene expression, of hours for cytokine production and up to a few days for cell differentiation. However, the remaining question is whether these responses can be correlated to each other? And in a quantitative or qualitative manner? It is relatively difficult to provide a simple "yes" or "no" answer because of the complex cell-cell signals and networks.

Shebl *et al.* [91] suggested that correlation between cytokine mRNA expression level and secreted protein levels relatively varied for different cytokines and chemokines in human recipients vaccinated with human papillomavirus-16 L1 virus-like particles. TNF-α mRNA and protein expression inTHP-1 monocytes challenged with Shiga toxin-1 (Stx1) were correlated in such a way that peak mRNA levels were reached at 2 h while peak protein levels were detected 3 h after toxin stimulation. Higher expression of TNF-α mRNA in Stx1 plus LPS stimulated THP-1 monocytes resulted in higher TNF-α secretion. However, this was not the case with the secretion of IL-1β in response to Stx1 although the IL-1β gene was abundantly expressed [92]. Similar to our findings in Chapter 2, the results showed that THP-1 macrophages later expressed inflammation-related genes compared to THP-1 monocytes, which also applied to cytokine production. The highest expressed inflammatory gene, IL-6, in both LPS-stimulated THP-1 monocytes and macrophages did not cause the highest protein secretion (IL-6) into the culture medium. These findings reflect the consequences of RNA stability, protein translation kinetics, and posttranslational processing which can differ per individual chemokine or cytokine gene.

The main propose of our studies was to develop an *in vitro* tool to screen and predict bioactivities from a variety of food components to better guide *in vivo* studies. Hence it is important to realize that such a screening set up requires high accessibility, safety, reliability, reproducibility and low time expenditure and costs. PCR or RT-qPCR is a common equipment which is installed in almost every molecular lab to study gene expression. Analysing secreted cytokines in culture medium will be more relevant in terms of biological functions. However, a rather long incubation time of *ca.* 18-24 h or more is required which makes it difficult to differentiate between food-cell signals and secondary cell-cell signals. Gene expression facilitates the possibility to investigate real effects from food-derived compounds, which are generally relatively mild. Such effects might have disappeared during long incubation time due to breaking down of mRNA or binding of molecules to cell receptors. Dedicated PCR array approaches with the focus on informative gene sets specific for biological functions, together with pathway based analysis and statistical tools for comparison of gene profiles can further support these strategies in which human cell lines can be used for mechanistic support or pre-screening of bioactive compounds. However, bioactive values from *in vitro* findings should be further confirmed through *ex vivo* or *in vivo* interventions. In the future, it is relatively crucial to link the data obtained from *in vitro* to those from *in vivo* to gain more predictive capacity to possible *in vivo* effects of food compounds based on *in vitro* findings.
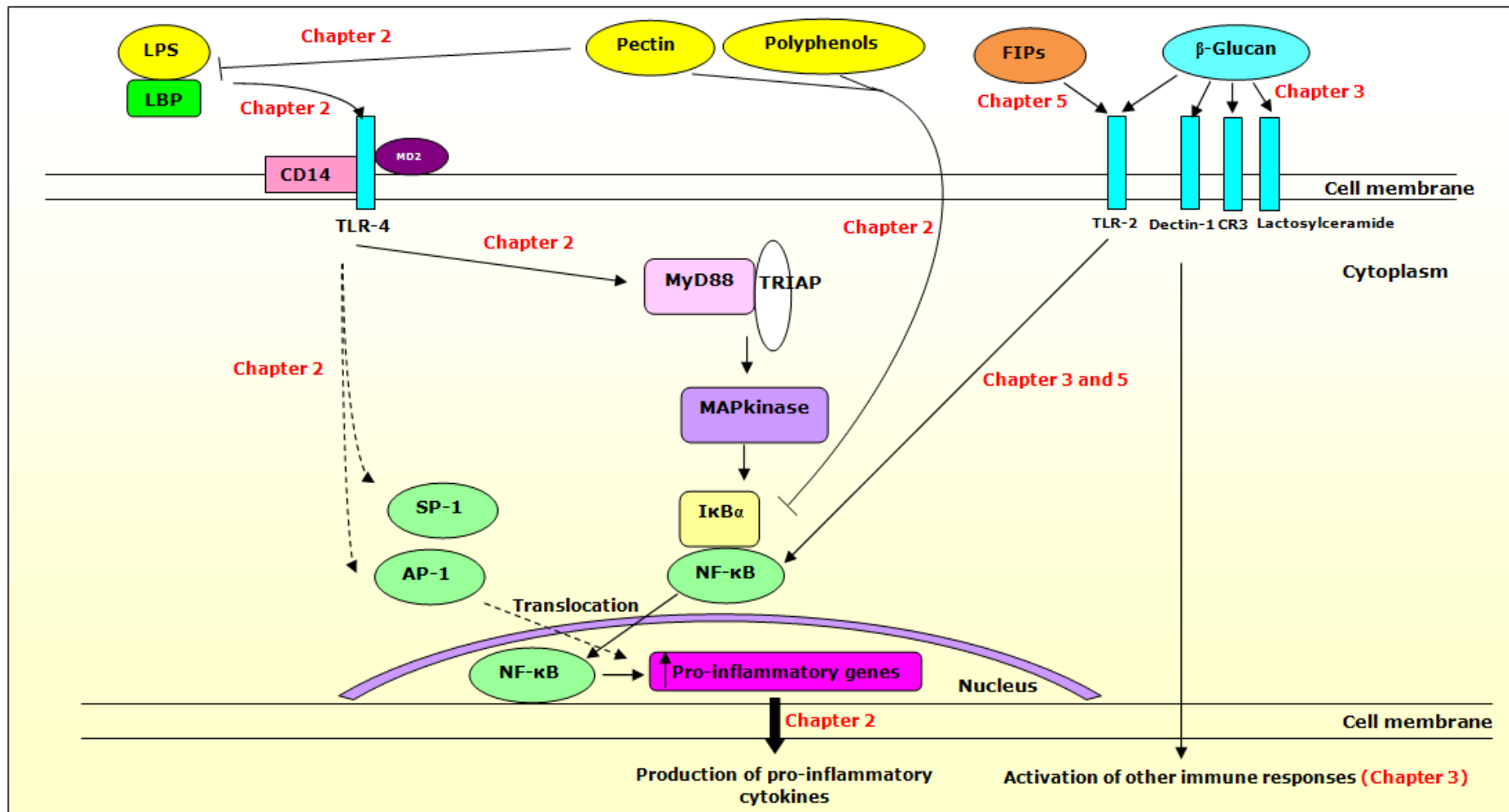
Figure 5 Simplified schematic cell responses of main interest in each experimental chapter (except chapter 4 which is indicated in Figure 2 as asterisks)

