RESEARCH ARTICLE

Particulate β-glucans synergistically activate TLR4 and Dectin-1 in human dendritic cells

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Scope: The major receptor for β(1-3)-glucans on immune cells is considered to be Dectin-1 receptor. Particulate β-glucans induce stronger immune responses than soluble β-glucans by clustering of Dectin-1 receptors. Here, it was hypothesized that activation of other pattern recognition receptors such as Toll-like receptor 4 (TLR4) can also contribute to enhanced activity of immune cells after exposure to particulate β-glucans.

Methods and results: To test this hypothesis, reporter cell lines were designed expressing TLR4 with either Dectin-1A or Dectin-1B, that is, one of the two transcript variants of human Dectin-1 receptors. Enhanced NF-κB activation was observed after stimulation with particulate β-glucans in both Dectin-1A-TLR4 and the Dectin-1B-TLR4 cell lines. This was different with soluble β-glucans, which enhanced activation in Dectin-1A-TLR4 cell lines but not in Dectin-1B-TLR4 cells. The synergistic activation of TLR4 and Dectin-1 by particulate β-glucans was confirmed in human dendritic cells. The effects of particulate β-glucan induced TLR4 binding were regulatory as blocking TLR4 enhanced pro-inflammatory cytokine IL-23, IL-4, IL-6, and TNF-α production.

Conclusion: These results suggest that TLR4 and Dectin-1 are synergistically activated by particulate β-glucans, wherein TLR4 activates an immune regulatory pathway in human dendritic cells. Our data suggest that β-glucan is an immune regulatory ligand for TLR4.

Keywords:
Dectin-1 / Dietary fiber / β-Glucan / Immunomodulation / TLR4

1 Introduction

Dietary carbohydrate fibers constitute an important part of our diet. Traditionally beneficial effects of dietary carbohydrate fibers were assumed to be associated with their water-holding capacity in the stomach, slowing down the digestion process and dilute toxic ingredients in the diet [1]. In the last decade, more beneficial effects of dietary fibers have been described such as immunomodulation. These immunomodulatory effects were suggested to be from their prebiotic effects [2] such as enhanced production of immune regulatory SCFAs by commensal microbiota [3]. More recently, another mechanism for immunomodulatory effects of dietary fibers has been described [4]. This is direct interaction of dietary fibers with pattern recognition receptors (PRRs) [4]. The dietary fiber inulin-type-fructan, for example, was shown to stimulate the PRRs Toll-Like receptor 2 (TLR2) and to induce strengthening of the barrier function of intestinal epithelial cells [5]. As these effects were observed in the absence of microbiota and SCFA, these effects are categorized as direct immunomodulatory effects [5, 6].

β-Glucans are one of the earliest discovered carbohydrate polymers interacting with PRRs such as C-type lectin domain family 7 member A receptor also known as the Dectin-1 receptor [7]. Also, complement receptor 3 (CR3) and lactosylceramide have been reported to be able to bind β-glucans [7, 8]. Dectin-1 is considered to be the major β-glucan receptor present on the immune cells that can recognize β-glucans with β(1-3) linkages [9]. β-Glucans come into the diet via consumption of fungi and cereal grains such as oats and barley [10, 11]. The dietary fungal β-glucans consist mainly of β(1-3, 1–6) linked glucans, whereas cereal grain β-glucans are composed of β(1-4, 1–3) linked glucans [10, 11]. Cellulose is β(1-4) linked glucan and constitutes the most common β-glucan in diet. However, cellulose has not been described as Dectin-1 activator [12]. These dietary β-glucans can be soluble or particulate depending on the linkages and size of the molecule [13, 14]. Interaction of β-glucans with the Dectin-1 receptor

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Abbreviations: **TLR**, Toll-like receptor; **PRR**, pattern recognition receptor; **SEAP**, soluble alkaline embryonic phosphatase

has been studied mainly with fungal β-glucans [8]. With these fungal β(1-3, 1-6)-glucans, it has been shown that immune effects are more pronounced with particulate than with soluble β-glucans [15]. Goodridge et al. [15] have shown that this can partly be explained by particulate β-glucan induced clustering of the Dectin-1 receptors and exclusion of negative regulators of immune responses such as CD45 and CD148 [15]. As such, soluble instead of particulate β-glucans induce only weak activation as they fail to cluster Dectin-1 receptors [15]. Another theory is that particulate β-glucans can synergistically bind to Dectin-1 and other immune stimulating receptors such as TLRs. Particulate β-glucans isolated from *Saccharomyces cerevisiae,* that is, zymosan, has been reported to bind to TLR2 in addition to Dectin-1 [16]. However, later this hypothesis was rejected as the TLR2 activation potential of zymosan could be removed by hot alkali treatments [16]. This suggests that the TLR2 activation by zymosan was not caused by β-glucans but by contaminating bacterial products present in the zymosan preparation [16]. It has been suggested that β-glucans and TLR4 agonist LPS can synergistically activate TLR4 in monocytes [17]. The role of TLR4 in β-glucan recognition is further substantiated by the observation that TLR4 knockout mice are more susceptible to pathogenic fungal infections such as by *Candida albicans* than wild-type mice [18]. However, true proof for real synergistic interaction in the absence of LPS or binding of β-glucans to the unstimulated TLR4 and Dectin-1 receptor is lacking.

