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Cellulose alters the expression of nuclear factor kappa B-related genes and Toll-like receptor-related genes in human peripheral blood mononuclear cells

Leonie M. Vogt [a], *Mark V. Boekschoten* [b], *Philip J. de Groot* [b], *Marijke M. Faas* [a,c], *Paul de Vos* [a,*]

[a] *Department of Pathology and Medical Biology, University of Groningen, University Medical Center Groningen, Hanzeplein 1, 9700 RB Groningen, The Netherlands*

[b] *Division of Human Nutrition, Wageningen University, P.O. Box 8129, 6700 EV Wageningen, The Netherlands*

[c] *Department of Obstetrics and Gynaecology, University of Groningen, University Medical Center Groningen, Hanzeplein 1, 9700 RB Groningen, The Netherlands*

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ABSTRACT

The immunomodulatory and epithelial barrier effects of cellulose as a dietary fibre were studied to analyse the potential for use in health promoting functional foods. Reporter assays demonstrated cellulose-mediated activation through TLR/MyD88 dependent-, and independent pathways. Microchip analysis of human PBMCs showed that cellulose induced upregulation of three NF-κB related genes, i.e. CD40 molecule, interleukin 1 receptor antagonist (IL-1Ra), and interleukin-1 receptor-associated kinase 1 (IRAK1). Five upregulated genes related specifically to TLR signalling were identified, i.e. interleukin 1 receptor antagonist (IL-1Ra), interleukin-1 receptor-associated kinase 1 (IRAK1), jun proto-oncogene, mitogen-activated protein kinase kinase 3 (MAP2K3), and mitogen-activated protein kinase 13 (MAPK13). Cellulose did not affect T84 intestinal epithelial cell resistance. Cellulose does not directly affect T84 cell barrier function. However, it alters gene expression in human immune cells and activates TLR and non-TLR related pattern recognition pathways, indicating the immunomodulatory potential of cellulose as major component of root pulp byproduct.

* *Corresponding author.* Department of Pathology and Medical Biology, Section of Immunoendocrinology, University Medical Center Groningen, Hanzeplein 1, HPC EA11, 9713 GZ Groningen, The Netherlands. Tel.: +31 503618043; fax: +31 50 3619911.

E-mail address: p.de.vos@umcg.nl (P. de Vos).

Abbreviations: ATM, ataxia telangiectasia mutated; AUC, area under the curve; CD, cluster of differentiation; ECIS, electric cell substrate impedance sensing; EU, endotoxin units; FBS, foetal bovine serum; HEK, human embryonic kidney; IL-1Ra, interleukin 1 receptor antagonist; IRAK1, and interleukin-1 receptor-associated kinase 1; JNK, c-Jun N-terminal kinases; LBP, lipopolysaccharide-binding protein; LTA, lipoteichoic acid; MAP2K3, mitogen-activated protein kinase kinase 3; MAPK13, mitogen-activated protein kinase 13; MyD88, Myeloid Differentiation Factor 88; NF-κB, nuclear factor kappa B; NK, natural killer; PIK3cG, phosphatidylinositol-4,5-bisphosphate 3-kinase; PIK3R1, phosphoinositide-3-kinase regulatory subunit 1; PBMC, peripheral blood mononuclear cell; PMA, phorbol 12-myristate 13-acetate; PRR, pattern recognition receptor; SEAP, secreted embryonic alkaline phosphatase; TEER, trans epithelial electrical resistance; TLR, Toll-like receptor; TNF, tumor necrosis factor; TRIF, TIR-domain-containing adapter-inducing interferon-β; VST, Variance Stabilisation Transformation

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1. Introduction

In today's society, sustainability and health are considered important assets. The health benefits of dietary fibres on the immune system are becoming more evident, leading to a renewed interest in root pulp byproducts for application in the functional food industry. One of the major byproducts derived from chicory root (Ramasamy, Gruppen, & Schols, 2013) and sugar beet pulp (Sutton & Doran Peterson, 2001) is cellulose. This cell wall polysaccharide consists of a linear chain with a varying number of β(1→4) linked D-glucose units with the formula $(C_6H_{10}O_5)_n$ (Crawford, 1981; Updegraff, 1969). Cellulose is water insoluble and is not processed by the digestive enzymes of the human small intestine (Joshi & Agte, 1995; Slavin, Brauer, & Marlett, 1981), but it is better known for its faecal bulking properties and moderate fermentability in the large intestine, leading to production of short chain fatty acids (SCFAs) (Lattimer & Haub, 2010).

Several dietary carbohydrates have shown the shared property of activating innate immune receptors (Brown et al., 2002; de Kivit, Kraneveld, Garssen, & Willemsen, 2011; Vogt et al., 2013), which we propose is also an important method of influencing health, by directly modulating the immune system. Activation of Toll-like receptor (TLR)2 by a specific *Lactobacillus* strain has demonstrated important intestinal homeostatic properties (Karczewski et al., 2010), such as regulation of human intestinal tight junction proteins and protection of the epithelial barrier, an important physical barrier belonging to the innate immune system. Previous studies in our group suggest that similar TLR-related mechanisms occur upon contact with specific dietary carbohydrates (Vogt et al., 2013, 2014), and evidence is emerging that dietary fibres can be beneficial for intestinal epithelial barrier function by activation of innate immune receptors of intestinal epithelial cells (Abreu, 2010; Cario, 2008; Hiemstra, Bouma, Geerts, Kraal, & den Haan, 2012; Karczewski et al., 2010; Vogt et al., 2014). This indicates that TLR activation is an important parameter to include in the study of cellulose-mediated immune effects. In addition, the same *Lactobacillus* strain was found to modulate cytokine profiles in human peripheral blood mononuclear cells (PBMCs) (van Hemert et al., 2010). The cytokine balance in the intestine is important in its homeostasis, and skewing this balance with dietary carbohydrates is in our opinion, one of the relevant immunological parameters to investigate in fibre studies (Vogt et al., 2013), similar to the study of probiotic induced immune effects. As nuclear factor kappa B (NF-κB) is an essential transcription factor which is activated upon ligation of Toll-like receptors, and it plays a central role in the production of cytokines by immune cells, it was selected as an additional immunological target in this study.

