RESEARCH ARTICLE

Resistant starches differentially stimulate Toll-like receptors and attenuate proinflammatory cytokines in dendritic cells by modulation of intestinal epithelial cells

Miriam Bermudez-Brito[1,2], Christiane Rösch[1,3], Henk A. Schols[1,3], Marijke M. Faas[2] and Paul de Vos[1,2]

[1] Top Institute Food and Nutrition, Wageningen, The Netherlands
[2] Department of Pathology and Medical Biology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands
[3] Laboratory of Food Chemistry, Wageningen University, AA Wageningen, The Netherlands

Scope: Main objectives of this study were (1) to demonstrate direct signaling of starch on human dendritic cells (DCs), (2) to study whether this is mediated by the pattern recognition receptors such as Toll-like receptors (TLRs) and (3) to study whether intestinal epithelial cells (IECs) are involved in modulating the starch induced immune activation of DCs.

Methods and results: Two different types of resistant starch, High-maize® 260 (RS2) and Novelose® 330 (RS3) were characterized for their starch content and particle size. Human DCs and reporter cells for TLRs were incubated with starches and analyzed for NF-kB/AP-1 activation. Complex coculture systems were applied to study the cross-talk. High-maize® 260 predominantly binds to TLR2 while Novelose® 330 binds to TLR2 and TLR5. The strong immune-stimulating effects of High-maize® 260 were attenuated by starch-exposed IECs illustrating the regulatory function of IECs. Despite these attenuating effects, DCs kept producing Th1 cytokines.

Conclusion: Resistant starch possesses direct signaling capacity on human DCs in a starch-type-dependent manner. IECs regulate these responses. High-maize® 260 skews toward a more regulatory phenotype in coculture systems of DCs, IEC, and T cells.

1 Introduction

Resistant starch (RS) represents a diverse range of indigestible starch-based dietary carbohydrates that are fermented by colonic microbiota [1]. Many beneficial health effects of RS have been described including the control of intestinal transit and bowel habits, a reduction in postprandial glycemia, increased insulin sensitivity, and stimulation of growth and function of gut microbiota [2].

Correspondence: Paul de Vos
E-mail: p.de.vos@umcg.nl
Fax: 0031-(0)50-3619911

Abbreviations: Caco-SM, Caco Spent Medium; **DC**, dendritic cell; **HLA-DR**, anti-human leukocyte antigen-DR; **IEC**, intestinal epithelial cell; **PRR**, pattern recognition receptors; **RS**, resistant starch; **RT**, room temperature; **SEAP**, secreted embryonic alkaline phosphatase; **TC**, T cell; **TLR**, Toll-like receptor

Four subtypes of RS have been identified based on their structure or source [3]. The first is *Resistant Starch type 1* (RS1) that is physically inaccessible to digestive enzymes. It is found in whole or partly milled grains, seeds, and whole-grain foods. *Resistant Starch type 2* (RS2) is highly resistant to digestion by α-amylases. It is found in raw potato, unripe banana, some legumes, and high-amylose corn. *Resistant Starch type 3* (RS3) is formed during food processing by the retrograde action of amylose and amylopectin and is found in cooked and cooled-down foods such as potatoes, bread, and cornflakes. The last is *Resistant Starch type 4* (RS4) that are chemically modified starches [3, 4].

Immunomodulatory effects of RS have not been studied in much detail. Immune effects of dietary fibers are often attributed to microbiota-dependent effects [5]. However, some dietary fibers have been reported to bind to specific receptors on immune cells, suggesting microbiota-independent, immune-modulatory effects [6, 7]. Recently, we

demonstrated that dietary fibers, acting via intestinal epithelial cells, have anti-inflammatory effects on DCs [8]. Toll-like receptors (TLRs) play a critical role in the innate immune response to invading pathogens by sensing microorganisms and endogenous danger signals, including those associated with a variety of food components. Signaling by TLRs may result in a variety of cellular responses including the production of proinflammatory cytokines, chemokines, IFNs, and effector cytokines that direct the adaptive immune response [9]. In a recent paper, we demonstrated that $\beta2 \rightarrow 1$-fructans mainly stimulate TLR2 resulting in NF-κB/AP-1 activation and cytokine release from human peripheral blood mononuclear cells [10].

Not all fibers have the same properties; therefore, the features and components of dietary fibers may determine their modes of action and effects on the immune system. As not much knowledge is available on the specific immunomodulating effects of RS, we investigated how High-maize® 260 and the product Novelose® 330, generated from the hydrolyzed products of corn starch [11], can affect the intestinal immunity via activation of TLRs. The effects of two types of starch on DCs activation, the release and expression of some proinflammatory cytokines and chemokines, and TLR activation were studied. By applying complex coculture systems the effects on T-cell polarization was studied.

2   Materials and methods

2.1   Substrates

High-maize® 260 (RS2) and Novelose® 330 (RS3) (National Starch and Chemical Company, Bridgewater, USA) are derived from high-amylose maize starch and retrograded starch from high amylose maize, respectively.

