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Dectin-1b activation by arabinoxylans induces trained immunity in human monocyte-derived macrophages

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ABSTRACT

Arabinoxylans of various structures and sources have shown to possess the ability to induce a range of immune responses in different cell types *in vitro* and *in vivo*. Although the underlying mechanisms remain to be fully established, several studies point towards the involvement of activation of pattern recognition receptors (PRRs). Activation of specific PRRs (*i.e.,* Dectin-1 and CR3) has also been shown to play a key role in the induction of a non-specific memory response in innate immune cells, termed 'trained innate immunity'. In the current study, we assessed whether arabinoxylans are also able to induce trained innate immunity. To this end, a range of arabinoxylan preparations from different sources were tested for their physicochemical properties and their capacity to induce innate immune training and resilience. In human macrophages, rice and wheat-derived arabinoxylan preparations induced training and/or resilience effects, the extent depending on fiber particle size and solubility. Using a Dectin-1 antagonist or a CR3 antibody, it was demonstrated that arabinoxylan-induced trained immunity in macrophages is mainly dependent on Dectin-1b. These findings build on previous observations showing the immunomodulatory potential of arabinoxylans as biological response modifiers and open up promising avenues for their use as health promoting ingredients.

1. Introduction

Arabinoxylan is the major component of dietary fiber found in cereals, such as oat, wheat, corn, rye and rice bran. Arabinoxylans consist of a backbone of β-(1,4)-linked xylose residues with branches of arabinose residues substituted on the xylose O-2 and/or O-3 position. The position of substitution and degree of branching differs between arabinoxylan preparations depending on their source [1]. Wheat and rye endosperm arabinoxylans are generally less branched with arabinose/xylose ratios of 0.5–0.7 and 0.5–0.6, respectively, compared to arabinoxylan in rice which contains an arabinose/xylose ratio of ~0.8 [2]. Studies have shown that variation in molecular weight and degree of branching are major determinants of their biological functionality [3,4]. Over the years arabinoxylans have gained attention as potential biological response modifiers (BRMs) [5]. Especially their interaction with immune cells is a topic of active research. Arabinoxylans were shown to induce the production of the immunoregulatory cytokine IL-10 by dendritic cells [6], to increase NK cell activity [7] and macrophage phagocytosis [8], and to induce monocyte-recruitment *in vitro* [9]. Moreover, arabinoxylans have been shown to stimulate the innate immune system *in vivo* through an increase in NK cell activity against tumor formation and neuroblastoma [7,10,11], and peripheral blood frequencies of myeloid-derived dendritic cells in multiple myeloma patients [12]. A dietary intervention study in healthy older individuals demonstrated that oral administration of an arabinoxylan preparation increased antibody titers and seroconversion rate in response to influenza vaccination [13].

Although the mechanism by which arabinoxylans enhance the immune system remains to be determined, it is conceivable that this occurs through pattern recognition receptor (PRR) ligation of immune cells

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[14]. Immune cell activation by β-glucans through ligation of specific PRRs has been shown to induce a non-specific memory response in innate immune cells, a process that has been termed 'trained innate immunity' [15,16]. Trained innate immune memory describes the ability of innate immune cells to enhance their immune responsiveness after a first microbiological or macro-molecular stimulus, which is attributed to epigenetic remodeling and rewiring of metabolic pathways [17,18]. This enhanced innate immune responsiveness is not only specific for the antigen of the initial trigger but also for heterologous secondary stimuli. Moreover, innate responses can also be rescued in situations where the secondary immune response is one of tolerance (*i.e.*, following lipopolysaccharide (LPS) exposure), a phenomenon we refer to as resilience [19]. Quintin and colleagues have shown that the BRM β-glucan derived from *Candida albicans* could induce trained immunity in purified human monocytes, depending on the direct interaction with Dectin-1 and complement receptor 3 (CR3) [16]. In previous work, we have shown that continuous exposure to β-glucans derived from bacteria and yeast can also induce training and resilience in human macrophages depending on structure and solubility of these β-glucans [20].

Based on our previous established training-model using human macrophages, we here investigate whether arabinoxylans also contain the capacity to induce innate immune training, *i.e.,* causing an enhanced response to a subsequent challenge, and to enhance resilience, *i.e.,* rescue macrophages from tolerance. In addition, we aimed to relate structure, particle size and solubility to the capacity of a series of arabinoxylans to induce innate immune training and resilience. Additionally, the involvement of Dectin-1a, Dectin-1b and CR3 in the immune activating properties of arabinoxylans will be determined. This research will contribute to characterize the potential of arabinoxylans to be applied as BRMs or nutraceuticals.

2. Materials and methods

2.1. Arabinoxylan preparations

We used six different arabinoxylan preparations: rice hull, wheat bran, corn cob, rice bran-1, rice bran-2 and rice bran-3. Rice hull is a preparation of arabinoxylans isolated from grounded rice hulls and was provided by RIBUS (St. Louis, MO, USA). Wheat bran is a preparation of wheat bran extracted arabinoxylan-oligosaccharides provided by Cargill (Minneapolis, MN, USA). Corn cob is a preparation of oligosaccharides constituting of chains of d-xylose molecules linked *via* β(1–4) bonds with a degree of polymerization (DP) ranging from 2 to 7. The preparation is obtained from corn cobs (*Z. mays* subsp. *mays*) and was provided by Shandong Longlive Bio-technology Co., Ltd. (Shandong, China). Rice bran-1 is a preparation of arabinoxylans isolated from rice bran (RiFiber) and provided by RiceBran Technologies (The Woodlands, TX, United States). Rice bran-2 is a preparation of arabinoxylans isolated from rice bran (Proryza® Gold) and provided by RiceBran Technologies (The Woodlands, TX, United States). Rice bran-3 is a preparation of arabinoxylans isolated from rice bran and provided by Urmatt Ltd (Wattana, Thailand).

2.2. Detection and removal of LPS from arabinoxylan preparations

To detect and quantify presence of LPS in arabinoxylan preparations the EndoZyme® test kit (Hyglos GmbH, Bernried am Starnberger See, Germany) was used according to the manufacturer's protocol and spike controls were included for all arabinoxylan preparations. All arabinoxylan preparations were found to be contaminated with LPS (> 0.002 ng/ml) and were treated as previously described [21]. Briefly, preparations (50 mg) were treated with alkaline-ethanol (0.1 M NaOH, 10 ml) at 56 °C for 5 h, followed by neutralization with HCl. After neutralization, absolute alcohol (29 ml) was added, and samples were stored overnight at 4 °C. Next, the arabinoxylan preparations were recovered by centrifugation for 20 min at 3320 ×*g* and washed three times with 60% ethanol. All arabinoxylan preparations were lyophilized and excluded for the presence of LPS prior to their use in further analyses.

