The impact of lemon pectin characteristics on TLR activation and T84 intestinal epithelial cell barrier function

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ABSTRACT

Sufficient dietary fibre intake reduces the risk of several diseases, but the mechanisms linking fibre structure and health effects remain unclear. To analyse the influence of the amount of methyl groups esterified to the backbone, lemon pectins of different degrees of methyl esterification (30, 56, and 74DM) were studied for immune receptor activating potential and epithelial barrier protection. In reporter assays, the pectins demonstrated TLR/MyD88 dependent activation of NF-κB/AP-1, which increased with increasing pectin DM. To analyse the importance of backbone structure, the pectins were enzymatically digested into oligomers, which abrogated TLR activating potential. The 30 and 74DM pectins induced strong protection of the epithelial barrier measured by T84 transepithelial electrical resistance (TEER). These results indicate that activation of immune cells by lemon pectins is TLR dependent, and the intact polymer backbone is indispensable for activation. In addition, DM is a determining factor in activating potential and epithelial barrier protective effects.

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1. Introduction

An important category of health promoting nutritional compounds that are under extensive study is that of dietary fibres (Landberg, 2012). Data from meta-analyses confirm that adequate intake of dietary fibres reduces the risk and incidence of diseases such as type 2 diabetes, cardiovascular diseases, cancer (Bruno-Barcena & Azcarate-Peril, 2015; Pereira et al., 2004; Schulze et al., 2007), several gastrointestinal disorders, and obesity (Mobley, Jones, Rodriguez, Slavin, & Zelman, 2014). Although dietary fibres have been shown to have a collective health promoting effect, it is largely unknown which chemical properties of which individual fibres are responsible for which of the observed effects. Dietary fibres comprise a spectrum of different molecules from a broad range of plant species, and can also be produced by chemical or enzymatic synthesis such as polydextrose or galactooligosaccharides (GOS) (Meyer, 2015). Moreover, within one category of dietary fibres, the physiological responses can differ depending on backbone chain length (Vogt et al., 2013) and other physicochemical properties such as the number and the type of side chains (Popov & Ovodov, 2013). In this manuscript, we describe a particular family of dietary fibre, i.e. pectins, and we evaluate which fundamental structural properties can be exploited to manufacture pectin-fortified functional foods with well-characterized beneficial effects on immune function and the intestinal barrier.

Pectins form a large family of dietary fibre molecules with a range of different structural properties. They are important structural components of plant cells, consisting of linear 1,4-α-D-galacturonan (homogalacturonan) segments and highly branched rhamnogalacturonan segments (McNeil, Darvill, Fry, & Albersheim, 1984). Industrially extracted pectins are used by the food industry as gelling agent. When pectin has more than 50% of the acid units in the backbone methyl-esterified (degree

of methyl esterification – DM), it is usually classified as "high methyl ester (HM) pectin" (Seymour & Knox, 2002). Saponification of pectins will reduce the degree of esterification and will yield "low methyl ester (LM) pectins" (DM < 50) (Rosenbohm, Lundt, Christensen, & Young, 2003). Examples of pectin structures with different DM are depicted in Fig. 1. Next to the absolute amount of methyl esters present within the pectin molecule, also the distribution of methyl esters over the galacturonan backbone may determine the techno-functional properties (Daas, Voragen, & Schols, 2000).

As previously shown, the degree of methyl esterification as well as the chain length of pectin molecules are important factors in inducing different biological effects (Gómez, Gullón, Yáñez, Schols, & Alonso, 2016), including effects on the consumer's immune system (Vos, M'Rabet, Stahl, Boehm, & Garssen, 2007). Up to now, these effects on the host immune system were mainly attributed to beneficial effects of the dietary fibres on the consumers' intestinal microbiota. However, we and others have recently demonstrated that dietary fibres can also have direct effects on immune cells; several prebiotic fibres have been shown to directly ligate sensors of the innate immune system, the so-called pattern recognition receptors (PRRs) (Vogt et al., 2013). Toll-like receptors (TLRs) are the best characterized PRRs and have been shown to be involved in signalling of dietary fibres (Vogt et al., 2013). We hypothesized that activation of TLRs may be one of the factors involved in the immunomodulatory properties of pectins. To address potential structure–function relationships, we compared lemon pectins that can be obtained in defined chemical compositions. We investigated the role of backbone chain length and degree of methyl esterification of pectins on TLRs. Also, we studied the effects of pectin with different degrees of methyl esterification on barrier function of human intestinal cells *in vitro*, as pectins might exert health effects by modulating the intestinal barrier (Daguet, Pinheiro, Verhelst, Possemiers, &
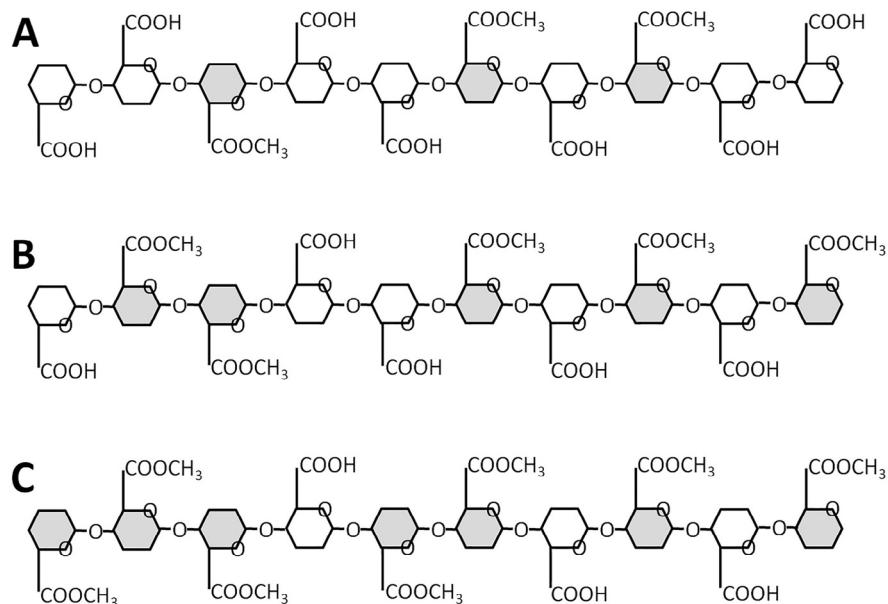

