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The impact of the level and distribution of methyl-esters of pectins on TLR2-1 dependent anti-inflammatory responses

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ABSTRACT

Pectins have anti-inflammatory effects *via* Toll-like receptor (TLR) inhibition in a degree of methyl-esterification-(DM)-dependent manner. However, pectins also vary in distribution of methyl-esters over the galacturonic-acid (GalA) backbone (Degree of Blockiness - DB) and impact of this on anti-inflammatory capacity is unknown. Pectins mainly inhibit TLR2-1 but magnitude depends on both DM and DB. Low DM pectins (DM18/19) with both low (DB86) and high DB (DB94) strongly inhibit TLR2-1. However, pectins with intermediate DM (DM43/DM49) and high DB (DB60), but not with low DB (DB33), inhibit TLR2-1 as strongly as low DM. High DM pectins (DM84/88) with DB71 and DB91 do not inhibit TLR2-1 strongly. Pectin-binding to TLR2 was confirmed by capture-ELISA. In human macrophages, low DM and intermediate DM pectins with high DB inhibited TLR2-1 induced IL-6 secretion. Both high number and blockwise distribution of non-esterified GalA in pectins are responsible for the anti-inflammatory effects *via* inhibition of TLR2-1.

1. Introduction

A lower intake of dietary fibres in Western society compared to more traditional diets is associated with a higher chance of developing diseases with a dysregulated immunity such as type 2 diabetes, obesity, inflammatory bowel disease, and autoimmune disorders (Berer et al., 2018; Maki & Phillips, 2015; Oliveira et al., 2013; Sonnenburg & Sonnenburg, 2014; Van Itallie, 1978). In contrast, a high dietary fibre intake in traditional societies coincided with a lower frequency of those diseases (Burkitt, Walker, & Painter, 1972; Sonnenburg & Sonnenburg, 2014). The mechanisms by which dietary fibre intake prevents immunity-related disease is not fully understood. Several studies have shown that dietary fibres can influence immunity by supporting intestinal microbiota and enhancing production of metabolic fermentation products such as short-chain fatty acids (SCFA), aryl hydrocarbon receptor (Ahr)-ligands or other microbial-derived molecules (Lamas, Natividad, & Sokol, 2018; Smith et al., 2013). Moreover, dietary fibres are also known to directly stimulate the immune system (Bermudez-Brito et al., 2015; Lépine et al., 2019) by binding to Toll-like receptors (TLRs) (Sahasrabudhe et al., 2018; Vogt et al., 2013, 2016).

TLRs are a family of pattern recognition receptors (PRRs) which play an important role in intestinal immune regulation (Hug, Mohajeri, & La Fata, 2018). PRRs serve as sensors for innate immunity and may after activation stimulate transcription factors Nf-κB and AP-1, which induce upregulation of pro- and anti-inflammatory genes, depending on the activated receptor interactions (Gay & Gangloff, 2007). This may activate not only innate immune responses but also activate adaptive immune responses (Inngjerdingen et al., 2017; Iwasaki & Medzhitov, 2010; Michallet, Rota, Maslowski, & Guarda, 2013). In the intestine, TLRs are expressed on most immune and gut epithelial cells (Abreu, 2010; Yiu,

Abbreviations: Ahr, aryl hydrocarbon receptor; AP-1, alkaline phosphatase-1; DAMP, damage associated molecular pattern; DB, degree of blockiness; DM, degree of methyl-esterification; DP, degree of polymerisation; GalA, galacturonic acid; HA, hemagglutinin; HB, high degree of blockiness; HEK, human embryonic kidney; HILIC, hydrophilic interaction liquid chromatography; HPAEC, high performance anion exchange chromatography; HPSEC, high performance size exclusion chromatography; LB, low degree of blockiness; Nf-κB, nuclear factor kappa-light-chain enhancer of activated B cells; PAMP, pathogen associated molecular patterns; PMA, phorbol 12-myristate 13-acetate; PRR, pattern recognition receptor; SCFA, short chain fatty acids; TLR, toll-like receptor.

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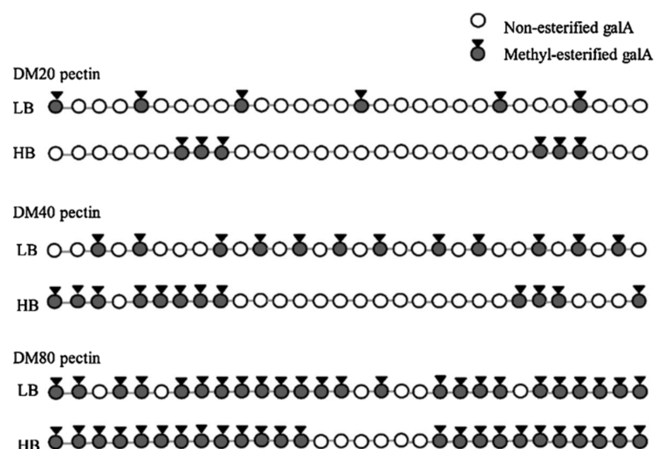

Fig. 1. Schematic representation of low DB and high DB structures of pectins with DM20, DM40 and DM80. Homogalacturonan pectins consist of a galacturonic acid (GalA) backbone structure in which GalA residues can be methyl-esterified (degree of methyl-esterification; DM). Low degree of blockiness (LB) pectins contain a more random distribution of non-esterified GalA residues, whereas high degree of blockiness (HB) pectins contain a more blockwise distribution of non-esterified GalA residues (Daas et al., 1999).

Dorweiler, & Woo, 2017). Each TLR recognizes specific pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs), or food associated molecules (Gay & Gangloff, 2007).

Pectin is one of the dietary fibre molecules with TLR binding capacity and has been shown to have anti-inflammatory effects depending on its chemical structure (Chen et al., 2006; Ishisono, Yabe, & Kitaguchi, 2017; Popov et al., 2013; Sahasrabudhe et al., 2018; Sun, He, Wang, Zhang, De Vos et al., 2017; Vogt et al., 2016). Native plant pectin consists of homogalacturonan, rhamnogalacturonan I (RG-I), and II (RG-II). RG-I segments consist of a backbone of repeating disaccharide backbone structures of alternating GalA and rhamnose residues. The rhamnose residues can be branched with neutral side chains. RG-II segments contain a backbone of GalA residues, with short side chains which contain 12 different sugar residues (O'Neill, Ishii, Albersheim, & Darvill, 2004; Voragen, Coenen, Verhoef, & Schols, 2009). These pectins consist mainly (≥ 70 %) of linear 1,4-D-galacturonic acid (GalA) (homogalacturonan) segments and minor amounts of branched rhamnogalacturonan segments (Caffall & Mohnen, 2009). The homogalacturonan backbone can be methyl-esterified (Fig. 1), and the amount of esters on the backbone is referred to as the degree of methyl-esterification (DM) (Thakur et al., 1997). Dependent on the DM, pectins have different functional properties. Sahasrabudhe et al. (2018) showed that TLR2-1 was inhibited in a DM-dependent manner by lemon pectins in which a gradual decreasing DM increased TLR2-1 inhibiting properties of pectins. In addition, TLR2 ectodomains bound stronger to pectins with a lower DM pectins than to pectins with a higher DM (Sahasrabudhe et al., 2018). However, pectins not only differ in DM but also in distribution of methyl-esters over the backbone. The degree of blockiness (DB) is a structural parameter for the distribution of non-esterified GalA residues in pectins (Fig. 1). When comparing pectins with similar DM, high DB (HB) pectins have a more blockwise distribution of non-esterified GalA residues compared to low DB (LB) pectins. This in contrast to LB pectins that have a more random distribution of non-esterified GalA residues (Fig. 1) (Daas, Meyer-Hansen, Schols, De Ruiter, & Voragen, 1999). When comparing pectins with a different DM (DM40 and DM80), but a similar DB, the total number of non-esterified GalA residues that are blockwise distributed is larger on pectins with DM40 than on pectins with DM80. This is because the DM40 pectin contains a larger number of non-esterified GalA residues than DM80 pectin (Fig. 1) (Daas et al., 1999). How DB contributes to TLR signalling is not known.

