RESEARCH ARTICLE

Comparative analysis of a large panel of non-starch polysaccharides reveals structures with selective regulatory properties in dendritic cells

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Scope: Structural-based recognition of foreign molecules is essential for activation of dendritic cells (DCs) that play a key role in regulation of gut mucosal immunity. Orally ingested non-starch polysaccharides (NSP) are ascribed many health-promoting properties, but currently we lack insight into the impact of structure and size for their capacity to affect immune responses.

Methods and results: This study addresses the importance of chemical structure, size, origin and presence of contaminants for the capacity of both dietary and non-food NSP to modulate DC. Of 28 NSP products, β-glucans of microbial and plant origin and the galactomannan guar gum were found to modulate the DC cytokine pattern induced by the Toll-like receptor 4-ligand LPS giving rise to reduced IL-12p70 and increased IL-10 levels, whereas IL-6 production was unaffected. A large proportion of the tested NSP were able to down-regulate LPS-induced IL-12p70 production. The most potent NSP induced up-regulation of CD86 on DC independently of LPS stimulation. Cereal-based β-glucans showed less potency than β-glucans of microbial origin, but proper molecular weight composition and preparation may improve effectiveness.

Conclusions: Collectively, this comparative study revealed that some plant-derived NSP besides those of microbial origin exert modulation of the DC phenotype, with the exact structure being important for the activity.

Keywords:
β-Glucan / Cytokine / Immune modulation / Mannan / Surface marker

1 Introduction

Non-starch polysaccharides (NSPs) are present in variable amounts in cell walls of plants and specific microorganisms, and several health-promoting properties are attributed the presence of certain NSP in food or in dietary supplements. It is widely accepted that many NSP hold prebiotic properties and improve gut transition time [1]. More recently, the attention on the direct immunoregulatory capacities of NSP has arisen; however, the knowledge regarding the immunoregulatory capacity of different NPS is limited. Improved

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Abbreviations: APC, allophycocyanin; **BBG,** barley β-glucan; **BM,** bone marrow; **CLR,** C-type lectin receptor; **DC,** dendritic cells; **EU,** endotoxic unit; **Mw,** molar weight; **NSP,** non-starch polysaccharides; **OBG,** oat β-glucan; **PE,** phycoerythrin; **PRR,** pattern recognition receptor; **Th,** T helper; **TLR,** Toll-like receptor; **TNF,** tumor necrosis factor

understanding of the impact of specific molecular properties for the regulation of immune cells may facilitate implementation and marketing of their use as dietary supplements or support development of future therapeutic products.

Dendritic cells (DCs) represent immune cells of importance for NSP regulatory properties. NSP recognition by DC is based on DC display of glycan-binding receptors, *e.g.* the C-type lectin receptors (CLR) [2]. CLR binding in DC has previously been reported to modify signals from other pattern recognition receptors (PRRs), such as Toll-like receptors (TLR) [3], and NSP binding to other glycan-binding receptors may exert similar effects. DCs play a decisive role in orchestrating the immune response by acting as sentinels of foreign material and in activation of naive $CD4^+$ T helper (Th) cells, thereby bridging innate and adaptive immune responses [4]. Due to the central role of DC in $CD4^+$ T-cell polarization, modulation of DC activity by any compound will instruct adaptive immunity into an immunogenic (Th1, Th2, Th17) or tolerogenic (Treg) functional $CD4^+$ T-cell effector type [5, 6]. Depending on the type of DC modification, the effectiveness of both innate and adaptive immune responses may be altered. In terms of DC modulation, the production of IL-12p70 and IL-10, which are potent mediators for development of Th1 and Treg subsets, respectively, is affected [7, 8]. Moreover, the surface display of MHC class II and the co-stimulatory molecules CD80, CD86 and CD40 on DC is required to induce activation of $CD4^+$ T-cells [9]. NSP may interact with DC at the gut mucosal epithelial surface upon ingestion of NSP containing foods or dietary supplements. Sites for gut mucosal interaction between NSP and DC are at the M-cell interface upon NSP internalization, or by direct paracellular uptake of NSP by DC that penetrate their dendrites through the gut epithelial tight junctions [10–12].

Various NSP from algae, fungi and higher plants have previously been shown to stimulate diverse components of the immune system, but with varying effect, in different experimental set-ups, and presumably through different mechanisms. Most knowledge exists on the fungal β-glucans that are reported to interact with DC through the CLRs dectin-1 [13] and CR3 [14, 15]. β-Glucans are polysaccharides containing glucose as the structural component, but with great variability in structural composition. Mixed-linked β-(1,3)(1,4)-D-glucans are abundant in cereals, mainly in barley and oat, whereas branched β-(1,3)(1,6)-D-glucans are found in fungi [16, 17]. β-Glucans are also found as straight β-(1,3)-glucans (*e.g.* curdlan and paramylon) and β-(1,4)-glucans (cellulose) [18]. NSP composed of other carbohydrates have attracted less attention than β-glucans, but may hold potent immunoregulatory properties as well. Not all NSP stimulate maturation of DC, but may nevertheless modulate the maturation of DC induced by a microbial signal, such as LPS.

In order to enhance our understanding of factors important for the immunoregulatory activities of these complex nondigestible polysaccharides, we here screened a range of different NSP addressing the influence of chemical structure, size, origin and purity of NSP for their capacity to interact with and regulate the phenotype of DC.

2 Materials and methods

2.1 NSP preparations

NSPs were obtained in purified form from different chemical companies, as experimental extract preparations, and in the form of commercially available food-grade preparations. Wheat arabinoxylan, xyloglucan from tamarind, arabinan from sugar beet, larch wood arabinogalactan, rhamno-galacturonan and galactan extracted from potato, konjac mannan from *Arnorphophallus konjac*, curdlan from *Alcaligenes faecalis*, lichenan from *Cetraria islandica*, pullulan from *Aureobasidium pullulans*, yeast β-glucan, high and medium viscosity oat β-glucan (OBG4, 5), and low, medium, and high viscosity barley β-glucan (BBG4-6) were purchased from Megazyme International, Wicklow, Ireland. Gum Arabic from Acacia, agar from Gracilaria seaweeds, xanthan from *Xanthomonas*, guar gum from *Cyamopsis tetragonolobus campestris*, locust bean gum from *Ceratonia siliqua*, ι-carrageenan from *Eucheuma spinosum*, κ-carrageenan from *E. cottonii*, λ-carrageenan from *Chondrus crispus*, Zymosan from *Saccharomyces cerevisiae*, Paramylon from *Euglena gracilis*, microcrystalline cellulose and amylose from potato were purchased from Sigma-Aldrich, St. Louis, MO.