Conclusion

The THP-1 cells were used in both the monocyte and the macrophage differentiation state, but mainly in the latter one. The results obtained during this *in vitro* study show that THP-1 gene expression can be modulated by specific food compounds such as β-glucans, pectin, polyphenols and fungal immunomodulatory proteins (FIPs) in both activation and resting stage. In the activation stage, these cells have been activated by LPS to mimic an inflammatory status, either observed by simultaneous stimulation of LPS and food compounds or pre-incubation with food compounds followed by LPS stimulation. In the resting state or non-inflamed situation, cytokine gene expression of resting PMA-differentiated THP-1 macrophages without LPS challenge can be also influenced by food compounds such as β-glucans and FIPs. Macrophage plasticity and polarization is one of the complex issues in the monocyte-macrophage cell lineage. The THP-1 macrophage model shows the ability to polarize from the resting M0 state to the activated M1 and M2 state. The versatility of macrophages in response to different situations and their participation in a number of pathologies challenges researchers to explore the underlying mechanisms and to find out how to modulate their plasticity. The use of the THP-1 cell line will allow to perform relevant mechanistic studies to gain insight in these processes.

In this thesis, we have attempted to investigate several immunological responses of THP-1 monocytes and macrophages challenged with either LPS or food-derived stimuli. The evaluation whether THP- 1 cells can be used as a reliable model to represent functions and regulations in freshly isolated cells from peripheral blood *ex vivo* and in real situation *in vivo* is also taken into consideration. The integration of results from this thesis with a review of recent publications leads to the conclusion that THP-1 cells present unique characteristics as a model to investigate/estimate immunomodulating effects of food compounds in both activated and resting situations.

Future perspectives

The growing awareness of people to remain healthy is a hot issue which draws interests among researchers and policy makers. Health of human and animals is strongly related to a proper balance of immune functions which can be directly mediated by diets: so called "immune-modulation by food". The studies on such topic have gained feasibility because of the use of *in vitro* cell lines which enables to minimize the effects of culture period or genetic variation, or the restraints posed by ethical issues, donor accessibility and availability. Food compounds are usually embedded into different food matrices. Hence, after oral consumption, a food matrix passes through the gastrointestinal tract where their bioactivities might have been modified. Therefore, a simulation of *in vitro* gastric digestion becomes necessary to study stimulation of immune cells *in vivo* and *ex vivo*. If food compounds are already extracted and purified from their matrices, the challenges for the use of cultured cells should be considered in relation to their relevance for the 'real situation' after oral ingestion or subcutaneous injection. As cells *in vivo* always work as a network, co-culture systems in *in vitro* or *ex vivo* conditions may present a step forward to mimic the *in vivo* situation. Examples are co-culture of intestinal cells-macrophages, dendritic cells-macrophages and T cells-macrophages etc.