In order to enhance the understanding of the working mechanisms and synergistic effects of TLR4 and particulate β-glucan induced Dectin-1 activation, we studied effects of particulate and soluble β-glucans on reporter cell lines expressing Dectin-1 in combination with TLR4. To this end, we developed reporter cell lines with two transcript variants of the Dectin-1 receptor; Dectin-1A and Dectin-1B combined with TLR4. Activation potential of both particulate and soluble β-glucans was compared in these reporter cell lines. Also, collaborative effect between TLR4 and Dectin-1 receptor was studied in human dendritic cells by quantifying cytokine production in the presence and absence of blocking antibodies for Dectin-1 and TLR4 in particulate β-glucan stimulated human dendritic cells.

2 Material and methods

2.1 Human Dectin-1 and Dectin-1-TLR reporter cell lines

The human Dectin-1 receptor has two transcript variants known as Dectin-1A and Dectin-1B [19]. Dectin-1 reporter cell lines with one of the two transcripts were developed using HEK-Blue™ Null1cells (InvivoGen, Toulouse, France), stably transfected with pUNO1-hDECTIN1a or pUNO1-hDECTIN1b plasmids (InvivoGen) as previously described [20]. Dectin-1-TLR4 reporter cell lines were developed in a similar fashion using HEK-Blue™ Null1cells (InvivoGen, Toulouse, France). Dectin-1-TLR4 expression plasmids were developed by inserting Dectin-1 and TLR4 expression genes in pDUO-mcs plasmid (InvivoGen). pDUO-mcs plasmid has two cloning sites for expression of two independent genes from the same plasmid, wherein the promoter strength for the two genes is similar. The combination of genes expressed in the reporter cell lines is listed in Table 1. The parental cell line HEK-Blue™ Null1 does not express Dectin-1 and TLR4.

The pDUO-mcs plasmid having Dectin-1A and TLR4 was developed by using GeneArt gene synthesis service (Life Technologies, Carlsbad, CA, USA). The plasmid was linearized using PacI FastDigest restriction enzyme (Thermo Scientific, Waltham, MA, USA). The linearized plasmid was purified and used for stable transfection in HEK-Blue™ Null1cells. For transfection, HEK-Blue™ Null1 cells (InvivoGen) were seeded at 500 000 cells/mL in 12-well culture plates (Corning Costar, NY, USA) and incubated overnight in DMEM culture media (Lonza, Basel, Switzerland) with 10 % decomplemented fetal calf serum (60°C for 1 h), 50 U/mL penicillin (Sigma, St. Louis, MO, USA), 50 μg/mL streptomycin (Sigma), and 100 μg/mL normocin (InvivoGen). Transfection was performed the following day by using Lipofectamine LTX® (Life Technologies). Purified 1 μg linear plasmid was diluted in low serum media Opti-MEM® (Life Technologies) and mixed with 3.5 μL of Lipofectamine LTX® (Life Technologies). The transfection mix was incubated at room temperature for 30 min and then added to the cells in the culture plate. Cells were incubated with transfection medium for 24 h and transfected cells were selected using antibiotic containing selection media containing 30 μg/mL blasticidin (InvivoGen) and 100 μg/mL Zeocin (InvivoGen). Single cell clones were selected by serial dilution in a 96-well plate for each newly developed Dectin-1 TLR4 cell line.

2.2 HEK cells soluble alkaline phosphatase reporter assay

The HEK-Blue™ Null1 reporter cell line for human Dectin-1A and Dectin-1B coexpressing TLR4 was cultured and maintained in DMEM culture media (Lonza) with 10 % decomplemented fetal calf serum (60°C for 1 h), 50 U/mL penicillin (Sigma), 50 μg/mL streptomycin (Sigma), and 100 μg/mL normocin (InvivoGen). The culture medium was supplemented with selection antibiotics to maintain stable expression of the PRR genes (see Table 1 for type of media). These reporter cell lines express the soluble alkaline phosphatase (SEAP) gene. This SEAP gene construct is expressed through NF-κB and the AP-1 transcription factor responsive promoter. Thus, when these reporter cell lines are activated with their respective agonists, NF-κB is transferred to the nucleus and the SEAP gene is expressed [20]. The SEAP from the supernatant can be measured using QuantiBlue reagent (InvivoGen). Particulate β-glucan zymosan depleted and soluble β-glucan laminarin were used for stimulation of Dectin-1 reporter cell lines. Zymosan depleted (InvivoGen)

Table 1. Expression plasmids and selection antibiotics for reporter cell lines.