Chicory inulin (Raftiline HP) was purchased from Orafti, Tienen, Belgium, sugar beet pectin from CP Kelco, Lille Skensved, Denmark, dextran (T2000) from *Leuconostoc mesenteroides* from Pharmacia Biotech Uppsala Sweden, whereas enzymatically modified barley β-glucan was provided by Crops & Food Research, Christchurch, New Zealand (BBG1-3). Isphagula (*Plantago ovata*) was bought in a local drugstore. PromOat (OBG1) was obtained from Biovelop, Höganäs, Sweden. Two barley β-glucans (BBG7, 8) were supplied by Novozymes, Bagsværd, Denmark.

Pilot plant scale preparations of oat β-glucan were kindly provided by Dr. Peter J. Wood, Agriculture Canada, Guelph, Canada (OBG2), and by Professor (emer.) Yrjö Mälkki, Cerefi, FI-02160 Espoo (OBG3).

The enzymatically modified barley β-glucans (BBG1-3) were prepared by the following procedure; barley flour was extracted in water (1:7.5) by stirring the mixture at 45°C for 45 min. After centrifugation, the supernatant was heated to 85–90°C for 30 min with occasional stirring. After a second centrifugation, the β-glucan extract was treated with cellulase (*endo*-1,4-β-D-glucanase) from *Trichoderma longibrachiatum* (Megazyme International, Wicklow, Ireland) at three different enzyme concentrations (0.6, 6 or 60 U/mL) at 55°C for 60 min with constant stirring. Following the incubation, the samples were frozen overnight and thawed the following day. The formed gel was collected on a 45 μm mesh, gently washed and freeze dried.

2.2 NSP analysis

Except for some of the β-glucan preparations, all fiber sources were analyzed for their content of neutral and acidic polysaccharides (uronic acids) as described by Knudsen [19].

2.3 Determination of molecular weight

Samples were dissolved in water containing 0.02% sodium azide for 2 h at 70°C after pre-wetting with 50% ethanol. The molecular weight was determined by gel-permeation chromatography using a Water HPLC Module 1 (Waters, Milford, USA) fitted with a series of three columns; TSKgel GMPWxl (Tosoh Bioscience LLC, Montgomeryville, PA, USA) Shodex B-806 HQ and SB-806M HQ (Showa Denka K.K., Tokyo, Japan) and a Water 2410 Refractive Index detector (Waters, Milford, USA) using 0.2 M acetate buffer containing 2 g/L oxalic acid as eluent at a flow rate of 0.5 mL/min. The samples were calibrated against pullulan (Shodex P-82) standards (5.8, 12.2, 23.7, 48.0, 100, 186 and 380 kDa measured by an ultra centrifugal sedimentation equilibrium method by the manufacturer, Showa Denko K.K., Tokyo, Japan) and in the case of β-glucan also by (1,3),(1,4)-β-D-glucan standards (40, 82, 123, 183 and 245 kDa as measured in 50 mM sodium hydroxide on Hydrogel 2,000, 500 and 200 columns at 70°C, and dual angle light-scattering detector fitted inside WATERS M411 refractive index detector by the manufacturer, Megazyme International, Wicklow, Ireland). Molar mass values calculated relative to the β-glucan and pullulan standards using the Waters Millenium32 software are reported in weight average molar weight (Mw).

2.4 Generation and stimulation of bone marrow-derived DC

Bone marrow (BM) cells were isolated from C57BL/6 mice (Taconic Europe, Denmark) as described previously [20]. To cultivate DC, 10 mL cell suspension containing 3×10^6 stem cells was seeded in 100-mm bacteriological petri dishes at day 0 (Greiner bio-one, Kremsmünster, Austria) and incubated for 8 days at 37°C and 5% CO_2. On day 3, additional 10 mL cell culture medium was added. At day 6, cell culture medium was replaced by fresh medium. On day 8, the non-adherent cells were gently pipetted from the Petri dishes and centrifuged for 5 min at 280 g. The cells were resuspended in fresh cell culture medium without granulocyte/macrophage colony-stimulating factor, and seeded in 48-well culture plates (Corning, Corning, NY) at 1×10^6 cells/600 μL well. DCs were cultured with various NSP with or without LPS (*Escherichia coli* O26:B6; Sigma-Aldrich) in a final concentration of 1 μg/mL. Cells added medium alone were used as untreated DC. After stimulation for 18 h, culture supernatants were collected and stored at −20°C until cytokine analysis.

All animals' studies were approved by The Danish Animal Experiments Inspectorate and were carried out according to the guidelines of ''The Council of Europe Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific purpose''. Permission number: 2007/561–1266.

2.5 Cytokine quantification in culture supernatants

IL-6, IL-10, IL-12p70 and tumor necrosis factor (TNF)-α were analyzed using commercially available ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instruction. Detection limits: IL-6: 4 pg/mL, IL-10:8 pg/mL, IL-12p70: 10 pg/mL, TNF-α: 10 pg/mL.

2.6 Analysis of surface molecule expression on DC

DCs were generated and stimulated as described above except for seeding in 12-well culture plates (Nunc, Roskilde, Denmark) at day 0. Upon stimulation at day 8, cells were treated with ice cold PBS-az; containing 1% v/v heat-inactivated fetal bovine serum and 1.5% w/v sodium azide (Sigma-Aldrich) to prevent internalization of surface markers during subsequent handling of the cells. DCs were thereafter kept at 4°C or below. To block non-specific binding of Abs, cells were incubated with anti-mouse FcγIII/II receptor antibody, clone 2.4G2 (3 μg/mL, BD Bioscience, San Jose, CA, USA) before addition of fluorochrome-conjugated Ab. Upon incubation, cells were washed twice in PBS-az before analysis on a BD FACCanto II (BD Bioscience). The analysis was based on 20 000 cells and gated on viable cells. Abs used: allophycocyanin (APC)-conjugated anti-mouse CD11c, clone N418, phycoerythrin (PE)-conjugated anti-mouse CD40, clone 1C10, PE-conjugated anti-mouse CD80, clone 16-10A1, PE-conjugated anti-mouse MHCII, clone NIMR-4, all eBioscience, San Diego, CA. APC-conjugated anti-mouse CD86, clone GL1 (Southern Biotech, Birmingham, AL, USA). Isotype-matched controls: APC-conjugated Rat IgG2a, clone R35–95 (BD Bioscience), PE-conjugated rat IgG2b, clone KLH/G2b-1-2 (Southern Biotech), PE-conjugated Armenian hamster IgG, clone eBio299Arm (eBioscience), PE-conjugated Rat IgG2a (eBioscience).

Data were analyzed using FCS Express (version 3.0, De Novo Software, Los Angeles, CA).

DC purity was verified by flow cytometry using the DC marker CD11c together with microscopic inspection. The proportion of $CD11c^+$ cells was 80–90%.