Evaluating the expression of specific marker genes in cultured cell lines might be useful to estimate bioactivity of test compounds on particular cell functions and responses. This suggests the option to use of such cells for compound screening purposes, product development and quality controls, for instance, when raw materials and production processes are changed and altered bioactivities of target compounds are expected. However, an investigation on how well the findings obtained from *in vitro* can be correlated to those from *ex vivo* or *in vivo* should be firstly considered. If good correlation could be found, the substitution of *in vitro* cell lines could then be used as a substitution to animal experiments. Future human interventions will be also required to achieve definite evidence of the bioactivity either for preventive or curative purposes of food compounds.

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Everybody is a genius. But if you judge a fish by its ability to climb a tree,

it will live its whole life believing that it is stupid.

Albert Einstein

Summaries

Summary

Samenvatting

สรุป

Summary

A number of innate immune cells with different functionalities are involved in immediate actions when infection or inflammation takes place. Monocytes can be differentiated into multi-functional macrophages both at the side of infection/inflammation and at lymph nodes situated throughout the body. There are limitations to study monocyte and macrophage functions and responses *ex vivo* due to the small number and donor-related differences of cells obtained from whole blood isolation and *in vivo* due to ethical and financial restrictions. Monocyte cell lines can be used to replace studies *ex vivo* and *in vivo*. A general introduction on the innate immune system, macrophage functions, the use of the THP-1 cell line in either the monocyte or the macrophage state, including examples of food-derived compounds which have immunomodulating properties, is given in **Chapter 1**.

In **Chapter 2**, we used LPS-stimulated THP-1 cells in either the monocyte or the macrophage differentiation state as a model to study gene expression during inflammation. We explored the differential inflammation-related gene expression between LPS-stimulated THP-1 monocytes and macrophages in terms of kinetics and magnitudes. At the translational level, secretion kinetics of the responsive cytokines was found to differ for these two cell types derived from the THP-1 cell line. Well-known immunomodulating food compounds from literature were applied to LPS-activated THP-1 monocytes and macrophages to investigate their modulating ability towards the inflamed status of the THP-1 cell model.

Chapter 3 detailed more immune-modulating effects of β-glucans, obtained from different origins (barley, oat and shiitake mushrooms), after hot water extraction. Commercially purified barley β-glucan and lentinan were also tested and their bioactivity was compared to crude β-glucan extracts. Inflammation-related gene expression kinetics after stimulation of resting PMA-differentiated THP-1 macrophages with 100 μg/ml β-glucan was investigated. A mild up-regulation of the observed genes was found in all β-glucan stimulations, however with different gene expression patterns. Pre-incubation of THP-1 macrophages with 100 μg/ml β-glucan before challenging with LPS decreased the induction of inflammation-related genes compared to the LPS treatment.

In **Chapter 4**, we aimed to examine the polarizing capability of resting PMA-differentiated THP-1 macrophages (M0) towards classical (M1) and alternative (M2) macrophages. A number of

M1 and M2 cytokine and chemokine marker genes have been analysed during polarization using RT-qPCR and human chemokine PCR array. The results showed that M0 THP-1 macrophages can be polarized to either the M1 or M2 state after stimulating with IFNγ+LPS and IL-4, respectively. LPS and food compounds (lentinan, vitamin D3 and combination of lentinan+vitaminD3) were also investigated for their polarizing ability. We observed a mild polarizing effect of these food compounds on M0-THP-1 macrophages after stimulation for 24 h.

