Pectin attenuates immune responses by directly blocking Toll-like receptor 2

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5.1 Abstract

Dietary carbohydrate fibers are known to prevent immunological diseases common in Western countries, although the underlying mechanisms of action are not well understood. Herein we show that the dietary fiber pectin binds and inhibits an innate immune receptor, Toll-like receptor 2 (TLR2), through specific inhibition of the proinflammatory TLR2-TLR1 pathway. This effect is most pronounced in pectins with a low degree of methyl esterification (DM). The anti-inflammatory effects of low DM pectins were confirmed in vivo in doxorubicin-induced ileitis in mice. Protective effects were shown to be TLR2-TLR1-dependent and independent of the short chain fatty acids produced by gut microbiota. These data suggest that low DM pectins as a source of dietary fiber can influence immune responses through direct interaction with the proinflammatory pathways induced by TLR2-TLR1 activation.
5.2 Introduction

A significantly increased intake of dietary fiber by Africans relative to Westerners [1] coincides with a virtual lack of certain diseases, such as diabetes [2], obesity [3], inflammatory bowel disease, and colorectal cancer [4]. A number of typical Western diseases, including asthma, allergies, and inflammatory bowel diseases [5], can also develop in youngsters and therefore, incidence cannot simply be explained by an extended lifespan. Development of such diseases may share a common basis, such as a deregulated immune system [5]. The underlying mechanisms that link dietary fibers and immunity are largely unknown. Short chain fatty acids (SCFAs) appear to be one of the mediators. Dietary fibers may serve as fermentation substrates for gut microbiota, which generate SCFAs that in turn attenuate inflammation by inducing immune regulatory Treg cells in the intestine [6]. However, beneficial effects of dietary fibers independent of SCFAs have also been reported [7, 8]. Immunomodulatory effects of dietary fibers were recently suggested to be accomplished through innate immune receptors, such as Toll-like receptors (TLRs) [9].

Pectin is one such heterogeneous family of dietary fibers with immunomodulatory effects in the gut [10]. Pectin as a dietary fiber confers different immune effects depending on its chemical and physical composition. However the mechanism for these immunomodulatory effects are unknown [11-13]. Structurally, pectin molecule is majorly composed of homogalacturonic regions which is a backbone of α(1-4)-linked galacturonic acid [14]. This homogalacturonic backbone can be methyl esterified at different positions and the percentage of methyl esterification is known as degree of methyl esterification (DM). Depending on the degree of methyl esterification, pectin is known to have different gelling properties [14]. However, role of DM in immunomodulatory effects of pectin has not been studied with respect to interaction of pectin with immune receptors like TLRs.

Toll-Like Receptors (TLR) are family of pattern recognition receptors (PRR) expressed in the intestine [15]. TLRs on immune cells present the first line of defense in the intestine, helping to differentiate between commensal and pathogenic bacteria in the gut. Whereas, the TLRs expressed on the intestinal epithelial cells play an important role in maintenance of epithelial barrier of the mucosal membrane [16]. Stimulation of TLR typically leads to activation of transcription factors NF-kB and AP1 eventually resulting in expression of pro-inflammatory or regulatory genes [17]. In the TLR family, TLR2 is a unique member in its ability to form heterodimers with other membrane receptors such as TLR1 or TLR6 [18]. TLR2-TLR1 heterodimer is activated by tri-acylated lipopeptide which lead to expression of pro-inflammatory cytokine profiles [19]. Whereas, TLR2-6 is activated by di-acylated lipopeptide and lead to expression of immune tolerance genes [19]. It was recently shown that inulin
type fructans could activate TLR2 receptor [9]. Continuing with this hypothesis, we checked the interaction of TLRs with carbohydrate fiber pectin.

Here, we studied the interaction of pectin with extra-cellular TLRs; TLR2, TLR4, and TLR5. The interaction of pectin with TLRs was studied using different DM values of pectin. Pectin showed TLR2 inhibition in a DM dependent manner wherein the mechanism of inhibition was confirmed by using an ELISA technique to test binding between pectin and TLR2. The TLR2 inhibition efficiency of pectin was also studied in human dendritic cells. To confirm efficacy of pectin in vivo, pectin was tested for suppression of TLR2-dependent ileitis induced by doxorubicin in a mouse model. Doxorubicin induces cell death in intestinal crypt and immune cells [20], which leads to a strong inflammatory response in a TLR2-dependent manner [20]. To further differentiate the direct immunomodulatory effect of pectin through inhibition of TLR2 from microbiota dependent effects, we measured SCFA concentrations in cecum samples of mice.

5.3 Materials and methods

5.3.1 Pectin Samples

Commercially extracted lemon pectins with different degree of methyl esterification (DM); DM7, DM22, DM45, DM60 and DM75) were obtained from CP Kelco (Lille Skensved, Denmark). The DM0 pectin (polygalacturonic acid) was obtained from MP Biomedicals (Fisher Scientific, Pittsburgh, PA, USA) and was also from citrus origin. Endotoxin levels in pectin samples were confirmed with endotoxin detection kit (Thermo Scientific, Sunnyvale, CA, USA) and endotoxin levels were below detection level of 0.1 ng/ml.

5.3.2 Characterization of pectins

High performance size exclusion liquid chromatography (HPSEC) was performed on an Ultimate 3000 HPLC system (Thermo Scientific, Sunnyvale, CA, USA). A Super AW-L guard column (4.6 mm i.d. × 35 mm, Tosoh Bioscience, Tokyo, Japan) and three TSK-gel columns (6 mm i.d. × 150 mm) connected in series (4000, 3000 and 2500 SuperAW Tosoh Bioscience, Tokyo, Japan) were used for analysis. A sample of 20 µL (2.5 mg/ml in 10 mM sodium acetate buffer, pH 5.0) was injected and eluted with 0.2 M sodium nitrate at a flow rate of 0.6 ml/min at 55 °C. The HPLC system was controlled by Chromeleon version 7. Detection was achieved with a refractive index detector Shodex R101 (Showa Denko, Japan). The molecular mass of polysaccharides was calculated using a calibration curve using pullulan standards (Sigma, St. Louis, MO, USA).
The DM of polysaccharides were determined by adding 0.8 mL of 0.4 N sodium hydroxide in 2-propanol/water (50/50 v/v) to 10 mg pectin for 4 hour and analyzing the acetic acid and methanol released by HPLC [21]. The DM was calculated as moles of acetic acid or methanol per 100 mol of UA.