2.3. Molar mass, solubility, monosaccharide and protein content of arabinoxylan preparations

High pressure size exclusion chromatography (HPSEC) was used to determine molar masses and solubility of the arabinoxylans present in the different preparations. Briefly, preparations were suspended in water (4 mg/ml) and heated to 100 °C for 10 min, followed by incubation with *endo*-xylanase 1 and 3 (1039, 1007–1) in 10 mM NaOAc buffer (pH 3) at 37 °C for 24 h. Samples were diluted to a concentration of 2 mg/ml and centrifuged (18,000 ×*g*) for 10 min at room temperature (RT). Supernatants were analyzed using an Ultimate 3000 HPLC (Dionex, Sunnyvale, CA, USA) equipped with a Shodex RI-101 refractive index detector (Showa Denko, Tokyo, Japan). Three TSK-Gel columns (Tosoh Bioscience, Tokyo, Japan) connected in series (4000–3000–2500 SuperAW; 150 × 6 mm) were preceded by a TSK Super AW-L guard column (35 × 4.6 mm). Ten microliters of sample was injected and eluted with 0.2 M $NaNO_3$ at 55 °C at a flow rate of 0.6 ml/min. Pullulan molecular-mass standards from 0.2 to 780 kDa (Polymer Laboratories, Palo Alto, CA, USA) were used for calibration. To determine the monosaccharide composition, arabinoxylan preparations were hydrolyzed with 1 M sulphuric acid at 100 °C for 3 h. The monosaccharides released, were derivatized to alditol acetates and analyzed by gas chromatography using inositol as an internal standard [22]. The presence of uronic acid (UA) was determined with the colorimetric *m*-hydroxydiphenyl assay automated on an autoanalyzer (Skalar, Breda, The Netherlands) as described before [23]. Solubility of the arabinoxylan preparations was assessed according to areas under the HPSEC curves compared to that of polygalacturonic acid (5 mg/ml), which is a highly soluble (> 99%) polymer. Preparations were solubilized in water (5 mg/ml) and shaken for 3 h followed by centrifugation at 15 ×*g* for 5 min, after which the preparations were subjected to HPSEC analysis.

Protein content in the arabinoxylan preparations was determined by the combustion method (Dumas) using a FlashEA 1112 Nitrogen and Protein Analyzer (Interscience, Breda, The Netherlands) according to the instructions of the manufacturer. Samples (5–10 mg) were weighted into aluminum cups and measured in triplicates using cellulose (Sigma-Aldrich, Zwijndrecht, The Netherlands) as negative control and methionine (Sigma-Aldrich) as standard. Protein content in the samples was calculated with 6.25 as nitrogen to protein conversion factor.

2.4. Quantification of (1,3;1,4)-β-glucan in arabinoxylan preparations

For quantification of cereal (1,3;1,4)-β-glucan levels in all arabinoxylan preparations, the Megazyme β-Glucan (Mixed Linkage) Assay Kit (Megazyme International Ireland Ltd., Wicklow, Ireland) was used according to the manufacturer's protocol (Protocol (A), AOAC method 995.16, AACC method 32–23 and ICC standard method no. 166). Briefly, preparations were weighed to calculate dry mass and milled using a Fritsch pulverisette 14® (Fritsch GmbH, Idar-Oberstein, Germany). Two independent samples and barley flour control samples were obtained and averaged for final cereal (1,3;1,4)-β-glucan quantification, calculated as % of dry weight (*w*/w). Two technical replicates were performed on all samples using the Megazyme kit including a standardized barley flour control (4.05%–4.15% w/w of (1,3;1,4)-β-glucan, Megazyme kit).

2.5. Particle size distribution

Particle size was determined by laser diffraction using the Mastersizer 2000 (Malvern Instruments, Worcestershire, UK). Briefly, 5 mg of the arabinoxylan preparations were suspended in 5 ml Milli-Q water by vortex mixing. This sample suspension was added into the Hydro 2000SM Dispersion Unit at a stirring speed of 112 ×g until a laser

obscuration factor within the range of 10–20% was achieved. The average size ($n = 3$) in μm was determined for all arabinoxylan preparations. Particle refractive and absorption indices were set to 1.51 and 0.01, respectively. These measurements were performed three times with independently prepared batches.

2.6. Isolation and culture of human monocytes

Primary human monocytes were isolated from peripheral blood mononuclear cells (PBMCs) of healthy donors who had provided written informed consent (Sanquin, Nijmegen, The Netherlands). Isolation of PBMCs was performed as described previously [20]. Briefly, blood was diluted 1:1 with sterile phosphate-buffered saline (PBS) (Sigma-Aldrich) containing 2% heat-inactivated fetal bovine serum (FBS) (HyClone™ Fetal Bovine Serum, Fisher Scientific, Loughborough, UK) followed by density centrifugation over 15 ml Ficoll-Paque plus (GE Healthcare Life Sciences) using Greiner Bio-One™ LeucoSEP™ Polypropylene Tubes. The interface layer, containing PBMCs, was isolated and the cells were washed three times in PBS containing 2% FBS. Isolation of monocytes was performed using the quadroMACS system and CD14 microbeads (Miltenyi Biotec, Leiden, The Netherlands). Monocytes were frozen in FBS with 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and stored in liquid nitrogen.