Chapter 5 describes the expression, isolation and purification of different recombinant FIPs (rFIPs); rLZ-8, rLZ-9, rFIP-*fve* and rFIP-*nha* in the *Pichia pastoris* expression system. These FIPs were investigated for hemagglutinating ability which revealed that rLZ-8, rLZ-9 and rFIP-*nha* were able to agglutinate rabbit, mouse and sheep red blood cells while rFIP-*fve* only agglutinated rabbit red blood cells. None of the rFIPs were able to agglutinate human red blood cells unless these cells were trypsinized. By analyzing the kinetics of modifications of transepithelial electrical resistance (TEER) values of Caco-2 monolayer after exposure to rFIPs or to commercial plant lectins, a TEER reducing effect has been found for rLZ-9, rFIP-*nha* and a lectin from wheat (WGA). Of the four tested rFIPs, rFIP-*fve* induced the highest up-regulation of the genes associated with pro-inflammatory responses and also of the TLR-2 gene in THP-1 macrophages. Due to LPS contamination in the purchased commercial plant lectins, only WGA and ConA type IV were tested for their pro-inflammatory activity. WGA massively up-regulated the observed genes in a dose-dependent manner. A mild expression of the TLR-2 and TLR-4 gene was also found after WGA stimulation.

The last chapter, **Chapter 6**, discusses the important results in each chapter of this thesis, taking into account the research limitations and future research perspectives. We propose the application of the THP-1 cell line as a screening tool to predict immunomodulating properties of food-derived compounds to better guide investigations in *ex vivo* and *in vivo* systems.

Samenvatting

Een aantal celtypes van het innate immuunsysteem, met verschillende functionaliteiten, is betrokken bij reacties als gevolg van infectie of inflammatie (ontsteking). Monocyten kunnen worden gedifferentieerd tot multifunctionele macrofagen zowel op de plek waar infectie of ontsteking zich voor doet, als in lymfeklieren op allerlei locaties in het lichaam. Het *ex vivo* bestuderen van monocyten en macrofagen wordt bemoeilijkt door het kleine aantal cellen en door donor-gerelateerde verschillen tussen deze cellen als ze worden verkregen uit volbloed. *In vivo* kunnen beperkingen ook nog samenhangen met financiéle en ethische randvoorwaarden. Cellijnen van monocyten kunnen worden gebruikt in plaats van *ex vivo* en *in vivo*-onderzoeken. Een algemene inleiding in het innate immuunsysteem en de functionaliteiten van macrofagen, inclusief voorbeelden van voedingsbestanddelen die immunmodulatoire eigenschappen vertonen, worden besproken in **Hoofdstuk 1**.

In **Hoofdstuk 2** gebruiken we LPS-gestimuleerde THP-1-cellen, gedifferentieerd tot monocyten of tot macrofagen, als model om genexpressie tijdens inflammatiereacties te bestuderen. We hebben de verschillen in kinetiek en sterkte van de genexpressie onderzocht tussen LPS-gestimuleerde THP-1-monocyten en –macrofagen. Op het niveau van translatie bleek de secretiekinetiek van de responderende cytokines te verschillen voor deze twee, van de THP-1-cellijn afgeleide, celtypes. Voedselbestanddelen, waarvan uit de literatuur bekend was dat ze immuunmodulerende eigenschappen bezitten, werden getest op LPS-geactiveerde THP-1-monocyten of –macrofagen om hun effect op de inflammatietoestand van het THP-1-celmodel te onderzoeken.

In **Hoofdstuk 3** wordt dieper ingegaan op de immuunmodulerende effecten van ß-glucanen van verschillende oorsprong (gerst, haver of shiitake-paddenstoelen), na extractie met heet water. Commercieel verkregen gezuiverde ß-glucanen uit gerst en lentinan werden ook getest en hun bioactiviteit werd vergeleken met die van de ruwe ß-glucanextracten. De kinetiek van de inflammatie-gerelateerde genexpressie, na stimulering van rustende PMA-gedifferentieerde THP-1-macrofagen met 100 µg/ml ß-glucan, werd onderzocht. Een milde verhoging van de expressie van de bestudeerde genen werd waargenomen in respons op alle stimuleringen met ß-glucan-bevattende preparaten, hoewel de genexpressiepatronen verschilden. Pre-incubatie van de THP-1-macrofagen met 100 µg/ml ß-glucan vóór activering met LPS verlaagde de inductie van inflammatie-gereleateerde genen ten opzichte van de LPS-behandeling.

Hoofdstuk 4 beschrijft het vermogen van rustende PMA-gedifferentieerde THP-1-macrofagen (M0) om te polariseren tot klassieke (M1) of alternatieve (M2) macrofagen. Een aantal merkergenen, die karakteristiek zijn voor M1-, dan wel M2-geassocieerde cytokines en chemokines, zijn gedurende de polarisatie geanalyseerd met behulp van RT-qPCR en een PCR-array voor humane chemokines. De resultaten lieten zien dat M0 THP-1-macrofagen kunnen worden gepolariseerd tot ofwel de M1-, dan wel de M2-toestand, na stimulering met respectievelijk IFNγ+LPS of IL-4. Ook LPS en voedselbestanddelen (lentinan, vitamine D3 en de combinatie van lentinan en vitamine D3) werden onderzocht op macrofaag-polariserend vermogen. We namen een mild polariserend effect waar voor deze verbindingen op M0-THP-1 macrofagen na een blootstelling van 24 uur.

