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Lipopolysaccharide associated with β-2,6 fructan mediates TLR4-dependent immunomodulatory activity *in vitro*

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ABSTRACT

Levan, a β-2,6 fructofuranose polymer produced by microbial species, has been reported for its immunomodulatory properties *via* interaction with toll-like receptor 4 (TLR4) which recognises lipopolysaccharide (LPS). However, the molecular mechanisms underlying these interactions remain elusive. Here, we investigated the immunomodulatory properties of levan using thoroughly-purified and characterised samples from *Erwinia herbicola* and other sources. *E. herbicola* levan was purified by gel-permeation chromatography and LPS was removed from the levan following a novel alkali treatment developed in this study. *E. herbicola* levan was then characterised by gas chromatography–mass spectrometry and NMR. We found that levan containing LPS, but not LPS-depleted levan, induced TLR4-mediated cytokine production by bone marrow-derived dendritic cells and/or activated TLR4 reporter cells. These data indicated that the immunomodulatory properties of the levan toward TLR4-expressing immune cells were mediated by the LPS. This work also demonstrates the importance of LPS removal when assessing the immunomodulatory activity of polysaccharides.

1. Introduction

Polysaccharides (PS) derived from plants and microbes, such as β-glucans or fructans, have been reported to modulate immune cell function *in vitro*, via interaction with immune cell receptors such as toll-like receptors (TLRs) (Porter & Martens, 2017; Ramberg, Nelson, & Sinnott, 2010; Vogt et al., 2013; Vogt et al., 2015; Zhang, Qi, Guo, Zhou, & Zhang, 2016). Studies in animal models and in humans have further demonstrated the immunomodulatory properties of PS from various sources (Ferreira, Passos, Madureira, Vilanova, & Coimbra, 2015; Fransen et al., 2017; Nie, Lin, & Luo, 2017; Patten & Laws, 2015; Ramberg et al., 2010).

Microbial fructan, levan, is an underexplored immunomodulatory PS comprising a glucose-primed β-2,6 fructofuranose linear chain with occasional β-2,1-linked branches (Öner, Hernández, & Combie, 2016). Levan is produced by a range of microbes, including commensal bacteria in the gut, such as *Lactobacillus reuteri* (Sims et al., 2011), or in the oral cavity such as *Streptococcus mutans* and *S. salivarius* (Burne, Schilling,

Abbreviations: AP-1, Activator protein 1; BMDCs, bone marrow-derived dendritic cells; ES, enzymatically synthesised; EU, endotoxin unit; GC-MS, gas chromatography–mass spectrometry; GPC, gel permeation chromatography; HBSS, Hanks's balanced saline solution; IL, interleukin; KO, knockout; NF-κB, nuclear factor kappa B; MD-2, Myeloid Differentiation protein 2; MyD88, myeloid differentiation primary response 88; PS, polysaccharide; SEAP, Secreted embryonic alkaline phosphatase; TLR4, toll-like receptor 4; TLRs, toll-like receptors; TNF-α, tumour necrosis factor alpha; TRIF, TIR-domain-containing adapter-inducing interferon-β; TRAM, TRIF-related adapter molecule; WT, wild type.

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Bowen, & Yasbin, 1987; Ogawa et al., 2011). Levan can also be found in fermented foods such as natto (fermented soybean) (Shih & Yu, 2005; Xu et al., 2006).

Levan is synthesised by the action of levansucrases (EC 2.4.1.10), fructosyltransferases that are generally secreted into the extracellular environment, but can also be found attached to the bacterial cell surface (Öner et al., 2016). While levan has been reported to have immunomodulatory properties both *in vivo* and *in vitro* (Young, Latousakis, & Juge, 2021), reports on the underpinning molecular mechanisms are scarce. *L. reuteri* levan was reported to increase the number of Foxp3+ regulatory T cells in the spleen as shown using mice gavaged with either wild type (WT) or fructosyltransferase knockout (KO) *L. reuteri* (Sims et al., 2011), while levan derived from *B. subtilis* natto was shown to induce cytokine production *in vitro via* TLR4 interaction as well as to modulate ovalbumin-induced IgE production and Th2-associated responses *in vivo* (Xu et al., 2006). Pathogen-recognition receptors, such as TLR4, are key players in innate immunity and are important for sensing microbes and initiating immune responses (Brubaker, Bonham, Zanoni, & Kagan, 2015). TLR4 is expressed by immune cells such as macrophages, monocytes and dendritic cells, including those found in the gut-associated lymphoid tissue and lamina propria (Hug, Mohajeri, & La Fata, 2018; Vaure & Liu, 2014), as well as intestinal epithelial cells (Price et al., 2018). TLR4 and its co-receptor Myeloid Differentiation protein 2 (MD-2) recognise lipopolysaccharide (LPS), a complex glycolipid found in the outer membranous layer of both commensal and pathogenic Gram-negative bacteria (Simpson & Trent, 2019; Steimle, Autenrieth, & Frick, 2016). LPS is made of lipid A, a core oligosaccharide region, and an O-antigen PS which is highly variable among bacterial species (Ranf, 2016; Steimle et al., 2016). Lipid A is primarily responsible for extracellular LPS recognition by TLR4/MD-2 on innate immune cells (Simpson & Trent, 2019; Steimle et al., 2016).

Here, we tested the hypothesis that the immunomodulatory properties of levan rely on its interaction with TLR4. Levans purified from *E. herbicola* (also known as *Pantoea agglomerans*) as well as other sources were structurally characterised by gas chromatography–mass spectrometry (GC–MS) and/or NMR and assessed for the LPS amount at different stages of purification. The purified levans were tested for their ability to activate TLR4 reporter cells and induce cytokine production in bone marrow-derived dendritic cells (BMDCs) from WT and TLR4-KO mice. We found that LPS contained in the *E. herbicola* levan rather than the levan itself induced cytokine production from BMDCs, suggesting that LPS is the molecular determinant for the immunomodulatory property of levan toward TLR4-expressing innate immune cells.

2. Materials and methods

2.1. Mice

C57BL6/6J WT mice were maintained at the University of East Anglia specific pathogen-free animal facility. Use of animals was performed in accordance with UK Home Office guidelines.

2.2. Polysaccharides

E. herbicola levan was purchased from Sigma Aldrich (St. Louis, USA) and resuspended in ultra-filtered sterile water (Lonza, Switzerland), subsequently heated at 60–70 °C in a water bath for up to 20 min till dissolved and briefly vortexed to homogeneity. Purified enzymatically synthesised (ES) levan prepared *in vitro* using the recombinant levansucrase Lsc3 from *Pseudomonas syringae* pv. tomato was obtained from Dr. Tiina Alamäe (Institute of Molecular and Cell Biology, University of Tartu, Tartu, Estonia) (Adamberg et al., 2014; Visnapuu et al., 2011) and was dissolved in ultra-filtered sterile water (Lonza). LPS from *Hafnia alvei*, used as an LPS control in the TLR4 reporter assays, was provided by Dr. Ewa Katzenellenbogen, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Wroclaw, Poland (Wittmann et al., 2016). Peptidoglycan from *B. subtilis* was from Invivogen (San Diego, USA). *B. subtilis* 168 levan was produced in-house (see supplementary methods).