2.7 Endotoxin test

NSPs were dissolved in endotoxin-free water to a final concentration of 1 mg dry matter/mL, mixed for 1 h by continuous shaking and afterwards centrifuged at 280 g for

10 min. Supernatants were collected and tested for endotoxin content using the commercially available test: Pyrochrome (Cape Cod, E. Falmouth, MA, USA) according to the manufacturer's instruction. The *Limulus* amebocyte lysate was reconstituted in Glucashield (Cape Cod) to prevent false positive results due to the presence of β-glucan.

2.8 Statistics

Data were analyzed for statistical significance (GraphPad Prism, version 4.03, GraphPad Software, San Diego, CA, USA) using one-way ANOVA and the Dunnett test (comparing individual treatments with that of a control treatment (LPS or medium)). A p-value < 0.05 was considered statistically significant.

3 Results

3.1 The influence of NSP structure and origin on modulation of LPS-induced cytokine production

Twenty-eight NSPs originating from different plant and microbial sources were characterized in regard to their DC modulating potency (Table 1). The NSPs were selected to obtain polysaccharides both of microbial and of plant origin in different structural categories of which some had previously been described as having potential immunoregulatory activities as well as NSP not former reported to be immunoregulatory but having structural familiarities. A high number of β-glucans were included with the aim of studying the effect of the main structure, origin and molecular size.

Assessment of the NSP content (Table 1) by the selected procedure, which is developed for determination in natural food and feedstuffs, led in general to slight underestimation of the content, but for some sources leading to severely low recovery, presumably due to degradation and transformation to furfural and 5-hydroxymethylfurfural.

To examine how NSP modulation of TLR-primed DC affects levels of proteins of importance for immune regulation and especially $CD4^+$ T-cell differentiation, we evaluated the secretion of IL-12p70 (Th1 polarization) and IL-10 (Treg polarization), and the levels of expression of MHC class II important for antigen presentation, as well as the co-stimulatory surface molecules CD80 (Th1), CD86 (not Th1) and CD40 (Th1) in DC. Moreover, we determined the levels of the general pro-inflammatory cytokine TNF-α, and the pleiotropic cytokine IL-6 which is involved in Th17 generation, and is generally up-regulated during pro-inflammatory conditions *in vivo*. To study how NSP modulate TLR-primed DC, the TLR4-ligand LPS was added to DC simultaneously with the individual NSP. TLR-triggering is needed as we have noticed that IL-10 and IL-12p70 is not measurable in DC upon incubation with TLR-free CLR-ligands. NSP from various sources can contain a variety of different TLR-ligands in varying amounts giving rise to variable effects in DC, but due to the nature of NSP it is not possible to purify for unknown microbial TLR-ligand contaminants. Addition of a high amount of LPS to all stimuli will level out these differences.

The NSPs differentially affected the LPS-induced production of the cytokines IL-12p70, IL-10, TNF-α and IL-6 in DC (Fig. 1). A diverse group of NSP was capable to suppress LPS-induced IL-12p70 production. Among those, the group comprising differently structured β-glucans and the galactomannan guar gum was at the same time able to augment the LPS-induced IL-10 production by a factor of 2–5. The microbial-derived β-glucans curdlan and zymosan showed the highest capacity to enhance LPS-induced IL-10 production, whereas lichenan, paramylon, yeast β-glucan, the cereal-derived β-glucans from barley and oat, and guar gum had a less pronounced but significant and dose-dependent effect. The 1,3-1,4-β-glucan lichenan and the β-glucans of microbial origin (curdlan, paramylon and zymosan) also enhanced LPS-induced TNF-α production by a factor of 4–9, with curdlan and paramylon being most potent. No significant effects of any of the tested NSP were observed in regard to modulation of LPS-induced IL-6. Importantly, the NSP inducing the highest level of IL-10 and TNF-α did not give rise to the strongest IL-12p70 inhibition. This is of importance as it has earlier been reported that enhanced levels of IL-10 inhibit IL-12p70 production [21]. The present data therefore indicate that the β-glucans and the galactomannan guar gum induce a specific regulatory pattern in DC leading to a synergistically increased IL-10 and TNF-α production and suppression of IL-12p70 upon LPS-stimulation.

3.2 Effects of NSP *per se* on cytokine production in DC

To evaluate the effect of NSP on DC without simultaneous presence of LPS, all NSP showing significant modulatory properties on LPS-induced IL-10 production in DC were examined for their capacity to stimulate DC *per se*. The production of IL-12p70, IL-10, TNF-α and IL-6 by DC upon exposure to 200 μg/mL of NSP was measured and compared with the concentration of cytokines produced by LPS-stimulated DC (Figs. 2A and B). Only curdlan gave rise to detectable amounts of IL-12p70 (Fig. 2B) with levels comparable to those obtained with LPS alone (Fig. 2A). The microbial-derived β-glucans, zymosan and curdlan were the only NSP that induced significant levels of IL-10 in DC *per se*. Lichenan, curdlan and paramylon gave rise to TNF-α induction similar to that induced by the NSP when co-administered with LPS, whereas most NSP gave rise to some IL-6 production by themselves. Only zymosan and