2.2   Determination of starch content

Total starch and resistant starch contents were analyzed according to AOAC method 996.11 and 2002.02 with enzyme kits from Megazyme (Megazyme international Ltd, Bray, Ireland), respectively. For the determination of the total starch amount, the sample was dissolved in 2 M KOH, neutralized with 1.2 M sodium acetate buffer (pH 3.8) and incubated with alpha-amylase and amyloglucosidase for 30 min. Aliquots were incubated with a GOPOD reagent, and the absorbance of the glucose was measured with a spectrophotometer at 510 nm. The determination of the resistant starch requires a predigestion with the two mentioned enzymes. In a washing step with ethanol, the degraded digestible starch was removed and the remaining pellet, consisting of the resistant starch, was treated as described for the total starch determination.

2.3   Constituent monosaccharide composition

The monosaccharide composition was determined using a prehydrolysis step with 72% w/w sulphuric acid at 30°C for 1 h. Followed by a hydrolysis with 1 M sulphuric acid at 100°C for 3 h. The monosaccharides released, were derivatized to alditol acetates and analyzed by GC using inositol as an internal standard [12]. The absence of uronic acid in the samples was substantiated by using the colorimetric m-hydroxydiphenyl assay [13] with an automated method as described previously [14].

2.4   Molecular weight distribution

The two starches were dissolved in 0.1 M malaeic acid buffer pH 6 at a concentration of 2 mg/mL, centrifuged (10 min, at RT, 18 000 x g) and the supernatant was analyzed for molecular weight distribution with high performance size exclusion chromatography on an Ultimate 3000 HPLC (Dionex, Sunnyvale, CA, USA) equipped with a Shodex RI-101 refractive index detector (Showa Denko K.K., Tokyo, Japan). Three TSK-Gel columns connected in series (4000-3000-2500 SuperAW; 150 × 6 mm) were used for the analysis. These columns were preceded by a TSK Super AW-L guard column (35 × 4.6 mm). All columns were from Tosoh Bioscience (Tokyo, Japan). Twenty microliters sample were injected and eluted with 0.2 M NaNO₃, at 40°C with a flow rate of 0.6 mL/min. Pullulan molecular-mass standards (Polymer Laboratories, Palo Alto, CA, USA) were used for calibration. Endotoxin concentrations of the dietary fibers samples were always below 0.3×10^{-3} μg^{-1} that has no effect on the responsiveness of the cells.

2.5   Particle size determination

The particle size of the two starch samples was determined using laser light diffraction (Mastersizer 2000, Malvern Instruments Ltd, Malvern, UK) equipped with a Hydro SM sample dispersion unit. The starch suspensions in water were analyzed in triplicate and averaged. To derive the particle size, the general purpose model was used, a refractive index of 1.529 and an absorption of 0.1 for the particles and a refractive index of 1.33 for water.

2.6   Pattern recognition receptor inhibition and activation

To investigate the involvement of different TLRs in immune effects by High-maize® 260 and Novelose® 330, human embryonic kidney (HEK)-cells overexpressing either TLRs 2, 3, 4, 5 were used [15]. HEK-Blue ™ TLRs cells stably coexpress a human TLR gene and a NF-kB-inducible SEAP (secreted embryonic alkaline phosphatase) reporter gene that

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can be monitored using the SEAP detection media QUANTI-Blue™ (InvivoGen, Toulouse, France). These cells selectively express TLR2, TLR3, TLR4, and TLR5. HEK-Blue™ cells were suspended in DMEM supplemented with 4.5 g/L glucose, 10% FCS (deactivated phosphatases), Pen-Strep (50 U/mL–50 μg/mL), 100 μg/mL Normocin™ and 2 mM L-glutamine at 280 000 cells/mL (hTLRs 2 and 3), 140 000 cells/mL (hTLRs 4 and 5) and 220 000 cells/mL (hTLRs 7), and plated in 96-well plates according to the manufacturer's instructions. Each well was stimulated with High-maize® 260 or Novolose® 330 and cultured overnight at 37°C and 5% CO_2 (50, 100, or 200 μg/mL). DMEM culture medium was used as a negative control and TLR signaling was always confirmed using the appropriate TLR agonist. NF-κB activation was assessed by measuring SEAP activity using QUANTI-Blue™. To study inhibition of the specific TLR, HEK-Blue™ hTLR-CD14 expressing the specific TLR and HEK-Blue™ Null1 TLR cells were pretreated for one hour with graded loads of High-maize® 260 or Novolose® 330 (50, 100, or 200 μg/mL) followed by treatment with TLR2 agonist FLS-1 (1 μg/mL InvivoGen, Toulouse, France), the TLR3 agonist polyinosine-polycytidylic acid (5 μg/mL, poly(I:C), InvivoGen, Toulouse, France), the TLR4 agonist lipopolysaccharide from *Escherichia coli* K12 strain (LPS-EK Ultrapure 100 ng/mL, InvivoGen, Toulouse, France), or the TLR5 agonist recombinant flagellin from *Salmonella typhimurium* (100 ng/mL RecFLA-ST, InvivoGen, Toulouse, France). Quantification of flagellin (TLR5 ligand) was performed with a human flagellin ELISA Kit (Qayee-Bio, Shanghai, China) according to the assay protocol. The plates were read in a spectrophotometer at a relative optical density of 450 nm within 15 min after adding the stop solution. All the incubations were performed at 37°C and 5% CO_2. Incubating the HEK-BlueTM hTLR-CD14 expressing the specific TLR and HEK-BlueTM Null1 TLR with 200 μg/mL High-maize® 260 or Novolose® 330 was used for stimulation studies. Endotoxin free H_2O was used as an additional negative control. Cells were cultured overnight at 37°C and 5% CO_2. Experiments were repeated at least five times.