Fig. 1 – Schematic representation of different DM pectins. (A) 30DM pectin. (B) 56DM pectin. (C) 74DM pectin. Methylated galacturonic-residues are indicated in grey, unmethylated in white.

Marzorati, 2016). To this end, we applied a phorbol 12-myristate-13-acetate (PMA) damage model to T84 intestinal epithelial monolayers.

2. Methods

2.1. Investigational compounds, enzymatic digestion, and chemical characterization with high performance anion exchange chromatography and high pressure size exclusion chromatography

The investigational compounds applied in this study were lemon pectins with different degrees of methyl esterification, i.e. 30% (30DM), 56% (56DM), and 74% (74DM), acquired from CP Kelco; Lille Skensved, Denmark (see also Fig. 1). The pectins have been described in detail by Daas et al. (2000) with the codes C30, C55, and C74, respectively. These compounds were applied to investigate structure–function relationships for the degree of methyl esterification. In some experiments, the pectins were digested to oligomers to confirm that the biological effects are dependent on an intact backbone of the molecule or whether they can also be achieved by the oligomers. To this end, the pectin substrates (30, 56, and 74D M; 500 mg) were dissolved in 50 mL of 50 mM ammonium acetate buffer, pH 5. The enzymes used were endopolygalacturonase (EC 3.2.1.15) from *Kluyveromyces fragilis* with an activity of 68.68 U/mL, 20.68 µL were added (Sakai, Okushima, & Yoshitake, 1984) (for 30 and 56DM pectin) and pectin lyase II (PL, EC 4.2.2.10), from *Aspergillus niger*, with an activity of 8.8 U/mL,

161.41 µL was added (Kester & Visser, 1994) for 74DM pectin. Enzyme incubations were performed for 10 h, rotating at 125 rpm at 30 °C. Substrate blanks and enzyme blanks were prepared as controls. Following incubations for 10 h, all samples were boiled for 5 min to inactivate the enzymes.

Chemicals were from Sigma-Aldrich Chemie B.V. (Zwijndrecht, Zuid-Holland, The Netherlands). Chemical characterization by high performance anion exchange chromatography (HPAEC) and high pressure size exclusion chromatography (HPSEC) of the untreated and treated pectins was performed as previously described (Vogt et al., 2013). Results of the chemical characterization are shown in Fig. 2.