Table 1. Characteristics of fiber preparations

	Chemical structure	Mw[a)] (kDa)	Mw relative to pullulan (kDa)	Mw relative to β-glucan (kDa)	NSP (% dm)	Purity[a)] (%)	Other supplier information[a)]
Pectin	Galacturonan	n.a.	784	n.r.	84	n.a.	
Gum Arabic	Arabinogalactan	250	n.a.	n.r.	97	n.a.	
Inulin	1,2-β-D-fructan	3.6	4	n.r.	99.9[b)]	n.a.	Average DP 25, distribution 11–60 without sugars or polymers <11 DP
Rhamno-Galacturonan	Rhamno-Galacturonan	n.a.	15	n.r.	47	>97	Galacturonic acid 62%, Rhamnose 20%, Arabinose 3.3%, Xylose 1%, Galactose 12%, other sugars <0.1%
Agar	β-1,3-alternating 1,4 linked 3,6-galactan, methoxyl, sulphate ester or pyruvate acetal substituted	n.a.	315	n.r.	94		89% dm, 3.1% ash, 0.2% foreign organic matter, 0.2% foreign insoluble matter, viscosity 18 cP
Arabinoxylan, wheat	1,4-β-D-xylose backbone with 1,2 or 1,3 linked L-arabinofuranosyl residues	n.a.	1600	n.r.	86	>97	
Isphagula	1,4-β-D-xylose backbone with 1,2 linked L-Ara*f* and 1,3 linked L-Ara*f*-α-(1,3)-D-Xyl*p*-β-(1,3)-L-Ara*f*	n.a.		n.r.	91		
Xanthan gum	1,4-β-D-Glcp backbone substituted with manno-glucuronic-mannosid units	n.a.	n.a.	n.r.	65	n.a.	
Xyloglucan	1,4-β-D-Glc*p* backbone substituted at O6 with mono-, di-, or triglycosyl units	202	3500	n.r.	94	>97	Sugar composition: 35% xylose, 45% glucose, 16% galactose, 4% arabinose
Arabinan	1-5-α-L backbone 1-3-α-L linked arabino-furanosyl residues	n.a.	70	n.r.	88	~ 95	Sugar composition: 88% arabinose, 3% galactose, 2% rhamnose, 7% galacturonic acid
Arabinogalactan	1,4-β-D-galactan with β-D-Galp or α-L-Araf and dimer/trimer galactosid-/arabino-galactosid residues	n.a.	18	n.r.	100	>95	Purified by ultrafiltration. Sugar composition: 81% galactose, 14% arabinose, 5% other sugars
Galactan	1,4-β-D-galactan	n.a.	240	n.r.	80	n.a.	Sugar composition: 88% galactose, 3% Arabinose, 3% rhamnose, 6% galacturonic acid
Konjac mannan	β-1,4-D-glucose and D-mannose	n.a.	3900	n.r.	94	>98	Protein (N x 5.7) <0.2%, sugar composition: 60% mannose, 40% glucose, galactose, arabinose and xylose undetectable
Guar gum	1,4-β-D-mannan substituted with single 1,6-β-D-galactoside	n.a.	n.a.	n.r.	92		89.5% dm, 0.65% ash
Locust bean gum	1,4-β-D-mannan substituted with single 1,6-β-D-galactoside	310	n.a.	n.r.	88		89% dm, 0.4% ash, viscosity 2700 cP
λ-Carrageenan	β1,4-α1,3-D-galactan, sulfated	n.a.	3700	n.r.	64[c)]	n.a.	
ι-Carrageenan	β1,4-α1,3-D-galactan, sulphated	n.a.	4000	n.r.	54[c)]	n.a.	
κ-Carrageenan	β1,4-α1,3-D-galactan, sulphated	n.a.	2300	n.r.	58[c)]		5.6% K, 2.7% Ca, 0.6% Na
Pullulan	1,4-1,6-α-D-glucan	n.a.	300	n.r.	94	>95	96% dm, <0.3% protein, ~3% ash. Viscosity 2–3 cP at 1% 30°C

Table 1. Continued

	Chemical structure	Mw[a)] (kDa)	Mw relative to pullulan (kDa)	Mw relative to β-glucan (kDa)	NSP (% dm)	Purity[a)] (%)	Other supplier information[a)]
Dextran	α-D-1,6-glucan with 1,3-a-D-glucopyranosyl side chains	2000	n.a.	n.r.	99	n.a.	
Curdlan	1,3-β-D-glucan	n.a.	n.a.	n.a.	93	>99	
Paramylon	1,3-β-D-glucan	~500	45	45	103	n.a.	
Zymosan	1,3-1,6-β-D-glucan	n.a.	n.a.	n.a.	83	n.a.	93% dm, 1.9% nitrogen, 0.8% phosphorus
Yeast β-glucan	1,3-1,6-β-D-glucan	n.a.	80	55	n.a.	>90	97.8% dm, <0.1% α-glucan, 0.1% protein, 0.1% ash
Cellulose	1,4-β-D-glucan	<50	n.a.	n.a.	110	n.a.	95–97% dm, <0.05%, <0.25% water soluble substances, <0.05% ether soluble substances, DP <350
Lichenan	1,3-1,4-β-D-glucan	n.a.	170	94	96	>85 (99.5% NSP)	98% glucose with 85% as 1,3-1,4-β-D-glucan, the remainder possibly isolichenan (α-linked form), <0.1% starch, 1.5% arabinose, undetectable mannose, xylose and uronic acids
BBG1	1,3-1,4-β-D-glucan	13	25	35	76	n.a.	
BBG2	1,3-1,4-β-D-glucan	65	110	75	80	n.a.	
BBG3	1,3-1,4-β-D-glucan	125	215	110	81	n.a.	
BBG4	1,3-1,4-β-D-glucan	137	350	150	n.a.	n.a.	98% dm, 1% protein, <0.0% starch. Viscosity 5 cSt at 1% 30°C
BBG5	1,3-1,4-β-D-glucan	260	620	215	n.a.	~96	97.8% dm, 0.4% protein, <0.3% arabinoxylan, <0.1% starch, 0.3% ash. Viscosity 28 cSt at 1% 30°C
BBG6	1,3-1,4-β-D-glucan	320	2300	430	n.a.	n.a.	96.4% dm, 1.2% protein, <0.1% starch. Viscosity >100 cSt at 1% 30°C
BBG7	1,3-1,4-β-D-glucan	4.6	5	20	n.a.	n.a.	
BBG8	1,3-1,4-β-D-glucan	8.6	10	25	n.a.	n.a.	
OBG1	1,3-1,4-β-D-glucan	>1500	1300	320	n.a.	n.a.	93.3% dm, 34–36% soluble β-glucan, 35–37% total DF, 54–56% maltodextrins, 2.5–3.5% protein, 3–4% ash, 0.5–1% fat
OBG2	1,3-1,4-β-D-glucan	1175	3200	485	n.a.	n.a.	81% β-glucan, 2% starch
OBG3	1,3-1,4-β-D-glucan	n.a.	75	60	n.a.	n.a.	
OBG4	1,3-1,4-β-D-glucan	n.a.	700	220	n.a.	>97	95% dm, <0.1% starch, <0.5% arabinoxylan, 0.35% protein, 0.5% ash. Viscosity 20–30 cSt at 1%
OBG5	1,3-1,4-β-D-glucan	n.a.	1000	290	n.a.	>97	98.8% dm, <0.1% starch, <0.5% arabinoxylan, 0.3% protein, 1.7% ash. Viscosity 69 cSt at 1%

a) Information provided by supplier, n.a.: not available, n.r.: not relevant, DP: degree of polymerization, cP: centipoise, cSt: centistokes.
b) Determined by HPLC.
c) Without sulfate. λ-, ι-, and κ-carrageenan were analyzed to contain 20, 30 and 26% ash, respectively.