By incubating Toll-like receptor reporter cells with cellulose, NF-κB-, and TLR activation as well as TLR/Myeloid Differentiation Factor 88 (MyD88) independent activation was investigated. Peripheral blood mononuclear cells are often applied as a model to study the immunogenic potential of dietary substances such as probiotics or prebiotics (van Hemert et al., 2010; Vogt et al., 2013), and are also known to express many TLRs (Chen et al., 2008; Siednienko & Miggin, 2009). By analysing cellulose-induced gene expression of isolated peripheral blood mononuclear cell (PBMCs) we aimed to identify genes within TLR-, and NF-κB pathways which are differentially regulated upon direct contact with cellulose. By analysis of the resistance across T84 human intestinal epithelial cell monolayers we studied whether addition of cellulose could improve the barrier function. Furthermore, a damage model was applied to study possible protective effects. By preincubation of the cells with cellulose, we studied whether a phorbol ester-induced decrease in trans epithelial electrical resistance (TEER) could be prevented or partially inhibited. These experiments were aimed to evaluate whether besides its bulking properties and the stimulation of SCFAs in the intestine, cellulose has the capacity to directly induce activation of immune cells and stimulate or protect the barrier function of intestinal cells.

2. Methods

2.1. Investigational compound

As an investigational compound to study the effects of well-characterised and pure cellulose, we used commercially available cellulose (Sigmacell® highly purified fibres, Type 101, Sigma-Aldrich Chemie B.V., Zwijndrecht, Zuid-Holland, The Netherlands). Cellulose consists of a linear chain with β(1→4) linked D-glucose subunits, characterised by the chemical formula $(C_6H_{10}O_5)_n$ (Crawford, 1981; Updegraff, 1969). An example of a Haworth projection of this molecule is depicted in Fig. 1. Endotoxin content (endotoxin units, EU) of cellulose was assessed by Toxikon (Leuven, Vlaams-Brabant, Belgium) and was 119 EU/g. This concentration has minimal effects on the responsiveness of the applied cell types.

2.2. Ethical statements

Isolation of PBMCs from human volunteers was conducted within the University Medical Center Groningen, The Netherlands. Written informed consent from the volunteers was obtained, and data were analysed and presented anonymously. This research and consent procedure have been approved by the ethical review board of the University Medical Center, Medisch Ethische Toetsingscommissie University Medical Center Groningen, as documented in the approved application

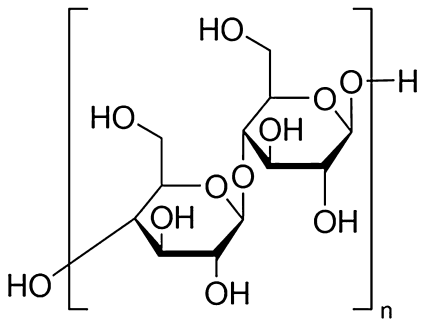

Fig. 1 – Haworth projection of cellulose (Haworth, 1939).

"2007/255". All clinical investigation was conducted according to the principles expressed in the Declaration of Helsinki.

2.3. Cell culture and assays reporter cell lines

The following reporter cell lines were acquired from InvivoGen (Toulouse, Haute-Garonne, France): THP1-XBlue monocytes with inserted constructs for MD2, CD14, and secreted embryonic alkaline phosphatase (SEAP); THP1-Xblue-defMyD reporter cells with inserted constructs for SEAP, and deficient in MyD88 activity; Human embryonic kidney (HEK-Blue) cells with inserted constructs for SEAP and human TLR2; and Human embryonic kidney (HEK-Blue) cells with inserted constructs for SEAP and human TLR4. These cell lines were cultured and maintained as previously described (Vogt et al., 2013). NF-κB activity of THP1 cell lines and HEK hTLR reporter cells by 24 h of stimulation with cellulose (dose range 100 μg/mL–2 mg/mL) was determined as previously described (Vogt et al., 2013). The rationale for the reporter cell experiments was as follows. First, cellulose was applied to THP1 monocytic cells which express all TLRs endogenously (Zarember & Godowski, 2002), and which overexpress MD2 and CD14 molecules, to boost TLR-mediated signalling. As NF-κB/AP-1 is a target of TLR activation, excretion of the SEAP into the media indicates that TLRs are activated. This cell type was thus specifically applied to identify TLR-mediated signalling, and to confirm the proof of principle that TLRs are involved in recognition of cellulose. To confirm full TLR-dependency of signalling, not only a THP1 cell line expressing all TLRs, but also a non-functional TLR-adapter molecule MyD88 was stimulated with cellulose. If the activation in this DefMyD cell line is absent, this indicates that signalling is fully dependent on activation of TLRs. If the activation in this cell line is present, but less compared to the THP1 cells with a functional MyD88, this indicates that both TLR-dependent and independent pathways are activated. Finally, if the activation is similar to the THP1 cells with a functional MyD88, this result indicates that signalling is not dependent on TLRs. Next, to determine which of the TLRs are activated by cellulose, Human Embryonic Kidney reporter cells were applied which express one type of TLR per cell line. HEK cells are typically used as reporter cells, as they are easily and efficiently transfectable with the relevant inserts. These cells are thus also specifically applied to confirm the proof of principle of activating a specific type of TLR.

2.4. Human peripheral blood mononuclear cell isolation, culture, and stimulation

To provide robust, representative outcomes in the microarray BeadChips studies, two independent experiments were performed with material from 5 male donors per experiment, which were different donors per experimental run. All samples were analysed in duplicate. In total, PBMCs of ten healthy male volunteers were isolated and cultured as previously described (Vogt et al., 2013). PBMCs were counted using a Z2 cell and particle counter (Beckman Coulter Nederland B.V., Woerden, Utrecht, The Netherlands) and 2×10^6 cells per well were transferred to a 24 well plate in a total volume of 1 mL per well. As a model for direct interaction of cellulose with immune cells (Hofmann et al., 2010; van Hemert et al., 2010; Vogt et al., 2013), PBMCs were incubated for 24 h (37 °C and 5% CO_2) with a thoroughly homogenised suspension of 1 or 100 μg/mL of cellulose (Sigma-Aldrich Chemie B.V.), or with normal culture medium, or with 2 ng/mL LPS (*Escherichia coli* serotype 0111:B4; Sigma-Aldrich Chemie B.V.).