Name of the cell line	Selection antibiotics	Expression plasmid	PRRs expressed on the reporter cells (human origin)
HEK-Null1 Dectin-1A	Blasticidin (12 μg/mL)	pUNO1-hDECTIN1a	Dectin-1A
HEK-Null1 Dectin-1B	Zeocin (100 μg/mL)	pUNO1-hDECTIN1b	Dectin-1B
HEK-Null1 Dectin-1A-TLR4	Blasticidin (30 μg/mL)	pDUO-Dectin-1A- TLR4	Dectin-1A TLR4
HEK-Null1 Dectin-1B-TLR4	Zeocin (100 μg/mL)	pDUO-Dectin-1B- TLR4	Dectin-1B TLR4

is a particulate β-glucan isolated from *S. cerevisiae*, which is treated with hot alkali to deplete its TLR2 activation potential. Zymosan majorly contains β(1-3) linkages and some β(1-6) linkages. Zymosan depleted will be named zymosan in this article. Laminarin (InvivoGen) is a soluble linear β(1–3)-glucan from *Laminaria digitata* having β(1–6) glucan (interchain) linkages. The molecular weight of Laminarin is 6 kDa. Particulate β-glucan zymosan and soluble β-glucan laminarin were dissolved in cell culture medium and mixed by vortex before applying to reporter cell lines or dendritic cells.

2.3 Stimulation of reporter cells with β-glucan

The Dectin-1 and Dectin-1-TLR reporter cell lines were seeded at 500 000 cells/mL in 100 μL/well in a 96-well culture plate and cultured overnight. The following day, reporter cell lines were treated with particulate β-glucan zymosan at 100 μg/mL or soluble β-glucan laminarin at 500 μg/mL. After 24 h of incubation, the media supernatant from different treatment groups was analyzed at 1:4 dilution with QuantiBlue solution. The color change was read at 650 nm using ELISA plate reader Versa Max (Molecular Devices, Sunnyvale, CA, USA). This color change in QuantiBlue reagent was measured as optical density at 650 nm and it is presented as NF-κB activation. The assay was performed at least five times. The endotoxin content in zymosan and laminarin were studied using a LPS-specific ELISA (ELISA kit; Clone-Cloud Corp, Houston, TX, USA) [21, 22]. The LPS concentration was lower than 4 ng/mL detection limit of the ELISA kit. To further confirm the absence of TLR4 activating agents in particulate β-glucan zymosan [23], we stimulated HEK blue™ TLR4-MD2-CD14 (InvivoGen) with LPS (10 ng/mL) as positive control and particulate β-glucan zymosan at 100 μg/mL. These reporter cell lines also have SEAP gene expression under a NF-κB promoter. The NF-κB activation was quantified similar to the above-mentioned procedure.

2.4 Human dendritic cell stimulation with β-glucans

Human dendritic cells (MatTek, Ashland, MA, USA) were seeded in a 96-well culture plate at 1 000 000 cells/mL in 100 μl/well volume of dendritic cell maintenance medium (DC-MM; MatTek) culture media [6]. After 24 h of incubation at 37°C, each well was treated with particulate β-glucan zymosan at 100 μg/mL concentration, dissolved in DC-MM (MatTek) culture media. To study the effect of TLR4 inhibition, TLR4 blocking antibody was pretreated at 10 μg/mL concentration for 1 h followed by treatment with 100 μg/mL particulate β-glucan zymosan in the same wells. As positive control for inhibition, Dectin-1 receptor was blocked by pretreatment with Dectin-1 blocking antibody (InvivoGen) at 10 μg/mL concentration for 1 h followed by the treatment with 100 μg/mL particulate β-glucan zymosan in the same wells. Untreated dendritic cells served as negative control for the experiment. After 24 h incubation at 37°C, media supernatant from dendritic cells was used to quantify cytokines/chemokines. The experiment was performed at least five times.

2.5 Cytokine detection

A multiplex kit was used to measure the concentration of IL-23, IL-12p70, IL-10, IL-6, and TNF-α. The antigen standards provided with the multiplex kit (Affymetrix, Santa Clara, CA, USA) were dissolved and diluted fourfold to have seven serially diluted standards. DC-MM (MatTek) culture media was used as blank control. The magnet beads were dispersed in a clear base, black 96-well plate, and washed with a hand-held magnetic plate holder with 150 μL of the wash buffer provided in the kit. The standards (duplicates) and samples (50 μL) were applied to the magnetic beads, mixed on a plate shaker, and incubated overnight at 4°C on a stable flat surface. Followed by overnight incubation, magnetic beads were washed three times as mentioned above and incubated with 25 μL/well of detection antibody mix for 30 min on a plate shaker at room temperature. The plate was washed three times and incubated with 50 μL/well streptavidin-PE for 30 min at room temperature on a plate shaker. In the last step, the 96-well plate was washed three times and beads were dispersed in 120 μL of reading buffer per well and read in a Luminex®100™ instrument with StarStation software.

2.6 Statistical analysis

Nonparametric distribution of data points was confirmed using the Kolmogorov–Smirnov test. Statistical differences were analyzed using two-way analysis of variance for grouped analysis and Mann–Whitney *U*-test for paired analysis. Data are expressed as mean ± standard deviation. p-values < 0.05 were considered to be statistically significant. The data were analyzed using Graphpad Prism5 program (La Jolla, CA, USA).