2.3. Human TLR4 reporter cell assay

HEK-Blue™ human TLR4 reporter cells were purchased from Invivogen. Binding to HEK-Blue™ human TLR4 reporter cells activates the NF-κB pathway producing secreted embryonic alkaline phosphatase (SEAP) which is detected in a colorimetric assay by the addition of HEK-Blue™ Detection medium (Invivogen, USA) (Wittmann et al., 2016). TLR4 reporter assays were performed using HEK-Blue™ detection medium and following the manufacturer's instructions with minor modifications. Typically, cells were grown to 50–80% confluency, the supernatant discarded, and the cells were washed with PBS. The cells were incubated with PBS for 5–10 min in an incubator at 37 °C and 5% CO_2 and gently tapped to remove adherent cells and harvested into a 15 or 50 ml tube. Cells were centrifuged at 250 ×*g* for 5 min, resuspended in D10 media - Dulbecco's modified Eagle medium (with 25 mM HEPES and 4.5 g/l glucose) (Lonza) supplemented with 10,000 Units/ml Penicillin/Streptomycin, 1× MEM Non-essential amino acids (Lonza) 4 mM L-glutamine, 10 μg/ml blastomycin, 1 μg/ml puromycin and 10% fetal bovine serum (FBS) - and counted using a haemocytometer. Cells were then centrifuged at 250 ×*g* for 5 min, resuspended in appropriate volumes of HEK-Blue™ detection medium and 2.5 × 10^4 cells were added to each well of a flat-bottomed 96 well plate (Sarstedt, UK). All treatments were prepared in HEK-Blue™ detection medium which was added to wells containing the cells in a total volume of 200 μl. Hafnia-LPS was used as a positive control in all TLR4 reporter assays. Final concentrations of all treatments are stated in the figure legends. Treated cells were incubated for 16 or 20 h (see figure legends) at 37 °C and 5% CO_2. Absorbance was read at 655 nm using a microplate reader (Benchmark Plus™, Bio-Rad, UK). For further details on TLR4 reporter cell culture see supplementary methods.

2.4. Isolation of bone marrow cells and generation of BMDCs

Mouse TLR4 KO bone marrow cells were provided by Dr. J.S. Frick (University of Tubingen, Germany). Mouse WT bone marrow cell isolation and subsequent BMDC generation were performed as previously described (Wittmann et al., 2016). Briefly, femur bones of C57BL6/6 J WT mice were isolated, washed with ethanol and then Hanks's balanced saline solution (HBSS, Lonza, Switzerland) supplemented with 3% fetal bovine serum (FBS), and crushed using a mortar and pestle and suspended in HBSS 3% FBS. The supernatant was transferred to a collection tube using a Falcon® 40 μm cell strainer and the process was repeated. The cell suspension was centrifuged at 270 ×*g* for 10 min, the cells were harvested and then incubated at room temperature in 3 ml of 1 X red blood cell lysis buffer (solution of 150 mM ammonium chloride, 10 mM sodium bicarbonate, and 1.27 mM EDTA) for 5 min. The solution was centrifuged at 270 ×*g* for 10 min, the cell pellet resuspended in HBSS 3% FBS and passed through a Falcon® 40 μm cell strainer. The cell suspension was again centrifuged at 270 ×*g* for 10 min, resuspended in HBSS 3% FBS and cells were counted using a haemocytometer. Cells were resuspended in cell freezing solution (10% Dimethyl sulfoxide [DMSO] in FBS) and 1 × 10^7 cells were added to cryogenic vials (Thermo Fisher Scientific, Waltham, USA) and stored at −80 °C.

BMDCs were generated *in vitro* from isolated bone marrow cells as described previously (Lutz et al., 1999; Wittmann et al., 2016). Briefly, bone marrow cells were thawed in a heating bath at 37 °C for 1–2 min. Cells were gently transferred into a new tube containing M10 media: RPMI-1640 media (25 mM HEPES and L-glutamine) (Lonza), supplemented with 10,000 Units/ml Penicillin/Streptomycin (Lonza), 50 mM 2-mercaptoethanol (Thermo Fisher Scientific, USA) 2 mM L-glutamine (Lonza) and 10% heat-inactivated FBS (Thermo Fisher Scientific), 1 mM

non-essential amino acids (Sigma Aldrich) and 1 mM sodium pyruvate (Lonza). The cells were then centrifuged at 270 ×*g* for 10 min, resuspended in M10 medium and cells were counted using a haemocytometer. Typically, cells were added to 10-cm culture dishes at 3×10^6 cells per dish. 20 ng/ml of granulocyte-macrophage colony stimulating factor (GM-CSF) (Peprotech, UK) was added to the culture dishes and cells were left for 6 days at 37 °C 5% CO_2 in M10 media to allow for differentiation into BMDCs.

2.5. Cytokine analysis of levan-treated BMDCs

After differentiation of bone marrow cells, adherent BMDCs were removed from the culture plates using PBS-EDTA (Lonza) and a sterile cell scraper. Cells were centrifuged at 270 ×*g* for 5 min, the pellet resuspended in M10 medium, and the cells counted using a haemocytometer. A total of 1×10^5 BMDCs per well in 200 µl were transferred to round-bottomed 96-well plates (Sarstedt, UK) and levans or positive control peptidoglycan (dissolved in M10 media) were added to the wells at different concentrations (as indicated in figure legends) and incubated for 18 h at 37 °C, 5% CO_2. The plates were centrifuged at 510 ×*g* for 3 min and the supernatant transferred into new 96-well plates. The levels of TNF-α or IL-6 in cell supernatants were measured by enzyme-linked immunosorbent assay (ELISA) kits for mouse TNF-α or IL-6 (Biolegend, San Diego, USA) following the manufacturer's instructions (see supplementary methods).

2.6. Gel permeation chromatography of levan

E. herbicola levan fractions were separated by size exclusion using a Superose™ 6 Increase 10/300 GL prepacked column for high-performance size exclusion chromatography (GE Healthcare Life Sciences, Chicago, USA). For *E. herbicola* levan collection, fractions were collected using a gel permeation chromatography (GPC) system and refractive index detector (Precision instruments, UK), and Trilution® Software (Version 3.0.26.0). All levan fractions were weighed and resuspended in ultra-filtered sterile water (Lonza). For the analysis of levan and dextran, a GPC system was used with refractive index detector (Series 200, PerkinElmer, Waltham, USA) connected to the Chomera software (PerkinElmer). Dextran from *Leuconostoc mesenteroides* of 5 kDa, 50 kDa, 410 kDa, and 1400 kDa molecular weight (Sigma Aldrich) were used as size standards. The purification was carried out at room temperature, all injection volumes were 1 ml, with a constant flow rate of 0.5 ml/min. Concentrations for all injections of *E. herbicola* levan, ES levan, and all dextrans were 5 mg/ml, 1 mg/ml, or 1 mg/ml, respectively.