2.5. Peripheral blood mononuclear cell RNA isolation

After stimulation, PBMCs were harvested and transferred to sterilised 1.5 mL Eppendorf containers, centrifuged for 5 min at 300 *g*, resuspended in RNAprotect Cell Reagent (Qiagen, Venlo, Limburg, The Netherlands) and stored at –20 °C. RNA was isolated using RNeasy Plus Mini Kit (Qiagen) according to the following protocol. PBMC suspension in RNAprotect was centrifuged as described above, supernatant was discarded and cell pellet was homogenised in 350 μL of 0.143 M β-mercaptoethanol/RLT buffer Plus by passing the lysate through a 20-gauge needle with an RNase-free syringe 5 times. Homogenised lysate was transferred to gDNA Eliminator spin columns and centrifuged for 30 s at 10,000 *g*. Three hundred fifty microlitres of 70% ethanol was added to the flow-through and samples were mixed by pipetting. Seven hundred microlitres of the sample was transferred to an RNeasy spin column and centrifuged for 15 s at 10,000 *g*. Flow-through was discarded and columns were washed once with 700 μL of RW1 buffer and once with 500 μL of RPE buffer with centrifuge steps of 15 s at 10,000 *g* and discarding flow-through after each centrifugation step. After washing the columns for a second time with 500 μL of RPE buffer and centrifuging for 2 min at 10,000 *g*, spin columns were transferred to fresh collection tubes and 30 μL of RNase-free water was directly added to the column membrane. Columns were centrifuged for 1 min at 10,000 *g* to elute the RNA. RNA quality was checked using an Agilent 2100 bioanalyser (Agilent Technologies, Amsterdam, Noord-Holland, The Netherlands).

2.6. Microchip analysis

Microchip analysis of PBMC gene expression was performed at the University Medical Center Groningen, The Netherlands. cDNA and cRNA synthesis were performed with an Ambion Illumina TotalPrep-96 RNA Amplification kit (Life Technologies, Bleiswijk, Zuid-Holland, The Netherlands) following the manufacturer's instructions. PBMC cRNA (300 ng) was labelled using an Epicentre TargetAmp Nano-g Biotin-aRNA Labeling kit (Westburg BV, Leusden, Utrecht, The Netherlands) and hybridised to Illumina Sentrix® HumanHT-12 v3 whole genome microarray BeadChips coding over 25,000 genes (Illumina, San Diego, CA, USA). Sample labelling, chip hybridisation, and image scanning were performed according to the manufacturer's instructions. All arrays met our criteria of the performed quality control.

The Bioconductor Lumi package was used for the quality check and normalisation of the Illumina arrays (Du, Kibbe, & Lin, 2008; Gentleman et al., 2004). The lumiR command was used to load the text-file as generated by the Beadstudio software. The NuIDs were automatically generated upon loading the text-file (Du, Kibbe, & Lin, 2007). The quality was checked by inspecting density plots, boxplots, array–array correlation plots, and the output of the lumiQ command (Du et al., 2008).

The normalisation procedure was done in 2 steps. First, the Variance Stabilisation Transformation (VST) was applied followed by the Loess normalisation procedure as it is available in the lumiN function (Lin, Du, Huber, & Kibbe, 2008). The normalised data were used for doing the statistics utilising the Limma package (Smyth, 2004). Pathway analysis was performed with Ingenuity Pathways Analysis software (Qiagen, Valencia, CA, USA).

2.7. T84 cell culture and epithelial cell barrier measurements

T84 human colon carcinoma cells (Sigma-Aldrich Chemie B.V) were grown to ca. 80% confluency at 37 °C, 5% CO_2, in culture medium consisting of 1:1 Ham's F12 medium:DMEM, acquired premade from Sigma-Aldrich Chemie B.V.), supplemented with 10% HyClone foetal bovine serum (FBS, Thermo Scientific, Breda, Noord-Brabant, The Netherlands) and gentamicin (50 μg/mL, Life Technologies Europe B.V.). Cells were maintained as previously described (Marcon, McCool, Forstner, & Forstner, 1990). Trypsin was acquired from MP Biomedicals, Eindhoven, The Netherlands, and ethylenediaminetetraacetic acid (EDTA) (Titriplex III) from Merck Millipore, Amsterdam, Noord-Holland, The Netherlands. Multiple electrode gold-plated 96 well chamber slides (96W20idf, Applied Biophysics, IBIDI, Munich, Bavaria, Germany) were coated with 300 μL/well of a 0.2% L-cysteine (Sigma-Aldrich Chemie B.V.) solution in DMEM (Life Technologies Europe B.V.) for 30 min at room temperature. Wells were washed twice with DMEM, and coated overnight at room temperature with 300 μL/well of 1% PureCol™ bovine tail collagen (Nutacon B.V., Leimuiden, Zuid-Holland, The Netherlands) and 0.1% BSA (Sigma-Aldrich Chemie B.V.) in culture medium. Wells were then washed twice with culture medium and cells were seeded at a density of 2×10^5 cells per well in a final volume of 300 μL/well. Prior to stimulation, the cells were maintained in the wells for 14 days to reach a stable TEER, and medium was changed every other day. Resistance was measured continuously at multiple frequencies (Lo, Keese, & Giaever, 1995) upon placing the chamber slides in an electric cell substrate impedance sensing (ECIS) incubator (Z-Theta model, Applied Biophysics, Troy, New York, USA). Measurements performed at 1000 Hz were used to calculate the area under the curve (AUC), as data acquired at relatively low frequency values specifically represent the tight junction mediated resistance in the 96W20idf plates. To establish whether cellulose exerts protective effects, a damage model was applied based on T84 cell incubation with phorbol 12-myristate 13-acetate (PMA, 10 nM, Sigma-Aldrich Chemie B.V.), which is a barrier disrupting agent (Tai et al., 1996). Briefly, T84 cells were incubated with a concentration series of thoroughly homogenised cellulose in culture medium (1 μg/mL–2 mg/mL) for 24 h, followed by addition of PMA. Cells were maintained in this stimulation medium for 24 h and this time frame was subsequently used to calculate the AUC relative to untreated controls.

2.8. Statistical analysis

Analysis of the microchip array data was performed using Genespring GX 9 software (Agilent Technologies, Santa Clara, CA, USA). Genes were defined as significantly changed when the False Discovery rate q-value of the intensity-based moderated t-statistics was <0.01 and fold change >1.2. Statistical analysis of the reporter cell data and the ECIS data was performed by Wilcoxon Signed Rank test. Significant differences as compared to control ($P < 0.05$) are indicated with an asterisk.