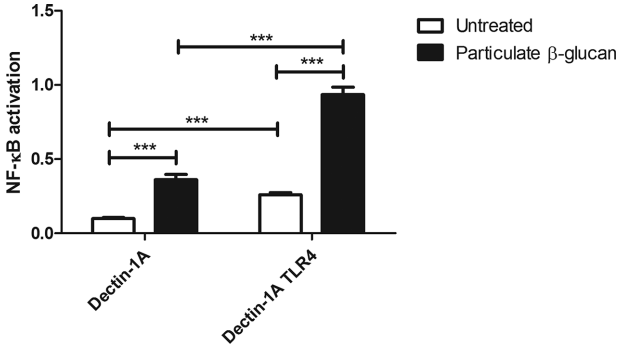

Figure 1. Particulate β-glucans synergistically stimulate Dectin-1A and TLR4. Dectin-1A and Dectin-1A-TLR4 reporter cell lines were stimulated with 100 μg/mL particulate β-glucan zymosan and NF-κB activation was measured at 650 nm using QuantiBlue reagent. Data are presented as mean ± standard deviation, and statistical differences were measured using two-way analysis of variance ($n = 5$; $^{***}p < 0.001$).

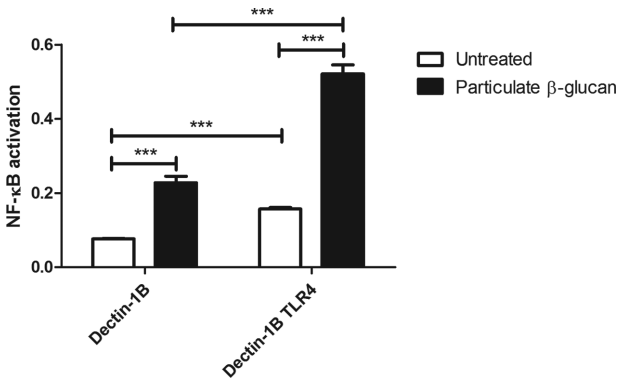

Figure 2. Particulate β-glucans synergistically stimulates Dectin-1B and TLR4. Dectin-1B and Dectin-1B-TLR4 reporter cell lines were stimulated with 100 μg/mL particulate β-glucan zymosan and NF-κB activation was measured at 650 nm using QuantiBlue reagent. Data are presented as mean ± standard deviation ($n = 5$) and statistical differences were measured using two-way analysis of variance ($^{***}p < 0.001$).

3 Results

3.1 Particulate β-glucans show increased NF-κB activation in Dectin-1 reporter cell lines coexpressing TLR4

To determine whether the particulate β-glucan zymosan has different NF-κB activation potential when Dectin-1 and TLR4 are simultaneously expressed, we studied the activation in reporter cell lines expressing either Dectin-1 or both Dectin-1 and TLR4. Activation of the receptors was quantified using the NF-κB activation assay. As mentioned in material methods, to exclude the potential influence of bacterial contaminations such as LPS on our findings, we tested and confirmed the absence of endotoxin contaminations in the β-glucan samples. To determine which transcript variants of the Dectin-1 receptor is synergizing with TLR4 in response to particulate β-glucan zymosan, it was studied separately for the 1A (Fig. 1) and 1B (Fig. 2) variant.

As shown in Fig. 1, particulate β-glucan zymosan has synergistic effects via TLR4 on Dectin-1A. The particulate β-glucan stimulated Dectin-1A reporter cell line coexpressing TLR4 showed 2.7-fold ($p < 0.001$) higher activation than its counterpart expressing only Dectin-1A (Fig. 1). The TLR4-induced enhancement of activation was also observed with Dectin-1B. Particulate β-glucan stimulated Dectin-1B activation was 2.2-fold ($p < 0.001$) higher in the presence of TLR4 compared to cells expressing only Dectin-1B (Fig. 2). While basal activation of Dectin-1A TLR4 and Dectin-1B TLR4 was 2.6- and twofold higher than Dectin-1A and Dectin-1B cell lines, respectively ($p < 0.001$).

To confirm that the higher activation seen in Dectin-1-TLR4 cell lines stimulated by particulate β-glucans is not because of TLR4 stimulating contaminants, we tested particulate β-glucans in TLR4-MD2-CD14 reporter cell lines. As observed in Fig. 3, particulate β-glucans did not stimulate

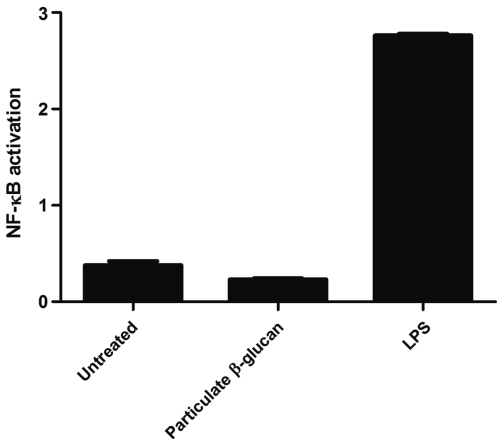

Figure 3. Particulate β-glucans do not activate TLR4 in the absence of Dectin-1. TLR4-MD2-CD14 reporter cell line was stimulated with 10 ng/mL LPS and 100 μg/mL particulate β-glucan and NF-κB activation was measured at 650 nm using QuantiBlue reagent. Data are presented as mean ± standard deviation ($n = 3$) and statistical differences were measured using Mann–Whitney *U*-test.

TLR4 in the absence of Dectin-1 receptors. Thus, we confirm that particulate β-glucans synergistically activate Dectin-1 and TLR4 receptors.