2.7. Structural characterisation of levan

2.7.1. GC–MS linkage analysis of E. herbicola levan

For glycosyl linkage analysis, the purified PS were permethylated using sodium hydroxide base and iodomethane as described previously (Black, Heiss, & Azadi, 2019). After extraction in dichloromethane (DCM) and water as described (Black et al., 2019), an initial mild acid hydrolysis (0.1 M, TFA, 80 °C, 0.5 h) of the samples was performed to allow for the depolymerization of the levan while minimising degradation, as keto sugars such as fructose are more sensitive to acidic degradation than aldo sugars such as glucose (Kamerling & Boons, 2007). The released monosaccharides were then reduced using sodium borodeuteride ($NaBD_4$, 400 µl of a 10 mg/ml solution in 0.5 M ammonium hydroxide). A more aggressive hydrolysis (2 M TFA, 120 °C for 2 h) was then employed to allow for the detection of any aldose sugars present in the samples. A second round of reduction using the same conditions was followed by acetylation of the free hydroxyl groups (250 µl acetic anhydride, 250 µl trifluoroacetic acid, 40 °C for 0.3 h). The resultant partially methylated alditol acetates (PMAAs) were analysed by GC–MS as described by Heiss, Klutts, Wang, Doering, and Azadi (2009) with minor modifications. For analysis of *E. herbicola* levan 0 and 3, a splitless injection into the GC–MS was performed, allowing for greater sensitivity to determine the presence of trace analytes (as compared to a split injection).

2.7.2. NMR

NMR analyses of levans were performed on a 600 MHz Bruker Avance spectrometer fitted with a 5 mm TCI cryoprobe and controlled by Topspin 2.0 software. ^1H NMR spectra were recorded in D_2O at 300 K and consisted of 64 scans of 65,536 complex data points with a spectral width of 12.3 ppm. The NOESYPR1D presaturation sequence was used to suppress the residual water signal with low power selective irradiation at the water frequency during the recycle delay (D1 = 3 s) and mixing time (D8 = 0.01 s) (Le Gall et al., 2011). Spectra were processed using Mnova 12.0 (Mestrelab) software. Interpretation of 1D spectra was assisted by use of 2D methods including standard Bruker COSY and HSQC parameter sets.

2.8. LPS quantification using the Endozyme recombinant factor C assay

LPS in levan samples was quantified using the Endozyme Recombinant Factor C assay (Hyglos, Germany) following the manufacturer's instructions. Briefly, LPS standard dilutions were prepared in Endozyme endotoxin-free water from 0.005 Endotoxin unit (EU)/ml to 50 EU/ml and 100 µl of the standards or samples were added to the wells of a 96 well black flat bottom microplate (ThermoFisher Scientific) followed by addition of 100 µl of Endozyme reaction mix (substrate, enzyme and assay buffer [volume ratio: 8:1:1]). The baseline fluorescence was measured at excitation 380 nm and emission 445 nm using a microplate reader (ClarioStar, BMG LABTECH, Germany) pre-heated at 37 °C. The plate was then incubated at 37 °C for 60 or 90 min in the plate reader and fluorescence was measured again at excitation 380 nm and emission 445 nm with the baseline fluorescence values subtracted. Data was processed using 4-parameter logistic non-linear regression analysis.

2.9. LPS removal from levan

LPS was removed by calcium silicate treatment using a commercially available lipid removal agent (LRA), as described in supplementary methods or following a bespoke treatment with sodium hydroxide as follows. Levan was lyophilised and resuspended in 0.9 M sodium hydroxide at a concentration of 4 mg/ml. The levan suspension was incubated at room temperature for 48 h and vortexed twice per day for 1–2 min, then dialysed in 4 l of Millipore water for 2 days using a 10 kDa molecular weight cut off (MWCO) dialysis membrane (ThermoFisher Scientific). Preliminary data showed that 0.9 M sodium hydroxide was the most effective to remove LPS when compared to 0.1 and 0.3 M. The water for dialysis was changed twice per day. Levan samples were collected from the inside of the dialysis membrane, freeze-dried and the dry product was redissolved in ultra-filtered sterile water (Lonza).

2.10. Statistical analyses

Statistical analyses are mentioned in the figure legends and were performed using Prism 6 (GraphPad Software, San Diego, USA). A p value <0.05 was considered as statistically significant.

2.11. Supplementary material and methods

Further material and methods for TLR4 reporter cell culture, ELISA, LPS removal by calcium silicate treatment, and *B. subtilis* 168 levan production are described in the supplementary methods. *B. subtilis* 168 strain was a kind gift from Professor Dr. Harry Gilbert (University of Newcastle, Newcastle, UK).

3. Results

3.1. Purification of E. herbicola levan and LPS removal

We first purified and characterised commercial *E. herbicola* levan (hereafter termed *E. herbicola* levan 0) using GPC (Fig. 1A). Using commercial dextran standards, the large F1 fraction showed an apparent molecular weight (Mw) estimated at ≥1400 kDa. Since *E. herbicola* is a Gram-negative bacterium, we next quantified LPS in *E. herbicola* levan using the recombinant Factor C assay (Fig. 1B). Before GPC purification, the concentration of LPS in *E. herbicola* levan 0 was found to be 82 EU/mg (from 4 independent tests). After purification by GPC, an approx. 2-fold increase in LPS was detected in the resulting F1 fraction (hereafter termed *E. herbicola* levan 1) corresponding to 238 EU/mg (from 2 independent tests). We then attempted to remove the LPS from *E. herbicola* levan 1 first using LRA, a calcium silicate hydrate with a high affinity for lipids (J. P. Zhang, Wang, Smith, Hurst, & Sulpizio, 2005). The treatment led to a 3.3-fold decrease in LPS to 55 EU/mg (from 3 independent tests), termed as *E. herbicola* levan 2 (Fig. 1B). Subsequently, since LPS has been shown to elicit immunostimulatory effects on immune cells *in vitro* as low as 0.02 ng/ml (Schwarz, Schmittner, Duschl, & Horejs-Hoeck, 2014), we developed a method to degrade LPS chemically in *E. herbicola* levan 2 using sodium hydroxide treatment. In this method, levan samples were lyophilised and resuspended in 0.9 M sodium hydroxide for 48 h at 4 mg/ml with occasional vortexing, dialysed using a 10 kDa Mw cut off membrane, freeze-dried, and re-dissolved in water. The alkali treatment was repeated for *E. herbicola* levan 2, resulting in a further reduction of LPS to obtain a levan fraction (termed *E. herbicola* levan 3) depleted of LPS (< 0.08 EU/mg) (Fig. 1B).