In some experiment the MyD88 inhibitor Pepinh-myd88 (InvivoGen, Toulouse, France), a 26 amino acid peptide that functions as a decoy by binding to the MyD88 TIR domain, was used to evaluate whether DC activation by the dietary fibers is mediated by TLRs, as described previously by Bermudez-Brito et al. [8]. Briefly, DCs were pretreated with 25 μM Pepinh-myd88 for 6 h at 37°C. Then, the starches or Caco Spent Medium (Caco-SM) were added and incubated for 24 h. Culture supernatants were collected for cytokine quantification.

2.7 Dendritic cells, T cells, and generation of an epithelial cell monolayer

Dendritic cells (DCs), autologous T cells, and Caco-2 epithelial cells were cultured and treated as previously described [8]. For coculture experiments, inserts containing fully differentiated Caco-2 cells monolayer were transferred to six-well plates. The transwell inserts were inverted and a drop containing 5×10^4 DCs was placed onto the membrane. DCs were allowed to adhere for 4 h at 37°C. Transwell inserts were transferred to 24-well plates holding DCs (2×10^5 DCs/well). The apical surface of intestinal epithelial cells (IECs) monolayers was challenged by addition of dietary fibers (400 μg/mL) in the upper chamber. Plates were incubated 24 h at 37°C in a 5% CO_2 atmosphere. Culture supernatants were collected from the basal compartment for cytokine analysis by Luminex [8].

In different sets of experiments, DCs were exposed to IECs released factors upon fibers stimulation (Caco-2 Spent Medium; SM) for 24 h, as described previously by Bermudez-Brito et al. [8]. Culture supernatants were collected for cytokine analysis and DCs were analyzed by flow cytometry.

2.8 DC and TC cocultures

DCs and T cells (TCs) were cultured according to the manufacturer's recommendation and cultured together in a proportion of 300 000 DCs/200 000 TCs. Then, RS was added to the culture and incubated for 24 h under standard conditions. Culture supernatants were collected for cytokine analysis. Negative control cultures contained cocultures of DCs and TCs.

In another set of experiments, DCs and TCs were challenged by the addition of the supernatant of IECs stimulated with RS for 24 h. Negative control cultures contained cocultures of DCs, TCs, and unstimulated Caco-2 SM. Plates were incubated at 37°C in a 5% CO_2 atmosphere for 24 h. Culture supernatants were collected for cytokine analysis.

2.9 Flow cytometry and antibodies

The following antibodies were used for flow cytometry staining: anti-human leukocyte antigen-DR fluorescein isothiocyanate conjugated (HLA-DR; FITC), anti-human CD86 phycoerythrin and cyanine dye conjugated (PE/Cy7) and anti-human CD83 allophycocyanin conjugated, with matched isotype controls (all from Biolegend, San Diego, CA). DCs were stained as described previously by Bermudez-Brito et al. [8] and later analyzed using the FACSCalibur Flow Cytometer platform (BD Bioscience, San Jose, CA) and Flowjo 7.6.5 software. For each analysis 20 000 counts, gated on a FSC versus SSC dot plot, based on viability, were recorded. CD83, CD86, or HLA-DR isotype controls were used to set the gate to 99% negative cells.

2.10 Cytokine expression

After 24 h of incubation cytokine levels in the supernatant were measured using a MilliPlex™ premixed cytokine

assay, according to the manufacturer's instructions (Linco Research Inc., MO, USA). This customized kit measures simultaneously several of the following molecules: human IFN-γ, IL-12p40, IL-10, IL-17, IL-1β, IL-1Ra, IL-2, IL-4, IL-6, IL-8, MCP-1/CCL2, MIP-1α/CCL3, RANTES/ CCL5, and TNF-α. Concentration series of cytokine standards were prepared for the appropriate concentration range, and coupled beads were diluted ten times, resuspended, and added to a prewetted filter plate. After washing the plate twice, standards, negative controls, and samples (all in duplicate) were transferred into the plate (50 μL per well), and the plate was sealed and incubated on a shaker at 4°C overnight (16–18 h) in the dark. After incubation, the plate was washed three times. Detection antibodies were resuspended, diluted ten times, and 25 μL was added to each well. The plate was incubated on a shaker at room temperature (RT) for 1 h in the dark. After washing three times, 50 μL of streptavidin-phycoerythrin was added to each well and the plate was incubated on a shaker at RT for 30 min in the dark. After washing the plate three times, 125 μL of assay buffer was added per well. The plate was incubated on a shaker for 5 min and fluorescence was measured using a Luminex 100 System and StarStation software.