3.2 Soluble β-glucans only stimulate Dectin-1A-TLR4 expressing cells and not Dectin-1B-TLR4 cells

Dectin-1 activation has been shown to be dependent on the particularity of the β-glucan agonist [15]. It has been suggested that only particulate β-glucans are able to activate Dectin-1 receptor by clustering of several Dectin-1 receptors.

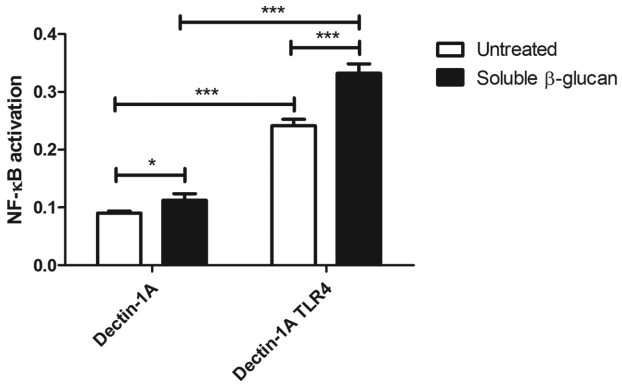

Figure 4. Soluble β-glucans synergistically stimulate Dectin-1A and TLR4. Dectin-1A and Dectin-1A-TLR4 reporter cell lines were stimulated with 500 μg/mL soluble β-glucan laminarin and NF-κB activation was measured at 650 nm using QuantiBlue reagent. Data are presented as mean ± standard deviation ($n = 5$), and statistical differences were measured using two-way analysis of variance (*$p < 0.05$, ***$p < 0.001$).

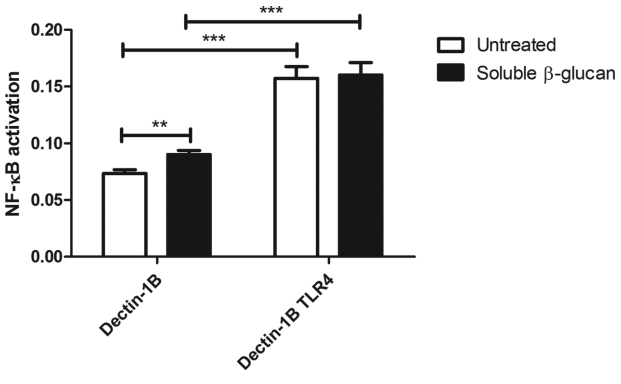

Figure 5. Dectin-1B and TLR4 are not synergistically activated by soluble β-glucans. Dectin-1B and Dectin-1B-TLR4 reporter cell lines were stimulated with 500 μg/mL soluble β-glucan laminarin and NF-κB activation was measured at 650 nm. Data are presented as mean ± standard deviation ($n = 5$), and statistical differences were measured using two-way analysis of variance (**$p < 0.01$, ***$p < 0.001$).

Soluble β-glucans in contrast to particulate β-glucans are not able to cluster Dectin-1 receptors and thus show weaker or no activation of Dectin-1 [15]. To determine whether soluble β-glucans just as particulate β-glucans acts synergistically with TLR4, Dectin-1A-TLR4 and Dectin-1B-TLR4 cell lines were stimulated with soluble β-glucan laminarin.

Similar to particulate β-glucans, soluble β-glucan laminarin induced higher NF-κB activation in reporter cell lines coexpressing Dectin-1A and TLR4. Reporter cell lines expressing Dectin-1A with TLR4 showed 2.9-fold ($p < 0.001$) increased NF-κB activation compared to cell lines expressing Dectin-1A (Fig. 4), while basal activation in Dectin-1A TLR4 cell lines was 2.6-fold ($p < 0.001$) higher than in Dectin-1A cell lines. However, in Dectin-1B-TLR4 cell lines, NF-κB activation after stimulation with soluble β-glucans was similar to untreated cells while there was 1.2-fold induction in Dectin-1B cell lines ($p < 0.01$) (Fig. 5). Thus, soluble β-glucans can stimulate Dectin-1A TLR4 but not Dectin-1B TLR4.

3.3 TLR4 blocking in particulate β-glucan stimulated human dendritic cells increased production of IL-23, IL-4, IL-6, and TNF-α but not IL-12p70 and IL-10

The reporter assays confirmed synergy between Dectin-1 and TLR4 after stimulation with particulate β-glucans. To determine the immune effects of this collaboration between Dectin-1 and TLR4, human dendritic cells were pretreated with TLR4 blocking antibody and stimulated with particulate β-glucans. The cytokine/chemokine production of anti-inflammatory cytokines IL-10, the Th2 promoting cytokines IL-4, IL-6, the Th1 stimulating cytokines TNF-α, IL-12p70, and the Th17 supporting cytokines IL-23 were measured in supernatants of these human dendritic cells.

First, the specificity of particulate β-glucan induced cytokine production was confirmed by application of Dectin-1 blocking antibody 1 h prior to stimulation. Dectin-1 blocking antibody could reduce production of IL-23 (4.6-fold, $p < 0.001$), IL-10 (4.9-fold, $p < 0.001$), IL-4 (2.4-fold, $p < 0.001$), IL-6 (8.6-fold, $p < 0.001$), and TNF-α (3.9-fold, $p < 0.001$) (Fig. 6).