Table 1
TLR reporter cell lines and selection antibiotics.

Cell line (Invivogen)	Selection antibiotics (Invivogen)
HEK-Blue hTLR2	HEK-Blue 1X
HEK-Blue hTLR3	Zeocin (100 μg/mL) Blasticidin (30 μg/mL)
HEK-Blue hTLR4	HEK-Blue 1X
HEK-Blue hTLR5	Zeocin (100 μg/mL) Blasticidin (30 μg/mL)
HEK-Blue hTLR7	Zeocin (100 μg/mL) Blasticidin (10 μg/mL)
HEK-Blue hTLR8	Zeocin (100 μg/mL) Blasticidin (30 μg/mL)
HEK-Blue hTLR9	Zeocin (100 μg/mL) Blasticidin (10 μg/mL)
HEK293 T TLR2-HA	Blasticidin (50 μg/mL)

In the present study, it was hypothesized that the level and distribution of methyl-esters in pectin determine the efficacy of pectins as TLR signalling molecule. Therefore, the relationship between pectin structures and Toll-like receptors signalling was determined by comparing the impact of pectins with different DM and DB on activation or inhibition of different TLRs in reporter cells expressing TLRs. First, structural different orange and lemon pectins were studied on having similar DM-dependent effects on activation and inhibition of TLR2, 2-1, 2-6, 3, 4, 5, 7, 8, and 9. Next, it was studied which combination of the structural parameters DM or DB induced most pronounced TLR2-1 inhibition. Furthermore, the effects of pectins on TLR2 binding was also studied. In addition to the effects of pectins on TLR2 reporter cell line, the stimulating or attenuating effects of pectins on cytokine secretion by human macrophages *in vitro* were studied.

2. Material & methods

2.1. Pectin samples

Commercially extracted pectins from orange origin (DM32, DM64) were obtained from Andre Pectin (Andre Pectin Co. Ltd., Yantai, China). Pectins from lemon origin DM18, DM19, DM33, DM43, DM49, DM52, DM84 and DM86) were obtained from CP Kelco (Lille Skensved, Denmark).

2.2. Cell lines

To study the influence of pectins on Toll like receptor (TLR) signalling various HEK-Blue™reporter cell lines (Invivogen, Toulouse, France) were used (Kiewiet et al., 2017; Vogt et al., 2016). These reporter cell lines express Soluble Embryonic Alkaline Phosphatase (SEAP). The SEAP reporter gene is placed under the control of a NF-κB and an AP-1 responsive promotor. Upon activation of the TLRs by a specific agonist, high levels of intracellular NF-κB will lead to secretion of SEAP which can be quantified by QUANTI-Blue (Invivogen, Toulouse, France). HEK-Blue cells containing a construct of human TLR2, 3, 4, 5, 7, 8, or 9 (Invivogen) were used to study the effect of pectins on single TLRs. HEK 293/hTLR2-HA (Invivogen) was used for studying the interaction of TLR2 and pectins. All HEK-Blue™ and 293/hTLR2-hemagglutinin (HA) cells were cultured in DMEM culture media (Lonza, Basel Switzerland) containing 10 % de-complemented Fetal Calf Serum, 50U/mL Penicillin (Sigma, St. Louis, MO, USA), 50 μg/mL Streptomycin (Sigma), 100 μg/mL Normocin (Invivogen) according to the manufacturer's instructions. The reporter cells were cultured for three passages before they were maintained in selection medium (Table 1). Human monocytic THP-1 cells (ATCC, Manassas, USA) were cultured in RPMI 1640 medium (Lonza, Bornem, Belgium) with 10 % fetal bovine serum (Sigma-Aldrich, MO USA), 2 mM L-glutamine (Lonza, Belgium), 1 mM sodium pyruvate (Lonza, Belgium), 0.05 mM 2-mercaptoethanol (Scharlau, Barcelona, Spain), 60 μg/mL gentamicin sulfate (Lonza, Belgium), 2.2 μg/mL amphotericin B solubilized (Sigma).

2.3. Determination of monosaccharide composition

Neutral sugar composition of the pectins was analysed after pre-hydrolysis with 72 % (w/w) H_2SO_4 (1 h, 30 °C) followed by further hydrolysis with 1 M H_2SO_4 (3 h, 100 °C) (Englyst & Cummings, 1984). Neutral sugars released were reduced with sodium borohydride to form their corresponding alditols and then acetylated to yield their volatile derivatives. These alditol acetates were separated and quantified by gas-liquid chromatography (GLC Trace 1300; Interscience Focus-GC, Thermo Fisher Scientific) as described by Englyst and Cummings (Englyst & Cummings, 1984) equipped with a flame-ionisation detector (FID) and a 15 m DB-225 column (Agilent J&W, Santa Clara, CA, USA). Inositol was used as internal standard. The uronic acid content was determined by the automated colorimetric m-hydroxydiphenyl method (Blumenkrantz & Asboe-Hansen, 1973).

2.4. Enzymatic hydrolysis

The enzymes used in this study were pectin lyase (EC 4.2.2.10) (Harmsen, Kusters-van Someren, & Visser, 1990) and endo-polygalacturonase from *Kluyveromyces fragilis* (Daas et al., 1999). All citrus pectins were dissolved in 50 mM sodium acetate buffer pH 5.2 (5 mg/mL). The hydrolysis was performed at 40 °C by incubation of the pectin solution with PL for 6 h followed by the addition of endo-PG and incubated for another 18 h. Enzyme doses were sufficient to degrade the pectin backbone within 24 h. Inactivation of enzymes was performed at 100 °C for 10 min and the digests were centrifuged (20,000×g, 20 °C, 15 min). The supernatants obtained were analysed by high-performance size exclusion chromatography (HPSEC), high-performance anion exchange chromatography (HPAEC-PAD/UV) and Ultra-High Pressure Liquid Chromatography HILIC-ESI-IT-MS^n.

2.5. High performance size exclusion chromatography (HPSEC)

Pectin before and after enzymatic digestion were analysed using an Ultimate 3000 system (Dionex, Sunnyvale, CA, USA). A set of four TSK-Gel super AW columns (Tosoh Bioscience, Tokyo, Japan) was used in series: guard column (6 mm ID × 40 mm) and the columns TSK super AW 4000, 3000 and 2500 (6 mm × 150 mm). The column temperature was set to 55 °C. Samples (10 μL, 2.5 mg/mL) were eluted with filtered 0.2 M $NaNO_3$ at a flow rate of 0.6 mL/min. The elution was monitored by refractive index detection (Shodex RI 101; Showa Denko K.K., Tokyo, Japan). The HPSEC system was calibrated using polydisperse pectin standards having molecular weights ranging from 10 to 100 kDa as estimated by viscosimetry (Deckers, Olieman, Rombouts, & Pilnik, 1986). To display clearly the molecular weight of pectins larger than 100 kDa, 150 kDa has been calculated from the standards. Molecular weights presented were estimated at the top of the curve, despite slight differences in elution patterns of the various pectins pointing to differences in polydispersity.

Table 2
Reporter cell seeding density and their agonists.