2.2. Cell culture

T84 human intestinal epithelial cells were acquired from Sigma Aldrich Chemie B.V. Cells were cultured as previously described (Vogt et al., 2014). THP1 MD2-CD14, THP1 DefMyD, HEK-Blue hTLR2, and HEK-Blue hTLR4 reporter cell lines; Normocin antibiotic; and selection media were all acquired from InvivoGen (Toulouse, Haute-Garonne, France). Reporter cells were cultured as previously described (Vogt et al., 2013). Briefly, the THP-1 cell lines were cultured in RPMI1640 supplemented with 10% heat inactivated FBS (60 °C, 60 min), glucose (4.5 g/L), NaHCO3 (Boom B.V., Meppel, The Netherlands; 1.5 g/L), HEPES (10 mM), L-glutamine (2 mM), penicillin/streptomycin (50 U/mL and 50 mg/mL), and sodium pyruvate (1 mM) all from Sigma-Aldrich Chemie B.V., and with Normocin (100 mg/mL, from InvivoGen). THP-1 cell lines were maintained at a concentration of 5×10^5 cells/mL. After culturing for 3 passages, THP1 cell
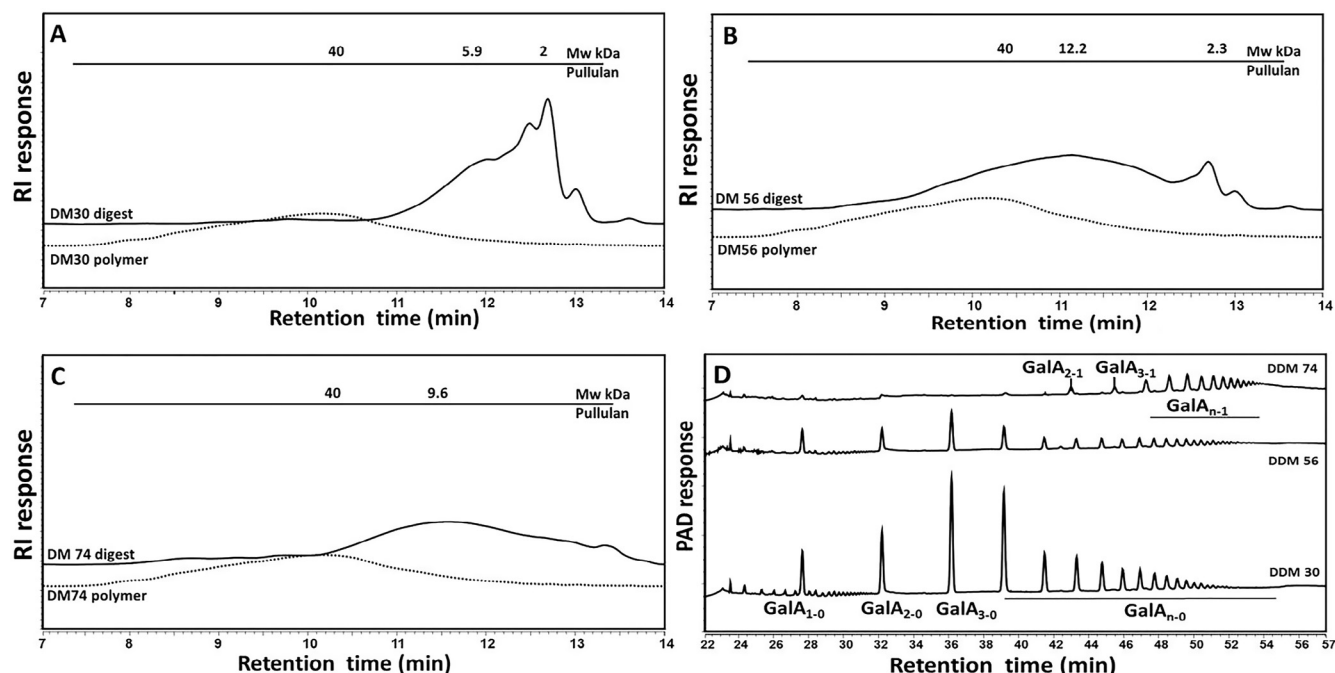
Fig. 2 – HPSEC elution patterns of the untreated pectin and of the corresponding enzyme digest, as a measure for their molecular weight distribution profiles in kDa for **(A)** DM30 pectin, **(B)** DM55 pectin, and **(C)** DM74 pectin. **(D)** HPAEC profiles of the pectin oligomers present in the enzyme digests of DM30, DM55, and DM74 pectins. GalA, galacturonic acid, uGalA, unsaturated galacturonic acid. Subscript number indicates length of oligomer. Mw, Molecular weight; DM, Degree of Methyl esterification; DDM30, digested 30DM pectin; DDM56, digested 56DM pectin; DDM74, digested 74DM pectin.

lines were maintained in selection media by adding Zeocin (200 µg/mL) and G418 (250 µg/mL) to the THP1 MD2-CD14 cell culture medium, and by adding Zeocin (200 µg/mL) and HygroGold (100 µg/mL) to the THP1 DefMyD cell culture medium. HEK-Blue cells were cultured in DMEM (Life Technologies Europe B.V., Bleiswijk, The Netherlands), supplemented with glucose (4.5 g/L), L-glutamine (2 mM), 10% heat inactivated FBS, penicillin/streptomycin (50 U/mL and 50 mg/mL), and Normocin (100 mg/mL). HEK cells were grown to ca. 80% confluency. After culturing for 3 passages, the HEK cell lines were maintained in selection media by adding 1X HEK-Blue Selection to the culture medium.

2.3. Reporter cell assays

To establish whether lemon pectin can activate PRRs, and more specifically TLRs, TLR reporter cell assays were performed according to the manufacturer's protocol. Briefly, THP-1 MD2-CD14 cells expressing all TLRs were resuspended to a cell density of 1×10^6 cells/mL, 100 µL of this cell suspension per well was stimulated for 24 h (37 °C, 5% CO_2) with untreated and treated pectic compounds (described in section 2.1), or 100 ng/mL ultrapure *Escherichia coli* LPS (InvivoGen) as a positive control. Culture medium was incorporated in the assay as negative control. Subsequently, 20 µL of supernatant of the stimulated reporter cells was incubated with 180 µL of Quanti-blue reagent (45 min, 37 °C, 5% CO_2), and NF-κB/AP-1 was measured by the amount of excreted SEAP and Quanti-blue colour conversion (OD) at 650 nm using a VersaMax microplate reader (Molecular Devices GmbH, Biberach an der Riss, Germany) and SoftMax Pro Data Acquisition & Analysis Software.

To study whether activation of NF-κB/AP-1 in the THP-1 MD2-CD14 cells was fully TLR-dependent, THP-1 cells expressing all TLRs but with a non-functional TLR adapter MyD88 were applied. These cells were resuspended to a density of 2×10^6 cells/mL and the reporter assay was performed as described in this section for THP-1 MD2-CD14 cells, with the exception that the positive control for MyD88 independent stimulation of THP1-DefMyD cells was 25 µg/mL Tri-DAP (InvivoGen).