The constituent monosaccharide content and composition was determined by GLC (Gas-Liquid Chromatography) [22]. Samples were pre-hydrolysed with 72 % (w/w) H2SO4 for 1 hour at 30 °C followed by hydrolysis with 1 M H2SO4 for 3 hour at 100 °C. The monosaccharides released were derivatized into their alditol acetates and determined by gas chromatography (Focus-GC, Thermo Scientific, MA, USA) using inositol as an internal standard. Uronic acid content was determined as anhydro-uronic acid content using an automated colorimetric m-hydroxydiphenyl assay [23] including 0.3 % (w/w) tetraborate in the sulphuric acid.

5.3.3 Cell Lines

To study the interaction of pectin with TLRs we used the HEK-Blue™ TLR cell based assays from InvivoGen, Toulouse, France. These HEK-Blue™ cells express Soluble Embryonic Alkaline Phosphatase (SEAP) gene which can be quantified using Quantiblue (InvivoGen, Toulouse, France). The SEAP gene is under the control of NFκB/AP-1 responsive promoter. These HEK-Blue™ cells also co-express single TLR genes. Upon activation with TLR specific agonists, NFκB is activated leading to SEAP expression. We used HEK-Blue™ hTLR4 and hTLR5 cells (InvivoGen, Toulouse, France) for studying interaction of TLR and pectin. HEK-Blue™ Null1 (InvivoGen, Toulouse, France) is the parental cell line for HEK-Blue™ TLR cells, expressing the SEAP gene, but not any TLR expression construct [24]. Cell lines, antibiotics, and concentration of agonists used to activate TLR signaling are shown in Table 1.

All cell lines were cultured in DMEM culture media (Lonza, Basel, Switzerland) with 10 % de-complemented Fetal Calf serum, 50 U/ml Penicillin (Sigma, St. Louis, MO, USA), 50 µg/ml Streptomycin (Sigma, St. Louis, MO, USA) and 100 µg/ml Normocin (InvivoGen, Toulouse, France).

5.3.4 TLR2 ectodomain expression

RNA was extracted from HEK-Blue™ hTLR2 (InvivoGen, Toulouse, France) cells using RNeasy® Plus Mini kit (Qiagen, Venlo, The Netherlands). cDNA was synthesized using OligodT primers (Life technologies, Carlsbad, CA, USA), dNTP mix (Life technologies, Carlsbad, CA, USA) and Superscript™III Reverse Transcriptase (Life technologies, Carlsbad, CA, USA). The first 586 codons of TLR2 were amplified from HEK-Blue™ hTLR2 cDNA using forward
primer 5'GCGCACCGGTATGCCACATACTTTGTGGATGG3' and reverse primer 5'GCGCGGATCCGTGACATTCCGACACCGAGAG3' with Pfu DNA polymerase (Thermo scientific, Waltham, MA USA). Primers were flanked by a 5' GC doublet for improved restriction enzyme digestion. Underlined letters represent AgeI and BamHI restriction sites for forward and reverse primers respectively. Fast digest restriction enzymes (Thermo scientific, Waltham, MA USA) were used to digest the PCR product and pSELECT-CHA-blasti (InvivoGen, Toulouse, France) to create sticky ends. After ligation using T4 DNA Ligase (Thermo scientific, Waltham, MA USA), the mixture was used to transform One Shot TOP10 Chemically Competent E.coli (Life technologies, Carlsbad, CA, USA). Transformed E.coli were selected on Blasticidin agar media (InvivoGen, Toulouse, France) and colonies were screened for correct orientation of the gene. Selected colonies were grown in Blasticidin liquid media (InvivoGen, Toulouse, France) after which the isolated plasmid (Midi prep kit, Qiagen, Venlo, The Netherlands) was sequenced (Baseclear, Leiden, The Netherlands) to confirm non-mutated clones.

Table 1: The TLR reporter cell lines, their agonists and selection antibiotics.

<table>
<thead>
<tr>
<th>Name of the cell line (InvivoGen)</th>
<th>Agonist (InvivoGen)</th>
<th>Selection antibiotics (InvivoGen)</th>
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<tr>
<td>HEK-Blue™ hTLR4</td>
<td>10ng/ml LPS</td>
<td>HEK-Blue™ Selection (1X)</td>
</tr>
<tr>
<td>HEK-Blue™ hTLR5</td>
<td>10ng/ml Flagellin</td>
<td>Blasticidin (30 µg/ml) Zeocin (100 µg/ml)</td>
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<tr>
<td>HEK-Blue™ Null1 TLR2</td>
<td>100ng/ml Pam3CSK4</td>
<td>Zeocin (100µg/ml) Hygromycin B (150µg/ml)</td>
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</table>

5.3.5 Plasmid transfection for stable cell line development

HEK-Blue™ Null1 TLR2 and HEK293T TLR2ectodomain-HA cell lines were obtained by transfecting cells with NotI (Thermo scientific, Waltham, MA USA) linearized plasmids. Plasmids, antibiotics, and parental cell lines are listed in Table 2. The parental cell line was seeded at 500,000 cells/ml in 12 well culture plates and incubated overnight. The following day, transfection was performed using Lipofectamine LTX® (Life technologies, Carlsbad, CA, USA). Purified, 1 µg linear plasmid was diluted in low serum media Opti-MEM® (Life technologies, Carlsbad, CA, USA) and mixed with 3.5 µl of Lipofectamine LTX® (Life technologies, Carlsbad, CA, USA). This transfection mix was incubated for 30 min at room temperature and then added to the previously seeded cells in the culture media. Cells were incubated with transfection medium mix for 24 hours and transfected cells were selected using
Pectin attenuates immune responses by directly blocking Toll-like receptor 2

antibiotics. Single cell clones were selected for each newly developed cell line.