Cell line (Invivogen)	Cell density for seeding	Agonist (Invivogen)
HEK-Blue hTLR2 (also expresses TLR1 and TLR6)	2.8 * 10^5 cells/mL (180 μL/well)	TLR2: Heat Killed Listeria Monocytogenes (10^7 cells/mL) TLR2-1: PAM3CSK4 (10 ng/mL) TLR2-6: FSL-1 (100 ng/mL)
HEK-Blue hTLR3	2.8 * 10^5 cells/mL (180 μL/well)	Poly-inosinic-olycytidylic acid (low molecular weight) (5 μg/mL)
HEK-Blue hTLR4	1.4 * 10^5 cells/mL (180 μL/well)	Escherichia coli K12 Lipopolysaccharide (10 ng/mL)
HEK-Blue hTLR5	1.4 * 10^5 cells/mL (180 μL/well)	Salmonella typhimurium derived flagellin (10 ng/mL)
HEK-Blue hTLR7	2.2*10^5 cells/mL	Imiquimod (5 mg/mL)
HEK-Blue hTLR8	2.2*10^5 cells/mL	Single stranded RNA (ssRNA40/LyoVecTM, 2 μg/mL)
HEK-Blue hTLR9	4.5 * 10^5 cells/mL (180 μL/well)	Type B CpG oligonucleotide (ODN2006; 0.25 μM)

2.6. High performance anion exchange chromatography (HPAEC)

The pectin digests were analysed and subsequently quantified using an ICS5000 High Performance Anion Exchange Chromatography system with Pulsed Amperometric detection (ICS5000 ED) (Dionex Corporation, Sunnyvale, CA, USA) equipped with a CarboPac PA-1 column (250 mm × 2 mm i.d.) and a CarboPac PA guard column (25 mm × 2 mm i.d.). The two mobile phases were (A) 0.1 M NaOH and (B) 1 M NaOAc in 0.1 M NaOH and the column temperature was 20 °C (Broxterman & Schols, 2018). GalA DP 1–3 (Sigma–Aldrich, Steinheim, Germany) were used as standards for quantification. UV-monitoring of the eluent at 235 nm allowed the identification of unsaturated oligoGalAs as released by the action of pectin lyase through the presence of a double bond. Before the analysis pectin digests were diluted using ultra-pure water to 0.5 mg/mL. They were injected (10 μl) and eluted at a flow rate of 0.3 mL/min. The gradient profile for elution was as follows: 0–55 min, linear 20–65 % B; 55.1–60 min column washing with 100 % B; finally, column re-equilibration with 20 % B from 60.1–75 min.

2.7. Ultra-high pressure liquid chromatography HILIC-ESI-IT-MS^n

Pectin digests were analysed using UHPLC in combination with ESI-IT-MS^n on a Hydrophilic Interaction Liquid Chromatography (HILIC) BEH amide column 1.7 μm, 2.1 × 150 mm (Thermo Scientific). Pectin digests were centrifuged (15000×g, 10 min, RT) and diluted (with 50 % (v/v) acetonitrile containing 0.1 % formic acid, to a final concentration of 1 mg/mL). The eluents used were (A) 99:1 % (v/v) water/acetonitrile (water/ACN); (B) 100 % ACN, both containing 0.1 % formic acid with a flow rate of 400 μL/min. The following elution profile was used: 0–1 min, isocratic 80 % B; 1–46 min, linear from 80 % to 50 % B; followed by column washing: 46–51 min, linear from 50 % to 40 % B and column re-equilibration; 51.1–60 min isocratic 80 % B with a flow rate of 400 μL/min. Oven and tray temperatures were kept at 40 °C. Mass spectra were acquired over the scan range *m/z* 300–2000 in the negative mode. Heated Electrospray Ionisation Ion Trap ionised the separated oligomers in an LTQ Velos Pro Mass Spectrometer (UHPLC-ESI-IT-MS^n) coupled to the UHPLC. The ratio of triGalA with or without methyl esterification was calculated from the peak area.

2.8. Determination of degree of methyl-esterification

Pectin samples (5 mg) were saponified using 1 mL of 0.1 M NaOH for 24 h (1 h at 4 °C, followed by 23 h incubation at RT). To the pectin blank, 1 mL of ultra-pure water was added. The head-space vials were immediately sealed with a Teflon lined rubber septum. To determine the degree of methyl-esterification (DM) a gas chromatography method was used as previously described (Huisman, Oosterveld, & Schols, 2004).

2.9. Determination of degree of blockiness

The degree of blockiness (DB) is calculated as the number of GalA residues present as non-methyl-esterified mono-, di- and triGalA released by endo-polygalacturonase related to the total amount of non-methyl-esterified GalA residues present and expressed as a percentage (Daas et al., 1999; Daas, Voragen, & Schols, 2000; Guillotin et al., 2005). The amount of mono-, di- and triGalA after the PG/PL digestion of pectins was determined by HPAEC-PAD. For the quantification GalA, $GalA_2$ and $GalA_3$ were used. Since the alkaline elution conditions

Table 3
Structural characteristics of pectins.

Pectin	origin	DB (%)	Mw (kDa)	Sugar composition (mol%)					Carbohydrate content (w/w%)*
				Rha	Ara	Gal	Glc	UA	
DM32	orange	35	77	1	3	6	1	85	57
DM64	orange	37	92	1	7	8	2	82	81
DM18	lemon	86	78	1	0	2	0	97	62
DM19	lemon	94	75	1	1	3	0	95	63
DM33	lemon	48	70	1	0	6	0	93	80
DM43	lemon	60	79	0	0	0	0	99	77
DM49	lemon	33	114	0	1	2	0	96	73
DM52	lemon	31	74	1	3	6	0	89	80
DM84	lemon	71	113	1	5	6	1	87	65
DM88	lemon	91	91	1	3	5	0	91	67

Degree of methyl-esterification (DM): mol of methanol per 100 mol of the total GalA in the sample. Degree of blockiness (DB): the amount of mono-,di- and triGalA per 100 mol of the non-esterified GalA in the sample. Molecular weight (Mw) as measured by HPSEC (Fig. A1). Rha = rhamnose, Ara = arabinose, Gal = Galactose, Glc = Glucose, UA = Uronic Acid.

removes all methyl esters from the oligo-uronides, no distinction could be made between methyl-esterified and non-methyl-esterified $GalA_3$. The amount of GalA3[1] (1 methyl ester) as measured by HILIC-ESI-IT-MS was used to calculate the amount of nonesterified $GalA_3$. DB was calculated using the following formula:

$$DB = \frac{\sum_{n=1-3}[\text{saturated GalA }_n\text{released}]_{\text{nonesterified}} \times n}{\text{total nonesterified GalA in the polymer}} \times 100$$

2.10. *Reporter cell assays*

To study whether pectins can activate TLRs or inhibit TLRs, activation or inhibition assays were performed with pectins using HEK-Blue™ cells expressing human TLRs (Invivogen). HEK-Blue™ hTLR cells were seeded in 96 wells plates at the indicated concentrations (Table 2) in 180 μL/well and were incubated overnight. The next day, the DMEM medium was replaced by DMEM medium containing pectins in the concentration 0.5 mg/mL, 1 mg/mL or 2 mg/mL. Experiments to compare lemon and orange pectins were tested at 1 mg/mL only. Activation of the TLRs was studied by treating the cells with the pectins for 24 h. Inhibition of the TLRs was studied by pre-treating the cells with pectins for 1 h followed by addition of 20 μL of the TLR specific agonist (Table 2). Culture medium was used as negative control and the TLR specific agonist was used as positive control for 24 h (Table 2). After 24 h of incubation, media supernatant was mixed with QUANTI-Blue (Invivogen) in a ratio of 1:10. After 1 h of incubation, NF-κB activation was quantified at 650 nm using a Versa Max ELISA plate reader (Molecular devices, Sunnyvale, CA, USA). Incubation steps were performed at 37 °C and 5 % CO_2. TLR activation data were represented as fold change compared to negative control. TLR inhibition data were represented as fold change compared to the positive control. Each experiment was performed at least five times.

2.11. *Protein immunoprecipitation and ELISA for binding of TLR2 to pectin*

hTLR2-HA protein was isolated from HEK 293/hTLR2-HA (Invivogen) as described before (Sahasrabudhe et al., 2018). HA-tagged proteins were immunoprecipitated using Pierce® anti-HA agarose (Thermo Scientific, Waltham, MA, USA). The proteins were eluted using 50 μg/mL HA peptide (Thermo Scientific) for 30 min at 30 °C. HA peptide was removed from the protein sample by using Zeba Spin Desalting Columns and Devices, 40 K MWCO (Thermo Scientific). Protein concentration was quantified using BCA protein assay kit (Thermo Scientific).