As a control, we analysed LPS levels in levan synthesised *in vitro* using recombinant *Pseudomonas syringae* levansucrase Lsc3 (Adamberg et al., 2014; Visnapuu et al., 2011), termed ES levan. ES levan contained LPS at 1 EU/mg, lower than that in *E. herbicola* levan 0. After treatment with sodium hydroxide, LPS levels in ES levan were further reduced to ≤0.02 EU/mg.

3.2. Structural characterisation of E. herbicola levan

Next, we characterised *E. herbicola* levan 0 and LPS-depleted *E. herbicola* levan 3 by NMR spectroscopy. ^{1}H NMR spectra of both levans showed identical spectra in the carbohydrate region (3–5 ppm), suggesting that the levan structure was not affected by the alkali treatment (Fig. 2A). *E. herbicola* levan 3 was further analysed by 2D COSY and ^{1}H,^{13}C HSQC (Fig. 2B and C). ^{13}C NMR chemical shifts for *E. herbicola* levan 3 (Table 1) were characteristic of a fructose-based levan polymer (Srikanth, Reddy, Siddartha, Ramaiah, & Uppuluri, 2015). ES levan pre- and post-alkali treatment showed similar ^{1}H NMR spectra to the *E. herbicola* levan 0 (Fig. 3A). ES and LPS-depleted ES levan confirmed that the predominant fraction had a high Mw ≥ 1400 kDa (Fig. 3B), as previously reported for ES levan (Mardo et al., 2017). No proteins were detected in *E. herbicola* levan 0 by SDS-PAGE with Spyro™ Ruby staining (Fig. S1).

To determine the carbohydrate linkage of *E. herbicola* levan 0 and 3, we used GC–MS methylation linkage analysis. *E. herbicola* levan 0 and 3 shared similar profiles, showing predominant β-2,6 fructose linkages (Fig. S2). Table 2 shows the relative proportions of components as follows: 2,6-linked fructose residues (labelled on the Fig. S2 GPC chromatogram as 6-Fructose), representing β-2,6 linkages, accounted for 86% and 82% of total residues for *E. herbicola* levan 0 and 3, respectively; 1,2,6-linked fructose residues (labelled on Fig. S2 GPC chromatogram as 1–6-Fructose), representing β-2,1 branched residues, accounted for 7.5% and 10.5% of linkage residues for *E. herbicola* levan 0 and 3, respectively; terminal fructose (labelled on Fig. S2 GPC chromatogram as t-Fructose) residues accounted for 6.2% and 5.8% for *E. herbicola* levan 0 and 3, respectively; a small amount of a terminal glucopyranosyl residue (0.5%) was detected in *E. herbicola* levan 3, but not in *E. herbicola* levan 0; and 1,4-linked glucopyranosyl residues accounted for 0.1% and 0.9% in *E. herbicola* levan 0 and 3, respectively which is likely due to minor contaminants.

Taken together, our data showed that *E. herbicola* levans 0 and 3 were high Mw fructofuranose polymers comprising a predominant linear chain of β-2,6-linked fructose with β-2,1 branching points.

3.2.1. E. herbicola levan induces cytokine production by BMDCs in a TLR4- and LPS-dependent manner

To gain molecular insights into the immunomodulatory properties of *E. herbicola* levan, we monitored the production of cytokines TNF-α and IL-6 in WT and TLR4 KO BMDCs following treatment with *E. herbicola* levans 0, 2 and 3. Peptidoglycan from *B. subtilis* was used as a non-TLR4 ligand control. *E. herbicola* levan 0 led to IL-6 production in WT BMDCs,

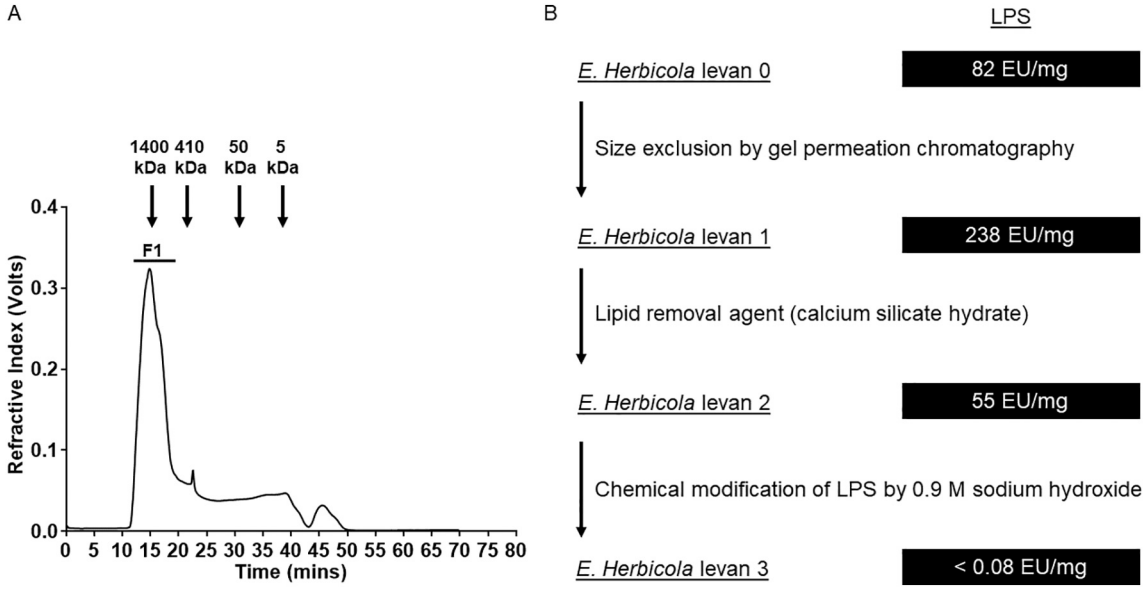

Fig. 1. GPC profile, LPS quantification and LPS purification of *E. herbicola* levan. A, representative fractionation and molecular weight determination of *E. herbicola* levan by GPC. Arrows on the chromatograph show the apparent molecular weights of the *E. herbicola* levan isolated fraction F1 based on dextran standards. B, illustration representing the purification of *E. herbicola* levan showing the subsequent stages of LPS quantification and removal.

