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RESEARCH ARTICLE

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

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Scope: We aimed to examine different immunological aspects of β-glucans derived from different food sources (oat, barley and shiitake) on phorbol myristate acetate (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, nuclear factor kappa B [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 upregulated 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 lipopolysaccharide (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.

Keywords:
β-Glucan / Immunomodulation / Inflammation / Pre-incubation / THP-1 macrophages

1 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 such as dectin-1, Toll-like receptor (TLR)-2, TLR-4, TLR-6, CR3, lactosylceramide and scavenger receptors that are located on innate immune cells such as macrophages, dendritic cells (DCs) and neutrophils [2–6]. The stimulation of these receptors results in upregulation of cytokine gene

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Abbreviations: HP-SEC, high-pressure size-exclusion chromatography; **MW**, molecular weight; **PAMPs**, pathogen-associated molecular patterns; **PMA**, phorbol myristate acetate; **PRRs**, pattern recognition receptors; **NF-κB**, nuclear factor kappa B; **NO**, nitric oxide; **RT-qPCR**, real-time quantitative-polymerase chain reaction; **Th**, T helper cells; **TLRs**, Toll-like receptors

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 characterized 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 gastrointestinal 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 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 nuclear factor kappa B (NF-κB) gene expression via binding to dectin-1 in association with TLR-2 and -4 in a MyD88-dependent signalling cascade [10]. It has been indicated in in vivo and in vitro studies that stimulation of β-glucan increased phagocytosis activity, nitric oxide (NO) 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. Phorbol myristate acetate (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.

2 Materials and methods

2.1 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) (*Escherichia coli* 0111:B4), commercial barley β-glucan (BG), α-amylase, β-glucanase, phenol red sodium salt and peroxidase from horseradish were purchased from Sigma (St. Louis, MO).

2.2 Extraction of β-glucan

The β-glucan extraction condition was slightly modified after Burkus and Temelli [22] and Ahmad et al. [23]. 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 30 U/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.

2.3 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 phosphate-buffered saline (PBS) pH 7 to reach the working concentration of 4 mg/mL β-glucan (dry basis) followed by sterilization by autoclaving. The sterilized 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, NJ) were determined in autoclaved samples.

2.4 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 × 7.8 mm), type GMHHR-M + Guard column running at 70°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™ version 4 (MD Scientific, Denmark) 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.

2.5 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°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 h 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.

2.6 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 (8.2×10^5 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°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 microlitres of fresh medium containing 5.5 mg/mL MTT were added into each well and incubated for 2 h at 37°C. After this incubation period, 10 μL dimethyl sulfoxide (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 enzyme-linked immunosorbent assay (ELISA) plate reader. The results were expressed relative to the control (non-stimulated cells).

2.7 Release of reactive oxygen

2.7.1 Nitric oxide

NO 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 tenfold serial dilution from 10 μg/mL to 0.1 pg/mL or β-glucan by twofold serial dilution from 200 to 1.56 μg/mL in 100 μL volume and incubated for 3 and 6 h in an incubator at 37°C in 5% CO_2. One hundred microlitre 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.

2.7.2 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 tenfold serial dilution from 10 μg/mL to 0.1 pg/mL or β-glucan by twofold serial dilution from 200 to 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°C in 5% CO_2, 10 μL of 1 M NaOH was added and subsequently the absorbance at 600 nm measured using an ELISA plate reader.

2.8 Phagocytic activity

Phagocytic assay was basically performed according to Shiratsuchi and Basson [24] 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°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 (4.1×10^4 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 [24, 25]:

$$\%\text{phagocytosis} = \frac{\text{number of cells with at least one intracellular bead}}{\text{total number of cells counted}} \times 100\%$$

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

2.9 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 real-time quantitative-polymerase chain reaction (RT-qPCR) were performed according to [26]. 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.

2.10 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 [26].

2.11 β-glucanase treatment

Briefly, the β-glucanase digestion was performed at 50°C in a heat block for 1.5 h with every 15 min vortex for 10 s. After that, the samples were heated for 90 min at 100°C in a heat block with every 15 min vortex for 10 s 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.

2.12 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.

3 Results

3.1 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.

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.

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 one endotoxin unit (EU) is equivalent to 0.2 ng of LPS [27], 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).