To confirm that specific pectins bind to TLR2, a capture ELISA was performed as described before (Sahasrabudhe et al., 2018). Isolated TLR2-HA was applied in the concentrations 0.1 μg, 1 μg and 10 μg/well. For each pectin, rat-anti pectin antibody LM20 (1:100; Plantprobes, Leeds, UK) was used as positive control for pectin binding, to confirm even pectin immobilization. Each experiment was performed at least five times.

2.12. *TLR2-1 inhibitory effect of pectins on IL-6 and IL-10 production*

In addition to the TLR2-1 inhibition assay on reporter cell lines, TLR2-1-dependent inhibition of immune responses by pectins was also tested on THP-1 cells differentiated to macrophages (Ren et al., 2016). THP-1 cell differentiation was induced by stimulation of THP-1 cells (1×10^6 cells/mL) with 100 ng/mL Phorbol 12-myristate 13-acetate (PMA, Sigma) in a 12 wells plate (in 0.5 mL medium) for 48 h at 37 °C and 5 % CO_2. The adherent cells were washed with PBS (Westburg, Grubbenvorst, the Netherlands) to remove PMA. Next, they were treated with pectins at 100 μg/mL dissolved in culture media. This concentration of pectins has previously been shown to be effective in activating and inhibiting macrophage responses (Sahasrabudhe et al., 2018). Non-treated THP-1 cells were used as negative control. After 1 h of pre-treatment with the pectins, 10 ng/mL of Pam3CSK4 was added. THP-1 cells treated with Pam3CSK4 or pectin only were used as control. After 24 h incubation, media supernatant was collected. IL-6 and IL-10 were quantified in the supernatant by ELISA according to manufacturer's protocol (eBioscience, San Diego, USA).

2.13. *Statistical analysis*

The results were analysed using Graphpad Prism program (La Jolla, CA, USA). Normal distribution was confirmed using the Kolmogorov-Smirnov test. Data that were not normally distributed were log transformed before analysis. Statistical comparisons were performed using two-way ANOVA was performed. Post-testing was performed with Tukey to test statistical differences between vehicle and pectins (* $p < 0.05$ was considered as statistically significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$), **** $p < 0.0001$) or to test statistical differences between week 1 and week 4 (# $p < 0.05$ was considered as statistically significant; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$), #### $p < 0.0001$). Values are expressed as mean ± standard error (SD).

3. Results

3.1. *Chemical composition of pectins*

Pectins obtained from lemon and orange were characterized for the degree (percent) of methyl-esterification (DM), molecular weight, and sugar composition. The degree of blockiness was calculated after enzymatic fingerprinting of the pectins and subsequent analysis of the released oligosaccharides by HPAEC and HILIC-MS. The characteristics

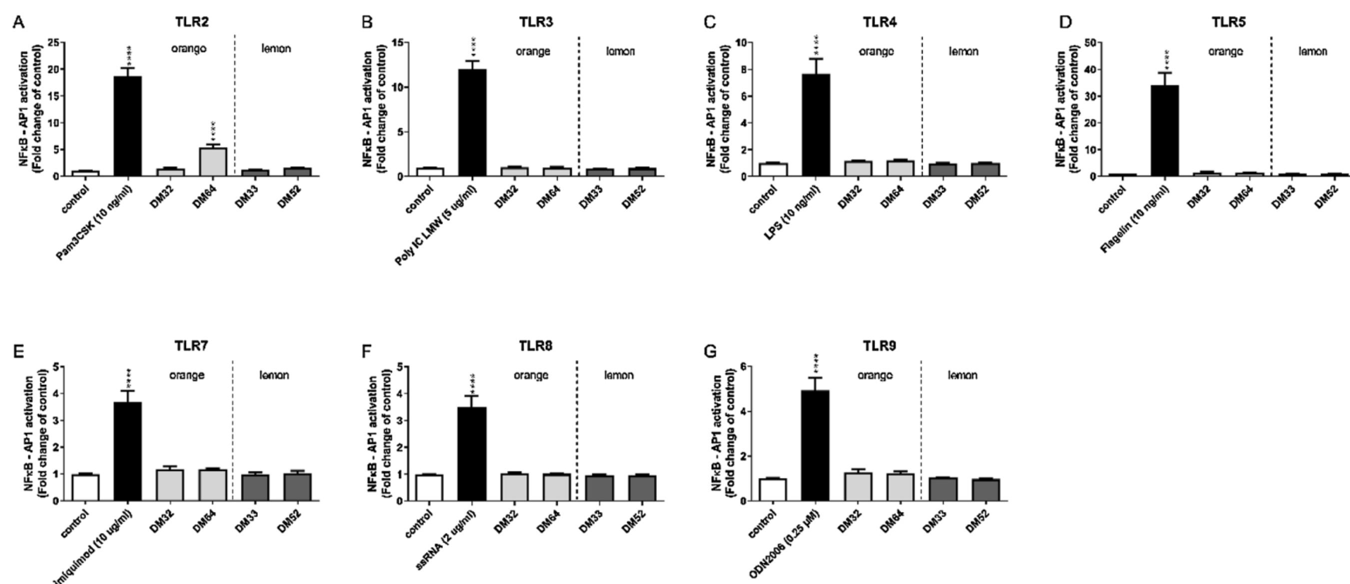

Fig. 2. TLR activation after stimulation of TLR-expressing reporter cells with orange and lemon pectins with a different DM. Activation of TLR2 (A), TLR3 (B), TLR4 (C), TLR 5 (D), TLR 7 (E), TLR8 (F), TLR9 (G) by orange and lemon pectins. The statistical differences between control and pectin samples were quantified using the one-way ANOVA test (**** $p < 0.0001$) (n=9).

of the pectins are given in Table 3. The type of citrus peel and the industrial processing conditions resulted in only small differences in the GalA content, the content and composition of neutral sugars, and in the molecular weight of the pectins. The molecular weight distribution of the pectins is shown in supplementary data (Fig. A1) and does not show major differences between the pectins. The orange pectins were characterised by a DM of 32 and 64 with a DB of 35 and 37, respectively. The methyl-esterification of the eight lemon pectins ranged from DM18 to DM88, whereas the blockiness varied between DB31 and DB93. DB is representing the charge density rather than the total charge of the molecule. For the two lemon pectins having a DM of 32 and 64, the (similar) DB values indicate that for both pectins, about 35 % of the non-esterified GalA residues are present in blocks, although the total number of non-esterified GalA residues differ. For the lemon DM52 and DM88 pectins, the distribution of the non-esterified GalA residues over blocks is quite different as they have respectively 33 % and 71 % of the non-esterified GalA residues distributed in blocks. Thus, the DB allows us to recognize different methyl ester distributions of pectins, even when the level of methyl esterification is similar.

3.2. TLR2 is activated by high DM orange pectins while TLR2-1 is inhibited in a DM-independent manner by orange and lemon pectins

Pectins might influence immunity through Toll-like receptor (TLR) signalling (Vogt et al., 2016). It has been shown for lemon pectins that the magnitude by which lemon pectin impact TLRs depends on the DM (Sahasrabudhe et al., 2018; Vogt et al., 2016). It is unknown whether other structural different pectins have similar DM dependent effects on TLR signalling. Therefore, the TLR activating and inhibiting effects of two orange pectins with a DM value of 32 and 64 with that of lemon pectins with a DM value of 33 and 52 were compared (Fig. 2). This was done by using HEK-Blue™ cells expressing either TLR 2, 3, 4, 5, 7, 8, or 9.

As shown in Fig. 2, TLR2 was activated, whereas TLR3, TLR4, TLR5, TLR7, TLR8, and TLR9 were not activated by any of the pectins. TLR2 was specifically activated by an orange pectin while lemon pectins did not have any TLR2 activating capacity. This TLR2-activation by orange pectin was DM dependent as high DM orange DM64 pectins activated TLR2 by 5.3-fold ($p < 0.0001$) while DM32 did not have such an effect.