According to recent literature reports, TLR2 and TLR4 appear to be two main TLRs by which dietary fibres can induce direct cellular activation (Vogt et al., 2013). Therefore, we next studied the activating capacity of the pectic compounds for activation of TLR2 and TLR4 in reporter cells. HEK-Blue hTLR2 reporter cells were resuspended to a cell density of 2.8×10^5 cells/mL, and HEK-Blue hTLR4 reporter cells were resuspended to a cell density of 1.4×10^5 cells/mL. One hundred eighty microlitres of cell suspension was stimulated for 24 h (37 °C, 5% CO2) with 20 µL of the pectic compounds described in section 2.1, or 20 µL of culture medium as negative control, or 20 µL of a suspension of 1×10^8 heat killed *Listeria monocytogenes* cells per millilitre (InvivoGen) or 100 ng/mL ultrapure *E. coli* LPS as positive control. Analysis of NF-κB/AP-1 activation was performed as described for the THP-1 cells.

2.4. Transepithelial electrical resistance of T84 human intestinal cells

Epithelial resistance across differentiated monolayers of T84 intestinal cells was determined using gold-plated electrode chamber slides and Epithelial Cell Substrate Impedance Sensing as described previously (Vogt et al., 2014) with some modifications. Briefly, multiple electrode gold-plated 96 well chamber slides (96W20idf, Applied Biophysics, IBIDI, München, 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, 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 ECIS incubator (Z-Theta model, Applied Biophysics, Troy, NY, USA). Measurements performed at 500 Hz were used to calculate the AUC, as relatively low frequencies are specifically representative for the tight junction mediated resistance (Lo et al., 1995). To establish whether pectins exert 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). T84 cells were incubated with DM30, DM56, and DM70 pectins for 24 h, followed by addition of PMA. Cells were maintained in this stimulation medium for at least 20 h, and this time frame of 20 h was subsequently used to calculate the AUC relative to untreated controls.

2.5. Statistical analysis

GraphPad Prism version 5.0 was used for statistical analysis of all reporter cell data and all data from the ECIS experiments. Significance levels were determined by Wilcoxon signed rank test and one-way ANOVA. Results are expressed as median and interquartile range. P-values < 0.05 are considered statistically significant and are denoted with an asterisk (*).

3. Results

3.1. Chemical characterization of lemon pectins

The pectins DM30, DM56, and DM74 used in this study have a similar molecular weight (Mw, 40–100 kDa) (Fig. 2A–C). All three pectins had a random distribution of their methyl esters. To study the effect of size of the pectin molecules, the pectins were degraded by enzymes to oligomeric fragments. Digests were studied with HPSEC and HPAEC (Fig. 2A–D). The 30DM digest after treatment with polygalacturonase consisted of saturated GalA oligomers of DP2-DP20, with the main fraction being DP2-DP6 (Fig. 2A, D). The 56DM polygalacturonase digest was less degraded by the enzyme but still consisted of lower Mw fragments (Fig. 2B, D). The digestion of 74DM pectin was performed by pectin lyase since polygalacturonase is hindered too much by methyl esters in the 74DM pectin. The HPSEC pattern in Fig. 2C demonstrates a successful degradation to low Mw
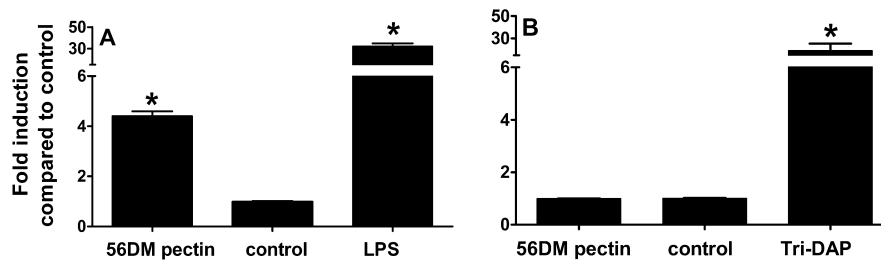
Fig. 3 – Stimulation of MyD88 proficient and deficient TLR reporter cells with 56DM lemon pectin. Figure A shows the results of fully functional THP-1 MD2-CD14 cells expressing all human TLRs, and Figure B depicts the results for THP-1 reporter cells expressing all human TLRs but which are deficient in MyD88 signalling (THP1 DefMyD cells). Both cell lines were incubated with 1 mg/mL 56DM lemon pectin, culture medium as negative control, and 100 ng/mL LPS or 25 μg/mL Tri-DAP as positive controls respectively. Statistical significance was determined with a Wilcoxon signed rank test, and activation compared to control is indicated with an asterisk. Median and interquartile range of activation is plotted as fold induction as compared to control (n = 8).

material. However, due to the working mechanism of the enzyme, mainly unsaturated GalA oligomers of DP2-DP14 were present in the digest (Fig. 2D).

3.2. Activation of THP1MD2-CD14 cells by lemon pectin polymers is dependent on TLR activation

To demonstrate the proof of principle that lemon pectins can activate immune cells, culminating in NF-κB/AP-1 induction, first 56DM pectin was co-incubated with THP1 MD2-CD14 cells equipped with a reporter gene. As shown in Fig. 3A, we observed that pectin activated the THP1 MD2-CD14 cells with a 4.4 ± 0.19-fold induction (median ± interquartile range, P < 0.05) as compared to control.

