



## Immune effects of $\beta$ -glucan are determined by combined effects on Dectin-1, TLR2, 4 and 5



Pochanart Kanjan <sup>a,b,\*</sup>, Neha M. Sahasrabudhe <sup>a</sup>, Bart J. de Haan <sup>a</sup>, Paul de Vos <sup>a</sup>

<sup>a</sup> Immunoendocrinology, Division of Medical Biology, Department of Pathology and Medical Biology, University of Groningen, University Medical Center Groningen, Hanzelplein 1, 9700 RB Groningen, The Netherlands

<sup>b</sup> Prince of Songkla University, Thailand

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### ABSTRACT

Particulate  $\beta$ -glucans enhanced NF- $\kappa$ B expression in cell-lines co-expressing Dectin-1A-TLR4 and Dectin-1B-TLR4, while soluble  $\beta$ -glucans only synergistically acted on Dectin-1A-TLR4. This was different with Dectin-1 co-expressing TLR2 and TLR5, which inhibited activation after particulate and soluble  $\beta$ -glucan stimulation. The co-acting effect of extracellular TLR2, TLR4 and TLR5 and Dectin-1 by particulate and soluble  $\beta$ -glucan on cytokine production was confirmed in THP-1 macrophages. Dectin-1 activation by particulate  $\beta$ -glucans during TLR2 and TLR4 but not TLR5 blocking was shown to enhance the pro-inflammatory cytokines IL-6, IL-8 and TNF- $\alpha$  in THP-1 macrophages. This effect was stronger with particulate than with soluble  $\beta$ -glucan. The data demonstrate that  $\beta$ -glucans is an immune regulatory ligand for TLR2 and TLR4.

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## 1. Introduction

The nutritional importance of dietary fibers is broadly recognized (Vogt et al., 2013). Dietary fibers are resistant to digestion and fermented in the colon into health promoting molecules (Gibson & Roberfroid, 1995). The consumption of dietary fiber is generally considered important for human health. Health care organizations therefore promote fiber consumption (Burkitt, Walker, & Painter, 1972). A high consumption of dietary fibers is associated with lower mortality and a lesser frequency of circulatory, digestive, and non-cardiovascular diseases. This includes diseases with an immune component such as inflammatory bowel disease and allergy (Arpaia et al., 2013). The mechanisms behind these beneficial immune effects are still not fully understood, but changes in gut microbiota composition and stimulation of short chain fatty acid (SCFA) profiles in the intestine have been suggested to be important. Attenuation of anomalous immune responses by enhanced SCFA production has been demonstrated in several mice studies (Kiely, Ajayi, Wheeler, & Malone, 2001; Lecerf et al., 2012) and clinical trials (Brown & Gordon, 2003; Estrada et al., 1997; Meijer, de Vos, & Priebe, 2010).

$\beta$ -glucan is a dietary fiber with a confirmed health benefit (Estrada et al., 1997). The effect of yeast, fungal, or cereal  $\beta$ -glucans on immune modulation has been studied extensively (Chanput et al., 2012).  $\beta$ -glucans from cereals, such as obtained from oat and barley, are linear  $\beta$  (1–3) and  $\beta$  (1–4) linked polysaccharide formed by  $\beta$ -D-glucopyranosyl units (Zielke et al., 2017), whereas yeast and mushroom  $\beta$ -glucans have similarities in the backbone structure with  $\beta$  (1–6) linkages in the side chains (Estrada et al., 1997). Dietary  $\beta$ -glucans are either soluble or particulate depending on the type of linkages and molecular weight of the molecule (Stier, Ebbeskotte, & Gruenwald, 2014; Tada et al., 2009) and also on the solubilizing agents applied (Tiwari & Cummins, 2009).  $\beta$ -glucan can have effects on the immune system by binding to pattern recognition receptors (PRRs) such as C-type lectin Dectin-1, complement receptor 3, and Toll-like receptors (TLRs) (Borchers, Krishnamurthy, Keen, Meyers, & Gershwin, 2008; Brown & Gordon, 2003; Chan, Chan, & Sze, 2009; Ujita et al., 2009). These receptors are expressed predominantly on macrophages, dendritic cells (DCs) and neutrophils (Brown & Gordon, 2003). Recently we discovered that also TLR4 recognizes  $\beta$ -glucan when co-bound together to Dectin-1 (Sahasrabudhe, Dokter-Fokkens, & de Vos, 2016).

Despite recognition by several immune receptors, Dectin-1 is still considered to be the most important receptor for  $\beta$ -glucans (Brown et al., 2002). The binding of  $\beta$ -glucans to Dectin-1 enhances

\* Corresponding author at: Immunoendocrinology, Division of Medical Biology, Department of Pathology and Medical Biology, University of Groningen, University Medical Center Groningen, Hanzelplein 1, 9700 RB Groningen, The Netherlands.

E-mail address: [pochanart.k@gmail.com](mailto:pochanart.k@gmail.com) (P. Kanjan).

the production of pro-inflammatory cytokines, induce phagocytosis and adaptive immunity (Brown & Gordon, 2003; Yadav & Schorey, 2006). Particulate  $\beta$  (1–3) glucans from fungal source were shown to be stronger stimulators of Dectin-1 than soluble  $\beta$ -glucans. Particulate  $\beta$ -glucans cluster Dectin-1 receptors on the cell membrane. This clustering leads to expulsion of neighboring negative regulators such as CD45 and CD148 of Dectin-1 induced immune activation (Goodridge et al., 2011) and leads to a strong activation of for example dendritic cells that subsequently produce pro-inflammatory cytokines (Yokota, Takashima, Bergstresser, & Ariizumi, 2001). Soluble  $\beta$  (1–3, 1–6) glucans is not able to cluster Dectin-1 receptors and fails to reduce the negative regulatory pathways resulting in lower activation (Goodridge et al., 2011).