2.7. Training and resilience model in primary monocyte-derived human macrophages

Monocytes (500,000 cells/well) were added to 24-well tissue culture (TC) treated plates (Corning Costar, New York, NY, USA) and incubated for 24 h at 37 °C in RPMI 1640 – Glutamax – HEPES medium (Gibco, Bleiswijk, The Netherlands) supplemented with 10% FBS, 1% MEM non-essential amino acids (Gibco), 1% Na-pyruvate (Gibco), 1% Pen/strep (Gibco) and 50 ng/ml macrophage colony-stimulating factor (M-CSF) (R&D systems, Minneapolis, MN, USA) in a total volume of 1 ml as described before [20]. After 24 h, monocytes used to apply the resilience model were stimulated by adding 10 ng/ml LPS (Sigma-Aldrich, *Escherichia coli* serotype O111:B4) to the culture medium and cells, while monocytes used to apply the training model were kept in culture medium. After 24 h of stimulation, cells tested for training and resilience were washed once with pre-warmed medium and culture medium with 50 ng/ml M-CSF was added for five days. During the five-day incubation period the monocytes were exposed to 5 μg/ml arabinoxylan preparation. This strategy of continuous stimulation with addition of M-CSF was previously validated and compared with the original training model as established by Netea and colleagues [17,20]. At day seven, cells tested for training and resilience were stimulated with 10 ng/ml LPS. After 24 h, supernatants were collected and stored at −20 °C for further analysis. For inhibition of Dectin-1 and CR3, monocytes were preincubated for 1 h with either 100 μg/ml laminarin (Sigma-Aldrich, L-9634), 5 μg/ml anti-CD11b antibody (R&D system, AF 1699) or 5 μg/ml control Goat IgG (R&D, AB-108-c) directly before supplementation with arabinoxylan preparations.

2.8. Culturing reporter cell lines

The NFκB reporter cell lines HEK-Blue™Null1-v cells, HEK-Blue™-hDectin-1a and HEK-Blue™-hDectin-1b (InvivoGen, Toulouse, France) were cultured and maintained in high glucose DMEM GlutaMAX™ (Gibco) supplemented with 10% heat-inactivated FBS (Gibco). These reporter cell lines contain the SEAP reporter gene which is expressed under control of an NF-κB transcription factor-responsive promoter. All cell lines were cultured and propagated according to the manufacturer's protocol. Briefly, cell passage was performed by trypsinization with 0.05% trypsin-EDTA (Life Technologies) using a split ratio of 1:10. All cell lines (passage 9–28) were seeded at 1×10^6 cells/ml in 100 μl/well in a poly-D-Lysine coated 96-well microplate (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) overnight at 37 °C and 5% CO_2. The following day, reporter cell lines were stimulated for 24 h with different concentrations of arabinoxylan preparations (5, 10, 100 or 1000 μg/ml) in a total volume of 200 μl/well. To study the inhibition capacity of laminarin for both Dectin-1 receptor isoforms, Dectin-1 reporter cells were preincubated for 1 h with 1000 μg/ml laminarin directly before stimulation with different arabinoxylan preparations. Stimulations with β-glucan yeast-b (Immitec, Tonsberg, Norway) [20] was used as positive control. After 24 h, a volume of 20 μl supernatant was transferred to a 96 well-plate (Corning Costar) and 180 μl QUANTI-Blue™ Solution (InvivoGen) was added and incubated for 2 h at 37 °C and 5% CO_2. SEAP activity was measured spectrophotometrically at 635 nm (TECAN, Giessen, The Netherlands).

2.9. Cytokine production analysis

The production of interleukin (IL)-6 and tumor necrosis factor alpha (TNF-α) in supernatant of macrophage cultures was determined by means of ELISA (BioLegend, San Diego, CA, USA) according to manufacturer's protocol.

2.10. Statistical analysis

All experiments were conducted with cells from a minimum of four human donors. All data were analyzed using GraphPad Prism software version 9.0 (Graphad, La Jolla, CA, USA). Results were analyzed using one-way ANOVA, two-way ANOVA or one-tailed Spearman's correlation test as applicable. A p value <0.05 was considered as statistically significant. Data are shown as means + standard deviation (SD).

3. Results

3.1. Physicochemical properties of arabinoxylan preparations

Initial endotoxin tests revealed that all dietary fibers selected were high in endotoxin-content, which hampers proper evaluation of any fiber-induced immunomodulatory effects and therefore has to be removed following a protocol as shown previously [24]. Following endotoxin removal, arabinoxylan preparations were analyzed for their protein and β-glucan content, monosaccharide composition and remaining endotoxin (LPS) content (Table 1). Arabinoxylan preparations were verified to contain ≤0.002 EU/mg LPS and protein content varied from 0 to 20% for the arabinoxylan preparations.

The solubility of arabinoxylan preparations was assessed by analysis of the areas under the curve obtained in HPSEC measurements. Corn cob and wheat bran contained the largest soluble fractions (*i.e.,* 82 and 80%, respectively), while rice bran-1 (19%), rice bran-2 (7%) and rice bran-3 (8%) components were mostly insoluble (Table 1), and rice hull was almost completely insoluble (1%). Next, we analyzed the monosaccharide composition of the arabinoxylan preparations. Monosaccharide composition analysis demonstrated that the arabinose/xylose ratio differed from 0.03 for corn cob arabinoxylan to 0.90 for rice bran-3 arabinoxylan (Table 1). Arabinoxylan preparations were mainly composed of arabinose and xylose, however, rice hull, rice bran-1, rice bran-2 and rice bran-3 also contained high amounts of glucose (> 20%) (Table 1). Only the wheat bran arabinoxylan preparation contains significant levels of β-glucan (15.4%), while corn cob contains 5.5% β-glucan. The high glucose levels in the rice bran and rice hull arabinoxylan preparations may originate from starch and/or cellulose. Finally, only small amounts of galactose and mannose were found in rice and corn-derived arabinoxylan preparations.

Particle size measurements of the arabinoxylan preparations were carried out using the Malvern Mastersizer. The volume-weighted mean particle diameter (D [3,4]), volume median diameter (Dv(50)) and span values for the arabinoxylan preparations are listed in Table 2 and the particle size profiles are shown in Fig. 1. Particle size distribution ranged

Table 1
Physicochemical characteristics of arabinoxylan preparations[a].

Arabinoxylan preparation	Relative Solubility (%)	Ara/Xyl ratio	Monosaccharides (mol%)							Total saccharide content (*w*/w%)	Total β-glucan content (w/w%)	Protein content (%)	LPS (EU/mg)
			Rha	Ara	Xyl	Man	Gal	Glc	Uronic acid				
Rice hull	1	0.17	0	5	30	2	2	59	4	39	0	2	0.002
Wheat bran	80	0.24	0	15	64	0	0	19	1	91	15.4	0	N.D.
Corn cob	82	0.03	0	2	84	0	2	11	0	92	5.5	0	N.D.
Rice bran-1	19	0.88	1	16	18	20	4	53	7	38	0	20	0.002
Rice bran-2	7	0.79	1	13	16	18	3	62	5	48	0	18	0.002
Rice bran-3	8	0.90	1	24	27	11	5	33	8	44	0	11	0.002

Rha rhamnose, *ara* arabinose, *xyl* xylose, *man* mannose, *gal* galactose, *glc* glucose, *LPS* lipopolysaccharide, *N·D* not detectable.
[a] Arabinoxylan preparations listed in this table were first treated to remove LPS and subsequently analyzed.