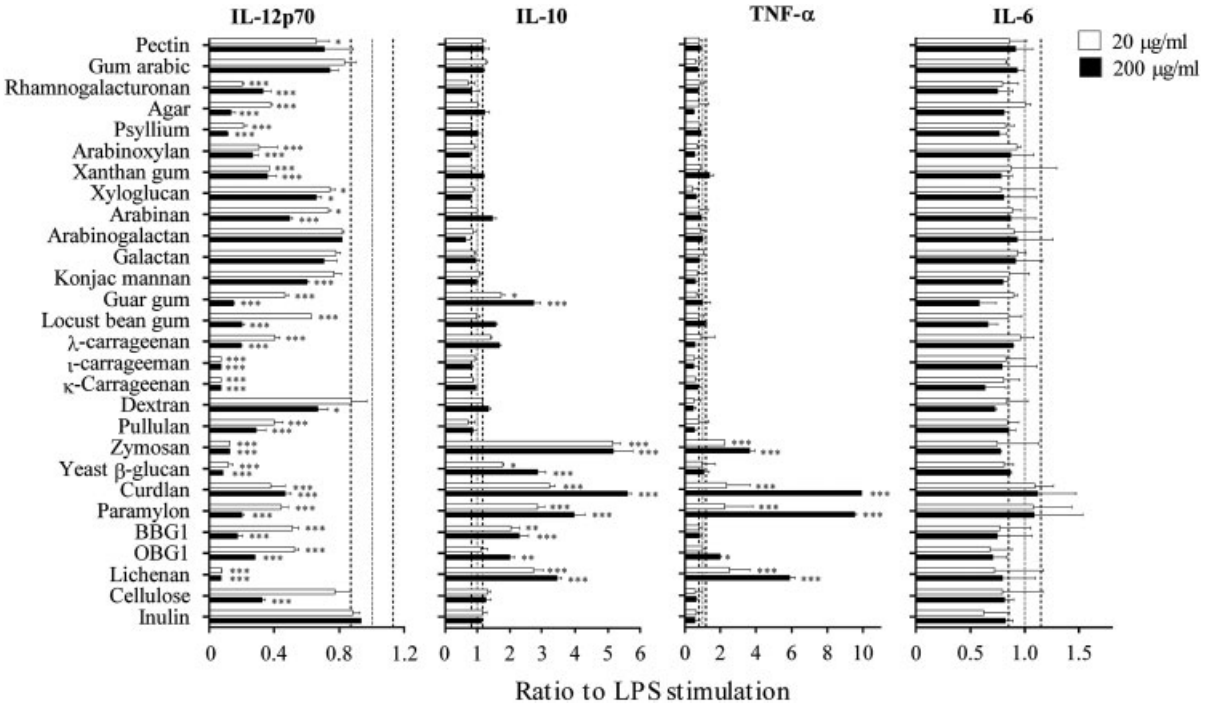

Figure 1. Influence of NSPs on LPS-induced cytokine production in DC. Levels of cytokines in culture supernatants from murine BM-derived DC upon culturing for 18 h with indicated NSP at 20 or 200 μg/mL in the presence of LPS (1 μg/mL) determined by ELISA. Data are presented as the ratio to the LPS stimulation alone (mean ± SD, $n = 2$). The solid line represents cytokine production from LPS-treated DC and the dotted line the SD from these cells. Absolute values in LPS-treated DC were as follows: IL-6: 80 ± 8 ng/mL, IL-10: 945 ± 150 pg/mL, IL-12p70: 570 ± 33 pg/mL and TNF-α: 21 ± 2 ng/mL. All cytokine concentrations in DC cultured with medium alone were below detection limit. Differences between dual-treated DC (NSP plus LPS) as compared to DC treated with LPS alone were tested by one-way ANOVA and the Dunnett test. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. Data are representative of three experiments.

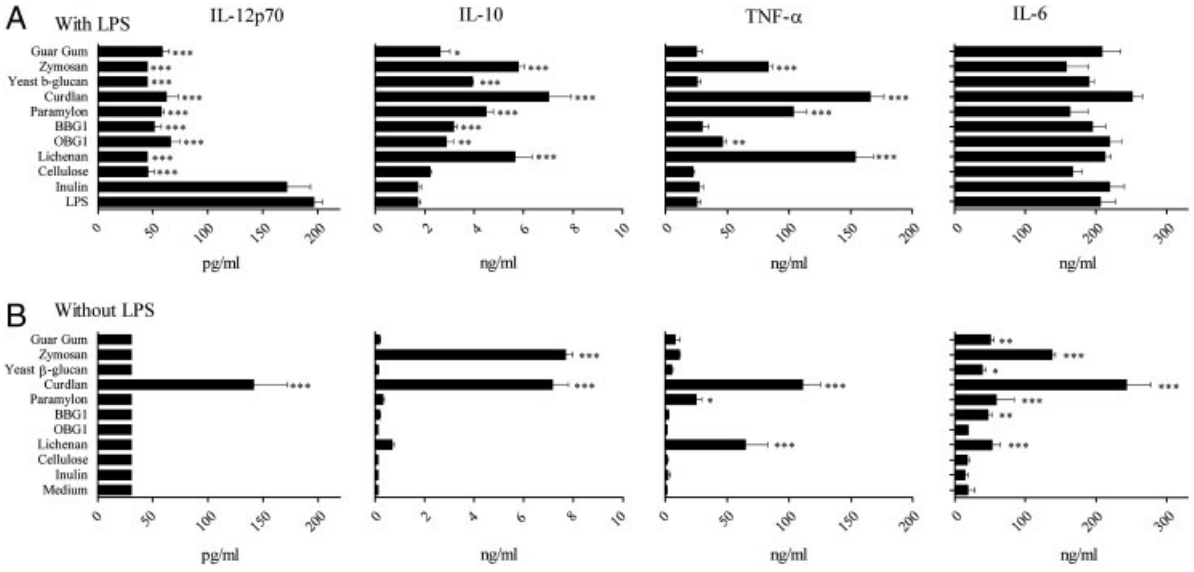

Figure 2. Cytokine production induced in DC by NSP *per se*. Murine BM-derived DCs were cultured for 18 h with NSP at 200 μg/mL with 1 μg/mL LPS (A) or without (B), and cytokines present in the culture supernatants were measured by ELISA. Data represent the mean ± SD ($n = 4$). Data were tested for statistical significance by one-way ANOVA and the Dunnett test. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. Data are representative of three experiments.

Table 2. Endotoxin content in selected fibers

Fiber	LPS (EU/mg fiber)
Guar gum	< 0.6
Zymosan	< 6
Yeast β-glucan	0.7
Curdlan	100
Paramylon	1
OBG1	< 0.6
OBG4	< 0.6
BBG4	18
BBG8	0.8
Lichenan	3

curdlan induced IL-6 comparable to the levels induced by LPS alone and did not exhibit additive effects with LPS. As the preparations, due to the microbial origin, and the manufacturing processes, may contain impurities with immunostimulating capacity, the endotoxin levels in the most potent preparations (Table 2) were tested. Among the products tested, the curdlan preparation contained the highest LPS content (100 endotoxin units (EU) *per* mg). Based on the approximation that 1 EU equals 0.2 ng LPS [22], we estimated the LPS contamination to correspond to administration of approx. 4 ng/mL LPS in curdlan stimulation of DC cultures, thus representing a minor, but stimulatory dose.