3. Results

3.1. Cellulose activates nuclear factor kappa B via Toll-like receptor 2 and Toll-like receptor 4

To gain insight in the TLR dependency of the effects of cellulose on PBMCs, activation of THP1 and HEK-Blue TLR reporter cells by cellulose was studied (Fig. 2). THP1 cell lines express all TLRs (Zarember & Godowski, 2002). By studying the activation of THP1 MD2-CD14 cells and comparing this with activation of THP1 DefMyD cells that express a non-functional MyD88, it is possible to determine the TLR dependency of the responses (Vogt et al., 2013). In THP1 MD2-CD14 cells, cellulose induced significant activation of NF-κB as compared to control, at the doses of 400 and 2000 μg/mL (Fig. 2A). Subsequently, to confirm whether this NF-κB activation was mediated by TLRs, THP1 DefMyD cells were incubated with cellulose. In this cell line also significant NF-κB activation was observed as compared to control, suggesting that the activation of NF-κB in THP1 MD2-CD14 cells was partly mediated through TLR/MyD88-dependent and partly through TLR/MyD88-independent signalling pathways (Fig. 2B). As TLR2 and TRL4 are important TLRs which recognise dietary carbohydrate oligomers and polymers (Capitan-Canadas et al., 2014; Kim, Muto, & Gallo, 2013; Lin et al., 2014a; Tsai et al., 2013; Vogt et al., 2013), we investigated whether NF-κB activation by cellulose was mediated via TLR2 or TLR4. To this end, HEK-Blue TLR reporter cells with individual TLR constructs were stimulated with cellulose. As depicted in Fig. 2C, cellulose induced statistically significant TLR2-mediated NF-κB activation at 400, 1000, and 2000 μg/mL. The dose-related pattern of TLR4-mediated NF-κB activation appeared to be biphasic (Fig. 2D), with the low doses inhibiting NF-κB, and cellulose at a dose of 2000 μg/mL inducing a small increase in activation (1.2-fold as compared to control, $P < 0.05$).

3.2. Cellulose alters the expression of nuclear factor kappa B and Toll-like receptor -related genes in human peripheral blood mononuclear cells

To gain insight in the expression of genes within Toll-like receptor-, and nuclear factor kappa B signalling pathways which are induced by cellulose, we performed microarray analysis on cellulose exposed human PBMCs. PBMC RNA was isolated and hybridised to whole-genome expression microarrays. Quality control of the hybridisations and primary data analysis were performed as previously described (van Baarlen et al., 2011) to ensure that the array data were of the highest possible quality. The in vitro stimulation of human PBMCs with cellulose resulted in the differential expression of a number of genes as compared to unstimulated controls. To specifically target genes

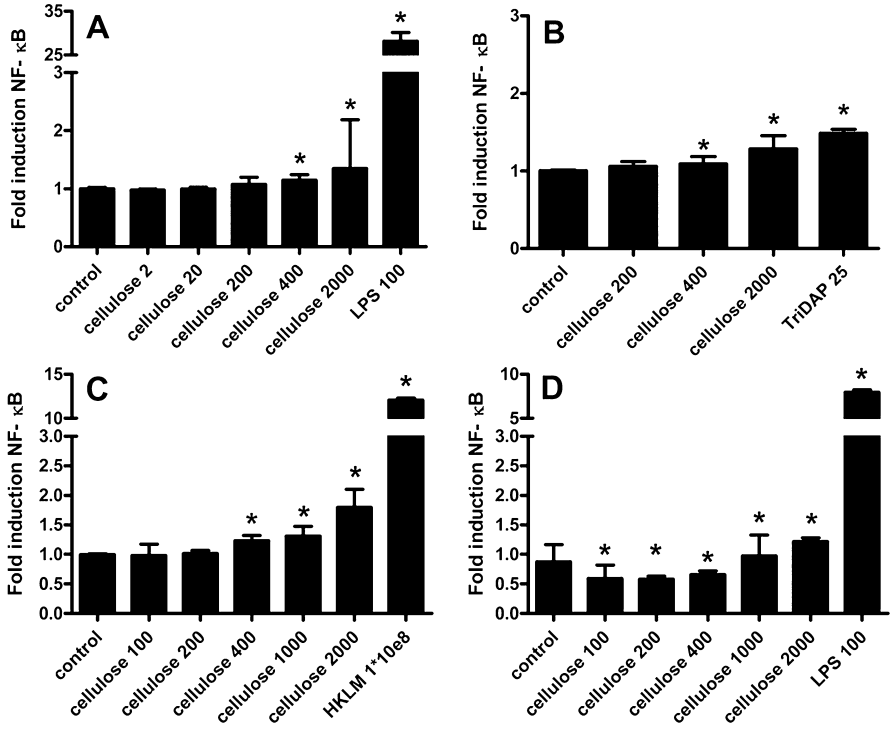

Fig. 2 – Reporter cell assays for TLR mediated NF-κB activation. (A) THP1 MD2-CD14 cells incubated with control medium, endotoxin free water, cellulose, and LPS (100 ng/mL). (B) THP1 DefMyD cells incubated with control medium, endotoxin free water, cellulose, and Tri-DAP (L-Ala-γ-D-Glu-mDAP, 25 μg/mL). (C) HEK-Blue hTLR2 cells incubated with control medium, endotoxin free water, cellulose, and heat-killed *Listeria monocytogenes* (HKLM, 1×10^8 cells/mL). (D) HEK-Blue hTLR4 cells incubated with culture medium, endotoxin free water, cellulose, and LPS (100 ng/mL, n = 8).

related to NF-κB-, and TLR-signalling, comparisons and pathway reconstruction of these transcriptional networks were made using an in silico approach.

Significant changes in expression of 8 genes related to NF-κB-signalling and 9 genes related to TLR-signalling induced by cellulose are visualised in Figs. 3 and 4, respectively. Within the NF-κB pathway, the following 3 genes were upregulated: CD40 molecule (Tumor Necrosis Factor receptor superfamily member 5), interleukin 1 receptor antagonist, and interleukin-1 receptor-associated kinase 1. In this pathway, 5 genes were downregulated, i.e. ataxia telangiectasia mutated (ATM serine/threonine kinase), phosphatidylinositol-4,5-bisphosphate 3-kinase, phosphoinositide-3-kinase regulatory subunit 1 (alpha), TLR5, and TLR7. Within the TLR signalling cascade, 5 genes were upregulated by cellulose. These were interleukin 1 receptor antagonist, interleukin-1 receptor-associated kinase 1, jun proto-oncogene, mitogen-activated protein kinase kinase 3, and mitogen-activated protein kinase 13. The 4 genes which were downregulated by cellulose were CD14 molecule, mitogen-activated protein kinase kinase kinase 1, TLR5, and TLR7. Results are summarised in Tables 1 and 2.