Hoofdstuk 5 beschrijft de expressie, isolering en opzuivering van diverse recombinante FIPs (rFIPs); rLZ-8, rLZ-9, rFIP-*fve* en rFIP-*nha* met behulp van het *Pichia pastoris* expressiesysteem. Deze FIPs werden onderzocht op haemagglutinerende activiteit. Hieruit bleek dat rLZ-8, rLZ-9 en rFIP-*nha* in staat waren om rode bloedcellen van konijnen, muizen en schapen te doen samenklonteren, terwijl rFIP-*fve* dit alleen kon met rode bloedcellen van konijnen. Geen van de rFIPs was in staat om humane rode bloedcellen te agglutineren, tenzij deze waren behandeld met trypsine. De kinetiek van de veranderingen in de transepitheliale elektrische weerstand (TEER) van een Caco-2-monolaag na blootstelling aan rFIPs of aan commerciële lectines werd geanalyseerd. Hierbij bleek dat de TEER werd verlaagd door rLZ-9, rFIP-*nha* en door een lectine uit tarwe (WGA). Van de vier geteste rFIPs induceerde rFIP-*fve* de sterkste verhoging van de expressie van genen die geassocieerd zijn met een pro-inflammatoire respons en eveneens van het gen voor TLR-2 in THP-1-macrofagen. Vanwege LPS-contaminatie in de aangeschafte commerciële plantaardige lectines konden alleen WGA en ConA-type IV worden getest op pro-inflammatoire activiteit. WGA veroorzaakte een sterke, dosis-afhankelijke, opregulering van de onderzochte genen. Een milde expressie van de TLR-2- en TLR-4-genen werd waargenomen na stimulering door WGA.

Het laatste hoofdstuk, **Hoofdstuk 6**, bediscussieert de belangrijke resultaten uit ieder hoofdstuk van dit proefschrift, met inachtneming van beperkingen in onderzoeksmogelijkheden en toekomstige onderzoeksperspectieven. Wij bepleiten het inzetten van de THP-1-cellijn als een screeningsmiddel om immuunmodulerende eigenschappen van voedselafgeleide verbindingen te voorspellen. Hiermee kunnen *ex vivo* en *in vivo*-onderzoeken beter opgezet worden.

สรุป

ด้วยหน้าที่ที่แตกต่างกันของเซลล์เม็ดเลือดขาวชนิดต่างๆ ในระบบภูมิคุ้มกันขั้นปฐมภูมิ (innate immunity) ทำให้ระบบภูมิคุ้มกันนี้มีความสามารถในการตอบสองต่อการติดเชื้อและการอักเสบด้วยความรวดเร็ว เมื่อมีการอักเสบหรือติดเชื้อเกิดขึ้นเซลล์โมโนไซต์ (monocyte) ซึ่งเป็นหนึ่งในเซลล์เม็ดเลือดขาวของระบบ ภูมิคุ้มกันขั้นปฐมภูมิสามารถเปลี่ยนเป็นเซลล์แมคโครฟาซ (macrophage) ทั้งในบริเวณที่มีการติดเชื้อหรือ อักเสบและภายในต่อมน้ำเหลือง เซลล์แมคโครฟาซนี้เป็นเซลล์ที่มีประสิทธิภาพการทำงานที่ดีกว่าเมื่อเทียบกับ เซลล์โมโนไซต์ ด้วยข้อจำกัดหลายๆ อย่างของการใช้เซลล์โมโนไซต์ที่คัดแยกมาจากตัวอย่างเลือด (ex vivo) ไม่ ว่าจะเป็นในแง่ของจำนวนเซลล์ที่มีไม่มาก, จำนวนผู้บริจาคเลือด และค่าใช้จ่ายที่สูง ทำให้การใช้เซลล์ไลน์ (cell line) เพื่อมาแทนที่การศึกษาในสิ่งมีชีวิต in vivo และการศึกษานอกสิ่งมีชีวิตแต่ใช้เซลล์จากสิ่งมีชีวิต ex vivo มี ความนิยมที่สูงขึ้น **บทที่ 1** ได้อธิบายถึงการทำงานของระบบภูมิคุ้มกันขั้นปฐมภูมิ, การทำงานของเซลล์แมคโคร ฟาส, การใช้เซลล์ไลน์ THP-1 ในรูปแบบของโมโนไซต์ และแมคโครฟาส รวมถึงตัวอย่างสารอาหารที่มี ความสามารถในการปรับเปลี่ยนการทำงานของระบบภูมิคุ้มกัน