In addition to clustering it has been suggested and recently also proven by our group (Sahasrabudhe et al., 2016) that particulate  $\beta$ -glucans can bind to Dectin-1 in a synergistically fashion and co-act with other immune-receptors such as extracellular TLRs (Sahasrabudhe et al., 2016). Toll-like receptors (TLRs) are involved in host defense against fungal  $\beta$ -glucan infections, and some TLRs may be abused by fungi to escape from immune attack (Kawai & Akira, 2011a; Kawai & Akira, 2011b). It has been shown that after  $\beta$ -glucan stimulation a complexation of TLR2 or TLR6 with the Dectin-1 receptor can stimulate release of pro-inflammatory cytokines such as TNF- $\alpha$  (Gantner, Simmons, Canavera, Akira, & Underhill, 2003). Likewise, it was reported that  $\beta$ -glucans and LPS can synergistically activate TLR4 in macrophages (Ferwerda, Meyer-Wentrup, Kullberg, Netea, & Adema, 2008) and that it actually, when synergistically bound to Dectin-1, also binds TLR4 (Sahasrabudhe et al., 2016). Also, it has been shown that TLR4 knockout mice are more susceptible to pathogenic fungal infection such as by *Candida albicans* than wild-type mice, illustrating the interplay between Dectin-1 and TLR4 (Netea et al., 2010). However, despite these observations there is a lack of knowledge on how  $\beta$ -glucans can synergistically bind to Dectin-1 and extracellular TLRs such as TLR 2 and 5.

To gain a better knowledge of the working mechanism and possible synergistic effects of TLR2, 4 and 5 and Dectin-1 after particulate and soluble  $\beta$ -glucans stimulation, we developed reporter cell lines expressing the extracellular TLRs; TLR2, TLR4 and TLR5 with either the transcript variant Dectin-1A or Dectin-1B. In addition, the collaboration between TLR2, 4 and 5 and the Dectin-1 receptor was studied in cytokine induction in human THP-1 macrophages in the presence and absence of blocking antibodies for Dectin-1 and TLR2, 4 and 5 after particulate and soluble  $\beta$ -glucans stimulation.

**Table 1**

Name of the cell line	Selection antibiotics	Expression plasmid	PRRs expressed on the reporter cells (Human origin)
HEK-Null1 Dectin-1A	Blasticidin (12 $\mu$ g/ml)	pUNO1-hDECTIN1a	Dectin-1A
HEK-Null1 Dectin-1B	Zeocin (100 $\mu$ g/ml)	pUNO1-hDECTIN1b	Dectin-1B
HEK-Null1 Dectin-1A-TLR2	Blasticidin (30 $\mu$ g/ml) Zeocin (100 $\mu$ g/ml)	pDUO-Dectin-1A-TLR2	Dectin-1A TLR2
HEK-Null1 Dectin-1B-TLR2		pDUO-Dectin-1B-TLR2	Dectin-1B TLR2
HEK-Null1 Dectin-1A-TLR4	Blasticidin (30 $\mu$ g/ml) Zeocin (100 $\mu$ g/ml)	pDUO-Dectin-1A-TLR4	Dectin-1A TLR4
HEK-Null1 Dectin-1B-TLR4		pDUO-Dectin-1B-TLR4	Dectin-1B TLR4
HEK-Null1 Dectin-1A-TLR5	Blasticidin (30 $\mu$ g/ml) Zeocin (100 $\mu$ g/ml)	pDUO-Dectin-1A-TLR5	Dectin-1A TLR5
HEK-Null1 Dectin-1B-TLR5		pDUO-Dectin-1B-TLR5	Dectin-1B TLR5

## 2. Materials and methods

### 2.1. Sample and chemicals

Zymosan depleted, particulate  $\beta$  (1–3) glucan with a mean molecular weight of 240 kDa obtained from *Saccharomyces cerevisiae* was applied. Particle size was 3  $\mu$ m. Laminarin, is a soluble linear  $\beta$  (1–3) glucan obtained from *Laminaria digitata* and is composed of  $\beta$  (1–6) glucan (interchain) linkages. It has a molecular weight of 6 kDa. These  $\beta$ -glucans were purchased from InvivoGen, Toulouse, France. Zymosan depleted was prepared by treating zymosan with hot alkali to remove its TLR2 stimulating properties. Endotoxin contamination was excluded by testing all  $\beta$ -glucans in a LPS-specific ELISA (ELISA kit from Clone-cloud corp, Houston, USA). The LPS concentrations were lower than the detection level of 4 ng/mL. None of the reporter cell lines applied in this study are responsive to these concentrations of endotoxin (Sahasrabudhe et al., 2016). Lipopolysaccharide (LPS) (*E. coli* K12 strain-TLR4 ligand) was purchased from InvivoGen (France).

### 2.2. Human Dectin-1 and Dectin-1 TLRs reporter cell lines

Dectin-1A and Dectin-1B were transfected with pUNO1-hDectin-1a or pUNO1-hDectin1b plasmids (InvivoGen) to obtain Dectin-1 responsive reporter cells as previously described (Sahasrabudhe, Schols, Faas, & de Vos, 2015). Dectin-1-TLR2, 4 and 5 expressing-plasmids were designed by insertion of Dectin-1 and TLR 2, 4 and 5 expression genes in a pDUO-mcs plasmid (InvivoGen, Toulouse, France). For this we used the GeneArt gene synthesis service (Life technologies, Carlsbad, CA, USA). The plasmids were linearized using PstI FastDigest restriction enzyme (Thermo scientific, Waltham, MA USA). Subsequently, the plasmid was purified and applied for stable transfection in HEK-Blue™ Null1 cells by using Lipofectamine LTX® (Life Technologies). Each newly developed Dectin-1 TLR2, 4 and 5 cell lines were selected by serial dilution in a 96-well plate. The cell lines were cultured and maintained in DMEM culture media (Lonza, Basel, Switzerland) and selection antibiotics as showed in Table 1.