Next, we studied whether the induced NF-κB/AP-1 activation in the THP1 MD2-CD14 cells was mediated by MyD88, i.e. the central adapter molecule for TLR signalling. This was done by comparing the activation of the THP1 MD2-CD14 cells that express all TLRs in conjunction with MyD88 (Zarember & Godowski, 2002), with THP1 DefMyD cells that only express all TLRs, but a truncated, inactive form of MyD88 (Vogt et al., 2013). As shown in Fig. 3B, the pectin induced no activation of the MyD88 deficient cell line, indicating that the observed activation in the THP1 MD2-CD14 cell line was dependent on MyD88 signalling and thus TLR-mediated.

3.3. TLR induced NF-κB/AP-1 activation by lemon pectin is dependent on the degree of methyl esterification

Next, the effect of the degree of methyl esterification on immunological properties of pectin was investigated by comparing the TLR-mediated NF-κB/AP-1 activation in THP1 MD2-CD14 cells after exposure to pectin polymers with a DM value of 30, 56, and 74.

As shown in Fig. 4A, the higher the degree of methyl esterification of the pectin polymers, the higher the TLR-mediated NF-κB/AP-1 activation in THP1 MD2-CD14 cells. The 30DM pectin polymers induced a 3 ± 0.86-fold induction of activation compared to control (median ± interquartile range, P < 0.05), the 56DM pectin polymers induced a 4.4 ± 0.19-fold induction of activation (median ± interquartile range, P < 0.05) and 74DM pectin polymers induced the strongest activation, i.e. a 5 ± 0.92-fold induction as compared to control (median ± interquartile range, P < 0.05). Comparison between the different DM fractions indicated that 56DM and 74DM pectins induced significantly stronger activation as compared to 30DM pectins, and 74DM pectins tended to induce stronger activation compared to 56DM pectins (P < 0.1). These results show that the structural property of degree of methyl esterification in pectins is an important feature determining the TLR-activating capacity of lemon pectins.
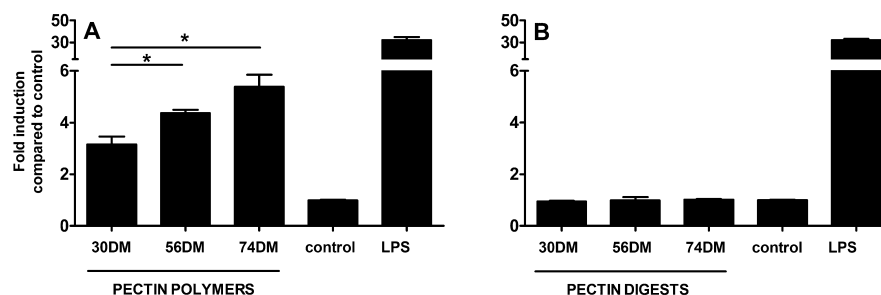

Fig. 4 – Activation of THP1 MD2-CD14 cells with polymeric pectins and digests of pectins of different DM (1 mg/mL). (A) Activation of the cell line upon stimulation with different DM pectin polymers. (B) The effect of oligomeric digests of the different DM fractions on THP1 activation. Statistical significance was determined with a one-way ANOVA and differences between the different DM fractions are indicated with an asterisk. Median and interquartile range of activation is plotted as fold induction as compared to control (n = 8).

3.4. TLR dependent NF-κB/AP-1 activation by lemon pectin requires an intact backbone

To determine whether the TLR-mediated NF-κB/AP-1 activation by lemon pectin is dependent on its molecular weight or can also be achieved by oligomers of pectins, the degree of polymerization was enzymatically reduced to pectin oligomers. Reduction of the polymer length resulted in loss of TLR activation (Fig. 4B). The oligomers did not have the same effect as the full pectins with an intact backbone, suggesting that backbone chain length is an important factor for the TLR-activating potential of these lemon pectins.

3.5. Different DM pectins show different activation patterns of TLR2 and TLR4

Next, it was studied whether TLR2 and TLR4 are involved in the activation. TLR2 and TRL4 can recognize polymeric carbohydrates (Oliveira-Nascimento, Massari, & Wetzler, 2012; Park et al., 2009) and are both implicated in the recognition of dietary fibres (Vogt et al., 2013). Therefore it was investigated whether the NF-κB/AP-1 activation by pectins is TLR2 or TLR4 dependent. HEK-Blue TLR reporter cells with either human TLR2 or TLR4 constructs were stimulated with concentration series of pectin polymers. As depicted in Fig. 5A–D, 30DM pectin only activated TLR2 at concentrations 200 and 400 µg/mL, and 56DM pectin only activated TLR2 at concentrations of 400 and 1000 µg/mL. In addition, both 30DM and 56DM pectins only induced moderate activation.

However, 74DM pectins induced a relatively strong and dose-dependent TLR2 activation over the whole concentration range tested, reaching a ca. 9-fold induction of activation at the highest concentration (1000 µg/mL, P < 0.05).