As a positive control for NF-κB-, and TLR-signalling, gene expression of LPS-stimulated PBMCs was analysed. Significant changes in expression of 24 genes related to NF-κB-signalling and 19 genes related to TLR-signalling were induced (online supplementary Figs. S1 and S2). Results are summarised in online supplementary Figs. S3 and S4.

3.3. *Results trans epithelial electrical resistance*

Trans Epithelial Electrical Resistance (TEER) measurements showed that preincubating differentiated monolayers of T84 human intestinal epithelial cells with different doses of cellulose did not significantly protect the cells against a phorbol ester (PMA)-induced decrease in TEER (Fig. 5A and B). The 2000 μg/mL dose appeared to induce a small ameliorating effect, however the area under the curve (AUC) was not significantly different from the condition with PMA only. Addition of cellulose only to T84 cell monolayers also did not change the TEER as compared to addition of control medium (data not shown).

4. Discussion

In this study, effects of cellulose on immune cells and intestinal epithelial cells were studied to obtain insight into the potential health promoting value of cellulose, as major component of root pulp byproduct. We hypothesised that cellulose can directly activate immune cells through Toll-like receptors as reported for other dietary fibres (Brown et al., 2002; de Kivit et al., 2011; Vogt et al., 2013). However, our results show both TLR/MyD88-dependent as well as TLR/MyD88-independent activation. Not all TLRs utilise MyD88 as adapter, TLR3 signals via the adapter molecule TRIF (Yamamoto et al., 2003). TLR3

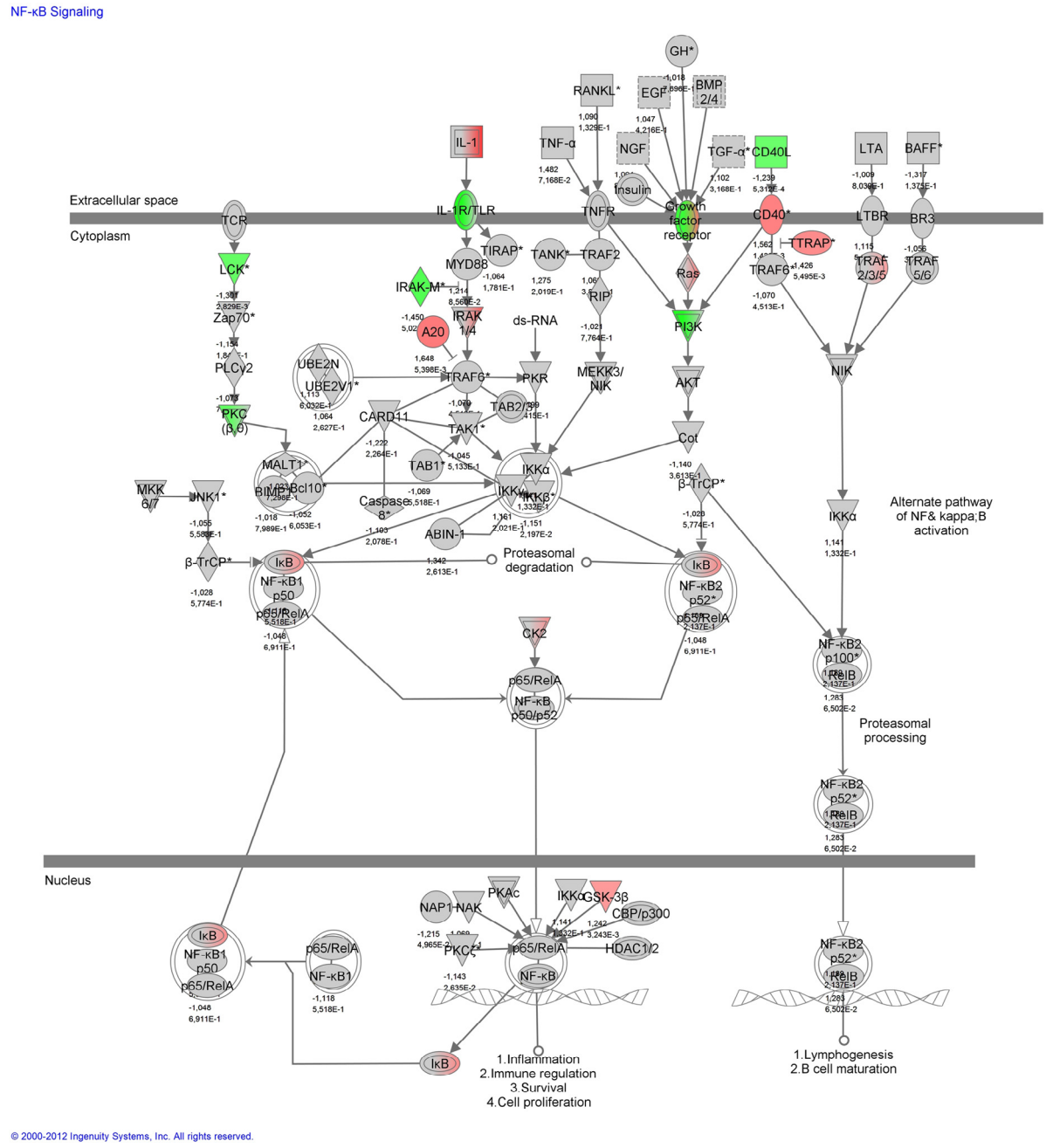

Fig. 3 – Ingenuity Pathway Analysis showing significantly up-, and down regulated molecules within NF-kB signalling pathways upon stimulation of human PBMCs with cellulose, as compared to control. Upregulated genes are indicated in red, downregulated genes are indicated in green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

could thus be one of the pattern recognition receptors (PRRs) which mediated the MyD88 independent NF-κB activation as measured in the THP1 DefMyD cells. However, this type of activation could also have occurred through other MyD88-independent PRRs expressed by the THP1 cells, such as c-type lectins (Rogers, Williams, Feng, Lewis, & Wei, 2013). Unfortunately, there are many c-type lectins and no reporter cells yet to identify their specific ligands. We were able to show that TLR2 and TLR4 are involved. The activation as elicited in TLR2 reporter cells occurred at the doses of 400 and 2000 μg/mL, and only showed weak activation as compared to the natural pathogen associated ligand (heat killed *Listeria monocytogenes*) which was used as a positive control. Compared to activation levels reported for other fibre ligands such as inulin-type fructans, this activation is also relatively weak (Vogt et al., 2013). However, we still feel that this TRL2 activation can occur in vivo, as in a normal Western diet, the average estimated daily intake is 3.2 g (Tungland & Meyer, 2002), which could provide concentrations of cellulose in the intestine that are within the range of the applied doses in these experiments.