To evaluate whether the dietary fibers induce a more anti-inflammatory or proinflammatory effect in the DCs, the IL-10/IL-12 ratio was calculated for both dietary fibers. A higher IL-10/IL-12 ratio is representative for a regulatory or anti-inflammatory effect [10].

2.11 Statistical analysis

Data were expressed as median and range. Statistical analyses were performed using NCSS2007 software by Kruskal–Wallis test followed by a Bonferroni multiple comparison's posthoc test. Differences were considered statistically significant when $p < 0.05$. p-values < 0.05 are denoted with *, p-values < 0.01 are denoted with **, and p-values < 0.005 are denoted with ***.

Differences between High-Maize® 260 and Novelose® 330 were also analyzed. $p < 0.05$ was considered statistically significant and is indicated with a pound sign (#). Statistical significant differences between Caco-SM and the dietary fibers were also indicated with a pound sign (#).

3   Results

3.1   Chemical substrate characterization

The total carbohydrate content of High-maize® 260 and Novelose® 330 is 85 and 82% w/w solely glucose (98 mol %). Both resistant starches consist of ca. 83% total starch and 36% resistant starch as defined to nondegradable using α-amylase as measured by the AOAC 2002.02 method. This results in both substrates to a calculated amount of 47% digestible starch. High-maize® 260, made from high amylose maize starch and having received a hydrothermal treatment is a RS type 2 and appears in natural granular form. The solubility at 95°C is ca. 2%. In contrast, the retrograded resistant starch, Novelose® 330 (RS3), has a solubility of 24% at 95°C [16]. This insolubility, which was 7–10 times lower at 25°C [16], was confirmed (data not shown). As stated by the supplier, both substrates consist of approximately 8–12% moisture and less than 1% protein and fat.

3.2   Particle size distribution

While the chemical characterization of the two starches was very similar, the particle size distributions of the two starch samples differ profoundly (Fig. 1). For both substrates one main population was observed. While the average median particle size of Novelose® 330 was 46.6 μm, it was 12.8 μm High-maize® 260. About 80% of the particles were in the size range of 18.6–95.5 μm for Novelose® 330, and 5.2–35.5 μm for High-maize® 260.

Besides the different particle size distribution of the two resistant starch samples, also a difference in morphology was observed. A scanning electron micrograph clearly shows that Novelose® 330 consists of destroyed, convoluted granules due to the retrogradation process. In contrast, High-maize® 260 particles have a high degree of molecular order and a rather smooth surface [17].

3.3   High-maize® 260 and Novelose® 330 are potent inducer of cytokine production in human dendritic cells

DCs were stimulated with High-maize® 260 or Novelose® 330 for 24 h to study direct effects of the dietary fibers on immune cells. As shown in Fig. 2 High-maize® 260 was a potent inducer of cytokines in human DCs. The proinflammatory cytokine IL-12 was increased by High-maize® 260 ($p < 0.005$), whereas Novelose® 330 had virtually no effect on this cytokine. High-maize® 260 also induced the proinflammatory IL-6 in DCs ($p < 0.005$). However, the IL-12 antagonist, IL-10, was also enhanced by High-maize® 260 ($p < 0.05$) (Fig. 2A) as well as the anti-inflammatory IL-1Ra (Fig. 2B). IL-8 production was increased by both High-maize® 260 ($p < 0.005$) and Novelose® 330 ($p < 0.01$) (Fig. 2B).

High-maize® 260 had a stronger effect on chemokine production by DCs than Novelose® 330 (Fig. 2C). High-maize® 260 enhanced the production of MCP-1, MIP-1α, and RANTES ($p < 0.005$) while Novelose® 330 only slightly induced the secretion of MIP-1α and RANTES ($p < 0.05$) and had no effect on MCP-1 production. The same superior effects of High-maize® 260 over Novelose® 330 were observed for the proinflammatory cytokine TNF-α ($p < 0.005$) (Fig. 2C).

The IL-10/IL-12 ratios were calculated [18] for both types of starch as a measure for the balance between anti-inflammatory and proinflammatory effects. As shown in
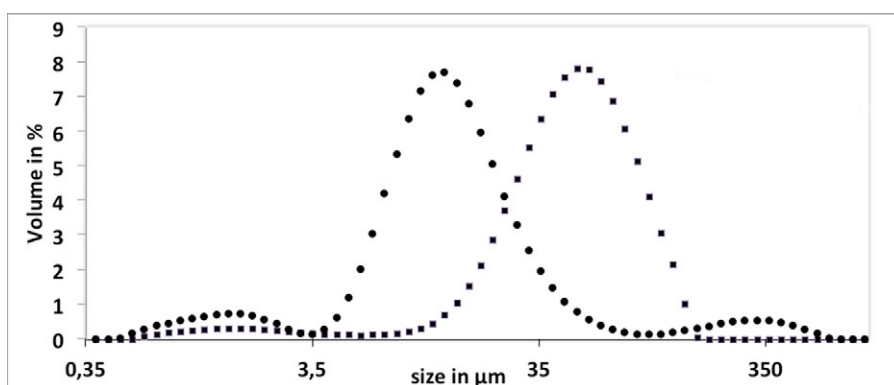
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Figure 1. Particle size distribution of High-maize® 260 (circles) and Novelose® 330 (squares).