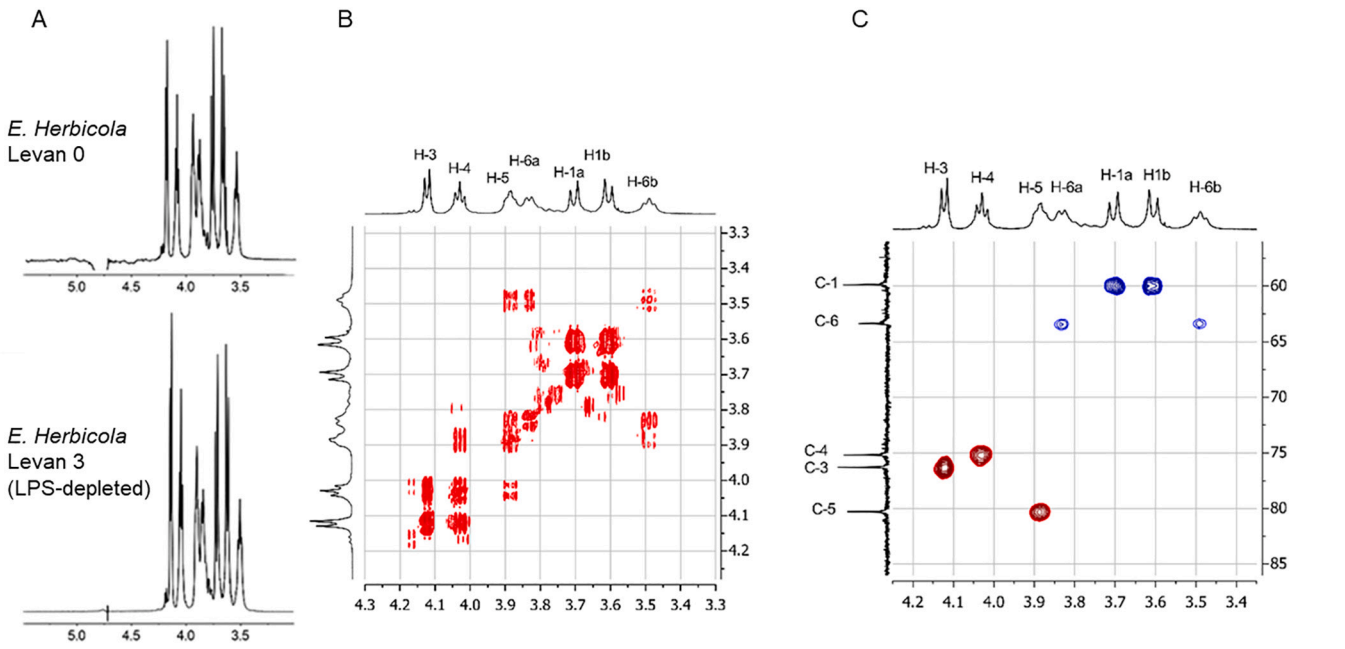

Fig. 2. NMR spectra of *E. herbicola* levan 0, and *E. herbicola* levan 3 which is LPS-depleted. A, Carbohydrate region of ^{1}H NMR spectra of *E. herbicola* levan 0 (top) as compared to purified *E. herbicola* levan 3 (bottom). B, COSY 2D NMR spectra of purified *E. herbicola* levan 3. C, ^{1}H—^{13}C HSQC 2D NMR spectra of purified *E. herbicola* 3 with proton carbon signals assignment shown on projections. NMR: 600 MHz, D_2O, 300 K.

Table 1
Chemical shifts for *E. herbicola* levan 3 in ^{1}H and ^{13}C NMR spectra.

H-1a	H-1b	H-3	H-4	H-5	H6a	H-6b
3.71	3.61	4.12	4.02	3.89	3.86	3.49

C-1	C-2	C-3	C-4	C-5	C-6
59.89	104.20	76.28	75.19	80.28	63.4

Legend: H, hydrogen. C, Carbon.

while IL-6 production was significantly reduced when using TLR4 KO BMDCs (Figs. 4A and S3A). *E. herbicola* levan 2 also induced IL-6 production in WT BMDCs, albeit to a lesser extent, as compared to *E. herbicola* levan 0, while no induction of IL-6 in TLR4 KO BMDCs was observed (Figs. 4A and S3A). However, there was no induction of IL-6 in WT or TLR4 KO BMDCs by LPS-depleted *E. herbicola* levan 3 (Figs. 4A and S3A). Further, *E. herbicola* levan 0 and 2 induced TNF-α in WT BMDCs in a TLR4-dependent manner, as there was no induction in TLR4 KO BMDCs (Figs. 4B and S3B). In contrast, stimulation of TNF-α by LPS-depleted *E. herbicola* levan 3 was not observed in either WT or TLR4 KOs (Figs. 4B and S3B). Overall, these data showed that the induction of cytokines in BMDCs by *E. herbicola* levan 0 was strongly dependent on TLR4. Importantly, there was no induction of either cytokine by LPS-depleted *E. herbicola* levan 3, suggesting that LPS was primarily responsible for the cytokine responses observed *in vitro*.

To further test the role of LPS in BMDC activation by the levan, we used ES levan (Adamberg et al., 2014; Visnapuu et al., 2011). We found that ES levan contained 1 EU/mg LPS, which probably arose from bacterial contamination acquired during the final steps of preparation such as the freeze-drying step. We next applied the alkali treatment procedure to this ES levan, resulting in ES levan containing negligible amounts of LPS (≤ 0.02 EU/mg). Unlike *E. herbicola* levan 0, ES levan or LPS-depleted ES levan did not significantly induce IL-6 (Figs. 5A and S4A) or TNF-α production (Figs. 5B and S4B). Taken together, these data suggest that the induction of cytokine production *in vitro* by levan in the conditions tested was due to LPS.

3.2.2. *E. herbicola levan activates TLR4 reporter cells in an LPS-dependent manner*

Next, we assessed the interaction of *E. herbicola* levans and LPS-depleted *E. herbicola* levan 3 with TLR4 using HEK 293 human TLR4 reporter cells. Ligand interaction with TLR4 on HEK-Blue human TLR4 reporter cells results in Nuclear factor kappa B (NF-κB) and Activator protein 1 (AP-1) activation, leading to the induction of secreted embryonic alkaline phosphatase (SEAP), which can be measured in a colorimetric assay (Wittmann et al., 2016). Here, TLR4 reporter cells were incubated with *E. herbicola* levan preparations of varying LPS concentrations (from Fig. 1B), *Hafnia alvei* LPS (positive control) or media alone (negative control). *E. herbicola* levans 0 and 2 but not *E. herbicola* levan 3 showed significant activation of TLR4 reporter cells (Figs. 6A, S5A and S5B), suggesting that the LPS was responsible for the activation of TLR4 reporter cells. We then tested the activation of TLR4 reporter cells by ES levans before and after alkali-based LPS removal treatment. ES levan containing lower amounts of LPS (1 EU/mg) was found to activate TLR4 reporter cells, whereas purified LPS-depleted ES levan (LPS: ≤ 0.02 EU/mg) showed no activation of TLR4 reporter cells (Figs. 6B, S5C and S5D). To further validate these findings, we used levan produced by LPS-free Gram-positive *B. subtilis* 168 grown in minimal medium (extracted using ethanol precipitation and purified by dialysis; see supplemental methods), and characterised by ^{1}H NMR (Fig. S6). As observed previously with LPS-depleted levans, *B. subtilis* levan did not activate TLR4 reporter cells (Figs. 6C and S5E).