Fig. 6 illustrates that low dosed pectins of different DM, all induced a ca. 4-fold induction of TLR4 activation, and that upon increase of the dose, only a slight increase in activation was observed as compared to the lowest dose. A subtle DM effect appears to be present suggesting that with increasing DM, pectins induce increased activation, however upon testing for multiple parameters, these differences proved not significant. Taken together, these results demonstrate that high DM pectins can dose-dependently activate TLR2, and can also activate TLR4 in a less pronounced manner, and that lower DM pectins can activate both TLR2 and TLR4, but TLR2 activation was much less pronounced as compared to high DM pectins.

3.6. Effect of different DM pectins on TEER of T84 intestinal epithelial cells

To study whether lemon pectins protect T84 cells against PMA-induced loss of TEER and whether the DM value is important in TEER modulation, T84 cells were incubated with lemon pectin polymers with a degree of methyl esterification of 30, 56, and 74, for 24 h. Then PMA was added and the AUC for a 20-h time period after PMA addition was plotted for the different pectin treatments, as a percentage of the AUC of untreated controls, which was set to 100% (Fig. 7).
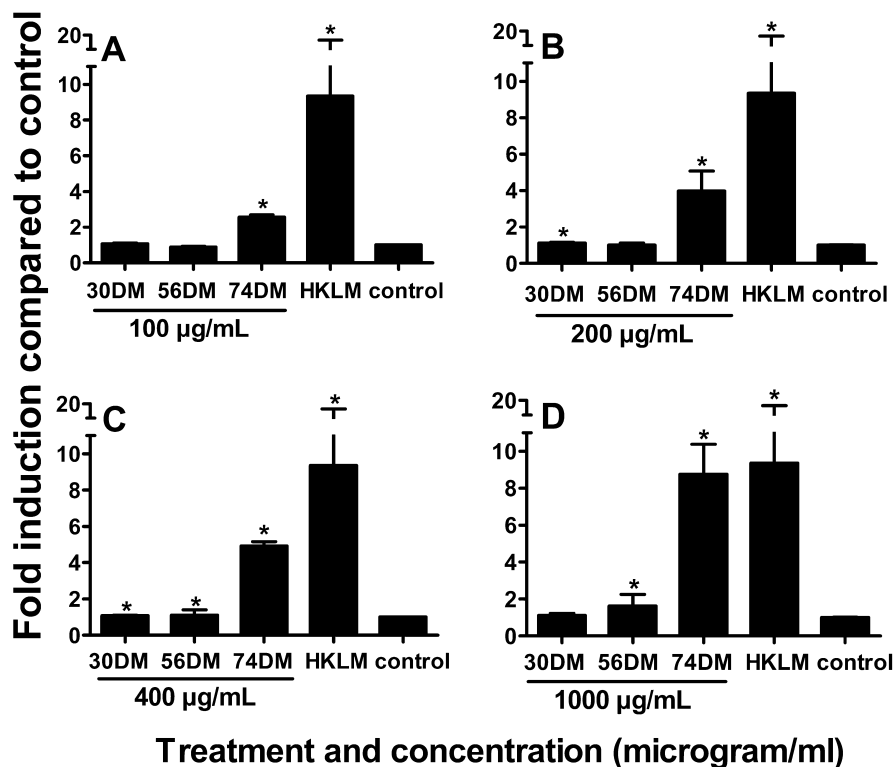

Fig. 5 – Stimulation of HEK-Blue hTLR2 cells with a concentration series of different DM pectin polymers. (A) 100 µg/mL dose. (B) 200 µg/mL dose. (C) 400 µg/mL dose. (D) 1000 µg/mL dose. Differences compared to control were assessed with Wilcoxon signed rank test, and are indicated by an asterisk. Median and interquartile range of activation is plotted as fold induction as compared to controls (n = 8).
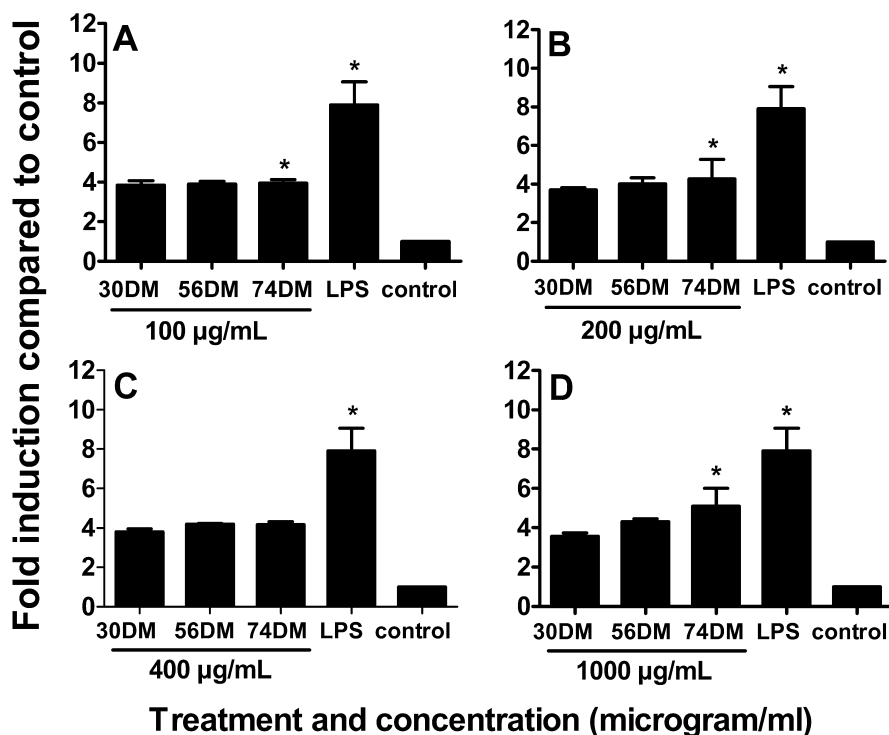
Fig. 6 – Stimulation of HEK-Blue hTLR4 cells with a concentration series of different DM pectin polymers. (A) 100 µg/mL dose. (B) 200 µg/mL dose. (C) 400 µg/mL dose. (D) 1000 µg/mL dose. Differences compared to control were assessed with Wilcoxon signed rank test, and are indicated by an asterisk. Median and interquartile range of activation is plotted as fold induction as compared to controls (n = 8).