3.2 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. Molecular weight distribution of non- and digested β-glucans

Sample	Peak 1		Peak 2	
	Retention volume (mL)	MW (Da)	Retention volume (mL)	MW (Da)
Barley				
Non-digested	11.152	1.59×10^6	17.338	3904
Digested	Ud	Ud	16.952	294
Oat				
Non-digested	10.728	1.55×10^6	14.668	8299
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	3671
Digested	11.245	9.66×10^5	17.227	2355
Lentinan				
Non-digested	12.412	6.87×10^5	Ud	Ud

Ud, undetectable.

(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.

3.3 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.

3.4 NO 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 NO nor hydrogen peroxide could be detected in all tested compounds in the concentration range of β-glucan 1.56–200 μg/mL and LPS 0.1 pg/mL to 10 μg/mL (data not shown).

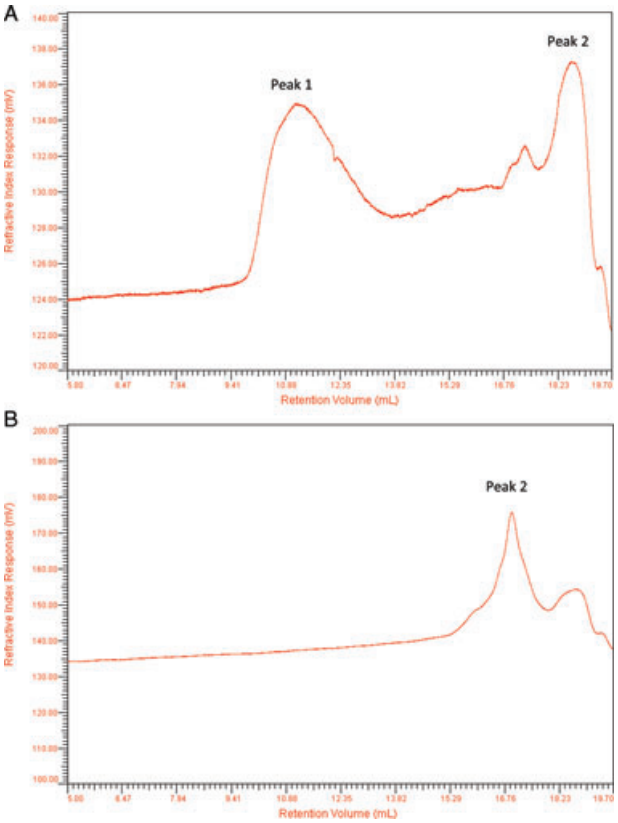

Figure 1. Molecular weight distribution of β-glucan from HP-SEC analysis; non-digested barley β-glucan extract (A) and β-glucanase-digested barley β-glucan extract (B).

3.5 Phagocytic activity

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

3.6 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

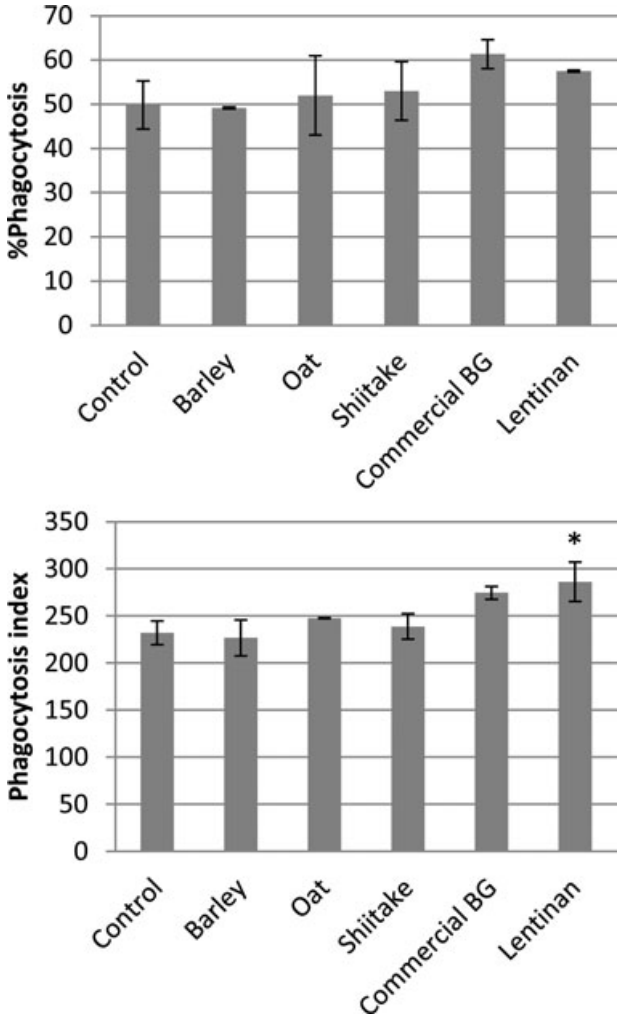

Figure 2. Phagocytic activity of 6 h pre-incubated THP-1 macrophages with 100 μg/ml β-glucan prior to 2 h incubation with serum-opsonized fluorescence-labeled latex beads (5 beads/cell). Cells were washed extensively with ice-cold PBS and fixed. Data were expressed as % phagocytosis and phagocytic index 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.