As shown in Fig. 3, the used sets of orange and lemon pectins have a different TLR inhibiting capacity. For the inhibition studies, TLR2-1 was studied by using Pam3CSK4 as agonist, TLR2-6 by using FSL-1 as agonist, and to study total TLR2 inhibition the agonist HKLM was used. Both lemon pectin and orange pectin specifically inhibited TLR2-1 and had no inhibitory effects on FSL-1 and HKLM induced TLR2 activation. Orange pectins did not inhibit TLR2-1 in a stronger way with gradual lower DM content, which is the opposite of what was observed before with lemon pectins (Sahasrabudhe et al., 2018): orange pectin with a DM64 had a higher inhibiting effect on TLR2-1 than orange pectin with a DM32 (50.0 ± 0.05 %, $p < 0.0001$ vs 40.4 ± 0.05 %, $p < 0.0001$, respectively). Lemon pectin inhibited just as reported before TLR2-1 stronger with lower DM. Lemon DM33 had a stronger inhibitory effect (44.3 ± 0.05 %; $p < 0.0001$) than lemon pectin with a DM of 52 (14.6 ± 0.05 %; $p < 0.05$). In addition to these opposite DM-dependent effects of orange and lemon pectin on TLR2-1, differences in inhibition of other TLRs were observed. Orange DM32 pectin inhibited TLR3 and TLR8 (TLR3: $p < 0.0001$; TLR8: $p < 0.001$) while orange DM64 pectin also inhibited TLR3, TLR5, TLR8 and TLR9 (TLR3: $p < 0.0001$; TLR5: $p < 0.001$; TLR8: $p < 0.001$; TLR9: $p < 0.05$). Lemon DM33 pectin had in addition to inhibition of TLR 2-1 ($p < 0.0001$) no other TLR inhibitory effects. However, lemon DM52 pectin inhibited in addition to TLR2-1 also TLR3 and TLR4 (TLR2-1: $p < 0.05$; TLR3: $p < 0.001$; TLR4: $p < 0.001$). None of the tested pectins did inhibit TLR2, TLR2-6, and TLR7. These findings suggest that the tested orange and lemon pectins have different inhibitory capacity towards the various TLRs, although both types do inhibit TLR2-1.

3.3. Pectin's degree of blockiness has overarching effects on DM induced effects on TLR2-1 inhibition

Here and in a previous study it has been demonstrated that TLR2-1 inhibition by lemon pectins was DM dependent with more pronounced inhibition of lower DM pectins (Sahasrabudhe et al., 2018). As orange pectins, with other structural features, did not seem to have this same DM dependent inhibitory effects on TLR2-1 with lowering of DM, it was questioned whether other structural properties of pectins may play a role in TLR2-1 inhibition. In search of such differences, the degree of blockiness (DB) of the tested orange and lemon pectins was determined (Table 3). Orange DM32 pectin has a lower DB than the lemon DM33 pectin (35 % and 48 %, respectively). Furthermore, orange DM64 pectin

Fig. 3. Inhibition of TLRs after stimulation with orange and lemon pectins with different degrees of methyl-esterification (DM). Inhibition of TLR2 (A), TLR2-1 (B), TLR2-6 (C), TLR3 (D), TLR4 (E), TLR5 (F), TLR7 (G), TLR8 (H), TLR9 (I) by orange and lemon pectins. HEK-Blue™ hTLR cells were first pre-incubated for 1 h with pectins (1 mg/mL) and subsequently stimulated with the TLR-specific agonist. The statistical differences between TLR ligand and pectin samples were quantified using the one-way ANOVA test (* $p < 0.05$, *** $p < 0.001$ and **** $p < 0.0001$) (n=9).

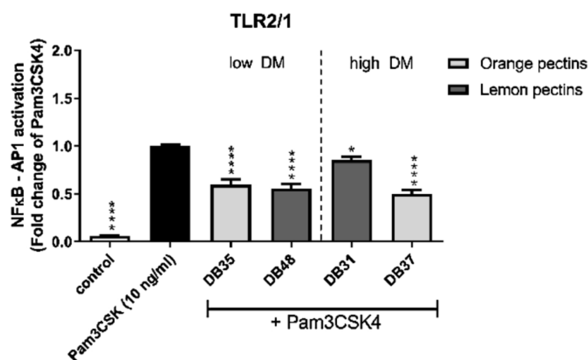

Fig. 4. Inhibition of TLRs after stimulation with orange and lemon pectins with different degrees of blockiness (DB). Inhibition of TLR2-1 by orange and lemon pectins with a different degree of blockiness. HEK-Blue™ hTLR cells were first pre-incubated for 1 h with pectins (1 mg/mL) and subsequently stimulated with the TLR-specific agonist. The statistical differences between TLR ligand and pectin samples were quantified using the one-way ANOVA test (* $p < 0.05$, *** $p < 0.001$ and **** $p < 0.0001$) (n=9).

has higher DB than the lemon DM52 pectin (37 % and 31 %, respectively). To visualize the impact of the DB more clearly the TLR2-1 inhibiting capacity was expressed according to the variations in DB (Fig. 4). Orange pectin with a DB of 37 compared to lemon pectin with a DB of 31, but rather similar DM, resulted in more inhibition of TLR2-1 (35,4 % $p < 0.0001$). Additionally, orange pectins with a DB of 35 compared to lemon pectins with a DB of 48, but similar DM, did not result in significant differences in TLR2-1 inhibition. These results suggest that pectins with a high DB induce more TLR2-1 inhibition, but the strength of this DB dependent TLR2-1 inhibition seems to be dependent on the DM of pectins as DB-dependent effects on TLR2-1 inhibition were stronger in high DM pectins than by low DM pectins. This may be related to the different structural patterns of low and high DM pectins with a high DB (Fig. 1). Low DM pectins with either a high or low DB contain larger blocks of non-esterified GalA acid residues than pectins with a higher DM and high DB. Furthermore, low DM pectins with a low and high DB both have large blocks of non-esterified GalA residues, while high DM pectins have a large difference in block size between low and high DB pectins (Fig. 1). This can be explained by the higher percentage of non-esterified GalA residues in low DM pectins (Daas et al., 2000). To study this in more detail, lemon pectins were used that varied in DM and DB with the aim to study which combination of the structural parameters DM and DB is responsible for the most pronounced TLR2-1 inhibition. This was not possible with orange pectin as these pectins could not

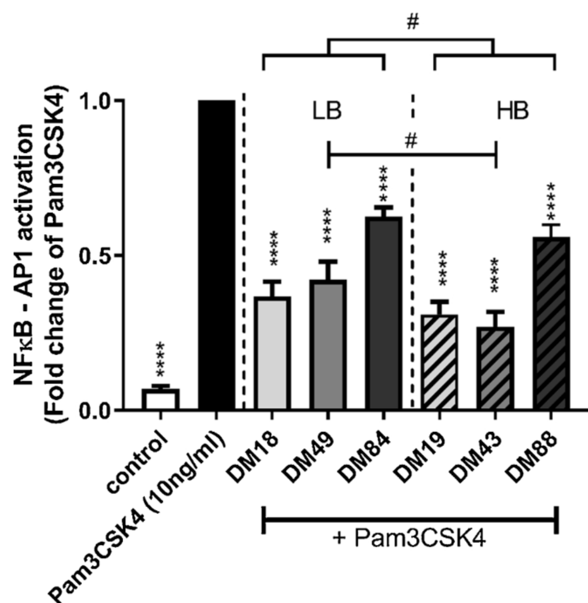

Fig. 5. Impact of the methyl-ester distribution (DB) of lemon pectins on inhibition of TLR2-1. Inhibition of Pam3SCK4 induced TLR2-1 activation by DM 18 (LB) pectin, DM 19 (HB) pectin, DM 49 (LB) pectin, DM 43 (HB) pectin, DM 84 (LB) pectin and DM88 (HB) pectin in the concentration 2.0 mg/mL. The statistical differences between Pam3SCK4 and pectin samples were quantified using one-way ANOVA test (* $p < 0.05$, *** $p < 0.001$ and **** $p < 0.0001$). Statistical differences between LB and HB pectins were tested by repeated measures two-way ANOVA (# $p < 0.05$) (n=6).