3.3 Up-regulation of surface molecule display on DC by specific NSP

The NSPs were also tested for their ability to induce or modulate the expression of the surface molecules MHC class II, CD40, CD80 and CD86 in DC. DCs were cultured with NSP (200 μg/mL) either alone or together with LPS (Fig. 3). The NSP showing most pronounced effects on LPS-induced cytokine production in DC (increase of IL-10 and decrease of IL-12) significantly enhanced CD86 display on DC *per se* (Figs. 3A and B). Only lichenan reduced CD80 surface expression significantly as compared to medium-treated DC (Figs. 3A and B). MHCII and CD40 were not affected by any NSP *per se* (Figs. 3A and B).

As regards modulation of LPS-induced maturation of DC, yeast β-glucan and lichenan enhanced the CD86 expression, whereas they reduced the expression of CD40 and CD80 (Fig. 3C and D). Curdlan reduced the expression of LPS-induced CD80, whereas zymosan reduced CD40 display on DC. Collectively, all β-glucans showed capacity to enhance surface expression of CD86 in immature DC by at least twofold, but in the presence of LPS, we found more diverse modulatory properties amongst the β-glucans, and observed a reduction of the CD80 and CD40 surface display by several of these compounds.

3.4 Effect of source and molecular weight of cereal β-glucans on modulatory capacity in DC

In our initial screenings of the different NSP preparations, the cereal β-glucans showed some regulatory potential, although their effects were clearly less potent than another β-(1,3),(1,4)-glucan; lichenan. To further identify factors of importance for the activity, we compared immunomodulatory activity of a number of cereal β-glucans to their Mw and supplier information (Table 1). Mw of all products to be compared were determined under identical analytical conditions by size exclusion chromatography against β-glucan as well as pullulan standards (Table 1).

The cereal β-glucan preparations exhibited great variability in their capacity to modulate LPS-induced cytokine production in DC. Five barley β-glucans (BBG1, 3, 4, 5, 8) and one oat β-glucan (OBG1) were able to enhance the IL-10 production concurrent with suppression of IL-12p70 (Fig. 4). The active BBG preparations were obtained from all three suppliers, and showed a size dependency, as only samples between 25 and 215 kDa exhibited immunoregulatory effect, whereas samples of higher or lower molecular weight did not. The TNF-α production in LPS-activated DC was enhanced by three of these products (BBG4, 5, 8), while the barley β-glucan products (BBG4, 5) from one provider also induced significant amounts of IL-6 (Fig. 4A). In order to assess a possible role of contaminating LPS in these preparations, the endotoxin content in BBG4 and BBG8 was analyzed (Table 2). We detected LPS in both products, and although the levels were below 20 EU/mg it cannot be excluded that this amount of LPS is capable of modulating especially TNF-α induction in DC.

For the different oat β-glucan preparations, we observed an IL-10 and IL-12p70 modulatory effect of OBG1 only (Fig. 4B). This effect is not seen in the other product of similar size (OBG5), or in lower or higher Mw products (OBG2, 3 or 4). Accordingly, it was not possible to relate the activity of the OBG products to the Mw. The endotoxin content in OBG1 and OBG4 was determined, and found to be < 0.6 EU/mg, and thus unlikely to induce significant production of TNF-α and IL-6.

4 Discussion

The central goal of our study was to address the structural and molecular basis for immunoregulatory capacity in DC amongst NSP that are part of our common foods or may hold potential uses as food supplements. We focused our evaluation on a broad panel of NSP with different structure, size and origin, in order to identify potent immunoregulatory molecules that may be able to modulate the functional phenotype of DC.

Our study demonstrated the existence of a large variability in terms of DC immunoregulatory properties among various NSP structures. We found that greatest DC modulatory potency lies within the group of microbial-derived β-glucans, with the cereal β-glucans and the galactomannan