Table 2
Particle size distribution of arabinoxylan preparations.

	D[4,3]	Dv(10)	Dv(50)	Dv(90)	Span
Rice hull	45.9 ± 0.1	7.5 ± 0.0	35.4 ± 1.0	99.9 ± 0.1	2.6 ± 0.0
Wheat bran	90.8 ± 2.9	22.9 ± 0.1	65.5 ± 1.3	186.4 ± 6.9	2.5 ± 0.1
Corn cob	2.3 ± 0.2	0.2 ± 0.1	2.2 ± 0.2	4.8 ± 0.1	2.1 ± 0.2
Rice bran-1	140.3 ± 4.8	17.9 ± 6.2	97.3 ± 0.8	309.6 ± 12.2	3.0 ± 0.1
Rice bran-2	163.1 ± 4.1	36.7 ± 0.2	122.8 ± 1.1	332.5 ± 6.0	2.4 ± 0.0
Rice bran-3	294.3 ± 3.6	90.2 ± 0.5	255.5 ± 1.0	556.1 ± 10.3	1.8 ± 0.0

All data are displayed in μm and represent the mean of three replicates, mean ± SD. D [4,3] = volume-weighted mean particle diameter. Dv(10) = particle size below which 10% of sample volume is found. Dv(50) = particle size below which 50% of sample volume is found. Dv(90) = particle size below which 90% of sample volume is found. Span = measurement of the width of the distribution calculated as ((Dv(90) – Dv(10)/Dv(50)).

from 2.3 to 294 μm for volume-weighted mean particle diameter (D [3,4]), in which the particle size is proportional to the volume of the particles (Fig. 1, Table 2). Rice bran-3 was found to have the largest particle size with a median diameter of 255 μm, followed by rice bran-2 at 122 μm and rice bran-1 at 97 μm (Table 2). Wheat bran, rice hull and corn cob had a median diameter of 65, 35 and 2 μm, respectively. All arabinoxylan preparation particle sizes were homogenous as the span values ranged between 1.8 and 3.0 (Table 2).

3.2. Arabinoxylan preparations induce training and resilience in macrophages

Arabinoxylan preparations were investigated for their potential to induce training or resilience in human macrophages. Continuous exposure of macrophages to the arabinoxylan preparations for 5 days did not result in increased levels of TNF-α when compared to non-exposed medium control macrophages (Supplementary Fig. 1). Applying the training protocol as previously described [20], macrophages exposed to rice hull, wheat bran, rice bran-1, rice bran-2 or rice bran-3 showed a significantly ($p < 0.05$) increased release of TNF-α upon LPS exposure compared to medium control on day 7 (Fig. 2A). This training effect was less apparent upon measuring IL-6 release, which was only significantly increased compared to medium control when macrophages were exposed to rice bran-1 and rice bran-3 (Fig. 2B). In contrast, macrophages exposed to corn cob did not significantly alter their TNF-α or IL-6 release when compared to control. Next, we investigated whether the arabinoxylan preparations could also induce resilience in macrophages tolerized by an LPS trigger on day 1. In line with the training results, macrophages stimulated with LPS at day 1 showed an enhanced TNF-α release compared to control upon re-stimulation with LPS at day 7 when they had been exposed to the three rice bran arabinoxylan preparations (Fig. 2C). This was not observed with the rice hull or wheat bran arabinoxylan preparations. Also, in this resilience setup, the observed effects of arabinoxylan exposure were less apparent when analyzing IL-6 release. A significantly increased IL-6 release was only observed following exposure of the macrophages to rice hull, rice bran-1 or rice bran-2 when compared to the LPS control (Fig. 2D).

To explore the relationships between particle size, solubility and impact on macrophage immune training and resilience, correlation analyses was performed. Arabinoxylan particle size significantly correlated with TNF-α release in the resilience protocol, while solubility correlated with TNF-α release in both training and resilience protocols

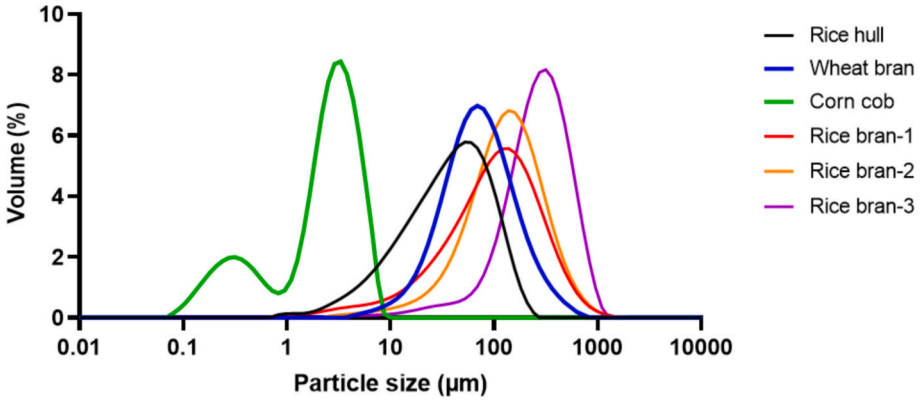

Fig. 1. Particle size distribution curves of arabinoxylan preparations. Particle size distributions are displayed of six arabinoxylan preparations, expressed as volume (%) and particle size (μm) using the laser diffraction method (LDM).