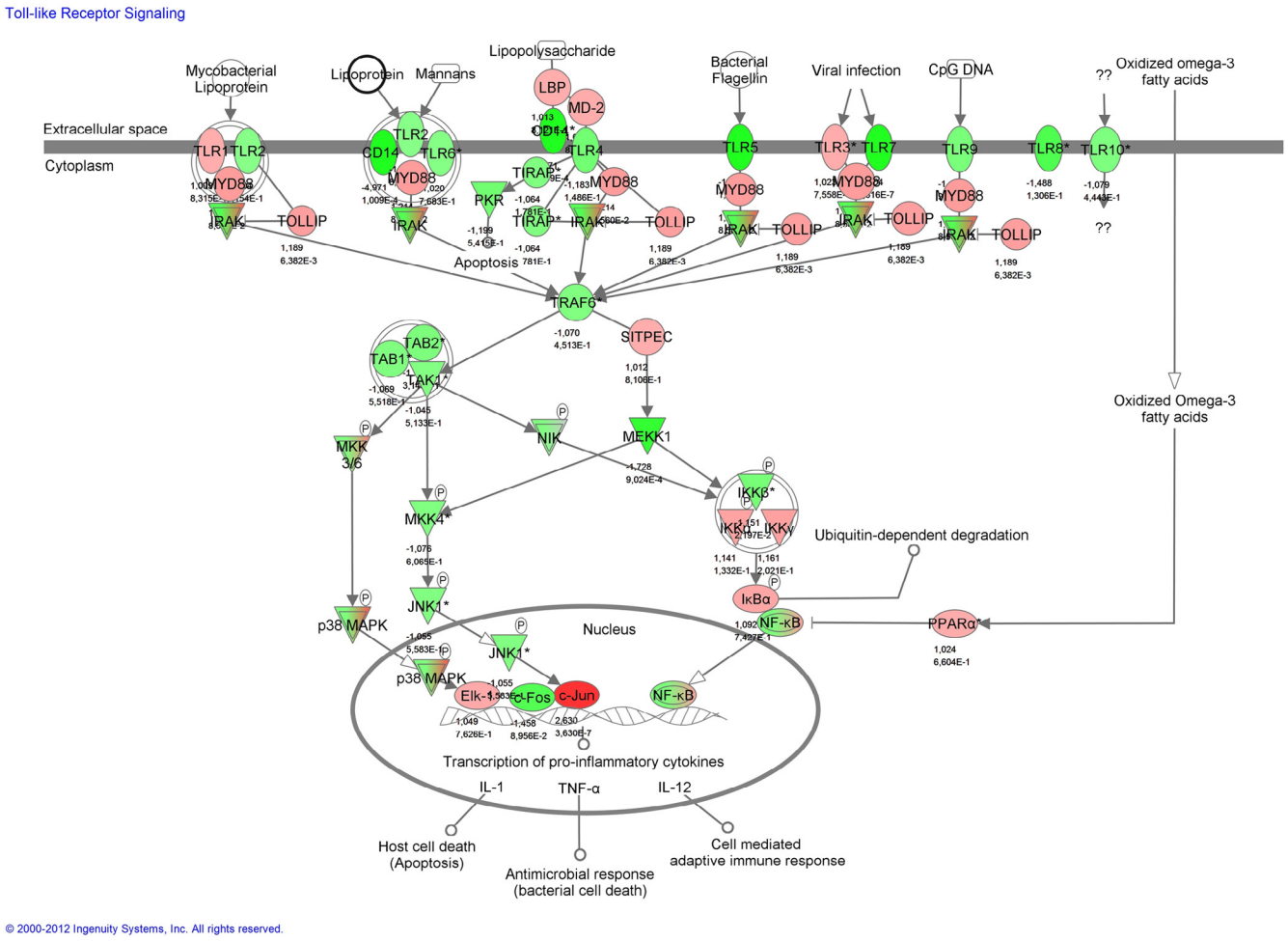

Fig. 4 – Ingenuity Pathway Analysis showing significantly up-, and down regulated molecules within TLR signalling pathways upon stimulation of human PBMCs with cellulose, as compared to control. Upregulated genes are indicated in red, downregulated genes are indicated in green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

A physicochemical difference between cellulose and other known PRR-activating carbohydrates is its insolubility in water. Although still subject of some debate, the notion that water insoluble particles cannot have any influence on cellular signalling is outdated. Examples in literature are present (Cooper & Petrovsky, 2011; Granum & Lovik, 2002), and with our experiments we add additional evidence that insoluble particulate matter can indeed induce cellular activation. Moreover, TLR activation, in specific, is not limited to water soluble agents as was demonstrated by Bachelder et al. (2010).

Peripheral blood mononuclear cells are often used to study immune effects of bioactive food components (Chen et al., 2008; Siednienko & Miggin, 2009; Vogt et al., 2013) as they express many PRRs, including TLRs and c-type lectins. The focus of this study was on the analysis of NF-κB-related changes in gene expression upon stimulation with cellulose in order to gain insight in the pathways which are activated by cellulose. Several genes were affected in their expression, demonstrating that cellulose induces transcriptional changes in several innate immune pathways in human PBMCs, as will be discussed below.