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 upregulated with a unique late upregulation 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 upregulation 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 ($p < 0.05$).

3.7 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 ($p < 0.05$).

3.8 β-glucanase treatment

Water with and without β-glucanase added was 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.

4 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

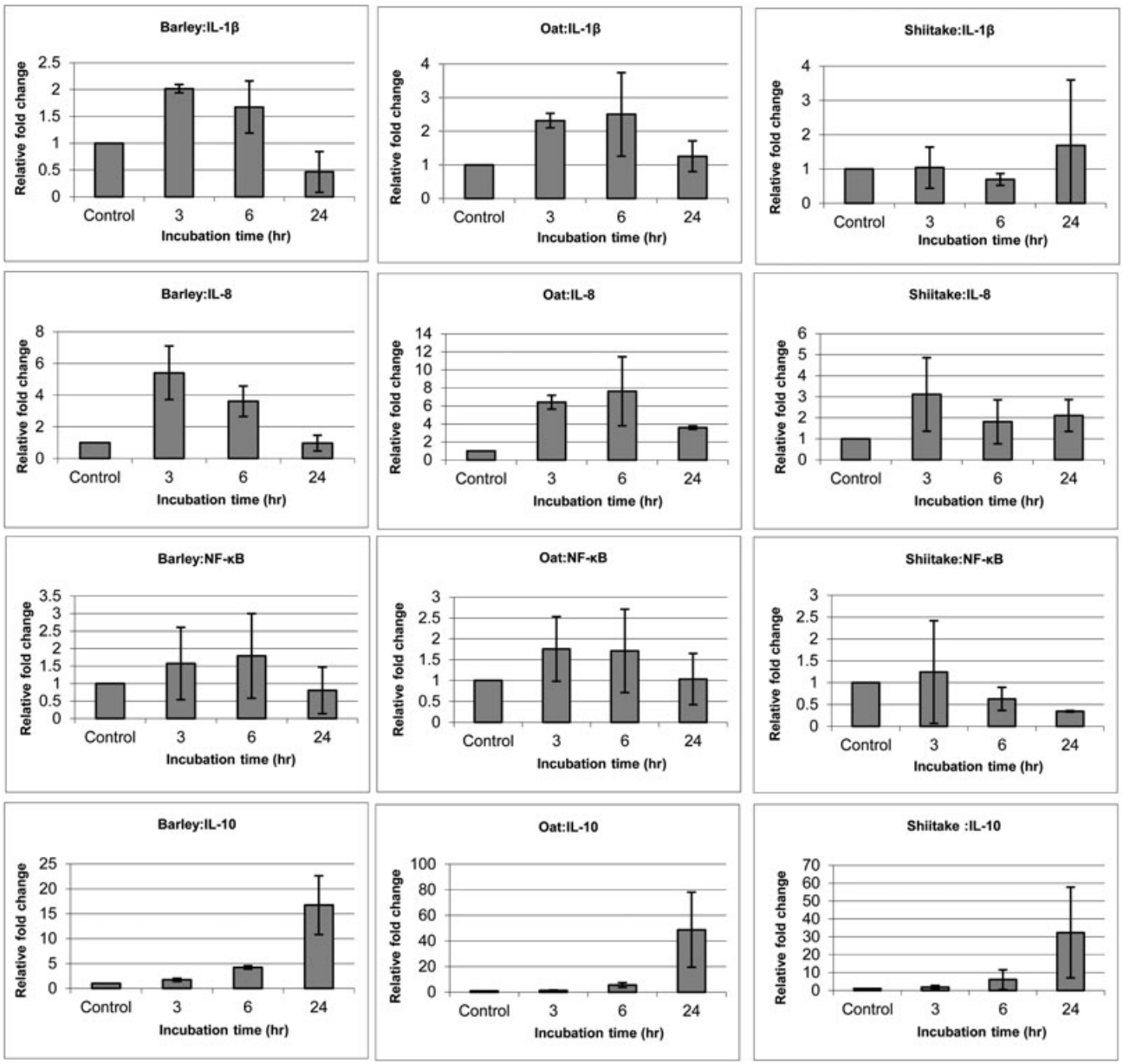

Figure 3. Differential inflammation-related gene expression kinetics of 100 μg/ml β-glucan stimulated THP-1 macrophages. Gene expression was expressed relative to GAPDH-expression and the control (non-stimulated cells) (ΔΔCt). Data shown from RT-qPCR are the means ± standard deviation (SD bars) from two biological and two technical replications.