<table>
<thead>
<tr>
<th>Name of the cell line</th>
<th>Parental cell line</th>
<th>Plasmid for transfection</th>
<th>Selection antibiotics (InvivoGen)</th>
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<tbody>
<tr>
<td>HEK293T TLR2ectodomain-HA</td>
<td>HEK293T</td>
<td>TLR2ectodomain in pSELECT-CHA blasti. This plasmid was developed in our lab</td>
<td>Blasticidin (50µg/ml)</td>
</tr>
<tr>
<td>HEK-BlueTM Null1 TLR2</td>
<td>HEK-BlueTM Null1</td>
<td>pUNO3-hTLR2 (InvivoGen)</td>
<td>Zeocin (100µg/ml) and Hygromycin B (150µg/ml)</td>
</tr>
</tbody>
</table>

Table 2: Parental cell lines, expression plasmids and selection antibiotics used to develop TLR2 expression cell lines.

5.3.6 TLR activation and inhibition assay

HEK-Blue™ cell lines were seeded at 500,000 cells/ml in 96-well plates at 100 µl/well. Cells were allowed to grow overnight. The following day, cells were treated with different pectins at 2 mg/ml and TLR agonists (Table 1). Inhibition of TLR was studied by pretreating HEK-Blue™ cells with pectin (0.5, 1, or 2 mg/ml) for 1 hour, followed by treatment with TLR agonists (Table 1). Activity of SEAP converts the pink QUANTI-Blue substrate to blue. Media supernatant was mixed with QUANTI-Blue in a ratio of 1:10 and NFkB activation was quantified at 650 nm using a Versa Max ELISA plate reader (Molecular devices, Sunnyvale, CA, USA). The assay was performed with five technical repeats. Each experiment was at least repeated three times.

5.3.7 Dialysis binding assay of pectin with TLR agonist

Pectin was dissolved in 1 mM CaCl2 and 150 mM NaCl in 0.05 M Tris buffer pH 8.2 at 0.5, 1, and 2 mg/ml. Rhodamine-labeled Pam3CSK4 (InvivoGen, Toulouse, France) was added at 10 µg/ml and incubated overnight at 37 °C. After incubation, 100 µl was applied to a 10 kDa molecular weight cut off microdialysis chamber (Thermo scientific, Waltham, MA USA). As pectin is larger than 10 kDa, it does not diffuse out of the micro dialysis chamber, while the smaller 2.15 kDa Pam3CSK4 will diffuse out. In the case of an interaction between the TLR2 ligand Pam3CSK4 and pectin, the complex will stay inside the dialysis chamber and fluorescence will increase.
micro dialysis chamber was washed with 1 mM CaCl2 and 150 mM NaCl in 0.05 M Tris buffer pH 8.2. Samples were dialyzed for 4 hours, with buffer change every hour. After dialysis, samples were recovered from the chamber and fluorescence intensity was measured at 566 nm (excitation at 549 nm) using a Varioskan reader (Thermo scientific, Waltham, MA USA). Non-dialyzed and dialyzed Pam3CSK4 rhodamine in buffer were used as controls. Dialysis was performed three times for each test sample.

5.3.8 Protein Immunoprecipitation

HEK293T TLR2ectodomain-HA cells were lysed using RIPA lysis buffer (Merck Millipore, Billerica, MA, USA) with a protease inhibitor cocktail (Sigma, St. Louis, MO, USA) at 4 oC for 10 min followed by two times sonication (Bandelin, Berlin, Germany) for 5 seconds at 50 % power. Supernatent was collected after centrifugation at 14000 g for 10 min. HA tagged proteins were immunoprecipitated using Pierce® Anti-HA Agarose in a micro centrifuge tube. Protein was eluted using HA peptide (Thermo scientific, Waltham, MA, USA) by incubating two times for 15 min with single bed volume of HA peptide at 30 oC. Isolated protein was desalted and HA peptide was removed using Zeba Spin Desalting Columns and Devices, 40K MWCO (Thermo scientific, Waltham, MA, USA). Isolated desalted protein was quantified using the Thermo scientific BCA protein assay kit (Thermo scientific, Waltham, MA, USA).