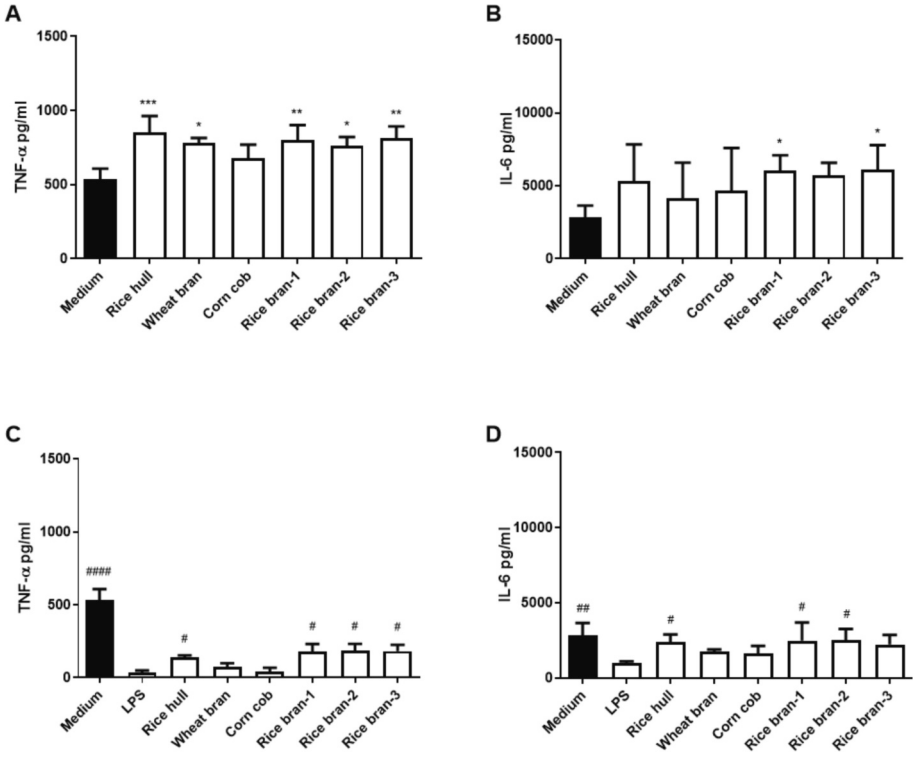

Fig. 2. Arabinoxylan preparations induced training and resilience in human macrophages. Monocytes were exposed to medium from day 1 to 6 or to medium on day 1 followed by 5 μg/ml arabinoxylan preparations from day 2 to 6. To test whether the arabinoxylan preparations induced innate immune training, the cells were challenged with 10 ng/ml LPS at day 7 for 24 h (A, B). To test the induction of resilience by the arabinoxylan preparations, cells were challenged with 10 ng/ml LPS at day 1 and 7 for 24 h (C, D). Supernatants of the day 7 stimulation were collected and TNF-α (A, C) and IL-6 (B, D) was measured by ELISA. Results are shown in bar graphs as averages (pg/ml ± SD) of $n = 6$ different donors. Data was analyzed using a one-way ANOVA test and statistical significances are shown as: * $p < 0.05$ compared to the no arabinoxylan (medium) control; ** $p < 0.01$ compared to the medium control; *** $p < 0.001$ compared to the medium control; $^{\#}$ $p < 0.05$ compared to the LPS control; $^{\#\#}$ $p < 0.01$ compared to the LPS control; $^{\#\#\#\#}$ $p < 0.0001$ compared to the LPS control.

(Supplementary Fig. 2). In contrast, neither particle size nor solubility of the arabinoxylan preparations showed a correlation with IL-6 release in either assay (data not shown).

3.3. Arabinoxylan preparations activate both dectin-1a and dectin-1b receptors

Since Dectin-1 activation is recognized as an important step in the induction of innate immune training, we measured arabinoxylan-induced Dectin-1 activation using Dectin-1a and Dectin-1b reporter cells. Both Dectin-1a (Fig. 3A) and Dectin-1b (Fig. 3B) reporter cells showed significant receptor activation upon exposure to the tested arabinoxylan preparations when compared to the control cell line Null1-v (Supplementary Fig. 3). Moreover, a concentration-dependent activation of both Dectin-1a and Dectin-1b was observed for all arabinoxylan preparations with a similar pattern of activation. Importantly, at a concentration of 100 and 1000 μg/ml, significant differences between arabinoxylan preparations were observed indicating rice hull, rice bran-1, rice bran-2 and rice bran-3 as most potent inducers of Dectin-1 activation (Fig. 3A, B).

A correlation analysis was performed to investigate possible links between Dectin-1 receptor isoform activation by arabinoxylan preparations and their capacity to induce training effects or induction of resilience in macrophages. In case of the training effects with arabinoxylan preparations, significant correlations found between cytokine release and Dectin-1b were more frequently observed than with Dectin-

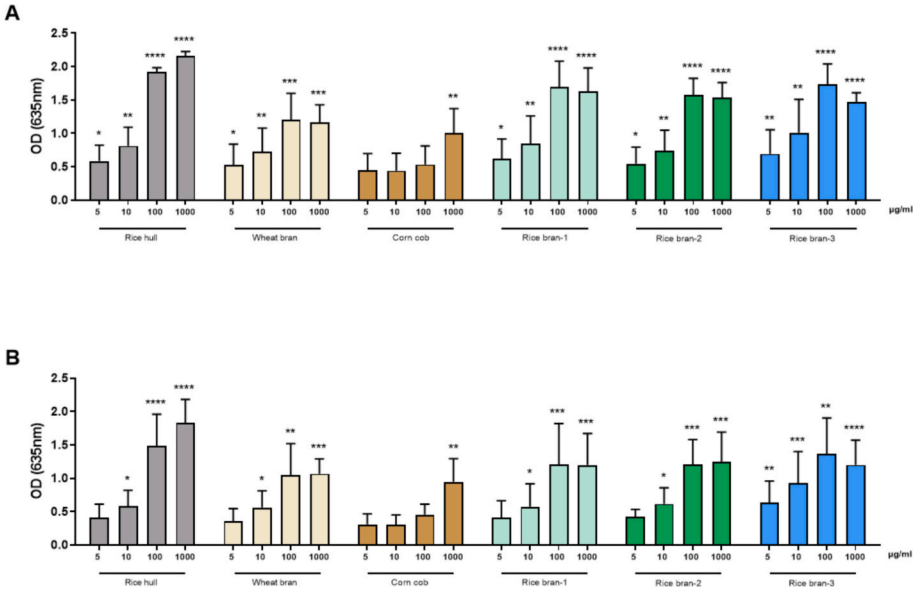

Fig. 3. Activation of both Dectin-1 isoforms by rice hull-, wheat bran-, corn cob- and rice bran-derived arabinoxylan preparations. HEK- Blue™ - Dectin-1a (A) and HEK- Blue™ - Dectin-1b (B) cells were stimulated with 5, 10, 100 and 1000 μg/ml of arabinoxylan preparations. After 24 h of stimulation, the secretion of SEAP was quantified in cell-free supernatants. Results of SEAP activity analysis are shown in bar graphs as average values ± SD of absorbance at 635 nm from $n = 3$ independent experiments. Data was analyzed using a two-way ANOVA test and statistical significances are shown as: * $p < 0.05$ compared to control HEK- Blue™Null1-v; ** $p < 0.01$ compared to control HEK- Blue™Null1-v; *** $p < 0.001$ compared to control HEK- Blue™Null1-v; **** $p < 0.0001$ compared to control HEK- Blue™Null1-v.