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% [22,23]. Other extraction procedures, such as use of alkaline conditions, resulted in a higher protein content in β-glucan extract [22]. 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

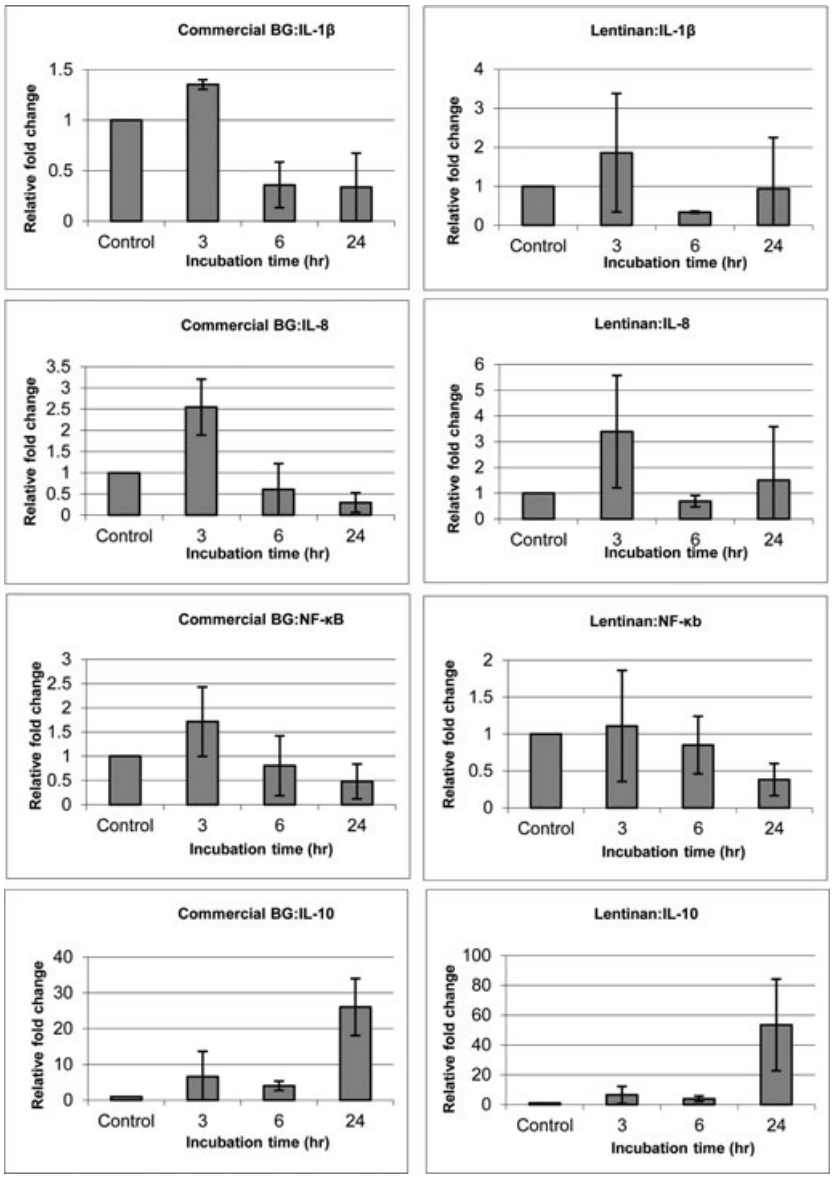

Figure 3. Continued.

also insoluble dietary fibres (cellulose, lignin, hemicellulose and waxes) can be present in the hot water extracted β-glucan preparations [22, 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 [26]. 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 [27]. 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