The differentially regulated genes by cellulose encode molecules of several functional categories. In respective order of strength of induction the upregulated genes within the NF-κB pathway were *IL-1Ra*, *IRAK1*, and *CD40*. IL-1Ra is an interleukin receptor antagonist, which is expressed as a secreted isoform from monocytes, macrophages, neutrophils, and other cells (sIL-1Ra), or as an intracellular isoform (icIL-1Ra1, 2, or 3) (Arend & Guthridge, 2000). Il-1Ra competitively inhibits IL-1 binding to cell-surface receptors, and the balance between IL-1 and IL-1Ra is important in preventing the development or progression of several inflammatory diseases (Arend & Guthridge, 2000). IRAK1 is one of two putative serine/threonine kinases that become associated with the interleukin-1 receptor (IL-1R) upon stimulation. It is rapidly recruited by MyD88 to the receptor-signalling complex upon TLR activation (Cao, Henzel, & Gao, 1996; Muzio, Ni, Feng, & Dixit, 1997; Neumann, Kollewe, Resch, & Martin, 2007), leading to phosphorylation and kinase activation by IRAK4 (Mamidipudi, Lin, Seibenhener, & Wooten, 2004; Qin, Jiang, Qian, Casanova, & Li, 2004). IRAK1 plays a critical role in initiating innate immune responses against foreign pathogens (Lin et al., 2014b). CD40 protein is a costimulatory receptor molecule, which is expressed by antigen presenting cells such as dendritic cells, macrophages, and B cells, and also by T cells (Banchereau et al.,

Table 1 – Fold induction of up-, and downregulated genes in the NF-κB pathway by cellulose.

Symbol	Entrez gene name	Illumina	Fold change	p-Value	Location	Type(s)	Entrez gene ID for human
IL1RN	Interleukin 1 receptor antagonist	ILMN_1774874	11.597	2.53E–05	Extracellular space	Cytokine	3557
IRAK1	Interleukin-1 receptor-associated kinase 1	ILMN_2379130	1.313	3.77E–01	Plasma membrane	Kinase	3654
CD40	CD40 molecule, TNF receptor superfamily member 5	ILMN_1779257	1.240	2.43E–01	Plasma membrane	Transmembrane receptor	958
TLR7	Toll-like receptor 7	ILMN_1677827	–2.288	3.24E–03	Plasma membrane	Transmembrane receptor	51,284
ATM	ATM serine/threonine kinase	ILMN_1779214	–2.136	4.53E–04	Nucleus	Kinase	472
TLR5	Toll-like receptor 5	ILMN_1722981	–1.648	1.02E–02	Plasma membrane	Transmembrane receptor	7100
PIK3CG	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit gamma	ILMN_1894072	–1.469	2.65E–03	Cytoplasm	Kinase	5294
PIK3R1	Phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	ILMN_1760303	–1.265	2.50E–01	Cytoplasm	Kinase	5295

Table 2 – Fold induction of up-, and downregulated genes related to TLR signalling by cellulose.

Symbol	Entrez gene name	Illumina	Fold change	p-Value	Location	Type(s)	Entrez gene ID for human
IL1RN	Interleukin 1 receptor antagonist	ILMN_1774874	28.118	2.17E–08	Extracellular space	Cytokine	3557
JUN	Jun proto-oncogene	ILMN_1806023	2.418	9.14E–06	Nucleus	Transcription regulator	3725
MAPK13	Mitogen-activated protein kinase 13	ILMN_1749327	2.098	8.15E–07	Cytoplasm	Kinase	5603
IRAK1	Interleukin-1 receptor-associated kinase 1	ILMN_2379130	1.851	2.05E–03	Plasma membrane	Kinase	3654
MAP2K3	Mitogen-activated protein kinase kinase 3	ILMN_1790534	1.537	4.11E–03	Cytoplasm	Kinase	5606
CD14	CD14 molecule	ILMN_2396444	–5.970	3.30E–03	Plasma membrane	Transmembrane receptor	929
TLR7	Toll-like receptor 7	ILMN_1677827	–3.126	2.29E–05	Plasma membrane	Transmembrane receptor	51,284
MAP3K1	Mitogen-activated protein kinase kinase kinase 1, E3 ubiquitin protein ligase	ILMN_1723020	–1.852	3.77E–03	Cytoplasm	Kinase	4214
TLR5	Toll-like receptor 5	ILMN_1722981	–1.800	8.89E–04	Plasma membrane	Transmembrane receptor	7100

1994; Bourgeois, Rocha, & Tanchot, 2002). It is required for their proliferation (O'Sullivan & Thomas, 2003), maturation (Aruffo et al., 1993; Elgueta et al., 2009; Hayden & Ghosh, 2014), and effective immune reactions (Hayden & Ghosh, 2014), including induction of chemokines and T helper 1 skewing cytokines (O'Sullivan & Thomas, 2003; Suttles & Stout, 2009). In addition, CD40/CD40L interaction of $CD4^+$ T cells and $CD8^+$ T cells is fundamental for induction of $CD8^+$ T cell memory (Bourgeois et al., 2002; Bourgeois & Tanchot, 2003). Upregulation of these genes, as observed in PBMCs, could thus represent a stimulatory effect on immune status by induction of immune cell recruitment and activation, antigen presentation processes, and subsequent adaptive immune responses.

However, not only upregulated but also downregulated genes within the NF-κB pathway were observed. Ataxia telangiectasia mutated (ATM) plays a role in cell cycle delay after DNA damage (Lee & Paull, 2007). It has a broad range of substrates related to DNA repair, apoptosis, G_1/S transition, intra-S checkpoint and G_2/M checkpoints, gene regulation, translation initiation, and telomere maintenance (Kurz & Lees-Miller, 2004). PIK3cG is involved in inflammatory and allergic responses (Collmann et al., 2013; Wymann & Solinas, 2013). It is thought to play a role in the regulation of development, proliferation, migration, and function of B cells, T cells, and NK cells (Beer-Hammer et al., 2010; Ladygina et al., 2013; Le Bouteiller et al., 2011), and controls motility of dendritic cells. Its regulatory subunit, PIK3R1 which was also downregulated, is necessary for the insulin-stimulated increase in glucose uptake and glycogen synthesis in insulin-sensitive tissues (Guo, Liu, & Yin, 2014). This protein is tightly involved in regulation of PIK3cG in B cells and T cells (Deau et al., 2014). Some TLRs were downregulated. TLR5 is an innate receptor which recognises bacterial flagellin (Hayashi et al., 2001), and TLR7 is an innate receptor which recognises single-stranded RNA in endosomes, represented by viral material which is internalised by macrophages and dendritic cells (Diebold, Kaisho, Hemmi, Akira, & Reis e Sousa, 2004). Another downregulated gene within the TLR pathway, not yet mentioned in the NF-κB pathway, is *CD14*. CD14 protein acts as a co-receptor, along with TLR4 and MD-2, in the detection of LPS and lipoteichoic acid (LTA) in the presence of lipopolysaccharide-binding protein (LBP) (Kitchens, 2000; Schroder et al., 2003). Downregulation of these genes