### 2.3. Human Dectin-1 reporter cell line assays

The cell lines co-expressing Dectin-1 and TLR2, 4 or 5 were cultured and maintained in DMEM culture media (Lonza, Basel, Switzerland) as previously described (Sahasrabudhe et al., 2016). NF- $\kappa$ B-induced SEAP activity was assessed using Quanti-Blue (InvivoGen). Cells were stimulated with 100  $\mu$ g/ml particulate and 500  $\mu$ g/ml soluble  $\beta$ -glucans for 24 h at 37 °C. Supernatant

from different treatments were mixed with Quanti-Blue in ratio of 1:4 and quantified at 650 nm using a Versa Max ELISA plate reader (Molecular devices, CA, USA). The assay was performed five times.

#### 2.4. THP-1 macrophage cell stimulation with $\beta$ -glucan

The human monocytic leukemia cell line THP-1 (American Type Culture Collection, Rockville, MD), was cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Invitrogen, UK), 10 mg/ml gentamycin (InvivoGen, Toulouse, France), 2.2  $\mu$ g/ml fungizone (InvivoGen, Toulouse, France), 200 mM glutamine (InvivoGen, Toulouse, France), 100 mM Na-pyruvate (InvivoGen, Toulouse, France) and 0.05 M  $\beta$ -mercaptoethanol (InvivoGen, Toulouse, France). The THP-1 monocytic cell was incubated at 37 °C in 5% CO<sub>2</sub> in a humidified incubator. The macrophage-state was induced by incubating THP-1 monocytes for 48 h with 100 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma) in 12-well cell culture plate (Greiner, Germany) as previously described (Ren et al., 2016). After 48 h of incubation, each well was washed twice with RPMI 1640 medium without PMA and incubated for 24 h in the culture medium.

The next day, each well was supplemented with particulate  $\beta$ -glucan or soluble  $\beta$ -glucan in RPMI 1640 culture medium. To determine the specificity of activation, TLR2, 4, and 5 blocking antibody was added at 10  $\mu$ g/ml and incubated at 37 °C for 1 h. After that, wells were treated with soluble or particulate  $\beta$ -glucan as the same concentration. As positive control for inhibition, 10  $\mu$ g/ml of Dectin-1 blocking antibody (InvivoGen, Toulouse, France) was first added for 1 h at 37 °C and then treated with soluble or particulate  $\beta$ -glucan. The negative controls were THP-1 macrophages incubated in culture medium containing phosphate-buffered saline (PBS). These experiments were repeated at least five times.

#### 2.5. Cytokine quantification

Supernatant from the THP-1 macrophages were collected for cytokine measurements after stimulation for 24 h. A multiplex kit (Affymetrix, Santa Clara, CA, USA) was used to measure the cytokines IL-23, IL-10, IL-12p70, IL-4, IL-6, and TNF- $\alpha$ . A Luminex-100 instrument with StarStation software was used according to the manufacturer's protocol (Luminex, Austin, TX, USA). The measurement of IL-8 was performed by using a commercial ELISA DuoSets (R&D systems), and applied according to the manufacturers instructions.

#### 2.6. Statistical analysis

The data were processed and analyzed by applying GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA). Data are expressed as means and the standard errors of the means (SEM). Data were tested to have non-parametric distribution. The statistical difference was analyzed using Mann-Whitney U-tests. Differences were considered statistically significant when the  $p$  value was  $\leq 0.001$ ,  $\leq 0.01$ , and  $\leq 0.05$ , respectively.

### 3. Results

#### 3.1. Particulate $\beta$ -glucans enhanced NF- $\kappa$ B in Dectin-1 reporter cell-lines co-expressing TLR4 but not when cell-lines co-express TLR2 and TLR5

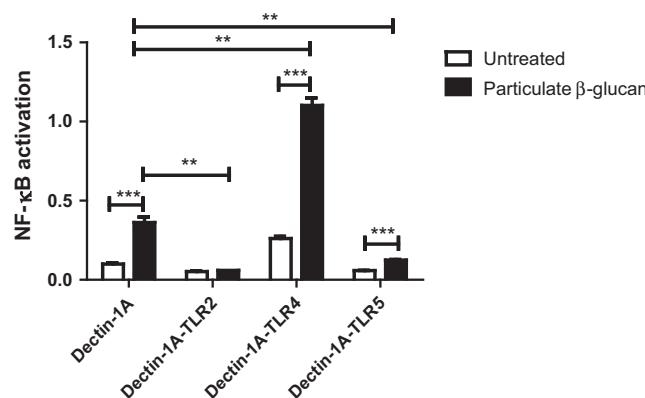
To investigate whether particulate  $\beta$ -glucan could stimulate NF- $\kappa$ B activation potential when Dectin-1 and the extracellular TLRs 2, 4 and 5, are simultaneously expressed, we developed reporter cell lines expressing either Dectin-1 or Dectin-1 combined with TLR2, 4 or 5. Activation of the receptors was determined by quantifying

NF- $\kappa$ B activation. The possible synergistic effects of the TLRs were studied separately for the Dectin-1 transcript variants 1A and 1B.

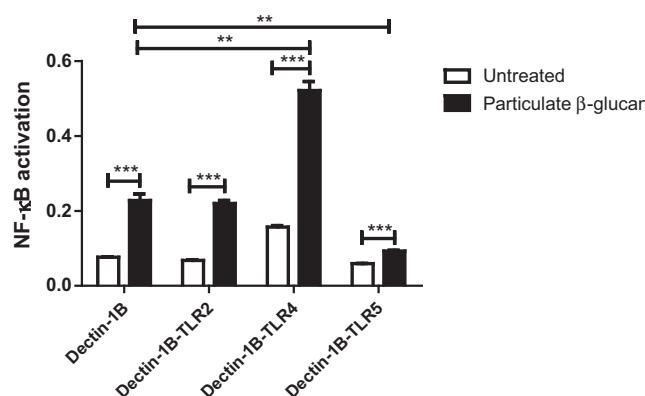
The co-stimulation was different with the transcription variant Dectin-1A and 1B. Particulate  $\beta$ -glucans enhanced NF- $\kappa$ B expression in cell-lines coexpressing Dectin-1A and TLR4 but inhibited particulate  $\beta$ -glucan induced activation of Dectin-1A when also TLR2 or TLR5 was expressed ( $p < 0.01$ ) (Fig. 1). With Dectin-1B the results were different. When particulate  $\beta$ -glucan binds Dectin-1B and TLR2 no effect was observed while with TLR4 enhanced activation was observed, and with TLR-5 inhibition of NF- $\kappa$ B was found ( $p < 0.05$ ) (Fig. 2).