บทที่ 2 ได้อธิบายถึงการกระตุ้น THP-1 ในรูปแบบของโมโนไซต์และแมคโครฟาสด้วย Lipopolysaccharide (LPS) ซึ่งเป็นส่วนประกอบของผนังเซลล์ E.coli เพื่อใช้เป็นโมเดลในการศึกษาการ แสดงออกของยีนส์ที่เกิดขึ้นระหว่างการอักเสบหรือติดเชื้อ จากการศึกษาพบว่าการแสดงออกของยีนส์จากเซลล์ใน รูปแบบของโมโนไซต์และแมคโครฟาสมีความแตกต่างกันในด้านของจลศาสตร์ (kinetics) และระดับของการ แสดงออกของยีนส์ (fold induction) นอกจากการแสดงออกของยีนส์ที่มีความแตกต่างกันแล้วรูปแบบของการ หลั่งของโปรตีนไซโตไคน์ (cytokine) ก็แตกต่างกันด้วย นอกจากนี้เรายังได้ศึกษาผลของสารอาหารบางชนิดที่มี ผลต่อการแสดงออกต่อเซลล์ที่ผ่านการกระตุ้นด้วย LPS (โปรดดูรายละเอียดในบทที่ 2) **บทที่ 3** แสดงผล การศึกษาจากการใช้เบต้ากลูแคน (β-glucan) ที่ผ่านการสกัดหยาบจากแหล่งต่างๆ เช่น ข้าวบาร์เล่ ข้าวโอ๊ต และ เห็ดหอม ที่มีต่อการแสดงออกของยีนส์ในเซลล์แมคโครฟาส THP-1 นอกจากนี้เรายังได้เปรียบเทียบผลการ ทดลองกับเซลล์แมคโครฟาส THP-1 ที่ผ่านการกระตุ้นด้วยเบต้ากลูแคนบริสุทธิ์ทางการค้าที่ความเข้มเข้นในการ กระตุ้นเดียวกันคือ 100 ไมโครกรัมต่อมิลลิลิตร จากการศึกษาพบว่ายีนส์ที่เกี่ยวข้องกับการอักเสบ (inflammation-related genes) ได้มีการแสดงออกในระดับอ่อนๆ จากการกระตุ้นเซลล์ด้วยเบต้ากลูแคน อย่างไร ก็ดีลักษณะการแสดงออกของยีนส์มีความแตกต่างกันตามแหล่งที่มาของเบต้ากลูแคน นอกจากนี้ยังพบว่าการให้ เบต้ากลูแคนกับเซลล์แมคโครฟาสด้วยความเข้มเข้น 100 ไมโครกรัมต่อมิลลิลิตร ก่อนที่จะนำเซลล์ไปกระตุ้นด้วย LPS สามารถลดระดับการแสดงออกของยีนส์ที่เกี่ยวข้องกับการอักเสบที่แสดงออกจากการกระตุ้นด้วย LPS

บทที่ 4 มีวัตถุประสงค์ในการศึกษาความสามารถในการปรับเปลี่ยนรูปแบบของแมคโครฟาสของเซลล์ ไลน์ THP-1 ไปยังเซลล์แมคโครฟาสแบบคลาสสิคัล (Classical M1) และแบบอัลเทอเนทีฟ (Alternative M2) ผล การศึกษาการแสดงออกของยีนส์ต้นแบบของแมคโครฟาสแบบ Classical M1 และ Alternative M2 ไม่ว่าจะเป็น

ยีนส์ที่เกี่ยวข้องกับไซโตไคน์และคีโมไคน์แสดงให้เห็นว่าแมคโครฟาสของเซลล์ไลน์ THP-1 สามารถปรับเปลี่ยน
รูปแบบไปยัง Classical M1 และ Alternative M2 หลังผ่านการกระตุ้นด้วย IFNγ+LPS และ IL-4 ตามลำดับ
นอกจากนี้เรายังได้ศึกษาผลของสารอาหาร เช่น เบต้ากลูแคนจากเห็ดหอม, วิตามินดี 3 และส่วนผสมระหว่าง
เบต้ากลูแคนจากเห็ดหอม+วิตามินดี 3 ที่มีต่อการปรับรูปแบบของแมคโครฟาส (โปรดดูรายละเอียดในบทที่ 4)