Next, the TLR4 blocking in particulate β-glucan stimulated human dendritic cells was studied. This increased production of IL-23 (3.2-fold, $p < 0.01$), IL-4 (1.9-fold, $p < 0.01$), IL-6 (3.4-fold, $p < 0.01$), and TNF-α (1.6-fold, $p < 0.01$) (Fig. 6). However, the anti-inflammatory cytokine IL-10 remained unaffected. Interestingly, the production of IL-12p70 also remained unaffected. IL-12p70 and IL-23 both belong to the IL-12 family of cytokines and both these heterodimeric cytokines have IL-12p40 subunits in their structure [24]. Thus, increased production of IL-23 but not of IL-12p70 confirms the specific role of TLR4 in particulate β-glucan induced IL-23 production in human dendritic cells.

4 Discussion

To the best of our knowledge, we show for the first time that particulate β-glucans can synergistically stimulate Dectin-1 and TLR4. We also show that the nature of this interaction is dependent on the physical–chemical structure of the β-glucans as particulate β-glucans had similar effects on both transcription variants of Dectin-1, that is, Dectin-1A and Dectin-1B, while soluble β-glucans only had synergistic effects when TLR4 was coexpressed with Dectin-1A. To our opinion, better insight into the structure–effector relationship between dietary fibers and PRRs may lead to design of more effective immunomodulatory food products.

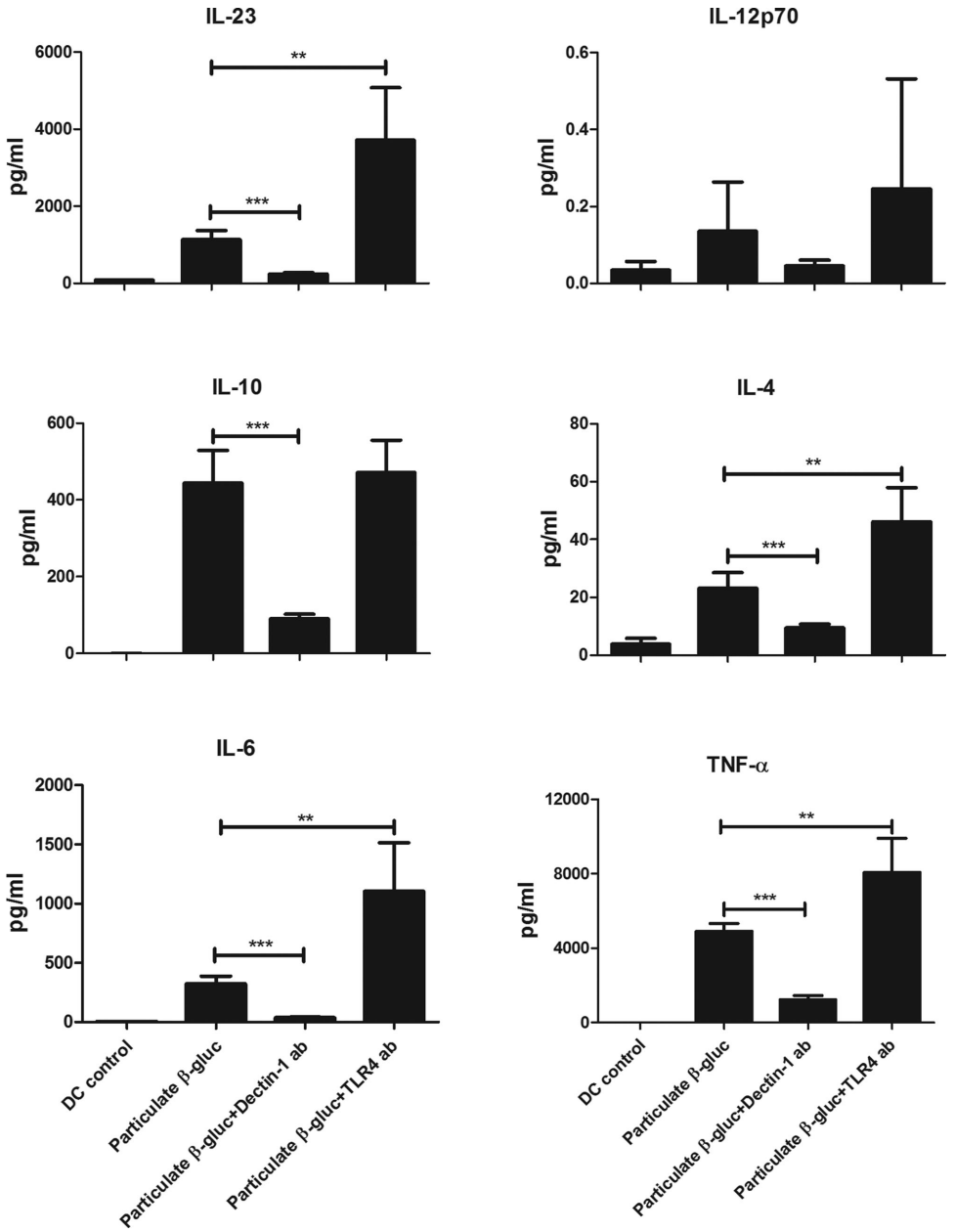

Figure 6. TLR4 blocking in particulate β-glucan stimulated human dendritic cells increases IL-23, IL-4, IL-6, and TNF-α production. Human dendritic cells were stimulated with 100 μg/mL particulate β-glucan zymosan. In separate experimental groups, the dendritic cells were pretreated with TLR4 blocking antibody or Dectin-1 blocking antibody at 10 μg/mL followed by stimulation with 100 μg/mL particulate β-glucan zymosan. The cytokine production was measured using multiplex assays. Data are presented as mean ± standard deviation ($n = 5$), and statistical differences were measured using Mann–Whitney *U*-test (**$p < 0.01$ and ***$p < 0.001$).