#### 3.2. Soluble $\beta$ -glucans only stimulate Dectin-1A co-expressing TLR4 and not Dectin-1B

Dectin-1 activation is determined by both the particle size and the solubility of  $\beta$ -glucans (Goodridge et al., 2011). It has been suggested that particulate  $\beta$ -glucans clusters different Dectin-1 receptors and is therefore a stronger activator than soluble  $\beta$ -glucans (Sahasrabudhe et al., 2016). An alternative explanation might be that soluble and particulate  $\beta$ -glucans have different binding capacities to Dectin-1 and may simultaneously bind to pattern



**Fig. 1.** TLR4 enhances while TLR2 and TLR5 inhibits particulate  $\beta$ -glucan stimulated Dectin-1A activation. Dectin-1A, Dectin-1A-TLR2, Dectin-1A-TLR4, and Dectin-1A-TLR5 reporter cell lines were stimulated with 100  $\mu$ g/ml particulate  $\beta$ -glucan and NF- $\kappa$ B activation was measured at 650 nm using QuantiBlue reagent. Data are presented as mean  $\pm$  standard deviation ( $n = 5$ ), and statistical differences were measured using Mann-Whitney U test (\*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).

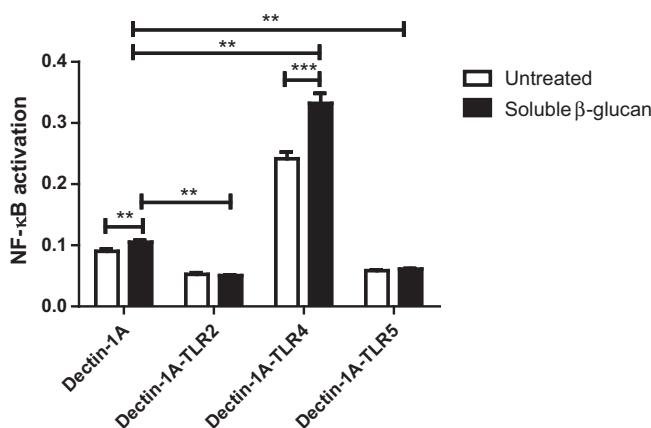


**Fig. 2.** TLR4 enhances while TLR5 inhibits particulate  $\beta$ -glucan stimulated Dectin-1B activation. Dectin-1B, Dectin-1B-TLR2, Dectin-1B-TLR4, and Dectin-1B-TLR5 reporter cell lines were stimulated with 100  $\mu$ g/ml particulate  $\beta$ -glucan and NF- $\kappa$ B activation was measured at 650 nm using QuantiBlue reagent. Data are presented as mean  $\pm$  standard deviation ( $n = 5$ ), and statistical differences were measured using Mann-Whitney U test (\*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).

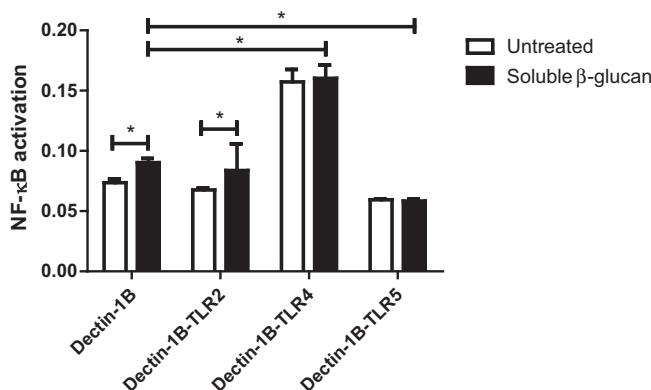
recognition receptors such as TLRs. To determine whether such a mechanism exist, we also studied activation of soluble  $\beta$ -glucans of Dectin-1A and 1B expressing cell-lines co-expressing TLR2, 4 and 5. As soluble  $\beta$ -glucan source we applied soluble  $\beta$ -glucans laminarin.

Soluble  $\beta$ -glucans laminarin induced high NF- $\kappa$ B activation in cell-lines co-expressing Dectin-1A and TLR4. The co-activation of Dectin-1A and TLR4 was strong as it showed a 2.1-fold ( $p < 0.01$ ) increase in NF- $\kappa$ B expression compared to cell lines expressing only Dectin-1A (Fig. 3). Surprisingly, the activation of Dectin-1A by soluble  $\beta$ -glucans was totally blocked when either TLR2 or TLR 5 was present in the cell-line ( $p < 0.01$ ).

The results were different and more variable with the splice variant Dectin-1B. Here we only found a complete blocking of the soluble  $\beta$ -glucan activation when TLR5 was expressed ( $p < 0.05$ ) but no effect was observed when either TLR2 or TLR4 was expressed (Fig. 4) compared to un-stimulated controls. Hence, we confirmed that soluble  $\beta$ -glucans can synergistically act via Dectin-1A and TLR4 but not via Dectin-1B and that TLR2 and 5 negatively impact soluble  $\beta$ -glucan activation via Dectin-1.



**Fig. 3.** TLR4 enhances while TLR2 and TLR5 blocks soluble  $\beta$ -glucan stimulated Dectin-1A activation. Dectin-1A, Dectin-1A-TLR2, Dectin-1A-TLR4, and Dectin-1A-TLR5 reporter cell lines were stimulated with 500  $\mu$ g/ml soluble  $\beta$ -glucan and NF- $\kappa$ B activation was measured at 650 nm using QuantiBlue reagent. Data are presented as mean  $\pm$  standard deviation ( $n = 5$ ), and statistical differences were measured using Mann-Whitney U test ( $^{**}p < 0.01$ ,  $^{***}p < 0.001$ ).