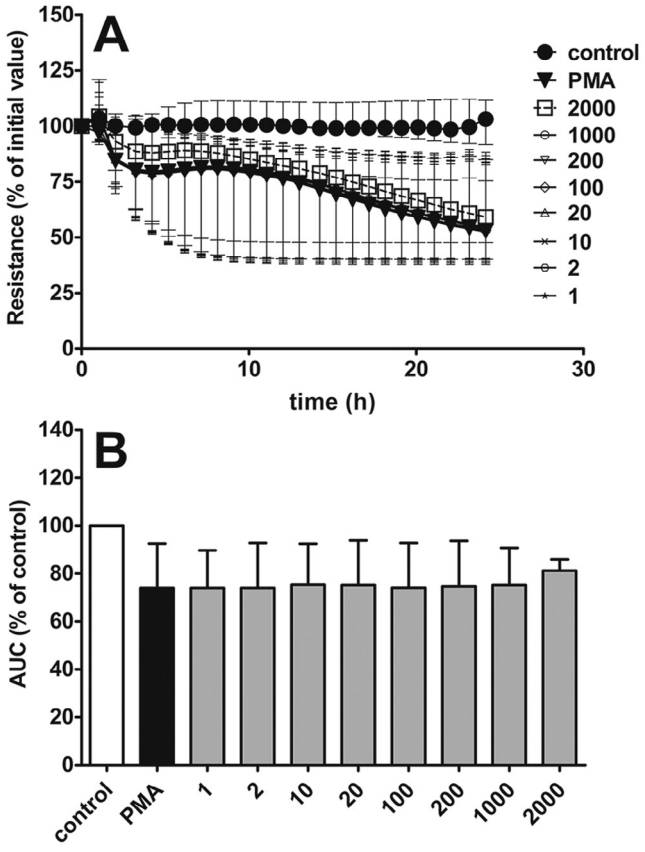

Fig. 5 – Relative TEER values and AUCs of T84 monolayers treated with cellulose and PMA. (A) Relative TEER values as percentage of starting value. (B) Area under the curve of TEER, calculated over a 24 h interval starting from the addition of PMA, shown as a percentage of the AUC of untreated control cells. Legends indicate concentrations of the applied cellulose in μg/mL (n = 6).

within the NF-κB-, end TLR-pathways could be part of a negative feedback loop to protect against overstimulation and unwanted reactions of immune cells (Oeckinghaus & Ghosh, 2009; Rothlin, Ghosh, Zuniga, Oldstone, & Lemke, 2007).

As expected because of the stimulatory effect on TLR2 and 4, upregulated genes within TLR-related pathways were observed. C-Jun, the encoded protein of *JUN*, combines with c-Fos to form the AP-1 early response transcription factor within the JNK pathway (Lopez-Bergami et al., 2007; Lories, 2006). C-jun plays a role in cell cycle progression through the G1 phase and stimulates proliferation (Angel, Hattori, Smeal, & Karin, 1988). It also exerts an anti-apoptotic effect, by cooperating with NF-κB to prevent apoptosis induced by TNFα (Angel et al., 1988). MAP2K3 is activated by mitogenic and environmental stress, and is regulated by NF-κB as a transcriptional cofactor (Gurtner et al., 2010). It can be activated by insulin, and is necessary for the expression of glucose transporter molecules (Fujishiro et al., 2001), indicating it also plays a role in metabolic processes. MAPK13 is a kinase in the p38 MAP kinase family, which can be activated by proinflammatory cytokines and cellular stress (Yang et al., 2014; Zhong et al., 2014). MAPK13 substrates include the transcription factor ATF2 and the microtubule dynamics regulator stathmin, and it is reported to play a role in differentiation, apoptosis, and inflammatory diseases (Inesta-Vaquera et al., 2009; O'Callaghan, Fanning, & Barry, 2014). Upregulation of these genes within TLR-related pathways demonstrates that cellulose not only exerts effects on genes which are directly related to innate immune reactivity but also impacts on genes involved in cell cycle regulation and metabolic processes of immune cells.

Results from the electrical cell substrate impedance sensing experiments indicate that cellulose does not exert direct barrier stimulating or protective effects in this in vitro model. TLR2 is an important regulator of epithelial resistance, and some dietary fibres indeed show barrier protective effects mediated by TLR2 binding (Vogt et al., 2014). Plausibly, the low binding and activation of TLR2 by cellulose could explain this lack of effect. Despite these results, in vivo studies indicate that cellulose confers other important protective effects on intestinal health, such as inducing production of SCFAs by the microbiota (Lattimer & Haub, 2010), and the capacity to adsorb a range of dietary carcinogens (Ferguson, Roberton, Watson, Kestell, & Harris, 1993).

The limitations of the experimental setup in this study consist of the fact that the human gastrointestinal tract is a highly complex system, which cannot be easily modelled. By making use of ex vivo PBMC stimulation and reporter cell assays we can only begin to form an idea of the effects which dietary fibres exert in the human body. Overall, these results show that cellulose is immunomodulating. Not only the expression of several categories of molecules within NF-κB and TLR-signalling was stimulated by cellulose, but also several genes within these pathways were downregulated in their expression. The activating potential of cellulose could be due to TLR activation and downstream signalling events, however this could also be an explanation for upregulation of inhibitory molecules within this pathway, which may be part of a negative feedback signal. This study comprises the first phase of the analysis of the immunomodulatory potential of cellulose and therewith warrants further studies into the effects of cellulose on TLR/NF-κB activation and regulation of expression in vivo. If the immunomodulatory effects of cellulose can be confirmed in vivo upon consumption, and the TLR-, and NF-κB activation proves to modulate intestinal cytokine profiles and immune status, those results may be relevant in studies aimed at protection against intestinal infections such as caused by *Salmonella*, and depending on the balance of induced cytokines, studies into stimulating tolerogenic dendritic cells, or inhibition of allergic (T helper 2) reactions are also warranted.

Although further studies are required to assess the functional impacts of cellulose and root pulp supplementation on the immune system, the immunostimulatory property of cellulose can be an additional health promoting characteristic contributing to the potential application of root vegetable byproduct in the functional food industry.

Author contributions

Conceived and designed the experiments: LMV PdV. Performed the experiments: LMV. Analysed the data: LMV PJdG

MVB. Contributed reagents/materials/analysis tools: LV PJdG MVB. Wrote the paper: LV PJdG MVB MMF PdV.

Conflict of interest

The authors have declared no conflict of interest.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jff.2015.08.011.

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