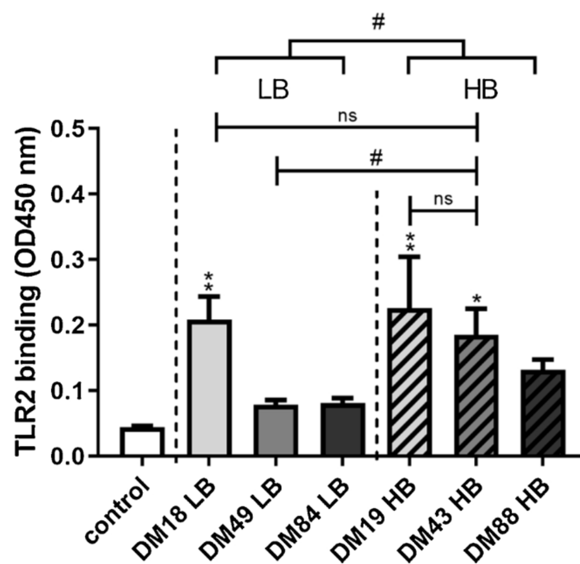

Fig. 6. LB and HB pectins bind differently to TLR2. TLR2 binding to DM 18 (LB) pectin, DM 19 (HB) pectin, DM 49 (LB) pectin, DM 43 (HB) pectin, DM 84 (LB) pectin and DM88 (HB) pectin. The statistical differences between control and pectin samples were quantified using the one-way ANOVA test (* $p < 0.05$ and ** $p < 0.01$). Statistical differences between LB and HB pectins were also quantified using repeated measures two-way ANOVA test (# $p < 0.05$) (n=9). Ns = not significant.

obtained with the same variation in DM and DB.

The TLR2-1 inhibitory capacity of six lemon pectins was compared. The pectins could be grouped into three levels of similar DM of 19 %, 46 % or 86 %, but with a different degree of blockiness (Table 3). For all three levels of DM there was one pectin with a lower degree of blockiness (DM18, DM49, DM84) and one with a higher degree of blockiness pectin (DM19, DM43, DM88) available. The pectins were first tested on TLR2-1 inhibiting and TLR2 activating capacities. For TLR2-1 inhibition there were clear DB dependent effects (Fig. 5). All high DB pectins together induced more inhibition of TLR2-1 than all low DB pectins together ($p < 0.01$). However, the magnitude by which the DB inhibited TLR2-1 was dependent on the DM. With low DM pectins, both high DB pectin (DM19) and low DB pectin (DM18) inhibited TLR2-1 strongly (69.1 ± 0.08 %, $p < 0.0001$ vs Pam3CSK4 and 63.2 ± 0.08 %, $p < 0.0001$ vs Pam3CSK4, respectively), but not significantly different. For intermediate DM pectins, the high DB pectin (DM43) inhibited TLR2-1 significantly ($p < 0.05$) stronger than the low DB pectin (DM49) (71.9 ± 0.07 %, $p < 0.0001$ vs Pam3CSK4 and 58.0 ± 0.07 %, $p < 0.0001$ vs Pam3CSK4) illustrating the impact of DB. The high DB pectin (DM43) did not inhibit TLR2-1 significantly less strong than the DM18 and DM19 pectins, indicating that pectins with a higher DM, but also a higher DB can reach similar levels of TLR2-1 inhibition as low DM pectins. Furthermore, the very high DM pectin with a high DB (DM88) did not inhibit TLR2-1 stronger than the very high DM pectin with a low DB pectin (DM84). Both high and low DB pectins with a high DM did not inhibit TLR2-1 as strong as the pectins the lower DM pectins (DM < 84) (44.1 ± 0.07 % vs Pam3CSK4 and 37.5 ± 0.07 %, $p < 0.0001$ vs Pam3CSK4) suggesting that probably above a certain DM threshold the DB does not enhance TLR2-1 inhibitory capacity anymore. Overall the results demonstrate that high DB pectin can inhibit TLR2-1 stronger than low DB pectins. This implies that the blockwise distribution of non-esterified GalA residues (high DB) of pectins with an intermediate DM can reach a similar level of TLR2-1 inhibition as low DM pectins. The effects of DB were most visible in intermediate DM pectins (DM43-49) and not in very low DM (DM18-19) or very high DM (DM84-88) pectins.

None of the six pectins activated TLR2 (Fig. A2).

3.4. Impact of DB and DM on binding to the TLR2 protein

To further substantiate the DB-dependent binding of pectin to TLR2 a capture ELISA was performed that measures the direct binding of pectins to TLR2. This approach allows us to determine true binding of pectin by the TLR2 receptor rather than neutralizing the agonist. All high DB pectins showed stronger binding to TLR2 than the low DB pectins ($p < 0.05$) (Fig. 6). This effect was concentration-dependent and most pronounced with 10 μg TLR2 protein (Fig. A3). However, similar to what was observed in the TLR2-1 inhibition assay, the DB-induced effects were dependent on the DM of the pectins. Both pectins with DM18 and DM19 having either a low and high DB showed the strongest binding to TLR2 (both $p < 0.0001$ vs control). The degree of binding between those pectins was similar and not significantly different, indicating that the difference in DB did not induce a difference in TLR2 binding at low DM. At an intermediate DM, high DB pectin (DM43) showed significantly stronger binding to TLR2 than low DB (DM49) pectin (Fig. 6; $p < 0.05$). The high DB pectin bound as strong as low DM pectins to TLR2. At a very high DM, there is no significant difference between the high and low DB pectins measured in TLR2 binding. Together, these findings suggest that pectins with blockwise distributed non-esterified GalA residues bind stronger to TLR2 than pectins with randomly distributed non-esterified GalA.

3.5. Pectin inhibited TLR2-1 induced IL-6 secretion, but not the TLR2-1 induced IL-10 secretion by in macrophages

The possible inhibiting effect of pectin's DM and DB on TLR2-TLR1 induced inflammatory responses was also investigated using THP-1 differentiated human macrophages. The cells were incubated with or without the TLR2-1 stimuli Pam3CSK4 in presence of the six pectins with a low DB and a high DB. The secretion of the pro-inflammatory IL-6 and anti-inflammatory IL-10 were quantified (Fig. 7). The pectins, without Pam3CSK4 stimulation, did not stimulate IL-6 or IL-10 secretion in the THP-1 differentiated macrophages. However, all pectins did

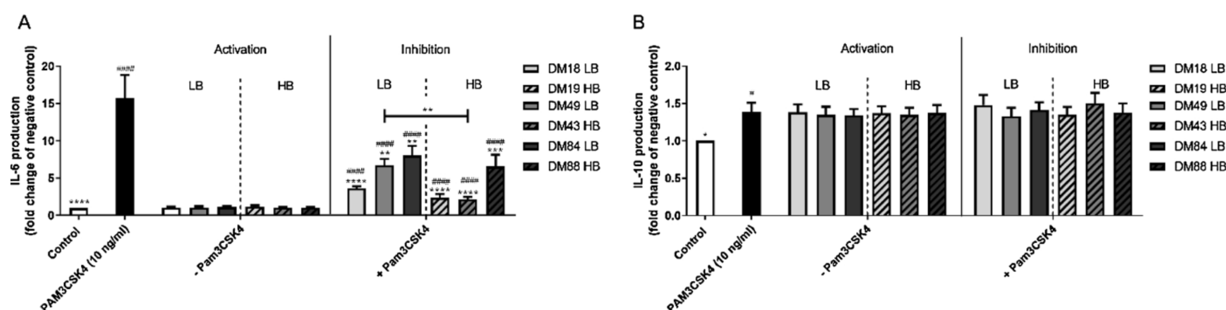

Fig. 7. IL-6 and IL-10 secretion by THP-1 macrophages stimulated with LB and HB pectins in presence or absence of Pam3CSK4. THP-1 macrophages were stimulated with DM 18 (LB) pectin, DM 19 (HB) pectin, DM 49 (LB) pectin, DM 43 (HB) pectin, DM 84 (LB) pectin and DM88 (HB) pectin in presence or absence of Pam3CSK4. The statistical differences between the Pam3CSK4 and pectin samples test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$) or between control and pectin samples test (#### $p < 0.0001$) were quantified using the two-way ANOVA test. (n=5).