**Fig. 4.** Absence of effect of TLR2 and TLR4 but complete blocking of soluble  $\beta$ -glucan stimulated Dectin-1B activation by TLR5. Dectin-1B, Dectin-1B-TLR2, Dectin-1B-TLR4, and Dectin-1B-TLR5 reporter cell lines were stimulated with 500  $\mu$ g/ml soluble  $\beta$ -glucan and NF- $\kappa$ B activation was measured at 650 nm. Data are presented as mean  $\pm$  standard deviation ( $n = 5$ ) and statistical differences were measured using Mann-Whitney U test ( $^{*}p < 0.05$ ).

### 3.3. TLR2 and TLR4 but not TLR 5 blocking stimulates IL-6, IL-8, and TNF- $\alpha$ production in THP-1 macrophages stimulated with particulate $\beta$ -glucans

The above presented study clearly demonstrates the synergistic action between Dectin-1 and TLRs after stimulation with particulate  $\beta$ -glucans. To investigate whether this holds consequences for human immune cells and cytokine production, we studied the effect of blockade of either Dectin-1, TLR2, 4 or 5 by using blocking antibodies in THP-1 macrophage cells stimulated with particulate  $\beta$ -glucans. After 24 h, supernatants were analyzed for presence of the innate chemokine IL-8, the anti-inflammatory cytokine IL-10, the Th2 promoting cytokine IL-6, the Th1 stimulating cytokine TNF- $\alpha$ , and the Th17 supporting cytokine IL-23.

As shown in Fig. 5, the specificity of particulate  $\beta$ -glucans induced cytokine release was determined by using a Dectin-1 blocking antibody which was added 1 h before stimulation. Dectin-1 blocking antibody could reduce production of IL-8 (2.2-fold,  $p < 0.05$ ), IL-6 (5.5-fold,  $p < 0.05$ ), and TNF- $\alpha$  (6.0-fold,  $p < 0.05$ ) (Fig. 5). However, there were no statistically significant effects of Dectin-1 blocking antibody on the anti-inflammatory cytokine IL-10 and the Th17 stimulating IL-23.

To study the effect of TLR inhibition, TLR2, 4 and 5 blocking antibody was added for one hour prior to stimulation. The TLR2 blocking antibody could enhance production of IL-6 (2.3-fold,  $p < 0.05$ ), IL-8 (1.5-fold,  $p < 0.05$ ), and TNF- $\alpha$  (4.3-fold,  $p < 0.05$ ) (Fig. 5). Also, TLR4 blocking antibody could increase production of IL-8 (1.6-fold,  $p < 0.05$ ), and TNF- $\alpha$  (6.1-fold,  $p < 0.001$ ) (Fig. 5). However, the anti-inflammatory cytokine IL-10 and Th17 supporting cytokines IL-23 showed no statistical significant difference after TLR2 or 4 blocking. TLR5 blocking had no effect on pro-inflammatory cytokines (Fig. 5).

### 3.4. TLR2 and TLR4 but not TLR 5 blocking enhanced IL-8 and TNF- $\alpha$ production in THP-1 macrophages after soluble $\beta$ -glucan stimulation

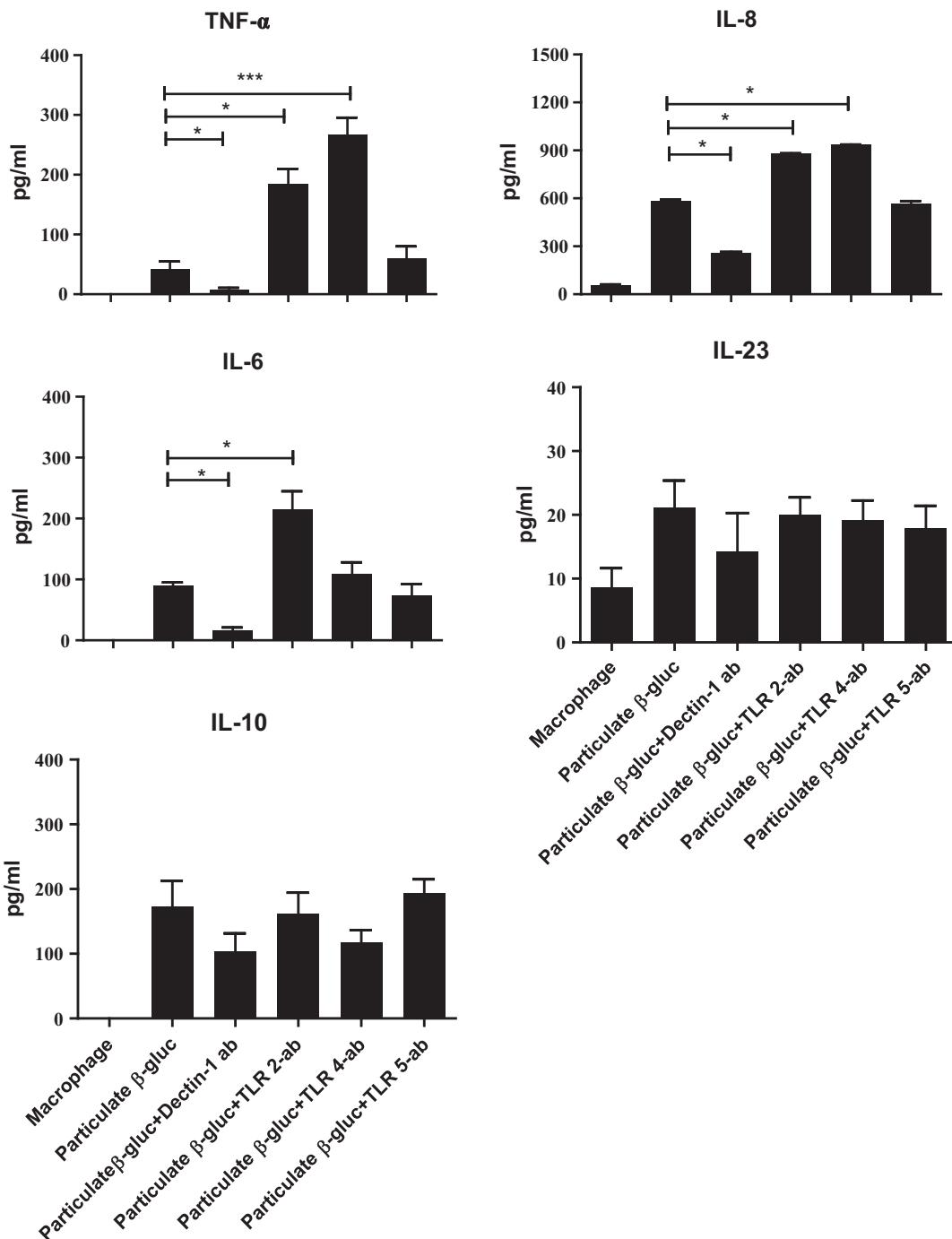
Next, we studied the impact of soluble  $\beta$ -glucan laminarin on cytokine release from THP-1 macrophages when THP-1 macrophages were pretreated with either Dectin-1, TLR2, 4 or 5 blocking antibody. Also, here after 24 h supernatants were analyzed for the presence of the innate chemokine IL-8, the anti-inflammatory cytokine IL-10, the Th2 promoting cytokine IL-6, the Th1 stimulating cytokine TNF- $\alpha$ , and the Th17 supporting cytokine IL-23.