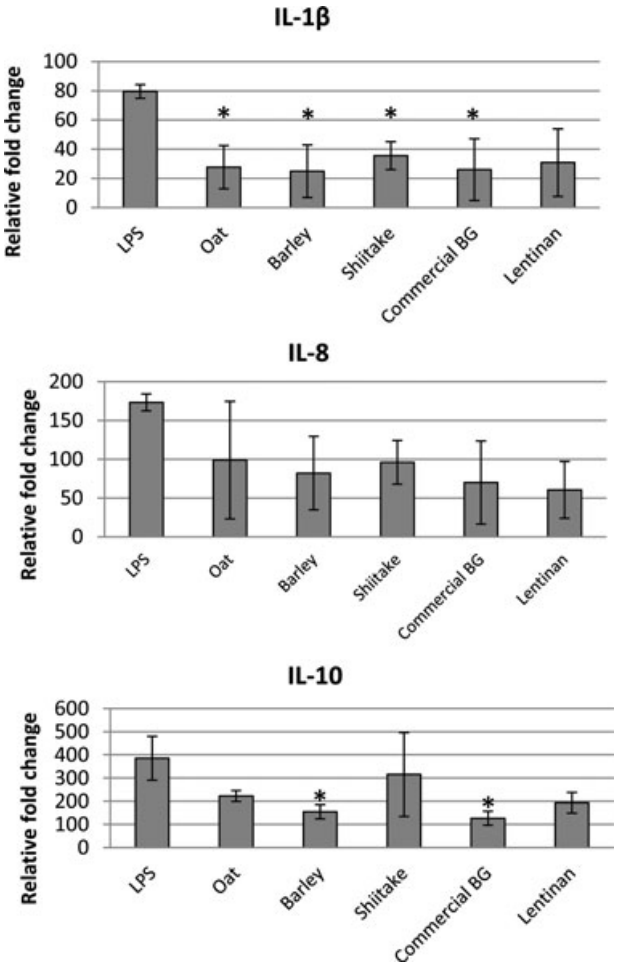

Figure 4. Inflammation-related gene expression of 3 h pre-incubated THP-1 macrophages with 100 μg/ml β-glucan prior to 6 h LPS exposure. Gene expression was expressed relative to GAPDH-expression and the control (non-stimulated cells) (ΔΔCt). Data shown from RT-qPCR are the means ± standard deviation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

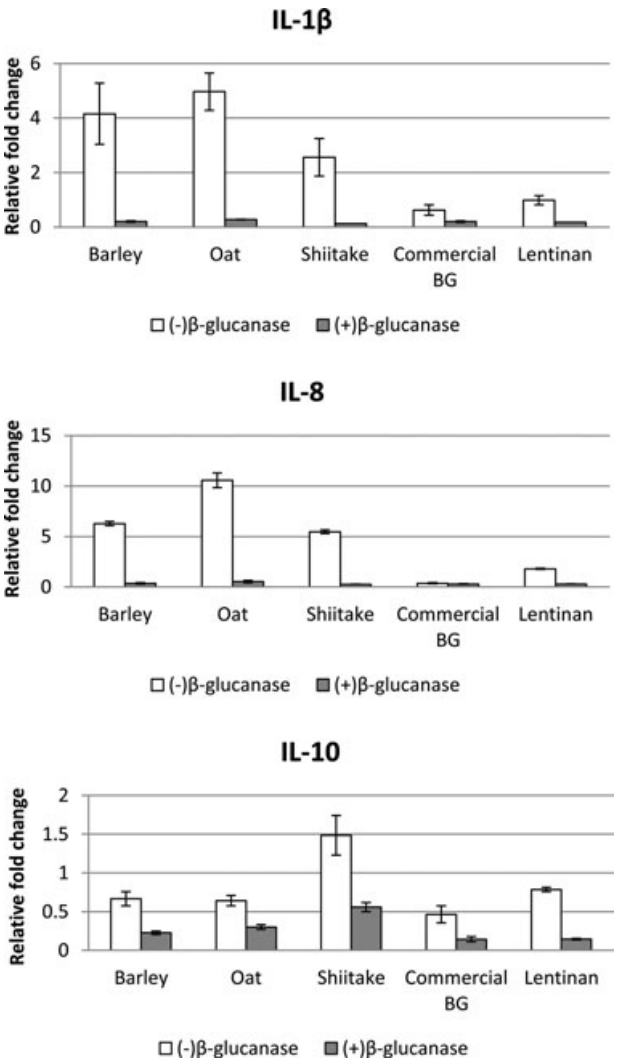

Figure 5. Inflammation-related gene expression of β-glucanase digested-β-glucan and non-digested β-glucan stimulated THP-1 macrophages. Gene expression was expressed relative to GAPDH-expression and corresponding controls (ΔΔCt) as described in "Materials and Methods" section. Data shown from RT-qPCR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

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 [26]. 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 baseline 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 immunostimulants 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 and 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 [26]. 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 with 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, MW, 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.

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