1a activation (Supplementary Table 1). Dectin-1b activation was found to correlate with TNF-α release in the training protocol across all arabinoxylan concentrations except 1000 μg/ml, while Dectin-1a activation only correlated with TNF-α release at a concentration of 100 μg/ml in the training protocol. At a concentration of 5 μg/ml Dectin-1a and Dectin-1b activation correlated with IL-6 release in the training protocol. Notably, TNF-α release in the resilience protocol also correlated with Dectin-1b activation, for 5 and 10 μg/ml. This was not found for Dectin-1a (Supplementary Table 1).

3.4. Dectin-1 and CR3 play a role in arabinoxylan-induced trained immunity

It is well established that β-glucans depend on both Dectin-1 and CR3 for their induction of training and resilience in innate immune cells [16]. To investigate whether these PRRs play a similar role in arabinoxylan-induced trained immunity, macrophages were incubated with the Dectin-1 antagonist laminarin or a CD11b blocking antibody before exposure to the different arabinoxylan preparations. TNF-α release was measured following LPS exposure on day 7. The β-glucan yeast-b was included as an experimental control. Supplementation of laminarin by itself did not induce enhanced TNF-α release, but it rather completely inhibited the arabinoxylan-induced enhanced TNF-α release following the secondary LPS trigger (Fig. 4A). Blocking CR3 had only a limited effect on the enhanced TNF-α release. Compared to isotype control, only partial inhibition of yeast-b or the three rice bran arabinoxylans-induced TNF-α release was observed (Fig. 4B).

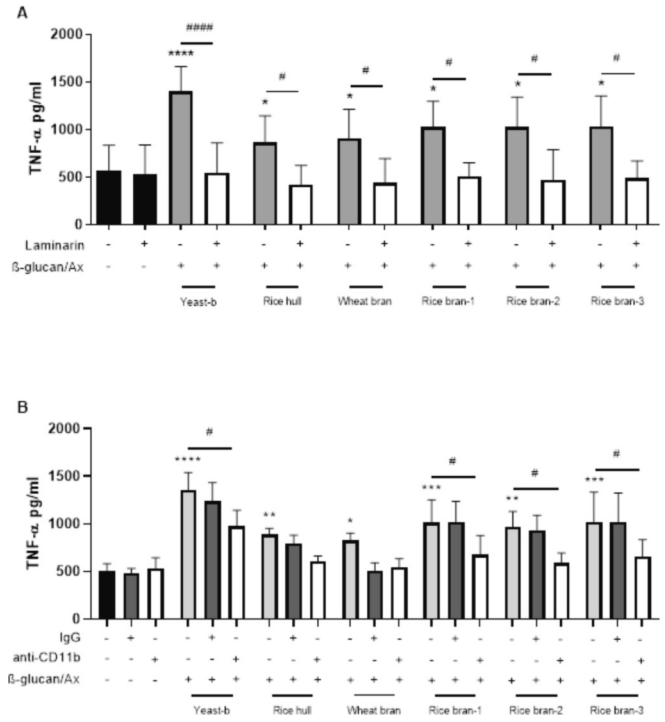

Fig. 4. Blocking Dectin-1b and CR3 inhibits macrophage training by arabinoxylan preparations. Monocytes were exposed to medium from day 1 to 6 or medium on day 1 followed by 5 μg/ml β-glucan (*i.e.,* yeast-b) or arabinoxylan preparations from day 2 to 6, in the presence or absence of 100 μg/ml laminarin (A), 5 μg/ml anti-CD11b antibody or anti-mouse IgG antibody (B). To test whether the arabinoxylan preparations induced innate immune training, the cells were challenged with 10 ng/ml LPS at day 7 for 24 h (A, B). Supernatants of stimulations were collected and TNF-α was measured by ELISA. Results are shown in bar graphs as averages (pg/ml ± SD) of $n = 4$ different donors. Data was analyzed using a two-way ANOVA test and statistical significances are shown as: * $p < 0.05$ compared to the medium control; ** $p < 0.01$ compared to the medium control; *** $p < 0.001$ compared to the medium control; **** $p < 0.0001$ compared to the medium control; $^{\#}$ $p < 0.05$ compared to Dectin-1 or CR3 blocking; $^{\#\#\#\#}$ $p < 0.0001$ compared to Dectin-1 or CR3 blocking.

Laminarin, as a soluble β-glucan, has been demonstrated to bind Dectin-1 but is unable to induce its activation. Based on our previous data, Dectin-1b rather than Dectin-1a correlated with enhanced cytokine release indicative of immune training. Because treatment with laminarin could prevent arabinoxylan-induced immune training we explored a possible role for Dectin-1a or Dectin-1b in the observed effect using the reporter cells lines. Surprisingly, activation of Dectin-1b, but not Dectin-1a, could be significantly blocked by the addition of laminarin. As shown in Fig. 5A, laminarin did not inhibit Dectin-1a receptor activation following stimulation with either the β-glucan control or arabinoxylan preparations. In contrast, pre-treatment with laminarin led to a significant inhibition of Dectin-1b receptor activation with the β-glucan sample yeast-b or all rice-derived arabinoxylan preparations (Fig. 5B).

4. Discussion

Arabinoxylans are considered BRMs with a variety of reported immunomodulatory effects. Here, we demonstrated for the first time that rice and wheat-derived arabinoxylan preparations are able to induce training and resilience in human macrophages. In analogy to the mechanism of action of β-glucan [16], our findings clearly demonstrate a role for CR3, Dectin-1, and more specifically Dectin-1b, in the induction of arabinoxylan-induced training in macrophages.