As shown in Fig. 6, the levels of TNF- $\alpha$ , IL-8, IL-6 and IL-10 after soluble  $\beta$ -glucan stimulation were in general lower than after particulate  $\beta$ -glucan stimulation, suggesting that particle size and solubility of  $\beta$ -glucans impacts stimulation and cytokine production. However, also here synergism between Dectin-1 and the extracellular TLRs was observed.

Blocking Dectin-1 with antibody reduced the production of the cytokine/chemokine IL-8 (1.5-fold,  $p < 0.05$ ), IL-10 (2.4-fold,  $p < 0.05$ ) and IL-6 (9.0-fold,  $p < 0.05$ ) (Fig. 6). The production of TNF- $\alpha$  and IL-23 were not statistically significantly different after soluble  $\beta$ -glucan stimulation. TLR2 blocking induced an enhanced production of IL-8 (1.8-fold,  $p < 0.05$ ) and TNF- $\alpha$  (8.2-fold,  $p < 0.05$ ) (Fig. 6). Also, TLR4 blocking antibody enhanced production of IL-8 (2.2-fold,  $p < 0.05$ ) and TNF- $\alpha$  (14-fold,  $p < 0.05$ ) (Fig. 6) after soluble  $\beta$ -glucan stimulation. Similar to particulate  $\beta$ -glucan, TLR5 blocking did not influence cytokines/chemokine production after soluble  $\beta$ -glucan stimulation (Fig. 6).

## 4. Discussion

We show here that particulate and soluble  $\beta$ -glucan can synergistically stimulate but also inhibit Dectin-1 via TLR2, 4 and 5. Synergistic