   บทที่ 5 อธิบายถึงการสกัด (extraction) และการทำให้บริสุทธิ์ (purification) ของ fungal
immumomodulatory proteins (FIPs) ที่ผ่านการแสดงออก (expression) และเพิ่มปริมาณในยีสต์ *Pichia
Pastoris* (recombinant FIPs, rFIPs) โปรตีนดังกล่าวเป็นโปรตีนที่มาจากเห็ดชนิดแตกต่างกัน; rLZ-8, rLZ-9,
rFIP-*fve* และ rFIP-*nha* (โปรดดูรายละเอียดในบทที่ 5) ผลการทดลองแสดงว่า rLZ-8, rLZ-9 และ rFIP-*nha*
สามารถทำให้เม็ดเลือดแดงของกระต่าย หนู และแกะตกตะกอนได้ในขณะที่ rFIP-*fve* สามารถทำให้เม็ดเลือด
แดงของกระต่ายเพียงเท่านั้นตกตะกอน rFIPs ทั้งสี่ชนิดไม่สามารถทำให้เม็ดเลือดแดงของมนุษย์ตกตะกอนได้
ยกเว้นแต่เม็ดเลือดแดงได้ผ่านการย่อยผนังโปรตีนออกเสียก่อน จากการทดสอบผลของ rFIPs ต่อการคงตัวของ
ชั้นเซลล์ลำไส้เล็ก Caco-2 พบว่า rLZ-9, rFIP-*nha* และโปรตีนจากข้าวสาลี (wheat germ agglutinin, WGA)
ทำให้ความคงตัวของเซลล์ลำไส้เล็กซึ่งวัดจากค่า TEER (transepithelial electrical resistance) มีค่าลดลง
นอกจากนี้เราได้ทำการศึกษาการแสดงออกของยีนส์ที่เกี่ยวข้องกับการอักเสบ (inflammation-related genes)
และยีนส์ของรีเซปเตอร์ (receptor) เช่น TLR-2 และ TLR-4 ภายหลังแมคโครฟาสของเซลล์ไลน์ THP-1 ผ่าน
การกระตุ้นด้วย rFIPs ชนิดต่างๆ ผลการศึกษาพบว่า rFIP-*fve* กระตุ้นการแสดงออกของยีนส์ดังกล่าวได้สูงสุด

   บทที่ 6 ได้แสดงการวิเคราะห์ผลการทดลองที่สำคัญของแต่ละบท รวมถึงการนำเสนอข้อจำกัดของ
งานวิจัยและข้อแนะนำสำหรับงานวิจัยในระดับถัดไป ซึ่งจากผลการวิจัยทั้งหมดพบว่าเซลล์ไลน์ THP-1 สามารถ
นำไปใช้เป็นเครื่องมือคัดแยกและประมาณการความสามารถของสารอาหารในการปรับเปลี่ยนระบบภูมิคุ้มกัน
ทั้งนี้เพื่อใช้เป็นตัวชี้นำการศึกษในระดับ *in vivo* และ *ex vivo*

Learn from yesterday, live for today and hope for tomorrow.

The important thing is not to stop questioning.

Albert Einstein

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The way is not in the sky. The way is in the heart.

Buddha

About authors

List of publications

Overview of completed training activities

Curriculum Vitae

List of publications

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Chanput W., Shuryo N. and Theerakulkait C. Introduction of new computer softwares for classification and prediction purposes of bioactive peptides : Case study in antioxidative tripeptides. **International Journal of Food Properties.** 2010 (13), pp. 947-959.

Chanput W., Theerakulkait C. and Shuryo N. Antioxidative properties of partially purified barley hordein, rice bran protein fractions and their hydrolysates. **Cereal Science**. 2009 (49), pp. 422-428.

Chanput W. Book Review. *In:* G.W. Tannock (Ed). Probiotics and prebiotics: Scientific aspects. **Food Research International.** 2006 (39), pp. 831-832.