5.3.9 ELISA for binding of TLR2 and pectin

ELISA buffer consisted of 1 mM CaCl2 and 150 mM NaCl in 0.05 M Tris buffer at pH 8.2. The buffer was used for washing as well as diluent for antibodies and pectin. Blocking buffer was made by adding 3 % milk powder (FrieslandCampina, Amersfoort, The Netherlands) to ELISA buffer. For antibody solutions, 1:2 dilution of blocking buffer with ELISA buffer was used. ELISA plates (Corning, Tewksbury, MA, USA) were treated with 50 µl of 50 µg/ml of Poly-L-lysine for 1 hour at 37 oC. Wells were washed once with 400 µl ELISA buffer. Pectins were dissolved in ELISA buffer at 1 mg/ml and 50 µl was applied to each well. The plates were incubated for 4 hours at 37 oC. Each well was then washed with 400 µl buffer and blocked overnight with 100 µl blocking buffer at 4 oC. After blocking, the plate was washed once with ELISA buffer. Isolated TLR2 ectodomain protein and HA peptide (Thermo scientific, Waltham, MA, USA) were applied to pectin-coated wells at 0.33 µg, 1 µg, 3 µg, and 9 µg /well; and incubated at 37 oC for 3 hours. HA peptide was used as a negative control. Pectin binding antibodies LM19 (DM0 and DM7) and LM20 (DM22, DM45, DM60 and DM75) (Plantprobes, Leeds, UK) were used as positive control for pectin binding at 1:100 dilutions. Afterwards, wells were washed with 400 µl of ELISA
buffer for 5 times and incubated with 50 µl primary HA tag antibody (Cell Signaling, Danvers, MA, USA) at 1:200 dilutions. Primary antibody was incubated for 2 hours at 37 oC. Afterwards, plates were washed 5 times with ELISA buffer and subsequently, 50 µl of biotin tagged secondary antibody (Southern Biotech, Birmingham, AL, USA) was applied to each well at 1:500 dilutions. Biotin tagged antibody was incubated for 1 hour at 37 oC followed by five washings with 400 µl of ELISA buffer. Streptavidin-HRP (100 µl) was applied to each well at 1:1000 dilutions. Anti-rat HRP antibodies (Cell Signaling, MA, USA) (1:500 for 1 hour at 37 oC) were used to detect pectin binding antibodies in control wells. In the final step, 100 µl TMB (Cell Signaling, MA, USA) was added as substrate for quantification and incubated at 37 oC for 30 min. The reaction was stopped by addition of 100 µl of stop solution (Cell Signaling, MA, USA). Read out was done in a Versa Max plate reader (Molecular devices, Sunnyvale, CA, USA) at 420 nm. The pectin loading control was confirmed to be at similar levels for all the pectin.

5.3.10 Doxorubicin induced ileitis in mice

C57BL/6 female mice (7-10 weeks) were obtained from Janvier laboratories, France. The experimental use of animals was approved by the Animal Ethical Committee of the University of Groningen. All the mice were acclimatized for 2.5 weeks prior to start of the experiment. Mice were fed ad-libitum with RMH-B (AB diets, Woerden, The Netherlands). The ingredients of the diet specified by supplier were: wheat, meat meal (80 % sterilized), yellow dent corn, whole oats, wheat middlings, alfalfa, soya oil, dried yeast, dicalcium phosphate, calcium carbonate, NaCl, dl-methionine, vitamins and trace elements. As pectin is present mainly in fruits and vegetables, the basal of level of pectin in diet was minimal. Mice were supplied with drinking water from the tap and the water bottles were changed once a week.

Ileitis was induced by administration of doxorubicin (Sigma, St. Louis, MO, USA). Doxorubicin was dissolved in sterile 0.9 % sodium chloride and stored in aliquots at 4 oC. Pectin was dissolved in sterile water and administered by gavage to mice for 10 days, twice a day at a dose of 1.5 mg. On day 8, doxorubicin was injected intra-peritoneally [2] at 10 mg/kg. Mice were sacrificed on day 10 (48 hour doxorubicin). Animals receiving water by gavage served as controls. After the collection of tissue samples, mice were sacrificed by cervical dislocation.

TLR2 blocking antibody, clone T2.5 (InvivoGen, Toulouse, France) was administered IP at 10 mg/kg one hour prior to doxorubicin treatment. The dose and concentration of TLR2 blocking antibody was used in accordance with Tye et al. [25]. TLR2 blocking antibody was also administered in additional controls to study possible immune effects of TLR2 blocking antibodies in mice.
5.3.11 Neutrophil counts in peritoneal lavage

Peritoneal lavage was collected by injection and aspiration of 2 ml PBS. The total number of living cells in the peritoneal lavage, lysed with lyserglobin, were counted using a Z™ Series coulter counter® (Beckman Coulter, Brea, CA, USA). The lavage was diluted to 500,000 cells/ml and 100 µl of cell solution was applied for cytospin preparation. The cytospin slides were stained with Giemsa (Merck Millipore, Billerica, MA, USA) for 1 hour at room temperature. The stained slides were scanned using a Hamamatsu slide scanner (Hamamatsu photonics, Hamamatsu, Japan) and neutrophils were counted using morphological features in 250 cells [20]. The total number of neutrophils were calculated using the total cell count of the peritoneum and the neutrophil counts from cytospin preparations.

5.3.12 Histology

Ileal samples from mice were fixed in 10 % formalin in PBS and embedded in paraffin. Paraffin blocks were cut in 4 µm sections. The tissue sections were also analyzed for apoptosis in crypts using a TUNEL assay. The TUNEL assay was performed using the ApopTag® Peroxidase in Situ Apoptosis Detection Kit (Merck Millipore, Billerica, MA, USA) according to the instructions of the manufacturer. Hematoxylin was used for counterstaining the slides. As peroxidase substrate, an incubation step of 15 minutes with 3-amino-9-ethylcarbazole (AEC) (5 % AEC stock; 95 % 0.05 M acetate buffer pH 4.9; 0.1 % of 30 % v/v H2O2) (Merck Millipore, Billerica, MA, USA) was used. The stained slides were scanned with a Hamamatsu slide scanner (Hamamatsu photonics, Hamamatsu city, Japan) and analysis was performed using NDP.view2 software. TUNEL positive cells were measured in 10 sequential crypts in the ileum per mouse.

5.3.13 Luminex assays

Peritoneal lavage from mouse was centrifuged to remove the immune cells and the supernatant was stored at -20 oC. Blood was collected from mice through heart puncture and stored on ice in EDTA coated tubes (Greiner Bio-One, Kremsmünster, Austria). The tubes were centrifuged at 1350 g for 10 min to separate plasma from the blood. Peritoneal lavage and plasma samples were analyzed in a luminex assay (Affymetrix, Santa Clara, CA, USA). TNF-α, MCP-1, IL-6, IP-10, and GRO-α were analyzed in the luminex assay.