Together these data support that LPS present in *E. herbicola* and ES levan was responsible for the activation of TLR4 reporter cells.

4. Discussion

Plant and microbial fructans including microbial levan have been proposed to have immunomodulatory properties (Young et al., 2021). However, the molecular mechanisms for the reported modulation of immune function by microbial levan remain largely unknown.

In this study, we thoroughly purified and characterised microbial levan in order to test its immunomodulatory properties *in vitro*. Using GPC, we found that the Mw of the primary fraction of levan obtained from *E. herbicola* (also known as *P. agglomerans*) was ≥1400 kDa, in line

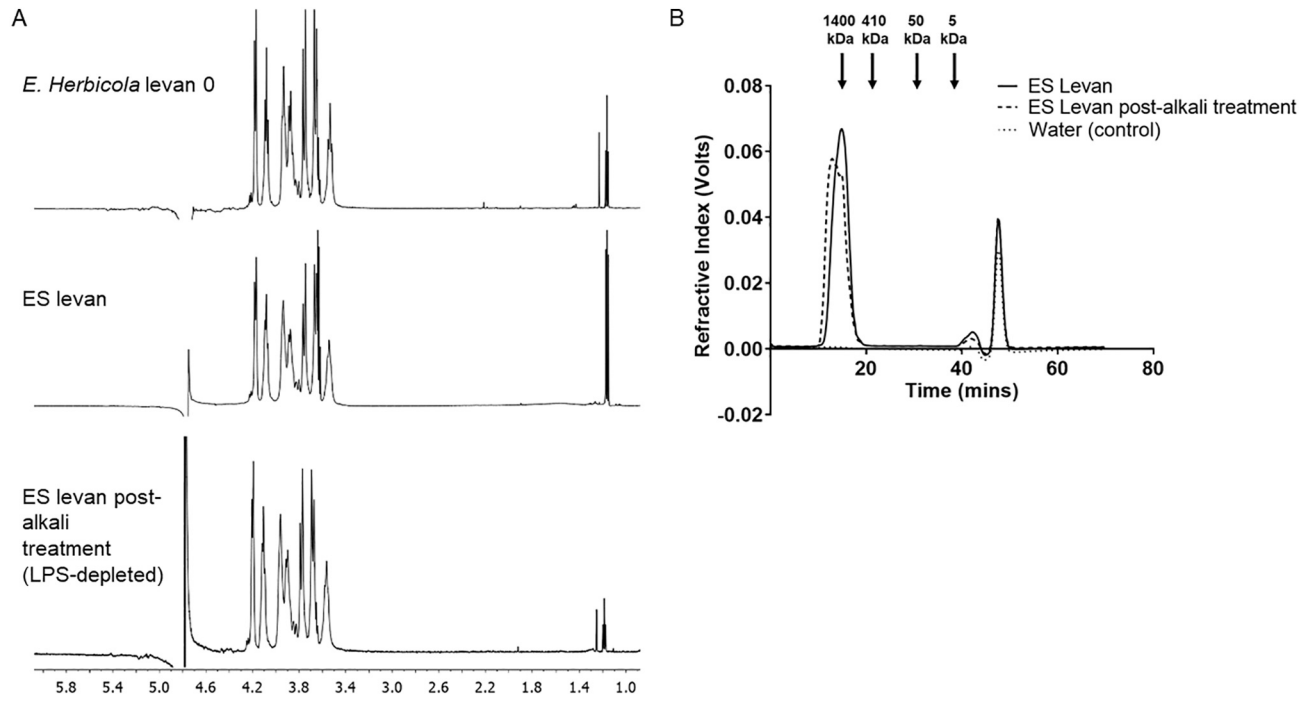

Fig. 3. Characterisation of ES levan and LPS-depleted ES levan by NMR and GPC. A, ^{1}H NMR spectra of ES levans are shown in comparison to ^{1}H NMR spectra of *E. herbicola* levan 0, ES levan (middle) and purified ES levan (ES levan post-alkali treatment [LPS-depleted] bottom). B, GPC profile of ES levan pre- and post-alkali treatment. Apparent molecular weights are based on the molecular elution times of dextran standards. Water (blank) was used as a control for known system contaminants (after 40 min).

Table 2
Relative percentage of each detected peak in *E. herbicola* 0 and 3 from GC–MS glycosyl linkage analysis [%] in relation to Fig. S2.

Peak	*E. herbicola* levan 0 [%]	*E. herbicola* levan 3 [%]
Terminal fructose residue #1 (t-Fruc)	2.2	2.0
Terminal fructose residue #2 (t-Fruc)	4.0	3.8
Terminal glucopyranosyl residue (t-Glc)	0	0.5
2,6 linked fructose residue #1 (6-Fructose)	46.2	37
2,6 linked fructose residue #2 (6-Fructose)	39.9	44.8
1,4 linked glucopyranosyl residue (4-Glc)	0.1	0.9
2,4,6 linked fructose residue #1 (4,6-Fructose)	0.0	0.3
2,4,6 linked Fructose residue #2 (1,6-Fructose)	0.0	0.2
1,2,6 linked Fructose residue #1 (1,6 Fructose)	2.7	4.1
1,2,6 linked Fructose residue #2 (1,6 Fructose)	4.8	6.4

(Brackets) refer to labelling on GPC chromatographs in Fig. S2.

with previous reports for *E. herbicola* levan ranging from 1.507 to 1.6 MDa (Keith et al., 1989; Keith et al., 1991; Liu, Kolida, Charalampopoulos, & Rastall, 2020; Mardo et al., 2017). The ^{1}H NMR spectra of *E. herbicola* levans were in agreement with those of L. *reuteri* levan (Sims et al., 2011). We further characterised *E. herbicola* levan by NMR and GC–MS linkage analysis, revealing a fructose polymer with a β-2,6-linked main chain and β-2,1 branching. For GC–MS linkage analysis: in order to observe ketose (fructose) as well as more typical aldose monosaccharides (glucose, *etc.*) in the same spectra, we employed a two-step hydrolysis and reduction procedure. An initial mild hydrolysis would limit degradation of the more labile ketose residues (Kamerling & Boons, 2007), while the second hydrolysis ensured we would also detect more recalcitrant residues. This is a common strategy employed to generate partially methylated alditol acetates of samples containing a mix of sensitive residues (*e.g.* KDO, anhydrogalactose) and non-sensitive residues (Stevenson & Furneaux, 1991; Willis et al., 2013). This detailed analysis provided finely detailed information on the branching of *E. herbicola* levan which is consistent with older reports using methylation analysis (Blake, Clarke, Jansson, & McNeil, 1982) in confirming *E. herbicola* levan as comprising β-2,1 branching. We also outline a methodology for generating linkage data for levans that simultaneously allows for analysis of other contaminating aldose residues.