Fig. 2D High-maize® 260 profoundly skewed the IL-10/IL-12 ratio in DCs more towards the proinflammatory IL-12 ($p < 0.005$) while Novelose® 330 had only a moderate but statistical significant enhancing effect on this ratio ($p < 0.05$). To determine whether this proinflammatory phenotype is relevant for DC activation, also CD83, CD86, and HLA-DR expression was quantified on the DCs. As shown in Fig. 2E, High-maize® 260 strongly upregulated the expression of CD83 and CD86 ($p < 0.005$), which was not observed on Novelose® 330 treated-DCs. Interestingly, HLA-DR was decreased by both fibers ($p < 0.01$).

3.4 High-maize® 260 and Novelose® 330 stimulate TLRs

As some dietary fibers have been shown to exert their immunomodulating effect via TLRs [10], the inhibition and stimulation of TLR1, 2, 3, 4, and 5 by High-maize® 260 and Novelose® 330 was studied. To this end, HEK reporter cell lines, each carrying one construct for a specific TLR, were stimulated with High-maize® 260 or Novelose® 330. As shown in Fig. 3, High-maize® 260 nor Novelose® 330 had any inhibiting effect on TLR2 to 5 induced activation of NF-kB/AP-1. This was different for the activating effects of High-maize® 260 and Novelose® 330. High-maize® 260 specifically stimulated TLR2 while Novelose® 330 stimulated both TLR2 and TLR5. The Novelose® 330-induced TLR5 activation was very strong. To exclude that this activation is caused by flagellin in the Novelose® 330 sample, a flagellin ELISA was performed. Flagellin was not found in the sample (data not shown).

3.5 IECs alleviate starch induced DCs activation

In the intestine IECs play an important role in regulation of DC responses [19]. Therefore, we investigated how IECs, stimulated with different resistant starches, can impact DC activation across an epithelial barrier. This was done by using a transwell coculture system of IECs and DCs. The DCs were

in this system on the basolateral site of the IECs or in between the epithelial layer but not on the apical site [8].

As shown in Fig. 2A, the High-maize® 260 and Novelose® 330 induced cytokine and chemokine response was not observed in the IEC-DC coculture system. IL-12 was even decreased and below the detection level when High-maize® 260 or Novelose® 330 was added. IL-1β was specifically inhibited by Novelose® 330. IL-10, IL-6, and IL-8 production was unaffected by both starches (Fig. 2A, B). The only small but statistical significant sign of a proinflammatory effect by High-maize® 260 was the enhanced MIP-1α and TNF-α production (Fig. 2C).

### 3.6 High-maize® 260 and Novelose® 330 modulate IECs to attenuate immune activation in DCs

To investigate whether the above described attenuation of the DC activation by High-maize® 260 and Novelose® 330 is due to modulation of the IECs or by other mechanisms, we compared the activation status of DCs after exposure to IECs-spend medium (Caco SM) exposed to starch (High-maize® 260-SM and Novelose®-SM). The responses were compared to that of control Caco SM. The difference with the previous experiment is that the IECs have never been in contact with DCs but only with the fiber or control medium lacking fibers.

Control Caco SM not exposed to any fiber enhanced the production of the proinflammatory cytokines IFN-γ, IL-12p40, IL-6, IL-8, MCP-1, MIP-1α, RANTES, and TNF-α in the DCs ($p < 0.005$) (Fig. 4). The anti-inflammatory cytokine IL-10 (Fig. 4B) was also enhanced but the overall effect was a profound proinflammatory phenotype.