Human umbilical cord dendritic cells (MatTek, Ashland, MA, USA) were seeded in a 96-well culture plates at 100,000 cells/ml in 100 µl dendritic maintenance medium provided by the supplier (MatTek, Ashland, MA, USA). After 24 hours at 37 oC, each well was treated with DM7 or DM75 pectin at 1 µg/ml, 10 µg/ml, and 100
μg/ml, dissolved in culture media. Similarly, TLR2 blocking antibody (InvivoGen, Toulouse, France) was applied as positive control at 10 μg/ml. Non-treated dendritic cells were used as negative control. After 1 hour of pretreatment, 10 ng/ml of Pam3CSK4 was added. Dendritic cells treated with Pam3CSK4 only were used as control. After 24 hours incubation, media supernatant was used to quantify IL-6 and IL-10 using the luminex assay (Affymetrix, Santa Clara, CA, USA).

The luminex assay was performed according to instructions from manufacturer. The antigen standards provided with the luminex kit (Affymetrix, Santa Clara, CA, USA) were dissolved and diluted 4-fold to have seven serially diluted standards. DC-MM (MatTek, MA, USA) culture media was used as blank. The magnetic beads were added to a clear base, black 96-well plate at 50 μl/well and washed with a hand-held magnetic plate holder with 150 μl of the wash buffer provided in kit. The plasma samples were diluted with universal assay buffer in 1:2 dilution. The standards and samples in duplicates were applied to the magnetic beads, mixed on a plate shaker and incubated overnight at 4 °C on a stable flat surface. The following day, magnetic beads were washed three times as mentioned above and incubated with 25 μl/well of detection antibody mix for 30 min on a plate shaker at room temperature. The plate was washed three times and incubated with 50 μl/well streptavidin-PE for 30 min at room temperature on a plate shaker. In the last step, the 96-well plate was washed three times and beads were dispersed in 120 μl of reading buffer per well and read in a Luminex-100 instrument with StarStation software.

### 5.3.14 Organic acids and SCFA measurement

Wet digests from caecum (200 mg) and colon of mice was mixed with 1.5 ml of 0.3 mg/ml 2-ethylbutyric acid (internal standard) in 50 mM H2SO4. After centrifugation (10 000 × g, 5 min, 20 °C), the supernatant was transferred into a vial for injection using an HPLC method as described [26].

### 5.3.15 Statistics

The results were analyzed using Graphpad Prism program (La Jolla, CA, USA). The parametric distribution of data was confirmed using Kolmogorov-Smirnov test. Values are expressed as mean ± standard deviation (SD) except where nonparametric, in which case median ± range was used. Statistical comparisons were performed using two-way ANOVA for grouped analysis of parametrically distributed data. Where no parametric distribution could be demonstrated, we applied Mann-Whitney U test or Kruskal-Wallis test. p<0.05 was considered as statistically significant (*p<0.05, **p<0.01, and ***p<0.001).
5.4 Results

5.4.1 Pectin inhibits TLR2 signaling in a degree of methyl esterification and concentration dependent fashion.

To study the possible effects of chemical composition on the immune properties of pectin, we chemically characterized citrus pectin with different degrees (percent) of methyl esterification (DM0, DM7, DM22, DM45, DM60, and DM75) and studied activation or inhibition of TLR signaling. The pectin samples had average molecular weights ranging from 50 to 250 kDa and were predominantly composed of D-galacturonic acid (Figure 1). The pectins were incubated with reporter cell lines expressing extracellular TLRs (TLR2, TLR4, and TLR5). As shown in Figure 2, pectins were unable to activate TLRs by more than 10% compared to controls (Figure 2A-2C), but had stronger inhibitory effects on TLR receptors.

Inhibitory effects of pectins on TLR signaling were studied by adding a TLR agonist to TLR reporter cell lines pretreated with pectin. As shown in Figure 3, this demonstrated pronounced inhibition of TLR2 and minimal inhibition of other extracellular TLRs, such as TLR4 and TLR5 (Figure 3A-3C). There was a clear DM-dependent effect, as inhibition of TLR2 due to lower DM pectins was more pronounced than pectins with higher DM. Pectin DM7 suppressed TLR2 activation by 91.1 ± 0.8% (p<0.001) at the lowest concentration tested (0.5 mg/ml) (Figure 3A). Increasing the concentration enhanced the efficacy of higher DM pectins to inhibit TLR2 (Figure 3A).

5.4.2 Pectin inhibits pro-inflammatory TLR2-TLR1 activation but not the tolerogenic TLR2-TLR6

TLR2 can only be activated in the presence of TLR1 and TLR6. As such, the TLR2 reporter cell line also expresses TLR1 and TLR6 [27]. TLR2 dimerizes with TLR1 and TLR6 and activates different pathways [18]. TLR2-TLR1 activation was shown to induce proinflammatory IL-17 dependent pathways whereas TLR2-TLR6 activation showed IL-10 inducing tolerogenic pathways [19]. TLR2-TLR1 is specifically activated by a P3CSK4 agonist. As shown in Figure 3A, the TLR2-TLR1 dimer was inhibited by pectin in DM- and concentration-dependent manners. The other heterodimer, TLR2-TLR6, is activated by di-acylated lipopeptides, such as FSL-1 [28]. To determine if pectin also inhibits TLR2-TLR6 activation, different DM pectins were evaluated with FSL-1 as an agonist. As shown in Figure 4, pectin did not substantially inhibit TLR2-TLR6. Thus, pectin can only inhibit proinflammatory TLR2-TLR1 but does not inhibit tolerogenic TLR2-TLR6 activation.
Table 1: Sugar composition (mol%) and carbohydrate content (w/w%) of pectin.

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Figure 1: HPSEC (high pressure size exclusion chromatography) elution patterns of pectin with monosaccharide composition (mol%) and carbohydrate content (w/w%).