significantly inhibit IL-6 secretion ($p < 0.0001$ vs Pam3CSK4) in Pam3CSK4 stimulated macrophages, but they did not inhibit TLR2-1 induced IL-10 secretion. The inhibitory effects of the pectins on IL-6 secretion corresponded with the trends of inhibition of TLR2-1 as was observed with the same pectins in the TLR2 reporter cell line. The low DM pectins (DM18 and DM19) did inhibit TLR2-1 strongly. Also, intermediate DM pectins combined with a high DB (DM43) did inhibit TLR2-1 induced IL-6 secretion stronger than intermediate DM pectin with a low DB pectin (DM49; $p < 0.01$). Very high DM pectins did not show this difference in TLR2-1 induced IL-6 secretion between low and high DB pectins corresponding to the inability to suppress TLR2-1 in the reporter cell-lines. These results show that the pectins not only inhibit TLR2-1 signalling, but also the subsequent initiation of pro-inflammatory IL-6 secretion after TLR2-1 stimulation with Pam3CSK4. The pectins with a blockwise distribution of non-esterified GalA residues were most effective in inhibiting TLR2-1 induced IL-6 secretion.

4. Discussion

Several studies have shown the protective effects of pectins on development of mucositis, pancreatitis, diet-induced obesity or auto-immune diabetes in mouse models (Jiang et al., 2016; Sahasrabudhe et al., 2018; Sun et al., 2017; Wu et al., 2019). The exact mechanisms responsible for these protective effects of pectins are not fully understood. One of the mechanisms by which pectin can protect against inflammatory disease is by modulating TLR signalling (Ishisono et al., 2017; Sahasrabudhe et al., 2018; Vogt et al., 2016). This modulation of TLRs depends on structural parameters of pectins, such as the DM (Sahasrabudhe et al., 2018; Vogt et al., 2016). However, the impact of other structural features such as the blockwise distribution of non-esterified GalA residues was so far unknown. Here, it was shown that the DB is an essential factor in the attenuating effects of pectins on TLR2-1 signalling and that the effects of the DB are most distinct in pectins with higher DM.

The current study shows that pectins strongly inhibit TLR2-1, whereas other extracellular or intracellular TLRs are inhibited to a much lower extend by pectins. This seems in contrast to other studies which showed inhibition of TLR2-6, 4 or TLR9 induced immune responses by pectins (Ishisono et al., 2017; Liu, Su, Wang, & Li, 2012). However, the inhibitory effects of those pectins may be related to the presence of RG-I and RG-II side chains, which are almost absent in pectins from the current study. These pectins are mainly homogalacturonan pectins (Ishisono et al., 2017; Liu et al., 2012). Sahasrabudhe et al. also confirmed that homogalacturonan pectins inhibit TLR2-1 specifically and not TLR2-6, TLR4, or TLR5 (Sahasrabudhe et al., 2018). Sahasrabudhe et al. provided evidence that homogalacturonan pectins interact with the TLR1 binding site on TLR2, preventing dimerization of TLR2-1 (Sahasrabudhe et al., 2018). The homogalacturonan pectins in that study were not able to inhibit dimerization of TLR2-6, which corroborates the current findings. Together, the current findings show that homogalacturonan pectins are very specific in inhibiting TLR2-1 immune responses, whereas RGI and RGII pectins can inhibit other TLR mediated immune responses (Ishisono et al., 2017; Liu et al., 2012).

Our data illustrate that the high DB strengthens the DM-dependent TLR2-1 inhibition. This suggests that not only the high level but also the blockwise distribution of non-esterified GalA residues of pectins (Fig. 1) is important for TLR2-1 inhibition. This is confirmed by the observation that both low DM pectins but also intermediate DM pectin with a high DB, which have a more blockwise distribution of their non-esterified GalA residues, inhibited TLR2-1 strongly. This argumentation is further supported by the observation that intermediate DM pectin with a low DB, having a more random distribution of its non-esterified GalA residues, inhibited TLR2-1 less efficiently. However, the very high DM pectins, which showed very low inhibition of TLR2-1, contain a very low number of non-esterified GalA residues (Daas et al., 1999). Together, these findings suggest that the blockwise distribution of non-esterified GalA residues in pectins induces more inhibition of TLR2-1 than pectins with a more random distribution of non-esterified GalA residues or having a very low number of non-esterified GalA residues.

The reason that a high DB in very high DM pectins is not leading to a significant inhibition, could be simply due to the fact that there is a limited number of non-esterified GalA residues present, despite that these non-esterified galA residues are blockwise distributed. The block size of DM88 pectin might be too small to induce a strong inhibition of TLR2-1, whereas the larger blocks in DM19 and DM43 pectins still are inhibitory. In general, a DB-value does not provide information whether the corresponding pectin may contain one big block or several smaller blocks of non-esterified GalA residues (Daas, Voragen, & Schols, 2001; Guillotin et al., 2005). Based on the absolute number of non-esterified galA residues present, DM19 and DM43 contain certainly more blocks than DM88 pectin. This suggests that a combination of block size and distribution (Daas et al., 2001; Guillotin et al., 2005) may be involved in TLR2-1 inhibitory capacity of pectins.

Next, the binding of low DB and high DB pectins to TLR2 was investigated to confirm true binding of pectins to the receptor rather than to the agonist. Binding of pectin to the receptor was confirmed and the DB-dependent patterns of binding were similar to what was observed for TLR2-1 inhibition in the reporter cell lines. Furthermore, in this capture ELISA less binding was observed for very high DM pectins which confirms the aforementioned reasoning that blockwise distribution and block-sizes of non-esterified GalA residues are important for the capacity of pectins to bind to TLR2 and preventing TLR1 to associate. In addition, it was confirmed that a more blockwise distribution of non-esterified GalA residues bind stronger to TLR2 than random distribution non-esterified GalA residues. This binding may be established through ionic binding between the blocks of non-esterified GalA residues and

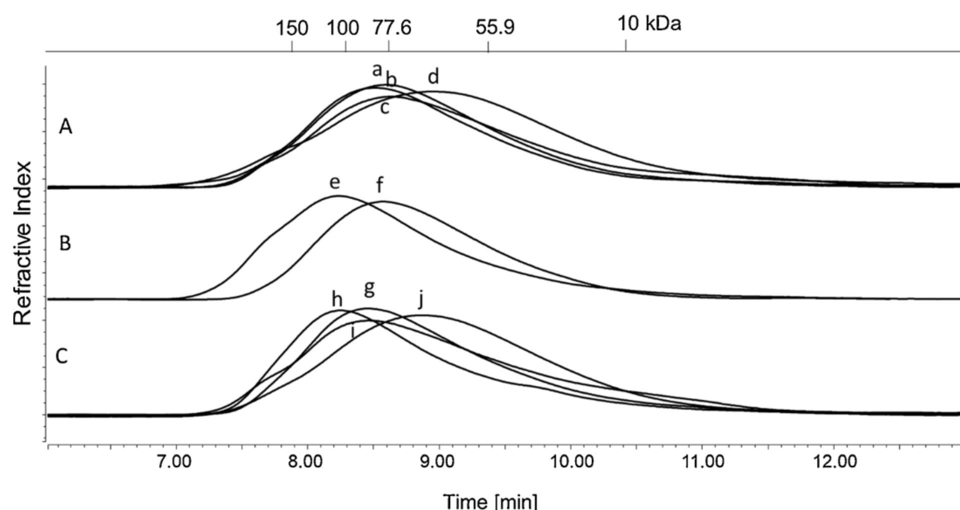

Fig. A1. HPSEC elution patterns of the pectins. Molecular weights of pectin standards (in kDa) are indicated. A = low DM pectins, B = intermediate DM pectins, C = high DM pectins. a = DM32, b = DM18, c = DM19, d = DM33, e = DM49, f = DM43, g = DM64, h = DM88, i = DM84, j = DM52.