Currently, it is assumed that the Dectin-1 receptor recognizes particulate β-glucans in a homotypic manner [25]. However, our data suggest that this has to be reconsidered and that the difference in immune modulating effects between soluble and particulate β-glucans might also partly be explained by differences in interaction with Dectin-1 and TLR4. Up till now the leading explanation for differences in efficacy between soluble and particulate β-glucans is the clustering of Dectin-1 receptors [15]. Our study was designed to investigate whether differences in costimulation of other receptors, in this case TLR4, might also be an explanation. As shown, the soluble β-glucans are not active in the Dectin-1B-TLR4 stimulation and might therefore be a contributing factor for its lesser immune stimulating effect [15] as reported before by us and others [9, 20].

The lack of activation by soluble β-glucans in activating Dectin-1B-TLR4 can be explained by pertinent differences in the Dectin-1A and 1B transcript variants. The Dectin-1 receptor is composed of an extracellular C-type lectin like domain, a neck region, an intermembrane region, and an intracellular immunoreceptor tyrosine-based activation motif unit [7]. The neck region connects the C-type lectin-like domain to the intermembrane region [26, 27]. Human Dectin-1B receptor does not have this neck region [26, 27]. It is plausible that the neck region in Dectin-1A helps to align better with TLR4 for binding with soluble β-glucans, whereas the absence of the

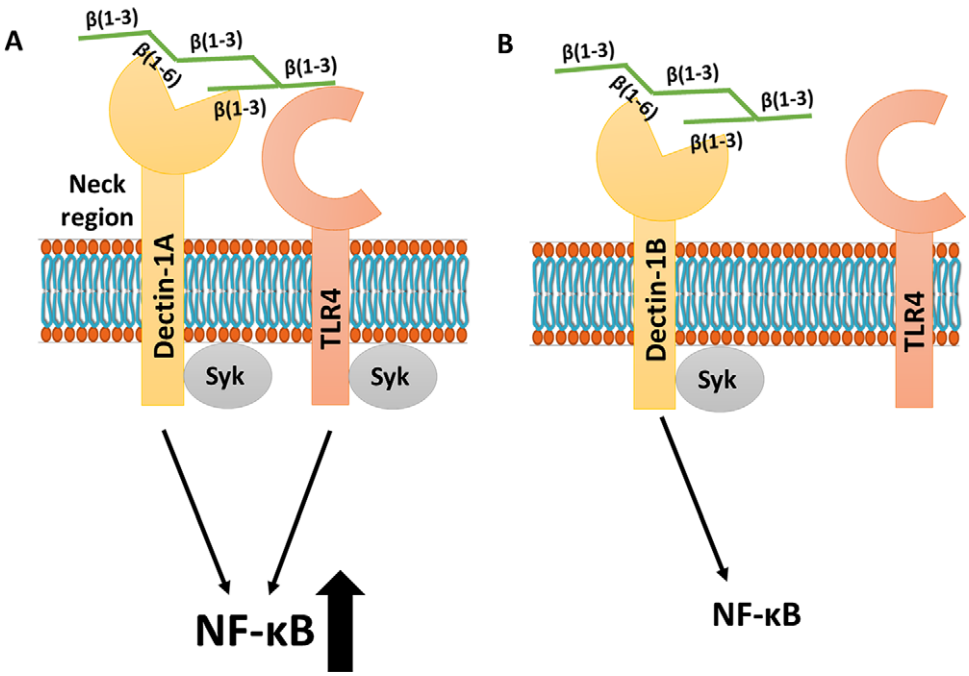

Figure 7. Proposed mechanism for synergistic activation of Dectin-1 and TLR4 by soluble β-glucans. (A) Soluble β-glucans having β(1-3, 1–6) linkages synergistically activate Dectin-1A and TLR4, which leads to enhanced NF-κB activation. (B) Soluble β-glucans fail to synergistically activate Dectin-1B and TLR4, thus do not enhance NF-κB activation. The presence of the neck region in Dectin-1A probably facilitates the interaction of Dectin-1A and TLR4 in enhanced activation.

neck region in Dectin-1B does not allow the alignment with TLR4 for synergism when stimulated with soluble β-glucans (Fig. 7).