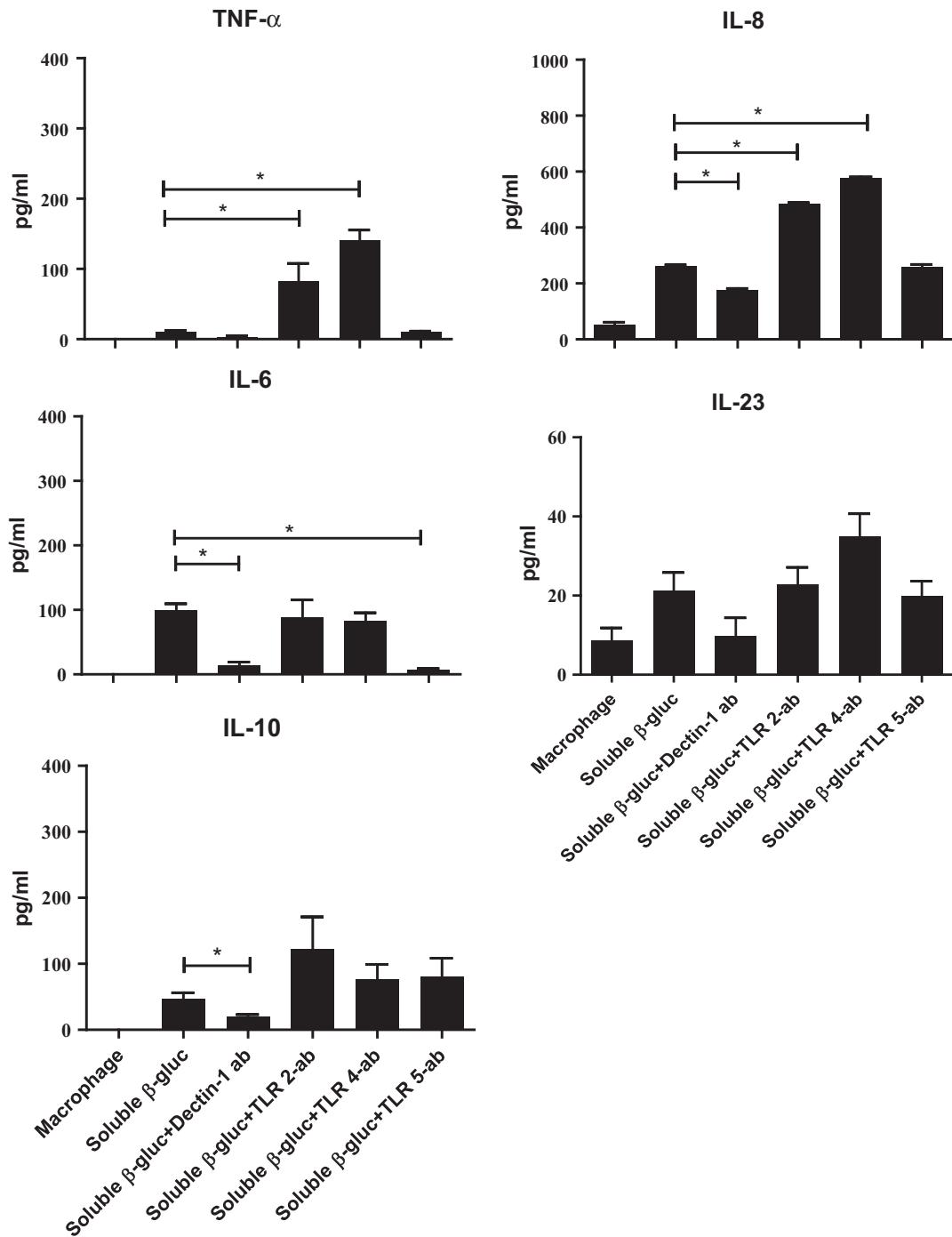


**Fig. 5.** TLR2 and TLR4 blocking in particulate  $\beta$ -glucan stimulated THP-1 macrophage increases IL-6, IL-8 and TNF- $\alpha$  production. THP-1 macrophage was stimulated with 100  $\mu$ g/ml particulate  $\beta$ -glucan zymosan. THP-1 macrophages were pretreated with either TLR 2, 4 and 5 blocking antibody or Dectin-1 blocking antibody at 10  $\mu$ g/ml followed by stimulation with 100  $\mu$ g/ml particulate  $\beta$ -glucan zymosan. The cytokine production was measured using multiplex assays. The data shown are the means and standard deviation of four different experiments, in triplicate. The statistical differences were measured using Mann-Whitney U test ( $^*p < 0.05$  and  $^{***}p < 0.001$ ).

ergy is present between Dectin-1 and TLR4 in NF- $\kappa$ B expressing reporter cell lines, but inhibiting effects were also demonstrated after particulate and soluble  $\beta$ -glucan stimulation for TLR2 or TLR5 when co-expressed with Dectin-1. The magnitude of the synergistic effect is determined by the physical-chemical composition of the  $\beta$ -glucans. The stimulation of the response in the reporter cell-lines was stronger with particulate than with soluble  $\beta$ -glucans. Our study demonstrate that particulate  $\beta$ -glucans had similar effects on both transcription variants of Dectin-1-TLR4, i.e. Dectin-1A and Dectin-1B. However, the synergistic effect of sol-

uble  $\beta$ -glucans was only present when TLR4 was co-expressed with Dectin-1A. These results clearly demonstrate the existence of structure-effector relationships between  $\beta$ -glucans and PRRs which may lead to design of more efficacious immune active food products containing  $\beta$ -glucans.

The difference in efficacy between particulate and soluble  $\beta$ -glucan in immune stimulation is usually attributed to clustering of Dectin-1 receptors by particulate  $\beta$ -glucan (Kawai & Akira, 2011a; Kawai & Akira, 2011b). This was indeed confirmed in the present study when Dectin-1 was co-expressed with



**Fig. 6.** TLR2 and TLR4 blocking in soluble  $\beta$ -glucan stimulated THP-1 macrophage increases IL-8 and TNF- $\alpha$  production. THP-1 macrophage was stimulated with 500  $\mu$ g/ml soluble  $\beta$ -glucan laminarin. THP-1 macrophages were pretreated with TLR 2, 4 or 5 blocking antibody or Dectin-1 blocking antibody at 10  $\mu$ g/ml followed by stimulation with 500  $\mu$ g/ml soluble  $\beta$ -glucan laminarin. The cytokine production was measured using multiplex assays. The data shown are the means and standard deviation of four different experiments, in triplicate. The statistical differences were measured using Mann-Whitney U test (\* $p < 0.05$ ).

extracellular TLR2, 4 or 5. Particulate  $\beta$ -glucans acted differently on the studied receptors than soluble  $\beta$ -glucans. Soluble  $\beta$ -glucan was not active in stimulation of Dectin-1B-TLR2, 4 or 5. This confirms and extends our previous study in human dendritic cells with Dectin-1 and TLR4 (Sahasrabudhe et al., 2016). Notably,  $\beta$ -glucans had no effect on TLR-4 alone when it was expressed in HEK-cells without Dectin-1 (Sahasrabudhe et al., 2016). The difference in efficacy of soluble  $\beta$ -glucans should probably be explained as follows. The Dectin-1 receptor has an extracellular C-type lectin like domain and stalk region, which is followed by

a single trans-membrane pass and a tyrosine based activation-like domain in the cytoplasm (Willment, Gordon, & Brown, 2001). The human Dectin-1B receptor lacks this stalk region (Willment et al., 2001; Yokota et al., 2001) and will be less available for the soluble form of  $\beta$ -glucan. This same stalk region in Dectin-1A probably facilitates the interaction of Dectin-1A and TLR4 in its enhanced activation. Thus, the lack of activation by soluble  $\beta$ -glucan in activating Dectin-1B-TLR4 can be explained by pertinent differences in the Dectin-1A and 1B transcript variants (Sahasrabudhe et al., 2016).

A role for extracellular TLRs such as TLR2, 4 and 5 in particulate and soluble  $\beta$ -glucan induced Dectin-1 activation was confirmed in THP-1 cells differentiated into macrophages. TLR2, 4 and 5 blocking in particulate and soluble  $\beta$ -glucan stimulated THP-1 macrophages lead to enhanced immune responses. By separately blocking Dectin-1 and TLR2, 4 and 5, we showed in THP-1 macrophages that different types and quantities of cytokines are induced by particulate and soluble  $\beta$ -glucan after binding to either Dectin-1 or TLR2, 4 and 5 or the combination. TLR2, 4 and 5 binding by particulate  $\beta$ -glucan during blockage of Dectin-1 reduced IL-8, IL-6, and TNF- $\alpha$ , while IL-10 and IL-23 reduction were minor and did not reach statistical significance. Similar to particulate  $\beta$ -glucan, TLR-2, 4 and 5 binding by soluble  $\beta$ -glucan during blockage of Dectin-1 reduced IL-8, IL-10, and IL-6, while TNF- $\alpha$  and IL-23 were not statistically significantly different.