PMA treatment caused a decrease in T84 TEER resulting in an AUC of 54.3 ± 3.4% (P < 0.01) as compared to control, confirming that damage was induced to the epithelial barrier. Upon 24 h of pre-incubation of T84 cells with 100 µg/L DM30, DM56, and DM74 polymers, all compounds conferred protection against PMA-induced loss of resistance with TEER AUC

values reaching 93.9 ± 24.6, 59.9 ± 2.2, and 77.6 ± 15.2% (all at P < 0.05), respectively (median ± interquartile range), as compared to control.

These results suggest that 30 and 74DM pectins confer considerable protection on T84 cells against a PMA-induced decrease in TEER, and that 56DM pectins exert moderate barrier protective properties.

4.   Discussion

The aim of this study was to investigate the structure–function relationship of lemon pectins by studying the effects of DP and DM on TLR-mediated cell activation, and on the barrier function of human intestinal epithelial cells. Since several dietary fibres are ligands for innate immune receptors (Sahasrabudhe et al., 2016; Vogt et al., 2013), we hypothesized that pectins could also be one of the dietary fibre types capable of activating TLRs, and that this would be one of the mechanisms by which pectins exert their immunomodulatory effects. Upon stimulating a THP1 reporter cell line, which expresses all TLRs, we observed that lemon pectins activate these human monocytic cells. Neither of the pectin compounds activated the THP1 DefMyD cells, confirming that the observed activity was mediated via TLRs. Immune activation through TLRs can also explain the immune effects of pectin described in vivo. Immune activating properties of lemon pectin have been reported in animal studies, which showed that supplementation prevented the induction of oral tolerance to OVA in rats, which was preceded by
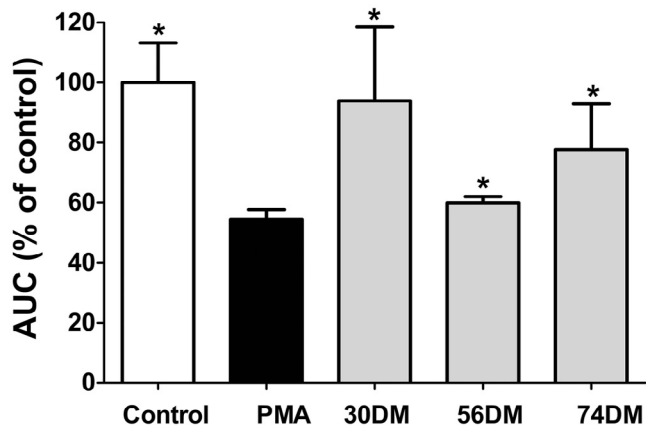

Fig. 7 – Barrier function of T84 human intestinal epithelial cells represented by AUCs of TEER values in time. Statistical significance levels compared to PMA were determined with a Wilcoxon signed rank test, and differences are indicated by an asterisk. Median and interquartile of the AUCs are plotted as percentage of control, which was set to 100% (n = 3).

enhanced protein antigen penetration to the blood and activation of macrophages (Khramova et al., 2009). Moreover, in that study, lemon pectin enhanced IFNγ and TNFα production by peritoneal macrophages. In a study by Suh et al. (2013), a lemon derived pectic-type polysaccharide was orally administered to mice, causing increased secretion of GM-CSF and IL-6 from Peyer's patches, indicating immune cell activation.

Some studies report immunomodulatory effects of the oligomer fractions that remain after enzymatic digestion of pectins (Popov & Ovodov, 2013). Although these oligomers often only induced immunomodulation in combination with galactooligosaccharides and fructooligosaccharides (Kerperien et al., 2014), we decided to test such digests for their TLR activating capacities. By incubating THP1 MD2-CD14 cells with untreated and digested pectin polymers, we observed that the intact polymers induced NF-κB/AP-1 activation, but the digested fractions showed no activating capacity. The absence of activation by the digests indicates that the intact polymeric lemon pectin molecules are required for TLR-activation, and that the oligomers derived after digestion had lost this property. This suggests that the direct immunostimulatory effects of pectins through TLRs will probably occur in the small intestine, as the pectins are likely to be digested by bacteria in the colon. The importance of the backbone of pectins has been thoroughly reviewed by Popov and Ovodov (2013). In a range of vegetables, the types of pectins were characterized and the effects on immune cells were evaluated. Important conclusions from the different types of pectins of different origins were that structural differences within pectin subtypes can induce different effects on immune cells. More specifically for lemon type pectins, the aforementioned study by Suh et al. (2013, this section) also confirmed that the intact backbone was necessary for immunostimulatory effects in Peyer's patches cells, as the hydrolysate of the pectin lost its activating potential. Our experiments subscribe that the intact backbone of lemon pectins is an important structural and functional feature, as it determines the TLR-activating capacity.