The role of TLR4 in particulate β-glucan induced Dectin-1 activation was also confirmed in human dendritic cells. TLR4 blocking in particulate β-glucan stimulated human dendritic cells leads to enhanced immune responses. By separately blocking Dectin-1 and TLR4, we investigated in human dendritic cells that cytokines are produced by particulate β-glucan binding to either Dectin-1 or TLR4 or the combination. TLR-4 binding by particulate β-glucans during blockage of Dectin-1 reduced IL-23, IL-10, IL-4, IL-6, and TNF-α, while IL-12p70 reduction was minor and did not reach statistical significance. Dectin-1 binding by particulate β-glucans during TLR4 blocking resulted in increased production of pro-inflammatory cytokines IL-23, IL-4, IL-6, and TNF-α, illustrating the regulatory or anti-inflammatory role of TLR4 after particulate β-glucan binding. Dendritic cells express a wide variety of immune receptors, thus a role of another regulatory receptor along with TLR4 cannot be excluded for increased cytokine production after TLR4 blocking [28]. The production of IL-12p70 remained unchanged after application of TLR4 antibody in particulate β-glucan treated dendritic cells, whereas IL-23 production was increased. IL-12p70 and IL-23 belong to the IL-12 family of cytokines. IL-12p70 is a heterodimer of IL-12p35 and IL-12p40 subunits, while IL-23 is a heterodimer of IL-12p40 and IL-23p19 subunits [29]. As both IL-23 and IL-12p70 share IL-12p40 subunit, we concluded that the role of TLR4 activation by particulate β-glucans is specific for the IL-23 production pathway, without affecting the IL-12p70 from the IL-12 family of cytokines. Reciprocal regulation of IL-23 and IL-12 is already recognized for murine bone marrow derived dendritic cells where costimulation of TLR2 and Dectin-1 by combined exposure to β-glucans and the TLR2 ligand P3CSK4 resulted in increased IL-23 production, but reduced IL-12 production [30]. Thus, similar reciprocal regulation processes may occur for IL-23 and IL-12p70 in human dendritic cells.

In addition to a synergistic effect of particulate β-glucan on Dectin-1 and TLR4, our data also suggest that β-glucan is a ligand for TLR4. However, β-glucan could only activate TLR4 signaling when accompanied by Dectin-1 receptor activation. Conformational changes in Dectin-1 and TLR4 might be responsible for this coactivation [31, 32]. To the best of our knowledge, this is not reported before. Dectin-1 receptors have an important role in recognizing pathogenic fungi and are involved in immune responses against such fungi by recognizing β(1–3) and β(1–6) linkages in the cell wall of the pathogens [11]. Our data suggest that TLR4 is needed in the fungal responses and that β-glucan is the principle ligand just as LPS is common ligand for responses against pathogenic bacteria [33]. This suggests that TLR4 is a more versatile receptor than considered up to now.

The differences in cytokines induced by particulate β-glucans via Dectin-1 and TLR4 in dendritic cells are plausibly caused by shared and interfering effects on shared intracellular pathways. One of the main mediators for activation of Dectin-1-dependent pathway in immune cells such as dendritic cells is Syk kinase [34]. Syk kinases act downstream of Dectin-1 receptors and activates NF-κB through the CARD9-Bcl10-MALT1 complex [35]. Interference in this pathway by simultaneous TLR4 activation might explain the synergistic effects. Syk kinase has also been associated with TLR4 activation and has been shown to activate the downstream pathway

of TLR4 [36, 37]. In addition, Syk kinase is involved in regulation of TLR4-induced inflammatory pathway [36, 37]. Thus, the regulatory role of Syk kinase in TLR4 activation might be responsible for the regulatory role of TLR4 in particulate β-glucan induced Dectin-1 activation [36, 37].

Dietary β-glucans from fungi or plant sources have been shown to pass through the intestinal barrier into the circulation [38, 39]. However, the route of transfer to circulation differs between soluble and particulate β-glucans. Orally administered soluble β-glucans can be detected in plasma within four hours and they are transported through internalization by intestinal epithelial cells and immune cells [38]. While particulate β-glucans are phagocytosed by macrophages and transported as a cargo through the circulation [39]. Thus, both soluble and particulate β-glucans can stimulate the immune cells in circulation. As Dectin-1 and TLR4 are involved in phagocytosis of antigens [40, 41], synergistic activation of Dectin-1 and TLR4 by soluble and particulate β-glucans could be one of the deciding factors for phagocytosis of β-glucans in vivo, and thus the immune responses by dietary β-glucans. Both types of β-glucans will also have an effect on the numerous dendritic cells in the small intestine that are located in between the intestinal epithelial cells, in the peyer patches, or in the lamina propria [42, 43].

In conclusion, in this study we present a new mechanistic regulation for β-glucan-induced Dectin-1 activation through synergism with TLR4. We have shown that TLR4 acts as a regulator for Dectin-1 activation as inhibition of TLR4 can lead to enhanced immune response against pathogenic fungi. Soluble and particulate β-glucans had different effects on the Dectin-1 isoforms coexpressed with TLR4 (Fig. 7). This different interaction might be an additional explanation for the well-known difference in immune stimulating effect of soluble and particulate β-glucans. Finally, our data suggest that by manipulating the particulate nature of β-glucans different PRRs may be activated resulting in variations in immune responses.

N. M. S. and P. D. V. conceived and designed the experiments, N. M. S. and J. D. F. performed the experiments, N. M. S. analyzed the data, and N. M. S. and P. D. V. wrote the paper.

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