Figure 2: Pectin does not activate TLR2, TLR4 and TLR5. TLR activation represented as NF-κB activation of HEK-Blue™ TLR stimulated with pectin at 2 mg/ml concentration and with P3CSK4 (A), LPS (B), and Flagellin (C) as positive control for TLR2, TLR4, and TLR5 activation respectively. Data are presented as mean ± SD. The statistical differences between pectin samples were quantified using Kruskal-Wallis test (*p<0.05 and **p<0.01).
Figure 3: Pectin inhibits TLR2 but is unable to inhibit TLR4 and TLR5. TLR activation represented as NF-κB activation of HEK-Null1 TLR2, HEK-BlueTM TLR4 and HEK-BlueTM TLR5 cell lines pretreated with pectin with varying DM-content and stimulated with P3CSK4, LPS, and Flagellin for TLR2, TLR4, and TLR5 cell lines respectively. The data are presented as mean ± SD. The statistical differences were quantified using two-way ANOVA (*p<0.05, **p<0.01, and ***p<0.001).

Figure 4: Pectin inhibits TLR2-TLR1, but not TLR2-TLR6, in a DM-dependent manner. NF-κB activation in HEK-Null1 TLR2 treated with TLR2-TLR6 agonist FSL-1 and pectin. Data are presented as the mean ± SD, and statistical significance was calculated using two-way ANOVA analysis (**p<0.01 and ***p<0.001).
5.4.3 Pectin inhibits TLR2 by direct binding to TLR2 ectodomain and not by binding its agonist

We performed two experiments to confirm an interaction between TLR2 and pectin and to exclude that inhibition of TLR2-TLR1 activation is caused by pectin binding to P3CSK4. In the first experiment, pectin was co-incubated with fluorescently labeled P3CSK4 and dialyzed using a 10 kDa selective dialysis membrane permissible to P3CSK4 but not to pectin. As shown in Figure 5, pectin did not bind P3CSK4, as fluorescence decreased profoundly upon dialysis. Also there was no difference between fluorescence levels of dialyzed P3CSK4 with and without pectin. Thus, with this assay we can conclude that pectin does not bind to TLR2-TLR1 agonist in order to impart its anti-inflammatory effects.

In the second experiment, we studied direct binding between pectin and TLR2. To test this, we developed cell lines expressing TLR2 ectodomain consisting of ligand binding site and it was C-terminally fused to a HA (hemagglutinin) tag. The TLR2 ectodomain protein was extracted, purified and applied to direct ELISA containing immobilized pectin (Figure 6A). TLR2 binding to pectin was determined using antibodies against the C-terminal HA tag. HA-tag peptide at similar concentrations was applied as negative control for analyze tag dependent binding shown by TLR2 ectodomain. As shown in Figure 6B, TLR2 binding to pectin was DM-dependent, wherein lower DM pectins showed stronger binding with TLR2 while minimal binding was shown by high DM pectin. The highest level of binding was observed by DM7 pectin and the binding gradually decreased with increase in DM wherein DM75 pectin showed the lowest level of binding. The binding by DM7 pectin was 74.5 fold higher than DM75 pectin (p<0.001) (Figure 6B). Among the low DM pectin samples, DM0 showed lower binding with TLR2 ectodomain than DM7, although DM0 has higher number of unesterified galacturonic acids. Differences in binding might be due to the presence of a low concentration of methyl esters, resulting in slightly different pectin conformations [14]. As observed in the reporter cell lines, DM7 showed the strongest inhibition of TLR2. Using ELISA, we confirmed that TLR2 inhibition was due to pectin binding.
Figure 5: Pectin does not bind to TLR2-TLR1 agonist P3CSK4. P3CSK4 rhodamine interactions with pectin presented as fluorescence retention in the dialysis membrane compared to non-dialyzed samples. Data are presented as the mean ± SD, and statistical significance was calculated using two-way ANOVA analysis.

A

- Streptavidin Horse Radish Peroxidase, for detection using color reaction with TMB substrate
- Biotin secondary antibody against primary antibody
- Primary antibody against HA tag on TLR2 ectodomain
- TLR2 ectodomain with HA tag
- Plastic well coated with pectin followed by overnight blocking with 3% milk in tris-NaCl-CaCl2 buffer at pH 8.2
Figure 6: Low DM pectin inhibits TLR2-TLR1 by directly binding to the TLR2 ectodomain
(A) Design of ELISA based system to demonstrate binding of pectin to TLR2. (B) TLR2 ectodomain binding to pectins with various DM. Isolated HA peptide was used as a negative control. Data are presented as the mean ± SD, and statistical significance was calculated using two-way ANOVA analysis (*p<0.05 and **p<0.001).
5.4.4 Pectin inhibits TLR2-TLR1 induced IL-6 and IL-10 production in dendritic cells

The effect of pectin DM on TLR2-TLR1-dependent inhibition of immune responses was also studied in human dendritic cells. Dendritic cells were stimulated with P3CSK4 in the presence of strong pectin inhibitors (i.e., pectin DM7) and a weak pectin inhibitor (i.e., pectin DM75). Production of the P3CSK4-dependent cytokines IL-6 [19] and IL-10 [29] were used to assess the effects. Concentration and DM-dependent effects were observed for TLR2 inhibition by pectin (Figure 7). At high concentrations (100 µg/ml), both DM7 and DM75 pectin reduced IL-6 and IL-10 production. However, at lower concentrations (10 µg/ml and 1 µg/ml) only DM7 pectin reduced IL-6 and IL-10 production (p<0.001) (Figure 7). Remarkably, the inhibition potency of DM7 pectin was similar, if not identical, to that of a TLR2 blocking antibody.

Figure 7: Low DM pectin inhibits P3CSK4 induced IL-10 and IL-6 production in human dendritic cells. IL-10 and IL-6 levels produced by human dendritic cells after P3CSK4 activation with or without pectin DM7 or DM75 pretreatment. Data are presented as the mean ± SD, and statistical significance was calculated using two-way ANOVA analysis (*p<0.05 and ***p<0.001).