This proinflammatory phenotype was attenuated when the DCs were exposed to High-maize® 260-SM or Novelose® 330-SM. The proinflammatory IL-12 production by the DCs was strongly reduced after High-maize® 260 or Novelose® 330 treatment of the IECs (Fig. 4A) suggesting that IECs were modulated by both starches and attenuate the DC's proinflammatory response. The proinflammatory IL-1β production was also slightly decreased by High-maize® 260-SM and Novelose® 330-SM (Fig. 4A) as well as its antagonist, IL-1Ra
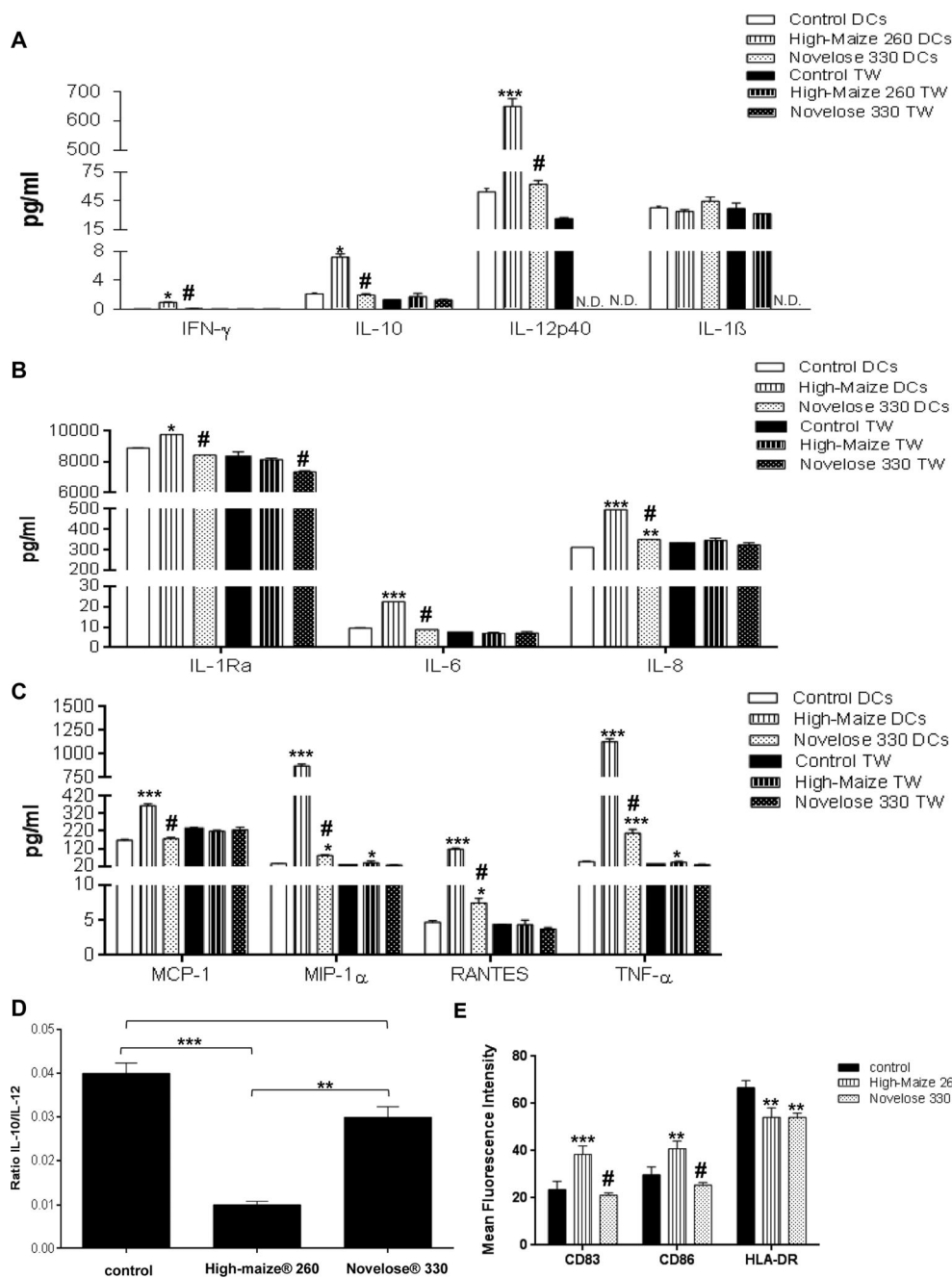
Figure 2. Resistant starches are potent inducer of cytokines in human DCs (A–C). The production of IFN-γ, IL-10, IL-12p40, IL-1β (Panel A), IL-1Ra, IL-6, IL-8 (Panel B), MCP-1/CCL2, MIP-1α/CCL3, RANTES/CCL5, and TNF-α (Panel C) by DCs and by DCs cocultured with IECs stimulated with High-maize® 260 and Novelose® 330 (400 μg/mL). The cells were incubated for 24 h with dietary fibers. Culture supernatants were collected and the cytokine levels were quantified by luminex. Statistical significance was determined with the Kruskal–Wallis test followed by the Bonferroni multiple comparison test. The data shown are the median and range of four different experiments and three different experiments in duplicates for DCs and DCs cocultured with IECs, respectively. p-values < 0.05 are denoted with * p-values < 0.01 are denoted with ** and p-values < 0.005 are denoted with ***. The # sign indicates differences between the starches ($p < 0.05$). N.D means not detected. (D) IL-10/IL-12 ratio decreases upon fibers stimulation. DCs were stimulated with fibers for 24 h. Statistical significance was determined with Kruskal–Wallis test followed by Bonferroni multiple comparison test. Median and range of the IL-10/IL-12 ratio is plotted for the different types of starch. Horizontal bars indicate the differences between the groups. p-values < 0.05 are denoted with * p-values < 0.01 are denoted with ** and p-values < 0.005 are denoted with ***. (E) High-maize® 260 elevated the expression of CD83 and CD86. Histogram plots of CD83, CD86, and HLA-DR surface marker expression of DCs stimulated with High-maize® 260 and Novelose® 330. Numbers indicate the median percentage of positive cells in the gate and range of four different experiments. p-values < 0.05 are denoted with * p-values < 0.01 are denoted with ** and p-values < 0.005 are denoted with ***. The # sign indicates differences between the starches ($p < 0.05$).