Using BMDCs derived from WT and TLR4 KO mice, we found that *E. herbicola* levan (*E. herbicola* levan 0) induced IL-6 and TNF-α by BMDCs in a TLR4- and LPS-dependent manner. Our results were consistent with the previous report showing macrophage activation by LPS from *E. herbicola* (*P. agglomerans*) (Kohchi et al., 2006). TLR4/MD2 associates with MyD88 or TRIF/TRAM which induces NF-κB transcription factor, resulting in the production of immune mediators such as IL-6 and TNF-α (Chow, Young, Golenbock, Christ, & Gusovsky, 1999; Park et al., 2009; Ranf, 2016; Stamatos et al., 2010; Steimle et al., 2016; Welcome, 2018). IL-6 along with TNF-α and IL-1β make up 3 critical cytokines involved with the acute inflammatory response, for example in mucositis particularly seen in the gut (Khan, Wardill, & Bowen, 2018). These cytokines contribute to further tissue damage/inflammation; however, it should be noted that IL-6 is suggested to play both anti- and pro-inflammatory role (Khan et al., 2018; Naugler & Karin, 2008; Xing et al., 1998). *In vitro*, other microbial levans have been reported to differentially modulate TNF-α and IL-6 production. For example, a high Mw (2 MDa) levan from *B. licheniformis* stimulated both TNF-α and IL-6 in human whole blood cells (van Dyk, Kee, Frost, & Pletschke, 2012) while *Paenibacillus* sp. nov BD3526 levan (> 2.6 MDa) induced TNF-α in murine splenocytes but no stimulatory effect of this levan was seen inducing IL-6 (X. Xu et al., 2016). *B. subtilis* natto levan stimulated TNF-α production in macrophages and peritoneal cells (Q. Xu et al., 2006), and increased TNF-α expression in human OVCAR-3 cells (Magri et al., 2020). This range of *in vitro* responses to microbial levans possibly

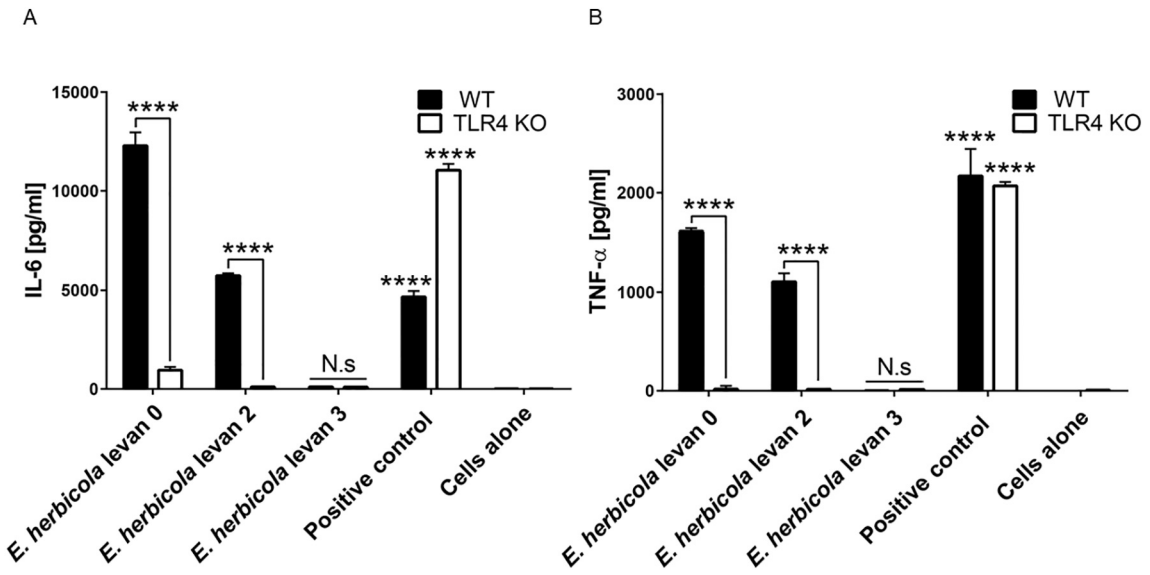

Fig. 4. Assessment of cytokine production in WT and TLR4 KO BMDCs in response to *E. herbicola* levans or LPS-depleted *E. herbicola* levan 3. BMDCs were incubated with 250 μg/ml of *E. herbicola* levans, 100 μg/ml of peptidoglycan (positive control) or left untreated in a 96 well plate. For each treatment, A, IL-6 or B, TNF-α in BMDC culture supernatants were measured by ELISA. Experiments were performed in triplicate. Error bars, + SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's test. ****, $p < 0.0001$ compared to cells alone. N.s. with straight line, all not statistically significant compared to cells alone. A repeated biological independent experiment is shown in Supplementary Fig. S3.

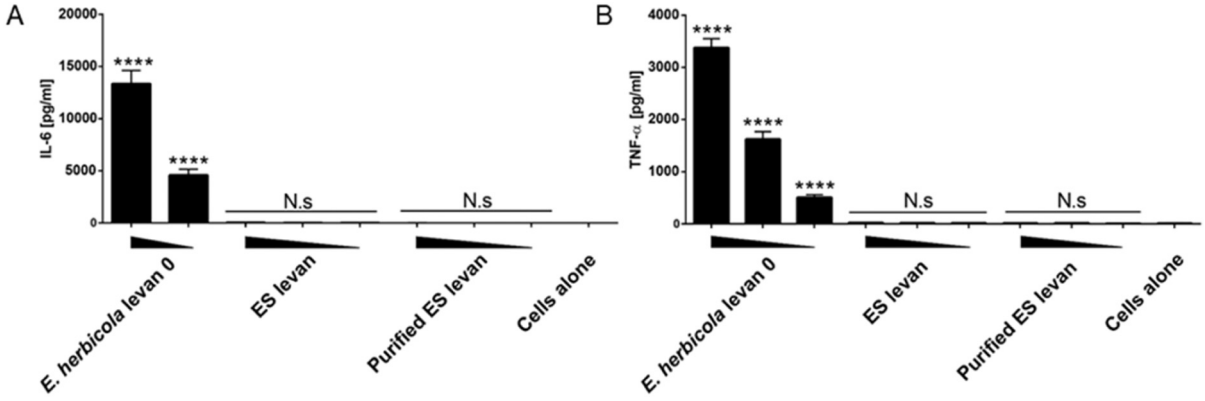

Fig. 5. Assessment of the cytokine production in WT BMDCs in response to ES levans before and after purification (LPS-depleted ES levan), and *E. herbicola* levan 0. BMDCs were incubated with ES levans before and after purification, or *E. herbicola* levan 0 in a 96 well plate. Cytokine production in the supernatant was measured by ELISA. Data show A, IL-6; B and TNF-α in the culture supernatant of BMDCs treated with *E. herbicola* levan 0, ES levan and purified ES levan (post-alkali treatment). In A, all ES levan concentrations were 250, 125 and 62.5 μg/ml and *E. herbicola* levan 125 and 62.5 μg/ml. For B, all levan concentrations were 250, 125 and 62.5 μg/ml. All experiments were performed in triplicate. Error bars, + SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's test. ****, $p < 0.0001$ compared to cells alone. N.s. with straight line, all not statistically significant compared to cells alone. For the repeated biological independent experiment see Fig. S4.

reflect variations in the type of mammalian cells used in these assays but may also be due to the structural composition or level of purification of microbial levans.