Submitted manuscripts

Bastiaan-Net S.[#], **Chanput W**. [#], Hertz A., Zwittink R.D. Mes J.J. and Wichers H.J. Biochemical and functional characterization of recombinant fungal immunomodulatory proteins (rFIPs). (submitted)

[#]Equal contribution

Overview of completed training activities

Discipline specific activities

Courses

- System Biology Course : Statistic analysis in Omics Data, VLAG, Wageningen, The Netherlands (2008)
- Nutrition in Omics Era, WIAS, Wageningen, The Netherlands (2008)
- Immunomodulation by Food and Feed, WIAS, Wageningen, The Netherlands (2009)
- 8th Master Class in Nutrigenomics, VLAG, Wageningen, The Netherlands (2009)
- 11th Summer Course Glycoscience, VLAG, Wageningen, The Netherlands (2010)
- 3rd International Advanced Proteomics, VLAG, Wageningen, The Netherlands (2011)
- Nutrigenomics in Clinical Intervention, VLAG, Kuopio, Finland (2011)

Conferences

- 14th World Congress on Food Science and Technology, Shanghai, China (2008)
- 11th European Nutrition Conference, Madrid, Spain (2011) [#]

General courses

- PhD Competence Assessment, WGS, Wageningen The Netherlands (2008)
- Information Literacy for PhD including Endnote, WGS, Wageningen, The Netherlands (2008)
- Advanced Course Guide to Scientific Artwork, WGS, Wageningen, The Netherlands (2008)
- Techniques for Writing and Presenting Scientific Paper, WGS, Wageningen, The Netherlands (2009)
- Basic Statistics, PE&RC, Wageningen, The Netherlands (2009)
- WIAS Introduction week, WIAS, Wageningen, The Netherlands (2010)

Additional activities

- Preparation PhD research proposal, Wageningen, The Netherlands (2008)
- Food Chemistry Study trip, Ghent University, Belgium (2009)
- PhD Study trip, China (2008)[#]
- PhD Study trip, Switzerland and Italy (2010) [#]
- Member of organizing PhD study trip to Switzerland and Italy (2010)
- Meeting in the groups at FBR and CBI (2008-2012)

[#] Oral presentation

Abbreviations

CBI Cell Biology & Immunology group

FBR Food & Biobased Research

PE&RC Production Ecology and Resource Conservation

VLAG Graduate School for Nutrition, Food Technology, Agrobiotechnology and Health Sciences

WGS Wageningen Graduate Schools

WIAS Wageningen Institute of Animal Sciences

Curriculum vitae

Wasaporn Chanput was born in Bangkok, Thailand on the 14th of May1980 and grew up in Samutprakarn, Thailand. After finishing her high school at Satree-samutprakarn School, she passed the entrance examination and could enter to the Faculty of Science, Mahidol University, Bangkok, Thailand. She chose the Department of Biotechnology in which she completed her Bachelor program. She did her internship at Nestlé, Thailand in the Department of Quality Assurance and her thesis at Erawan brand where she optimised formulas to make instant Thai noodles (Ka-nom Jean). She worked for one and a half years at the Mad Science Group, the world's leading provider of science enrichment for children. After that, she decided to continue her Master program at the Department of Food Science and Technology, Faculty of Agro-Industry, Kasetsart University (KU), Bangkok, Thailand. During her Master study, she received a scholarship from University of British Columbia (UBC), Vancouver, Canada where she conducted her thesis for a full academic period. By that, she had to change her thesis topic when it was already half-way from improving functional properties of rice bran protein concentrate to computer-based prediction anti-oxidative peptides in different protein sources. During her master thesis, she was mainly supervised by Prof.dr. Shuryo Nakai (UBC) and Dr. Chockchai Teerakulkait (KU). Her master thesis took longer than expected but it was worth it, since three publications came out of it and her English was improved enormously.

After graduating Master degree in 2006, she fell in love with research and wanted to continue with her PhD abroad. After half a year of searching scholarships/funding to come abroad and being appointed as a research assistant, she went to the yearly examination, organized by the Thai government, in which they provide full funding for the whole PhD period abroad. The contract of the funding is to work for the Thai government as a lecturer/researcher in one of the universities in Thailand for certain number of years. She passed the exam and the interview and received the PhD scholarship to come to any country within Europe. She started her PhD in June 2008 at Laboratory of Food Chemistry and Laboratory of Cell Biology & Immunology in Wageningen University and Research Centre, The Netherlands, in the discipline of "Immunomodulation by Food" in which she looks at the interaction between food compounds and responses of innate immune cells, monocytes and macrophages. The research was carried out at Food & Biobased Research (FBR), Wageningen, under supervision of Prof.dr. Harry J. Wichers (chair of

Immunomodulation by Food), Prof.dr.ir. Huub J. Savelkoul. (head of the Department of Cell Biology & Immunology) and Dr. Jurriaan J. Mes (senior researcher at FBR). During her PhD project, she was involved in teaching activities of two courses. Under her supervision, three master students performed their theses in the subject of her PhD.

She will start her career as a university lecturer/researcher at the Department of Food Science and Technology, Faculty of Agro-Industry, Kasetsart University, Bangkok, Thailand in January 2013.

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Where there's a will, there's a way.