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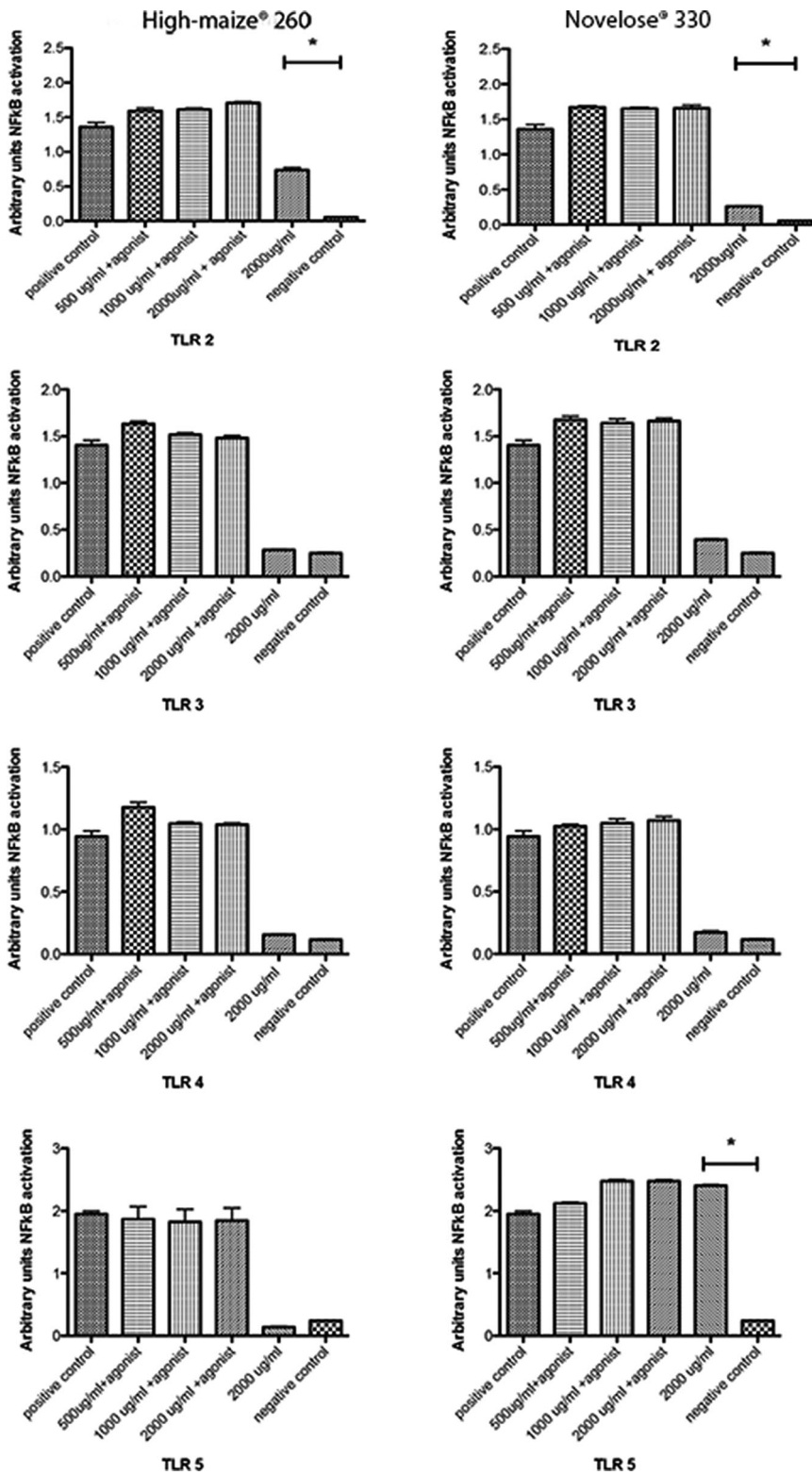
Figure 3. High-maize® 260 stimulates TLR2 and Novelose® 330 stimulates TLR2 and 5. NF-kB/AP-1 activation of HEK cell lines overexpressing separate TLRs. Statistical significance levels were determined with a nonparametric Mann–Whitney *U*-test for unpaired observations (two-tailed). Median and SD of NF-kB/AP-1 activation in HEK cell. Dosages are plotted in μg/mL. Endotoxin-free H_2O was used as an additional negative control and per cell line the relevant agonists were applied as mentioned in the materials to study the inhibitory effect of High-maize® 260 and Novelose® 330. *p*-values < 0.05 are denoted with *, ($n = 9$).
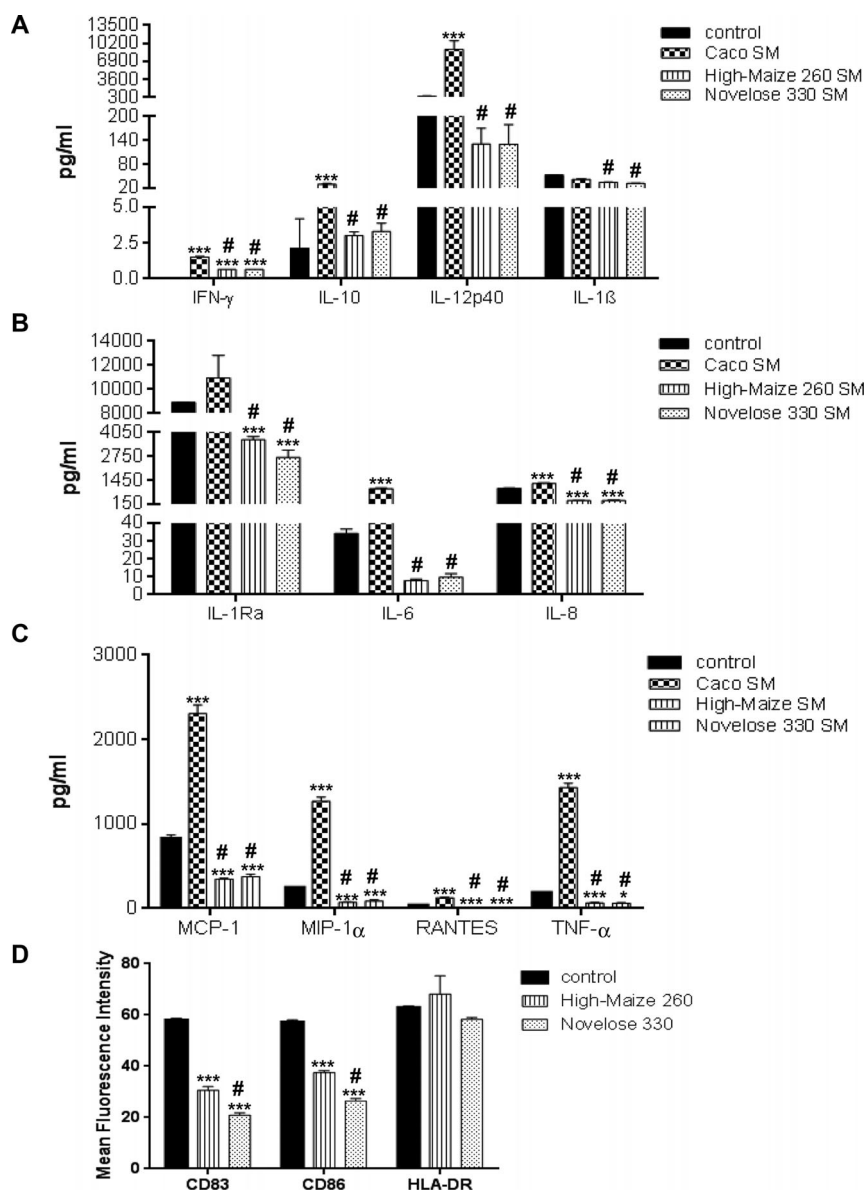
Figure 4. IECs derived factors produced after High-maize® 260 and Novelose® 330 stimulation induced regulatory responses in DCs (A–C). The production of IFN-γ, IL-10, IL-12p40, IL-1β (Panel A), IL-1Ra, IL-2, IL-4, IL-6, IL-8 (Panel B), MCP-1/ CCL2, MIP-1α/ CCL3, RANTES/ CCL5, and TNF-α (Panel C) by DCs incubated for 24 h with fibers-stimulated IECs supernatant (Caco SM). Culture supernatants were collected and the cytokine levels were quantified by immunoassay. Statistical significance