In this work, we showed that concentrations of LPS < 0.08 EU/mg did not interfere with the *in vitro* assays conducted using TLR4 reporter cells and BMDCs. However, LPS immunostimulatory potency or activity is dependent on the nature and affinity of LPS for TLR4 (Sandle, 2012; Steimle et al., 2016), the cells used in the bioassays (see below examples), and experimental conditions. Other studies reported that some pg/ml of LPS would be sufficient to modulate biological assays *in vitro*. For example, LPS has been shown to stimulate cytokine production from human dendritic cells as low as 20 pg/ml (Schwarz et al., 2014), and 10 pg/ml and 20 pg/ml of LPS led to the induction of IL-6 and TNF-α by BMDCs, respectively (Tynan, McNaughton, Jarnicki, Tsuji, & Lavelle, 2012). Another study investigating mesenchymal cell differentiation by endotoxins in culture found that 1.0 ng/ml of LPS enhanced osteoblast differentiation (Nomura, Fukui, Morishita, & Haishima, 2018). It is therefore essential to determine and report LPS concentrations in EU in samples tested *in vitro*.

The presence of LPS has been shown to influence the immunomodulatory activities of other PS *in vitro* (Dong et al., 2016; Pugh et al., 2008; Rieder et al., 2013). Therefore, in addition to existing commercial methods to deplete LPS such as LRA (also used in this study) or the reported limited use of LPS-inhibitor polymyxin B (Tynan et al., 2012), several LPS-depletion strategies have been developed including the use of Triton-X-114 (Teodorowicz et al., 2017) or methods using both alkali and acids (Govers et al., 2016; Lebre, Lavelle, & Borges, 2019). Here, treatment with LRA was not sufficient to remove LPS from GPC-purified *E. herbicola* levan, prompting us to develop a method based on an alkali treatment cleaving the lipid moieties (Lipid A) with no heating or acid

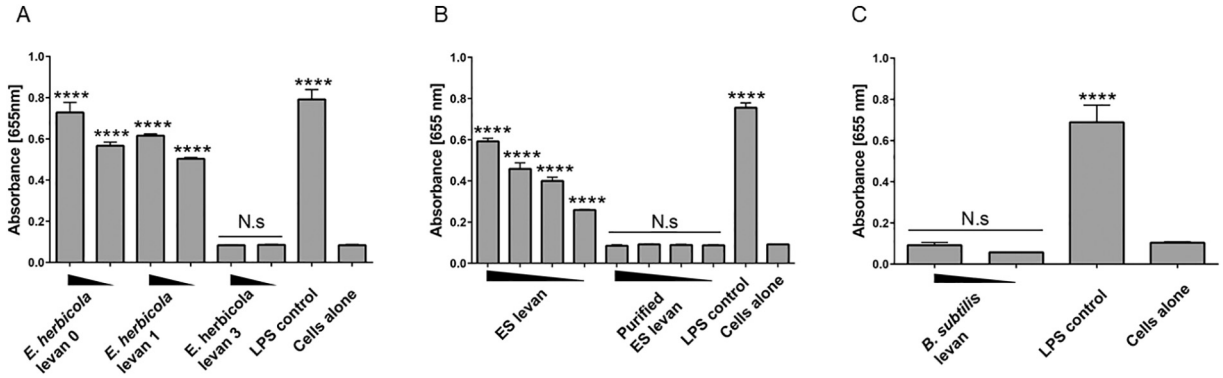

Fig. 6. Activation of TLR4 reporter cells by various levans. TLR4 reporter cells were incubated with levan, control (LPS) or left untreated (cells alone) in HEK blue medium in a 96 well plate for 20 h (A) or 16 h (B and C). Absorbance values were read at 655 nm. A, *E. herbicola* levans were used at 100 μg/ml and 50 μg/ml, and positive control LPS at 0.1 μg/ml. B, ES levans were used at 250 μg/ml, 125 μg/ml and 62.5 μg/ml and 31.25μg/ml and positive control LPS at 1 μg/ml. C, TLR4 reporter assay with Gram-positive *B. subtilis* levan compared to controls. Concentrations for *B. subtilis* levan were 100 μg/ml and 10 μg/ml, and LPS at 1 μg/ml. Experiments were performed in triplicate. Error bars, + SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's test. ****, $p < 0.0001$ compared to cells alone. N.s with straight line, not statistically significant compared to cells alone. For repeated biologically independent experiments see Fig. S5. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

treatment, resulting in at least a thousand-fold reduction of LPS levels in the levans from *E. herbicola* levan 0. Furthermore, use of our LPS depletion technique combined with our TLR4 reporter cell assays, we unequivocally showed that the LPS in *E. herbicola* and ES levan preparations was responsible for the activation of TLR4 similar to that seen with levan and BMDC cytokine induction. As such, our work constitutes a practical framework for assessing the immunostimulatory properties of not only levan or fructans but also other microbial and food polysaccharides, especially when evaluating their immunomodulatory properties toward TLR4-expressing innate immune cells *in vitro*.

5. Conclusion

The work reported herein describes the thorough characterisation and modulation of immune function by *E. herbicola* levan *in vitro*. We showed that LPS mediated the induction of cytokine production by *E. herbicola* levan in BMDCs as well as TLR4 activation by both *E. herbicola* and ES levan. Our data highlight the importance of thorough LPS depletion in levan and other microbial PS preparations when investigating their immunomodulatory function *in vitro* especially toward TLR4-expressing innate immune cells. Further work is warranted to decipher the health benefits of microbial levan both *in vivo* and *in vitro* using structurally characterised and highly-purified levan from diverse sources.

CRediT authorship contribution statement

I.D.Y, N.J, R.A.F and N.K overall interpreted data and conceived the study; I.D.Y, N.J, R.A.F and N.K wrote and edited the manuscript; I.D.Y performed most experiments, methods and experimental design. N.K, N. J, R.A.F and A.W supervised this work. I.D.Y and R.A.F conceived the LPS removal technique using sodium hydroxide. S.A.N and G.L carried out all NMR experiments, interpretation and/or NMR figures (S.A.N), and helped with relevant manuscript editing. S.A.N helped with GPC experiments and data interpretation; I.M.B and P.A carried out GC-MS carbohydrate linkage analysis and interpretation. I.M.B helped with relevant manuscript editing and writing. T.V made and provided ES levan and helped with relevant manuscript editing. D.L helped with SDS-PAGE experiments and biochemistry support. A.W helped with experimental design and technical advice for cell experiments.

Declaration of competing interest

The authors report no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carbpol.2021.118606.

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