5.4.5 Pectin is protective against TLR2 dependent ileitis in mice

To confirm efficacy in vivo, DM7 pectin was tested for suppression of TLR2-dependent ileitis induced by doxorubicin in a mouse model. Doxorubicin induces massive cell death in intestinal crypt and immune cells [20], which leads to the release of damage-associated molecular pattern molecules (DAMPs) that induce a strong inflammatory response in a TLR2-dependent manner [20]. Pectin DM7 was administered for one week at 3 mg/day followed by doxorubicin treatment for 48 hours. Pectin administration was continued until the animal was sacrificed.
Initial symptoms of doxorubicin-induced ileitis included neutrophil influx in the peritoneum [30] and cell death in crypts of the small intestine [20], as shown in doxorubicin-treated controls (Figure 8A and 8B). Neutrophil influx and cell death in the crypts were statistically significantly lower in mice with doxorubicin-induced ileitis treated with pectin DM7 (p<0.01) (Figure 8A and 8B) than in mice treated with doxorubicin only, suggesting an active protective role. Strong inhibitory pectin, DM7 effects are illustrated by the observation that reductions in neutrophil influx and cell death in the crypt were similar to effects in mice treated with TLR2 blocking antibodies (p<0.01) (Figure 8A and 8B).
Figure 8: Low DM pectin reduces the doxorubicin induced inflammation. (A) Neutrophils (indicated by arrows) in peritoneal lavage stained with Giemsa. (B) Ileal apoptotic cells (indicated by arrows) determined using the TUNEL assay. Stained cells were counted in 10 sequential crypts per mouse. Data are presented as the median ± range, and statistical differences were calculated using the Mann-Whitney U-test (**p<0.01).

5.4.6 Pectin inhibits pro-inflammatory cytokine production in peritoneum and also in plasma.

TLR2-TLR1 activation has been reported to lead to proinflammatory responses [19, 31]. The cytokine profile in mice demonstrated that pectin indeed specifically inhibited the TLR2-TLR1 induced proinflammatory response. In the peritoneum of mice treated with DM7 pectin, doxorubicin induced production of the proinflammatory cytokines TNF-α (p<0.05), MCP-1 (p<0.01), GRO-α (p<0.05), and IL-6 (p<0.01) were reduced (Figure 9A), with a similar efficacy to TLR2 blocking antibodies. The immune regulatory cytokine IL-10 remained unchanged after pectin treatment in doxorubicin-treated mice (Figure 9A). The anti-inflammatory effect of pectin was not limited to the peritoneal fluid and intestine, but was also observed in systemic circulation. IP-10, MCP-1, GRO-α, and IL-6 levels (p<0.05) were also lower in the plasma of mice pretreated with DM7 pectin than in non-pretreated mice (Figure 9B). The regulatory cytokine IL-10 was unaffected in plasma as well (Figure 9B), suggesting that the immune regulatory pathway was not affected by pectin.
Pectin attenuates immune responses by directly blocking Toll-like receptor 2.
Figure 9: Low DM pectin is protective against doxorubicin-induced ileitis through TLR2-1 inhibition. (A) and (B) Cytokine and chemokine levels in peritoneal fluid and plasma, respectively. Data are presented as the median ± range, and statistical differences were calculated using the Mann-Whitney U-test (*p<0.05 and **p<0.01).
Figure 10: The anti-inflammatory effect of low DM pectin is due to TLR2-1 inhibition versus SCFAs. SCFA concentrations in cecum samples from mice after 48 hours of doxorubicin treatment. Data are presented as the median ± range, and statistical differences were calculated using the Mann-Whitney U-test (*p<0.05).
5.4.7 Anti-inflammatory effects of pectin are not through SCFA production

Attenuation of immune responses in mice may not be entirely caused by direct interaction with TLR2, but might be supported by SCFA production by microbiota that possibly metabolize pectin. To exclude this possibility, we quantified the concentration of organic acids (lactic acid and succinic acid) and short chain fatty acids (acetic acid, propionic acid, iso-butyric acid, butyric acid, iso-valeric acid, and valeric acid) in cecum samples from mice (Figure 10) after doxorubicin and/or pectin treatments. SCFAs were not enhanced and therefore cannot be responsible for attenuation of immune responses. Butyric and valeric acid were significantly reduced by doxorubicin treatment (p<0.05), which was not prevented by pectin and doxorubicin treatment (p<0.05) (Figure 10). Administration of a TLR2 blocking antibody alone also reduced butyric and valeric acid levels (p<0.05) (Figure 10), suggesting that these changes might be due to TLR2 blocking in the colon.

5.5 Discussion

Immunomodulatory effects of pectin in vivo have been described to be because of microbiota dependent effects and microbiota derived SCFA [32, 33]. Apart from these beneficial effects, SCFA and microbiota independent effects have also been described in vitro [9, 34]. However, a true binding between dietary pectin and immune receptor has not been investigated yet. Here, to the best of our knowledge, we show for the first time that the immunomodulatory effects of pectin are through inhibition of TLR2 and the inhibition of TLR2 was dependent on DM value of pectin. We also showed that the anti-inflammatory of pectin in small intestine is mainly through TLR2 inhibition and is independent of SCFA production. Thus, in addition to the microbiota and SCFA dependent anti-inflammatory effects, dietary fibers like pectin can also interact directly with innate immune receptors in intestine like TLR2. This novel mechanism for immunomodulatory effect of pectin can help in designing functional food and feed products to improve and maintain immune homeostasis.

In this chapter we showed that the anti-inflammatory effects of pectin were primarily through inhibition of TLR2 and not by inhibition of other extra cellular TLRs as TLR4 and TLR5. Pectin has been shown to inhibit TLR4 activation in cell lines and mouse models [12, 35]. However, in our reporter cell line assays we did not observe substantial decrease in NF-κB activation after application of pectin to LPS induced reporter cells. We observed only a minimal inhibition of TLR4 by all the DM values of pectin tested in the assay at range of concentrations. These differences might have been due to different models used in previous publications and in our
Pectin attenuates immune responses by directly blocking Toll-like receptor 2 assays. Apart from that, the articles showing TLR4 inhibition by pectin have used pectin oligosaccharides [12]. Thus, it might be that molecular weight and degree of polymerization might also be important for TLR4 inhibition.