TLR2. Ionic binding has been shown to play an important role in the interaction of pectins and TLR2 (Jin et al., 2007). More negatively charged pectins (low DM) bound stronger to TLR2 than less negatively charged pectins (high DM). The binding between negatively charged pectins became stronger to mutant TLR2 proteins with more positively charged amino acids (Sahasrabudhe et al., 2018). Pectins with a blockwise distribution (high DB) of non-esterified GalA residues have larger areas with negative charge (higher charge density) compared to random distributed non-esterified GalA residues (Jiang, Liu, Wu, Chang, & Chang, 2005). This suggests that the larger negative charge areas of the blockwise distributed non-esterified GalA residues in pectins may be of importance in the binding of pectins to TLR2.

The current study also showed that high DB pectins were more effective in suppressing TLR2-1 induced IL-6 responses than low DB pectins, which is in line with the TLR2-1 inhibition as was observed in TLR2-1 inhibition assays. This suggests that pectins not only affect TLR2-1 signalling, but also the subsequent initiation of IL-6 secretion. Inhibition of IL-6 responses may be beneficial under inflammatory conditions, as high levels of IL-6 play an important role in intestinal inflammation (Atreya & Neurath, 2005; Nishimoto & Kishimoto, 2004). The secretion of IL-6 strongly depends on TLR2 activation (Chiu et al., 2009; Flynn et al., 2019). This has been observed in mice with mucositis in which high activation of TLR2 induces inflammation characterized by high IL-6 levels (Kaczmarek, Brinkman, Heyndrickx, Vandenabeele, & Krysko, 2012; Meirovitz et al., 2010). Low DM pectins were able to reduce this inflammatory response in mucositis by inhibiting TLR2 signalling and IL-6 secretion (Sahasrabudhe et al., 2018). As HB pectins were able to inhibit the TLR2-1 induced IL-6 secretion, they may also serve as a dietary component with potential anti-inflammatory effects on mucositis.

5. Concluding remarks

In the current study, we hypothesized that the level and distribution of methyl-esters in pectin determine efficacy of pectins to impact TLR signalling. The current study demonstrates that the high number and blockwise distribution of non-esterified GalA residues in pectins is responsible for the TLR2-1 inhibitory effects. Such pectin structures were most effective in preventing the induction of pro-inflammatory cytokine responses in human macrophages. This knowledge is important for a better understanding of structural characteristics of pectins with TLR2 inhibiting properties and can be instrumental in the design of functional food applications with strong TLR2-blocking properties. Consumers may benefit from consuming pectins with a high DB as the blockwise distribution of non-esterified GalA residues in those pectins may limit the development of small intestinal inflammation induced by high activation of TLR2 (Kaczmarek et al., 2012; Sahasrabudhe et al., 2018), whereas pectin may stimulate microbial-derived SCFA production in the colon (Tian et al., 2016). Ultimately, understanding which specific pectin structures protect the intestinal immune barrier may contribute to the prevention of the development of immune-related disorders.

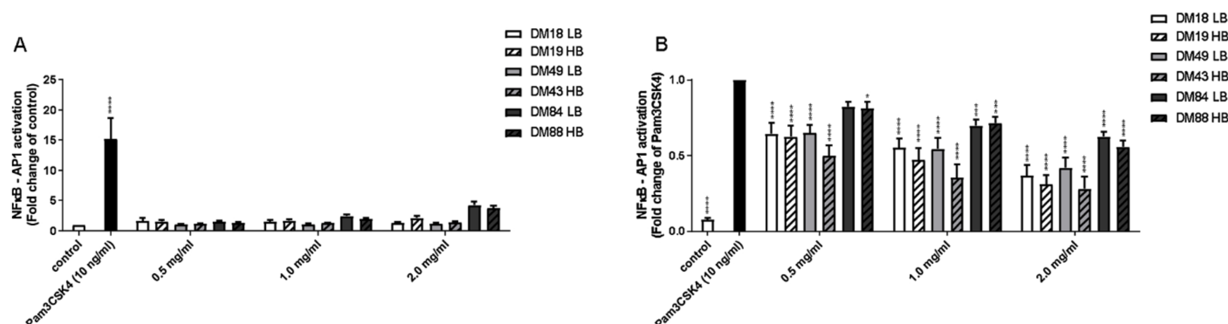

Fig. A2. Impact of the DB of pectins on TLR2 activation and TLR2-1 inhibition in a concentration dependent manner. TLR2 activation (A) and TLR2-1 induced inhibition (B) by DM 18 (LB) pectin, DM 19 (HB) pectin, DM 49 (LB) pectin, DM 43 (HB) pectin, DM 84 (LB) pectin and DM88 (HB) pectin in the concentrations 0.5, 1.0 and 2.0 mg/mL. The statistical differences between control and pectin samples (A) or Pam3CSK4 and pectin samples (B) were quantified using the two-way ANOVA test (**** $p < 0.0001$) (n=6).

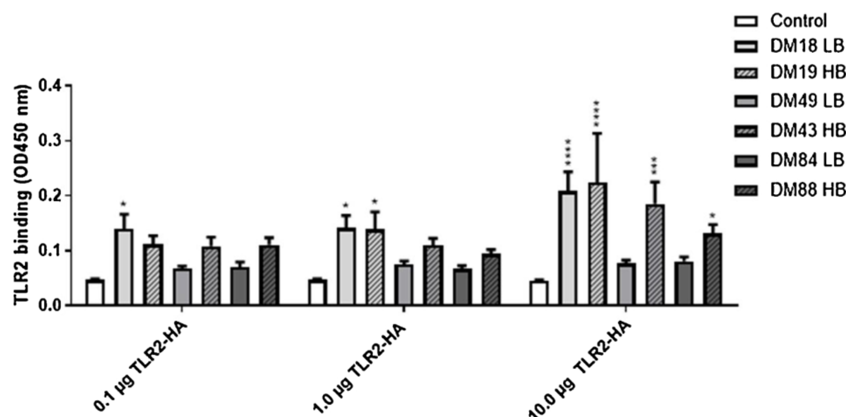

Fig. A3. LB and HB pectins bind to TLR2 in a concentration dependent manner. TLR2 binding to DM 18 (LB) pectin, DM 19 (HB) pectin, DM 49 (LB) pectin, DM 43 (HB) pectin, DM 84 (LB) pectin and DM88 (HB) pectin. The statistical differences between control and pectin samples were quantified using the two-way ANOVA test (* $p < 0.05$, *** $p < 0.001$ and **** $p < 0.0001$) (n=9).

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CRediT authorship contribution statement

M. Beukema: Conceptualization, Investigation, Formal analysis, Project administration, Writing - original draft, Visualization. **É. Jermendi:** Conceptualization, Investigation, Formal analysis, Writing - review & editing. **M.A. van den Berg:** Conceptualization, Resources, Writing - review & editing. **M.M. Faas:** Conceptualization, Writing - review & editing. **H.A. Schols:** Conceptualization, Funding acquisition, Writing - review & editing, Supervision. **P. de Vos:** Conceptualization, Funding acquisition, Writing - review & editing, Visualization, Supervision.

Declaration of Competing Interest

The authors report no declarations of interest.

Appendix A

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