Although a number of research articles have been published detailing the immunomodulatory effect of dietary arabinoxylans, the relationship between their activity and structure is still unclear as these polysaccharides exhibit great structural heterogeneity. The lack of insights in this structure-activity relationship may be due to the use of arabinoxylans derived from various sources with different molecular weights and degree of branching. Furthermore, the method of extraction can add even more structural diversity [25], yielding arabinoxylans of various molecular weights, level of arabinose substitution and consequently solubility [4]. In order to increase our knowledge on the

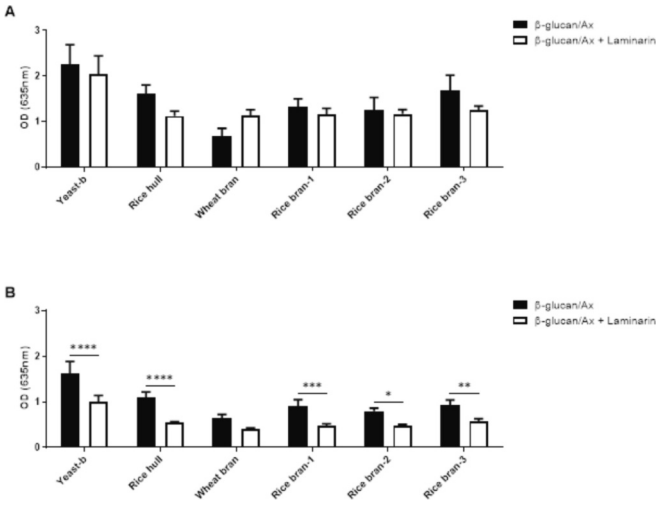

Fig. 5. Laminarin inhibits activation of Dectin-1b by arabinoxylan preparations. HEK- Blue™ - Dectin-1a (A) and HEK- Blue™ - Dectin-1b (B) cells were pre-treated with 1000 μg/ml laminarin for 1 h after which they were stimulated with control β-glucan (*i.e.,* yeast-b) and arabinoxylan preparations at 100 μg/ml. After 24 h of stimulation with β-glucan or arabinoxylan preparations, the secretion of SEAP was quantified in cell-free supernatants. Results of SEAP activity analysis are shown in bar graphs as average values ± SD of absorbance at 635 nm from $n = 3$ independent experiments. Data was analyzed using a two-way ANOVA test and statistical significances are shown as: * $p < 0.05$; ** $p < 0.01$. *** $p < 0.001$; **** $p < 0.0001$.

structure-activity relationship of arabinoxylans, we tested six different rice-, wheat- or corn-derived arabinoxylan preparations. Our findings indicate that rice-derived arabinoxylan preparations with a high arabinose/xylose ratio and larger particle size accompanied by a low solubility in medium [4,26], elicit a strong induction of both innate immune training and resilience as measured by the increased release of TNF-α and IL-6. In contrast, wheat and corn cob-derived arabinoxylan preparations with a lower arabinose/xylose ratio, high solubility and smaller particle size induced neither immune training nor resilience. These results are in line with literature describing that yeast β-glucans with a high degree of branching, large molecular weight and low solubility induce the highest activity [6,27]. This may be due to the ability of larger fibers to form insoluble triple helices capable of inducing immunomodulatory effects [5,20,28–31]. However, we cannot exclude a role for structural differences in the arabinoxylan preparations between corn, wheat and rice. Therefore, further research is needed to determine whether other structural characteristics, such as glycosidic linkages, particle size, molecular weight and arabinose/xylose ratio are equally important contributors to the immunomodulatory effects of the arabinoxylan preparations.

Similar to arabinoxylans, β-glucans occur in varying structures and sizes. Despite these differences, it has been shown that many β-glucans depend on Dectin-1 and CR3 for their direct immunomodulatory effects [15–17,32–34]. Although PRRs are prime candidates in the direct interaction of cells and dietary fibers, only a limited number of PRRs are now proven to be bound by or activated by dietary fibers, namely Dectin-1 [14,35], Toll-like receptor 4 (TLR4) [35,36], TLR2 [37] and CR3 [38]. Since our data indicate induction of immune training by arabinoxylan preparations, we sought to determine the Dectin-1 activating capacity of the various preparations and sources of arabinoxylan. Both Dectin-1a and Dectin-1b receptor isoforms could be activated by the tested arabinoxylan preparations. Arabinoxylan preparations derived from rice and wheat showed the highest activities in the reporter cell lines compared to the arabinoxylan preparation derived from corn. Degree of solubility was previously indicated to be important for Dectin-1 activation, which is in line with our and published observations that indicated soluble corn arabinoxylan and soluble β-glucans as poor inducers of Dectin-1 activation [14,39]. However, our wheat-derived arabinoxylan preparation had a similar solubility to that from corn but induced a Dectin-1 activation comparable to the insoluble arabinoxylan preparations. This would suggest that next to solubility, other factors determine Dectin-1 activation, such as particle size and number of binding sites [40]. Compared to wheat, average particle size from corn-derived arabinoxylan preparation was significantly lower. However, it remains to be determined if they differ in number of binding sites which could potentially explain the observed discrepancy. The presence of β-glucans in the wheat arabinoxylan preparation could also be an explanation for the observed difference in Dectin-1 activation. However, since the wheat arabinoxylan preparation was highly soluble, the β-glucan present in this preparation is likely to be soluble too. Soluble yeast and cereal β-glucans, compared to insoluble β-glucans are poor inducers of Dectin-1 activation [20,39]. Moreover, soluble β-glucan might even inhibit Dectin-1 activation by Dectin-1 agonists. Future studies should therefore consider treating arabinoxylan preparations with specific glucanases to remove contaminating β-glucans to better evaluate their Dectin-1 activating properties.

We also determined whether Dectin-1 activation by arabinoxylan preparations was responsible for the innate immune training results we observed. Indeed, in line with the underlying mechanism of β-glucans [16], blocking Dectin-1 activation using laminarin completely inhibited the enhanced TNF-α release following training with arabinoxylan preparations. Interestingly, using Dectin-1 reporter cells, we determined that laminarin could significantly inhibit Dectin-1b activation but not Dectin-1a. This would suggest that arabinoxylan-induced immune training is dependent on Dectin-1b activation rather than Dectin-1a. As far as we know this has not been demonstrated before and could be a relevant finding in the translation from *in vitro* to *in vivo*. Mouse studies show that the expression of Dectin-1 isoforms on peritoneal macrophages are mouse strain dependent [41]. Mouse strain C57BL/6 predominantly expresses isoform Dectin-1b while, for instance, BALB/c mice express both Dectin-1 isoforms equally which could influence the capacity to induce TNF-α following arabinoxylan supplementation. This is even more relevant when mixtures of soluble and insoluble arabinoxylan preparations, with potential differences in inhibiting/activating Dectin-1 isoforms [42], are used with the intention to modulate immune activity. In humans the different isoforms of Dectin-1 are selectively expressed. For instance, Dectin-1b, but not Dectin-1a, is significantly expressed on macrophages and immature dendritic cells [43,44]. However, the importance for arabinoxylan or β-glucan functionality and possible synergistic interaction of Dectin-1 with other PRRs remains to be determined as data on this topic are very scarce.