Next, when comparing the untreated polymers with different DMs for their activating potential, we observed that higher DMs were associated with higher activation of THP1 MD2-CD14 cells. This activating effect for high DM lemon pectins, but also inhibitory effects on immune cells were previously reported by Chen et al. (2006) and Popov et al. (2013). In the latter study, different DM pectins were studied for their LPS-inhibiting effect on macrophages, and the highest DM pectin demonstrated the greatest inhibition. As the pectins were not studied without the presence of LPS, the intrinsic activation capacity of the pectins themselves was not determined in that study. Nevertheless, the inhibitory mechanism suggests that there may be a competitive interaction from the high DM pectins with TLR4. From our results it can be concluded that besides DP, DM is also a structural feature that plays a role in the immunomodulatory potential and outcome upon immune cell contact with this particular type of pectins.

Subsequently, we studied whether the TLR-mediated activation could be attributed to TLR2 and/or TLR4 by applying the pectin compounds to HEK-Blue hTLR2 and HEK-Blue TLR4 cells. TLR2 and TLR4 are the TLRs that have so far been identified as innate receptors important in carbohydrate fibre recognition. In addition, for apple pectin, an LPS-inhibiting interaction

with TLR4 has been reported (Liu et al., 2010). As the digests showed no TLR-activating properties in the THP1 cells, only the intact polymeric pectins of different DMs were applied to the HEK reporter cells. We observed that only the higher DM pectins were capable of TLR2 activation, but that all pectins, already at low doses, readily activated TLR4. These results suggest that the activating potential of lemon pectins as observed in the THP1 cells could be attributed to both TLR2 and TLR4 activation. Several mechanisms for TLR-mediated immune modulation by pectins can be suggested. As a TLR4 ligand, pectin could bind to TLR4 and in this way cause competitive inhibition of LPS-mediated activation. Although TLR4 ligation is best known for its stimulating effect on immune reactions, and TLR2 is involved in defence against several categories of pathogens, TLR2 ligation can also induce anti-inflammatory responses, depending on its co-signalling molecules and also depending on tissue type or even type of organism (Cario, 2008; Vogt et al., 2014). In addition, TLRs have been shown to interact strongly with each other's signalling pathways (Trinchieri & Sher, 2007; van Bergenhenegouwen et al., 2013) so the different outcomes in immune modulation by different DM pectins could also be related to the ratio between TLR2 and TLR4 activation.

TLR2 serves as an important barrier regulatory receptor (Cario, 2008), and TLR2 ligation by certain types of fibres can induce beneficial effects on intestinal epithelial cells (Vogt et al., 2014). Therefore, we investigated whether incubation of T84 intestinal epithelial cells with pectins could protect the barrier function against the barrier damaging agent, PMA. In previous studies in our group, the TLR2-activating capacity of the dietary fibres short chain inulin-type fructans exerted preventive effects against barrier loss in human intestinal epithelial cells (Vogt et al., 2014), and TLR2 is an important innate immune receptor with a crucial role in intestinal barrier regulation (Cario, 2008). When we applied PMA to the T84 cells, a decrease in TEER was observed, and by pre-incubating with pectins, this decrease was ameliorated, confirming a protective effect of pectins on epithelial barrier function. In two studies, bacterial infection or NSAID treatment was applied to broiler chicks and cats respectively, as challenge to the intestinal barrier (Wils-Plotz, Jenkins, & Dilger, 2013). In these studies, protective effects of pectin supplementation regarding intestinal integrity and barrier function were also observed (Wils-Plotz et al., 2013), suggesting that pectin-mediated protection of the intestinal barrier is not restricted to one damage model and can be beneficial against several toxins or pathogens.

In conclusion, DP and DM are physicochemical properties of lemon pectin that can determine their immunostimulatory properties and therefore may be relevant in the use of pectins for improvement of immune status. The implications of our results for functional food applications are that pectin fibres are promising immunomodulatory agents, but that studies into the physiological and more specifically the immunomodulatory effects of pectins should be meticulously designed. The origin, the extraction method, and the full chemical characteristics such as molecular weight and level and distribution of methyl esters may explain deviating observations for the immunomodulatory effects of pectins and should be included in publications in the future. Ultimately, this will facilitate side-by-side comparisons of reported immunomodulatory

effects of all the different types of pectins available, providing an opportunity to develop tailored pectin fortified foods, with well described effects supported by knowledge of the underlying physiological responses.

Author contributions

LMV, UR, HAS, and PdV designed the research; LMV and UR conducted the research; LMV and UR analysed the data; and LMV, NMS, UR, DM, GP, MMF, KV, HAS, and PdV wrote the paper. LMV had primary responsibility for final content. All authors read and approved the final manuscript. This study was performed within the framework of the Carbohydrate Competence Center.

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