The TLR2 inhibition by pectin was both dependent on concentration and DM value of pectin. The low DM pectin showed the highest efficiency in TLR2 inhibition at low concentrations. Whereas at high concentrations, both high and low DM pectin could inhibit TLR2 with similar efficiencies. The concentration dependent effect can be explained by charge densities on pectins at different concentrations. Low DM pectins have higher levels un-esterified regions and are more charged compared to high DM pectins [36]. At high concentrations, high DM pectins have higher effective concentration of un-esterified galacturonic acid patches than at low concentrations. Thus, probably due to this higher effective concentration of un-esterified regions on pectin, high DM pectin inhibit TLR2 more efficiently at higher concentrations. Similar phenomena have also been observed in enhanced gel formation by high DM pectin at increased concentrations due to more effective free carboxyl groups in pectin [36, 37].

The TLR2 inhibition by pectin was specific for TLR2-TLR1 and not for TLR2-TLR6. This difference may be due to the different interaction forces between TLR2-TLR1 and TLR2-TLR6 interfaces. TLR2-TLR1 binding is governed both by ionic and hydrophobic forces [38], whereas binding at the TLR2-6 interface is mainly through hydrophobic forces [39]. Pectins, and especially low DM pectins being highly charged molecules that are hydrophilic in nature [40], might therefore interfere with ionic TLR2-TLR1 interactions without hampering TLR2-TLR6 hydrophobic interactions. This argument is supported by our observation of DM-dependent binding of pectin to the TLR2 ectodomain (Figure 6B). TLR2 ectodomain showed a high level of binding with low DM pectins but a minimal binding to high DM pectin. Thus, we can suggest that the binding between TLR2 and pectin is mainly due to electrostatic forces.

In human dendritic cells, where we measured cytokine production after pectin and TLR2-TLR1 agonist treatment, we observed that both pro-inflammatory IL-6 and regulatory cytokine IL-10 production was reduced by pectin. In this in vitro assay we observed a simple blocking mechanism of pectin for TLR2. However, when we measured cytokine production in peritoneal fluid and plasma in vivo, regulatory cytokine IL-10 levels remained unchanged after pectin administration and only proinflammatory cytokines were reduced by pectin. This discrepancy in inhibitory effects of pectin for regulatory cytokine might be due to different mechanism of interaction of pectin in the intestine compared to with dendritic cells in vitro. In the intestine, the TLR2 blocking effect of pectin might prime intestine epithelial cells to produce immune regulatory IL-10 cytokine [41]. Also, it might be that the complex
system of immune cells in intestine interact with pectin to induce a regulatory cytokine profile, thus showing protection against doxorubicin induced ileitis.

Another interesting observation was that the proinflammatory cytokine production in both peritoneal fluid and plasma was reduced after pectin administration in mice treated with doxorubicin. Thus, pectin was able to protect against inflammation both locally in peritoneum and in systemic immune system. Dietary fibers like β-glucan have been shown to pass through intestinal barrier to circulation. This transport of fibers from intestine is performed by macrophages [42]. Similarly, pectin might as well have passed to the circulation by macrophages and thus could impart its anti-inflammatory effects in plasma. Additionally, the anti-inflammatory effect of pectin in intestinal immune cells which might have influenced the cytokine profiles in immune cells in the circulation.

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Doxorubicin induced ileitis model has already been described to have protective effect by blocking of TLR2 [20]. Thus to confirm TLR2 inhibiting capacity of low DM pectin, we used this model in combination with pectin. Pectin administration in doxorubicin induced inflammations in mice could reduce the symptoms. Strikingly, the anti-inflammatory effects of pectin were similar to TLR2 blocking antibody administration which confirmed that the anti-inflammatory effects of pectin are mainly through TLR2 inhibition. To ascertain that the protective role of pectin in ileitis is through TLR2 inhibition and not because of SCFA production by commensal microbiota, we measured SCFA concentrations from cecum of mice. Pectin administration did not increase SCFA levels in mice, thus confirming that the anti-inflammatory role of pectin is independent of SCFA.

The TLR2 blocking antibody reduced butyric acid and valeric acid concentrations in cecum of mice. This might be due to TLR2 inhibitory side-effect of TLR2 blocking antibody in cecum and colon of mice. However, low DM pectin which can inhibit TLR2, did not reduce butyric and valeric acid concentrations. Dietary pectin is known to pass through the small intestine almost undigested and is easily digested as soon as it is accessible to commensal microbiota [43]. Thus, pectin is not able to impart its TLR2 inhibition effects in cecum and colon where it gets degraded by commensal microbiota, and so pectin does not show TLR2 inhibition side-effects in cecum and colon.

In this article we have shown that dietary pectins with a low DM can effectively bind to TLR2 at low concentrations and prevent proinflammatory responses, even in the presence of a TLR2-TLR1 stimulus. The TLR2-TLR6 heterodimer remained intact, and tolerogenic immune responses remained unaffected. In Figure 11, we propose a mechanism by which pectins suppress TLR2-TLR1. TLR2-TLR1 inhibition
by pectin was also effective in preventing an intestinal disorder in which intestinal inflammation is exacerbated by TLR2 activation. Inflamed intestinal lesions release DAMPs, such as HMGB1 (high-mobility group box-1), leading to proinflammatory cytokine release [44] and intestinal damage through TLR2 [45]. The anti-inflammatory effects of pectin via TLR2-TLR1 inhibition can be an effective strategy in designing foods to prevent or cure intestinal inflammation and may even provide an explanation as to why increased dietary fiber intake is associated with a lower frequency of immunological disorders [5, 13].

Figure 11: Proposed mechanism of anti-inflammatory effects due to low DM pectin.
References:

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