Next to Dectin-1, CR3 has a prominent role in the immune effects elicited by specific dietary fibers. In fact, CR3 and Dectin-1 collaborate to activate the Syk-JNK-AP-1 signaling pathway leading to a synergistic production of pro-inflammatory cytokines in macrophages [45]. Huang and colleagues demonstrated that Dectin-1-deficient macrophages stimulated with both iC3b-coated beads and depleted zymosan only minimally activates Syk. They reported that the lack of inside-out signaling provided by Dectin-1 may account for the failure for iC3b-coated beads and depleted zymosan to activate CR3 [45,46], which might be an additional explanation for the difference in blocking the training effects *via* Dectin-1 and CR3. It was previously determined that rice bran arabinoxylan-containing fractions have anti-complementary functions against foreign invasive organisms such as bacteria, fungi, and viruses, indicating a role for the complement system in their immunomodulatory capacity [47,48]. Our data support these observations and specifically, for the induction of immune training, blocking of the CR3 receptor resulted in a partial inhibition when cells were exposed to insoluble rice bran-derived arabinoxylan preparations, but not in case soluble wheat bran-derived arabinoxylan preparations were used. These differences might be explained by the fact that rice-bran arabinoxylan preparations, in contrast to wheat bran arabinoxylan preparations, contain mannose and/or galactose units which are important for the binding to CR3 [49]. CR3 is a heterodimeric complex consisting of the protein components CD11b and CD18. Complete blocking of β-glucan binding to human neutrophils and monocytes was achieved by blocking the CD11b chain, while blocking CD11b along with the CD18 chain further inhibited binding to other β2 integrins [50]. This is in agreement with previous studies showing that CD11b harbors a carbohydrate-binding lectin-like site that binds fungal-derived soluble β-glucan and does not require CD18 for ligation to soluble polysaccharides [51,52]. In our studies we used blocking antibodies that exclusively blocked interactions with CD11b but not the β2 integrin CD18, because CD18 is not an exclusive binding partner for CD11b [53]. Additional blocking of CD18 might lead to a further inhibition of cytokine release or even explain the difference between wheat and rice bran-derived arabinoxylans, however, this remains to be determined in future studies.

Clinical trials have demonstrated that trained immunity induced by Bacillus Calmette-Guérin (BCG) vaccine responses can have beneficial effects through priming of macrophages for a stronger response to subsequent infection [54,55]. Furthermore, clinical studies have demonstrated that dietary fibers support influenza vaccination efficiency by increasing titers when orally consumed pre-vaccination [13,56]. Based on the immune supportive effects of β-glucans, several clinical studies have evaluated the oral application of β-glucans for the prevention of upper respiratory tract infections. A meta-analysis of the results indicated a positive effect for yeast β-glucans on human upper respiratory tract infections, however, the number of studies included was small and suffered from high heterogeneity [57]. One of the contributing factors to this heterogeneity could be the source and preparations of the different β-glucans which could impact the bioavailability. Yamada et al. suggested that only superfine dispersed

(particle size <1 μm) are taken up by Peyer's patches and epithelial cells, in contrast to larger particles [58]. Particle size of the β-glucan preparations, nor uptake are currently reported in the majority of studies and could potentially offer an explanation for the lack of effect in studies aimed to measure improved immune responses following β-glucan administration [59]. Also, for arabinoxylan preparations it remains to be determined in how far beneficial effects are determined by particle size. However, animal studies have demonstrated that arabinoxylans induce NK cell activity, macrophage phagocytosis and pro-inflammatory cytokine secretion and stimulate the antibody-mediated immune responses [8,10,60–62]. Nutritional interventions might be promising strategies to modulate immune system responsiveness, thereby improving health and reducing disease risk. Our findings, albeit *in vitro,* are in line with these results showing both enhanced as well as recovered responses of pro-inflammatory cytokines in macrophages. Induction of immune training effects is considered promising in relation to defense against viral-induced upper respiratory tract infections and cancer immunotherapy, however, a persistent hyperactivation of the innate immune system may also contribute the pathology of cardiovascular disease [63–65]. Given the epidemiological evidence and systematic reviews showing a reduction in the risk of cardiovascular disease, LDL cholesterol and type 2 diabetes through dietary fiber interventions [66], it can be suggested that trained immunity induced by dietary fibers may differ from those induced by other dietary components, such as the Western-type diet [19]. Furthermore, dietary fiber may have other additional immunomodulatory effects in addition to the induction of trained immunity [67,68]. This merits a more thorough investigation of the structure-activity relationship between arabinoxylan, Dectin-1 and CR3 binding resulting in immune training in order to find dietary arabinoxylans as potential therapeutic candidates [69,70].

In conclusion, our study demonstrates that wheat- and rice-derived arabinoxylan preparations induced trained immunity in macrophages, which is mainly dependent on Dectin-1b. Moreover, rice bran-derived arabinoxylan preparations also induced resilience in macrophages, providing both enhanced responses as well as enhanced recovery. These effects correlated strongly with the degree of insolubility of the rice-derived arabinoxylan preparations. Induction of innate immune training is a promising mechanism to utilize in the defense against, for instance, virus-induced upper respiratory tract infections and cancer immunotherapy. Methods used in our research provide options for rapid screening of arabinoxylan preparations for their immunomodulatory activities. The current study provides a better understanding of the molecular mechanisms underlining these effects, which is highly beneficial in unlocking the immunomodulatory potential of arabinoxylans.

CRediT authorship contribution statement

Bart G.J. Moerings: Conceptualization, Formal analysis, Investigation, Visualization, Writing – original draft. Jeroen van Bergenhenegouwen: Supervision, Writing - Review & Editing. Matthew Furber: Writing - Review & Editing. Suzanne Abbring: Writing - Review & Editing. Henk A. Schols: Writing - Review & Editing. Renger F. Witkamp: Writing - Review & Editing. Coen Govers: Supervision, Writing - Review & Editing. Jurriaan J. Mes: Supervision, Writing - Review & Editing.

Declaration of competing interest

M.F. and J.v.B. are employed by Danone Nutricia Research.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2022.04.071.

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