In contrast to the Dectin-1 blockade, Dectin-1 binding by particulate and soluble  $\beta$ -glucan during TLR2 and 4 blocking resulted in increased production of the pro-inflammatory cytokines TNF- $\alpha$ , IL-8, and IL-6, illustrating the regulatory or anti-inflammatory role of TLR2 and 4 after particulate and soluble  $\beta$ -glucan binding, indicating that Dectin-1 is the major receptor for particulate  $\beta$ -glucans. However, Dectin-1 binding by particulate and soluble  $\beta$ -glucan during TLR5 blocking did not have such an effect. These data suggest that TLR5 binding by  $\beta$ -glucan does not lead in THP-1 macrophages to downstream signaling and modulation of the cytokines studied. Although soluble  $\beta$ -glucan could stimulate THP-1 macrophages to produce cytokines, the levels were in general lower than after particulate  $\beta$ -glucan stimulation. It can be concluded and confirms our findings in cell-lines that different structures of  $\beta$ -glucan determine the type and mode of action on regulating immune responses via co-stimulation via extracellular TLRs (Goodridge et al., 2011; Yokota et al., 2001). This corroborates the *in vivo* findings of Yap and Ng (2005), who reported that TNF- $\alpha$ , IL-8 and IL-6 levels were higher in blood from mice fed with particulate  $\beta$ -glucan than those fed with soluble  $\beta$ -glucan. Notably however, THP-1 macrophages express in addition to TLRs also other regulatory receptors along with TLR2, 4 and 5 and it can therefore not be excluded that other receptors are responsible as well for the increased cytokine production (Diebold, 2009). However, the blockade studies in the macrophages as well as the cell-line studies demonstrate a pertinent role for especially TLR2 and 4 in stimulation of Dectin-1 by both soluble and particulate  $\beta$ -glucans.

We demonstrate that Dectin-1 and TLR2 and 4 in THP-1 macrophages are important partners in enhancing pro-inflammatory cytokine production after particulate and soluble  $\beta$ -glucan stimulation. The variations in cytokines induced by particulate and soluble  $\beta$ -glucan via Dectin-1 and TLR2 and 4 are most likely the consequence of common and interfering influences on shared intracellular pathways. This suggestion is supported by previous studies with yeast cell wall zymosan demonstrating that Dectin-1 and TLR2 act synergistically in zymosan-induced TNF- $\alpha$ , IL-23 and IL-6 production (Rogers et al., 2005). A major mediator for activation of Dectin-1-dependent pathways in immune cells such as macrophages is Syk tyrosine kinase (Rogers et al., 2005). The Syk tyrosine kinase acts downstream of Dectin-1 receptors and activates NF- $\kappa$ B through the CARD9-Bcl10-MALT1 complex (Gross et al., 2006). In addition,  $\beta$ -glucans can induce nuclear NF- $\kappa$ B via binding to Dectin-1 in association with TLR2 and 4 in a MyD88-dependent signaling cascade (Adachi et al., 2004). Thus, the activation of Syk kinase or MyD88 in TLR2 and 4 might be responsible for the regulatory role of TLR2 and 4 in particulate and soluble  $\beta$ -glucan induced Dectin-1 activation. The activation of the Dectin-1 receptor in the reporter cell lines is demonstrated only via NF- $\kappa$ B mediated production of SEAP. Although, NF- $\kappa$ B is an important transcription factor, it is not the only downstream pathway activated through Dectin-1 activation in normal immune cells such

as macrophage. In macrophage, other transcription factors like nuclear factor of activated T-cells (NFAT) are also activated downstream and contribute to production of pro-inflammatory cytokines (Kawai & Akira, 2011a; Kawai & Akira, 2011b). This explains why the reporter cell lines showed no effect of Dectin-1 co-activation TLR2.

Up to now, it was assumed that TLR2 only recognizes lipoproteins of various infectious pathogens. This included peptidoglycan and lipoteichoic acid of Gram positive bacteria as well as from mycoplasma and spirochetes. A TLR4/MD2 complex is the receptor for bacterial lipopolysaccharides, which is enforced by co-expression of CD14 (Kawai & Akira, 2011a; Kawai & Akira, 2011b). In the present study,  $\beta$ -glucan could also activate TLR2 and 4 signaling when accompanied by Dectin-1 receptor activation. This is most likely due to conformational variations in Dectin-1 and TLR2 or TLR4 (Adachi et al., 2004; Wang et al., 2016). Dectin-1 plays an essential role in recognizing fungal pathogens and is involved in immunity against fungi by recognizing  $\beta$ (1–3) and  $\beta$ (1–6) linkage on pathogens (Stier et al., 2014).

The role of physicochemical characteristics of  $\beta$ -glucans in the activation of innate or adaptive immune responses is well-known (Chan et al., 2009). The effects are probably both intestinal and systematic. The dietary  $\beta$ -glucans from fungi or plant sources can pass the intestinal barrier after binding to gut epithelial cells and to the gut-associated lymphoid tissue (GALT) (Brown & Gordon, 2003; Estrada et al., 1997). Subsequently,  $\beta$ -glucans can be phagocytosed by macrophages and transported into the bloodstream (Rice, 2005). Collaboration between Dectin-1 and extracellular TLR2 and 4 are inducing phagocytosis of antigens (Hayashi et al., 2001; Hong et al., 2004) which might be the mechanism behind uptake of  $\beta$ -glucan. The co-activation of Dectin-1 and TLR2 and 4 by particulate and soluble  $\beta$ -glucans might therefore be crucial for the type and magnitude of the immune responses induced by dietary  $\beta$ -glucans.

## 5. Conclusion

Our data provide new mechanistic insight in  $\beta$ -glucan-induced Dectin-1 activation. Soluble and particulate  $\beta$ -glucans induce different quantities and types of cytokines when stimulating macrophages. We have shown that TLR2 and 4 acts as regulators for Dectin-1 activation and inhibition of TLR2 and 4 can lead to enhanced immune responses. Soluble and particulate  $\beta$ -glucans interact differently with TLR2, 4 and 5 and this different mode of interaction might be involved in the difference in immune stimulating effect of soluble and particulate  $\beta$ -glucans. This insight might assist and facilitate development of effective  $\beta$ -glucan preparations to guide immune responses.

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