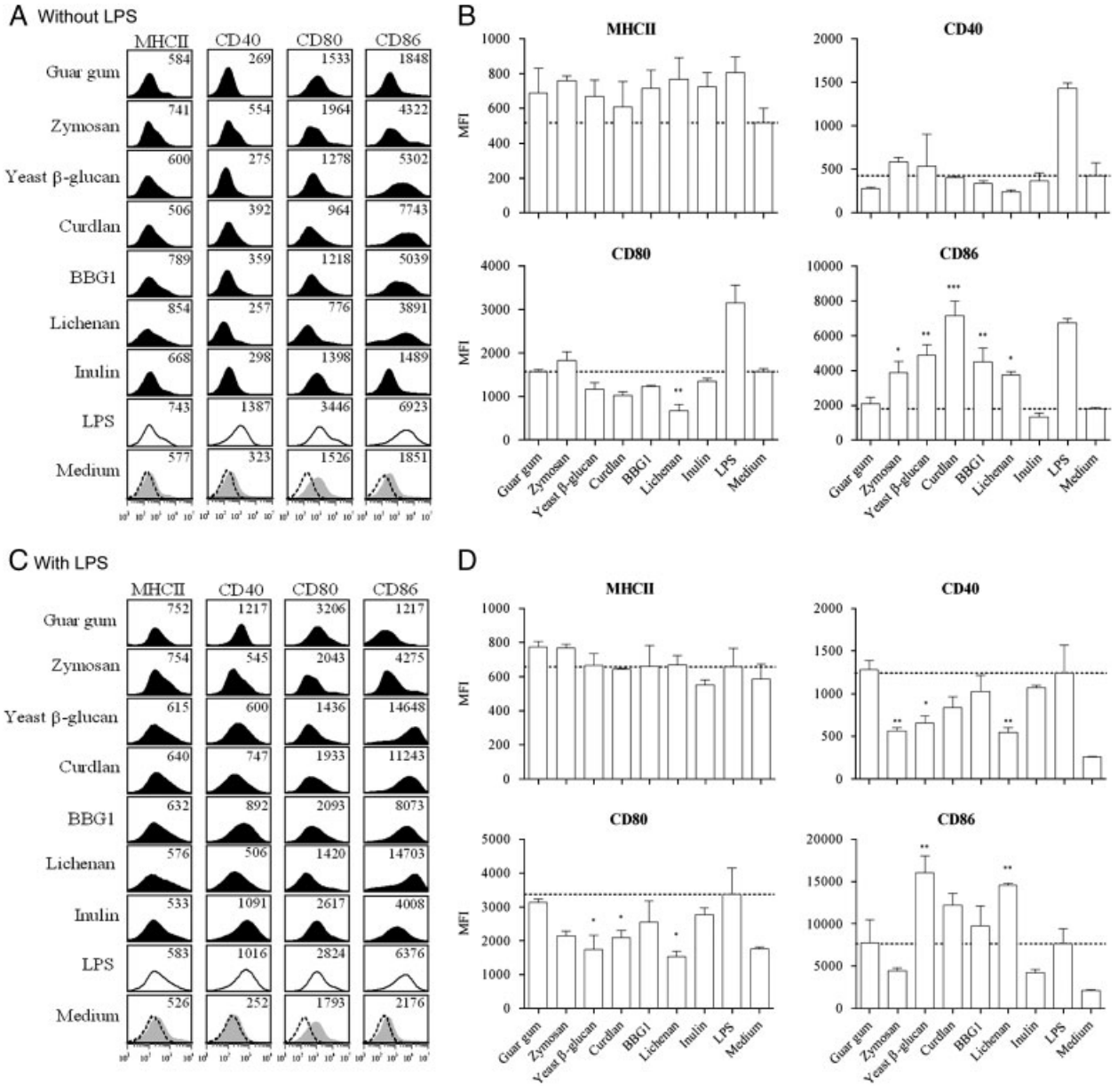

Figure 3. Modulation of MHC class II, CD40, CD80 and CD86 surface display on DC. Murine BM-derived DC were cultured for 18 h with NSP at 200 μg/mL without (A, B) or with (C, D) addition of LPS at 1 μg/mL. Surface phenotype of DC was determined by flow cytometry after staining cells with PE-MHC class II (MHCII), PE-CD40, PE-CD80 or APC-CD86 mAbs. (A, C) Histograms of surface expression levels of MHCII, CD40, CD80 and CD86. Isotype controls for each antibody-conjugate are shown by the dashed line in the medium-treated DC diagrams. (B, D) The mean fluorescence intensity of the surface markers. Data represent mean+SD from two independent experiments. Differences between treatments and medium-treated DC (A, B) or LPS-treated DC (C, D) were analyzed by one-way ANOVA and Dunnett test. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

guar gum carrying a less but still effective structural basis for DC regulatory potential. Of specific notice is the fact that the regulatory pattern found in DC upon interaction with the potent NSP structures concomitant with LPS stimulation, is balanced towards high IL-10 production with very low level IL-12p70 secretion, a DC signature that suggests priming towards a tolerogenic milieu, involving Treg subset generation in the microenvironment surrounding the NSP-triggered DC [23]. Such regulatory profile may be particularly valuable in the highly exposed gut epithelium environment. However, the concomitant induction by some NSP of the pro-inflammatory cytokine TNF-α may disturb polarization into this regulatory Th-cell subset. We did not focus on this issue here, and further studies are therefore needed in order to define the specific functional Th-cell types that are generated by NSP-triggered DC.

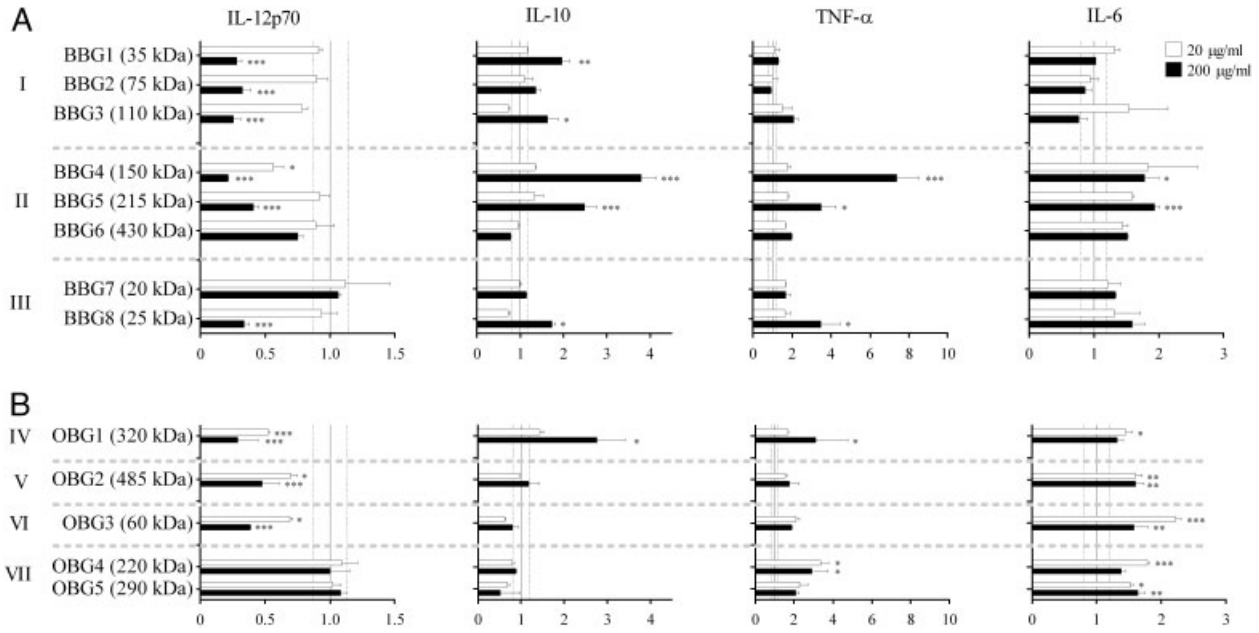

Figure 4. Effect of different molecular sizes and supplier of cereal β-glucans on cytokine-inducing potency in DC. Cereal β-glucans were obtained from different suppliers, as indicated by the roman numerals (I: GraceLinc, II and VII: Megazyme, III: Novozymes, IV: Biovelop, V: Agriculture Canada, VI: Cerefi), and molecular sizes were determined by size-exclusion chromatography (Table 1). The immunomodulating property of these cereal β-glucans was measured by culturing of BM-derived DC for 18 h with 1 μg/mL LPS together with the different BBG (A) or OBG (B) at 20 or 200 μg/mL. Data are presented as the ratio to the LPS stimulation alone (mean ± SD, $n = 2$). The solid vertical line represents the cytokine production in LPS-treated DC and the dotted vertical line indicates ± SD. Differences between treatments were analyzed by one-way ANOVA and Dunnett test (as compared to LPS-treated DC). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. Data are representative of two experiments.

Interestingly, a broad range of glycan polymers was observed to hold IL-12p70-inhibitory potential, including so different structural components as cellulose and arabinoxylan. Whether this effect is caused by binding of the rather distinct structures to a single or a number of glucan recognizing receptors on DC, all resulting in IL-12p70 inhibition, cannot be ruled out from the present results. From our data on β-glucans, it was, however, clear that none of the NSP that inhibited LPS-induced IL-12p70 in DC without simultaneous modulation of other cytokines, stimulated cytokine production in DC by itself, whereas NSP structures that modulated both IL-10 and IL-12 production were all capable to a varying degree of stimulating IL-6 and TNF-α production *per se*. The mechanistic basis for this observation is currently unclear.

Especially, the co-stimulatory molecule CD86 on DC was up-regulated by all the β-glucans exhibiting ability to modulate TLR4-induced IL-12p70 and IL-10 production. CD86 engagement with CD28 on T-cells results in T-cell activation provided a simultaneous MHC class II up-regulation takes place [24]. In the presence of LPS, especially lichenan and yeast β-glucan/zymosan exhibited regulatory functions, leading to reduced CD40 and CD80 display along with enhanced CD86 expression on DC. We are currently in the process of evaluating the effect on Th cell polarization of the specific DC phenotype (IL-10(hi), IL-12p70(lo), CD86(hi), CD80(lo), CD40 (interm)) induced by lichenan and yeast β-glucan.

The varying immunomodulatory activity of different β-glucans may relate to the structural basis of the compound. The β-glucans can be divided into groups based on their overall structure: (i) β-glucans containing β-(1,3)-D-glucopyranose units only, (ii) β-glucans containing (1,3)- and (1,4)-D-glucopyranose units, (iii) β-glucans containing β-(1,4)-D-glucopyranose units only and (iv) β-glucans with (1,3)- and (1,6)-D-glucopyranose units. Curdlan and paramylon, representative of β-glucans in group 1 [18], were observed to possess a medium to strong IL-6 and TNF-α-inducing capacity in DC *per se*, and curdlan additionally induced IL-10 production. This capacity may well be promoted by the LPS present in the curdlan preparation, and the LPS presence here may as well explain the different effects of curdlan and paramylon.

β-Glucans from lichenan and cereals both belong to group 2, containing β-(1,3)- and (1,4)-bonds. However, despite their structural similarities, they do not share the same capacity to induce cytokine production or surface marker display in DC. Lichenan exhibited a more potent immunomodulatory capacity than oat- and barley β-glucans. This may be explained by a considerable diversity in the ratio of cellotriose (DP3) to cellotetraose (DP4), the amount of longer cellulose oligomers, and the ratio of (1,4):(1,3)

linkages. In general, the ratio of cellotriose to cellotetraose units for barley β-glucan is 1.8–3.5 and for oat β-glucan 1.5–2.3, whereas for lichenan β-glucan it is 24.5 [25]. The ratio of the two types of linkages, (1,4) to (1,3), is for both oat and barley between 1.9 and 2.8 [26], whereas lichenan has a ratio of 1.5–1.6 [27]. This indicates that β-glucans with a low (1,4):(1,3) ratio and a higher content of cellotriose *versus* cellotetraose units possess higher immunomodulating capacity, hence, signifying that a greater amount of β-(1,3) glucan structures are central for the immunomodulating potency. This is further supported by the fact that curdlan and partly paramylon, which, together with lichenan, were found to be among the most potent β-glucans tested in the present study, contain β-(1,3) linkages only. In contrast, cellulose that contains β-(1,4) linkages only, did not affect the production of IL-10, TNF-α or IL-6 production, although it did inhibit LPS-induced IL-12p70 production.

The group 4 structures are represented by yeast β-glucan and zymosan, both derived from baker's yeast. Zymosan besides β-glucan also contains mannan [28] that additionally holds immunoregulatory properties [29]. The similar regulatory effects of the galactomannan guar gum and β-glucans on the functional DC cytokine profile indicate that these compounds may interact *via* the same receptors on DC, or, alternatively, with receptors having identical signaling pathways. Guar gum and β-glucans are recognized to bind to the mannose receptor and also to CR3 (CD11b/CD18) [30] both of which have previously been linked to IL-12 suppression in DC [31]. In this study, we compared a large panel of NSP and consequently we did not focus on identifying the explicit receptor-mediated mechanisms behind the β-glucan and galactomannan effects in DC.

Presently, it is difficult to estimate whether the weaker effect of the cereal-derived β-glucans as in contrast to those of microbial origin is physiologically important, but as the ingestion of plant-derived NSP usually far exceeds that of the β-glucan-containing microorganisms, we cannot exclude that they may play a role in maintaining a tolerogenic milieu in the small intestinal environment. As cereal-derived NSP vary greatly in terms of molecular sizes, we assessed the relevance of the molecular size for immunoregulatory potential by testing β-glucans of varying molecular size generated by enzymic degradation. The cereal β-glucan products differed in cytokine modulating effects and, in general, the products with intermediary molecular weight (25–215 kDa) displayed activity, while higher and lower molecular weight products did not. The inactivity of the smallest (most degraded) β-glucans may suggest that a certain number of repeats in the pattern of alternating (1,3)- and (1,4)-D-glucopyranose units is required for the β-glucans to modulate the LPS-induced cytokine production. Why the highest molecular weight β-glucan had no effect may be due properties related to solubility or viscosity that may vary with size. In general β-glucans from oat were much poorer immunoregulators than β-glucan from barley. This may be due to variations in the specific structure of β-glucans from these cereals. Other factors relating to specific processes at individual suppliers, such as varieties and growth conditions of cereals used for production, water-holding capacities, and enzymes may also play a role in relation to the immunomodulatory capacity of the products, but our results points towards molecular pattern and size to be major determinants of the immunomodulating activity.

Besides molecular size, one factor that may also influence the immunomodulating potential of NSP is the solubility of the compounds. Some of the tested NSP are insoluble or only slightly soluble under the analytical conditions employed, and there is a great variability within the different preparations. In order to take into account both soluble and insoluble parts, we here suspended all products in growth medium, and soaked the preparations for 1 h before adding a representative suspension of the sample to cells. Based on this approach, we cannot decipher whether the solubility of a given product is of essential importance for the bioactivity. However, in a pre-study where DC was treated with the soluble fraction only, we found a reduced or no modulatory effect, suggesting that solubility is not a prerequisite for the bioactivity. We cannot, however, from this study conclude whether solubility is a critical parameter for immunomodulation by NSP *in vivo*.

Conclusively, we here demonstrated that, among the various NSP products tested, superior DC immunomodulatory activities are found within the group of β-glucans, and in the galactomannan guar gum. Comparisons between different cereal-based β-glucans made it clear that these products vary greatly in terms of bioactivity, and that size may be of specific importance in relation to the immunoregulatory properties of the product. Enhanced insight into the structural requirements for NSP efficacy, such as set forth in this larger comparative study, may facilitate development of more effective NSP-based products and promote their use as dietary supplements.

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The authors have declared no conflict of interest.

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