levels were determined with the Kruskal–Wallis test followed by the Bonferroni multiple comparison test. The data shown are the median and range of three different experiments, in duplicates. *p*-values < 0.05 are denoted with * *p*-values < 0.01 are denoted with ** and *p*-values < 0.005 are denoted with ***. The # sign indicates differences between the Caco SM and starches-SM (*p* < 0.05). (D) Reduction of CD83 and CD86 by High-maize® 260-SM and Novelose® 330-SM. Histogram plots show CD83, CD86, and HLA-DR surface markers staining of DCs and fibers-stimulated IECs supernatant (High-maize® 260-SM and Novelose® 330-SM). Numbers indicate the percentage of positive cells in the gate. *p*-values < 0.05 are denoted with * *p*-values < 0.01 are denoted with ** and *p*-values < 0.005 are denoted with ***.

(Fig. 4B). Also the DC-production of the proinflammatory IL-6 was strongly reduced after exposure to High-maize® 260-SM and Novelose® 330-SM (Fig. 4B). The chemokines IL-8 and MCP-1 were profoundly reduced (Fig. 4B, C) as well as TNF-α production by both starch types (Fig. 4C). These results were corroborated by a strong reduction of the surface activation markers CD83 and CD86 in DCs by both High-maize® 260-SM and Novelose® 330-SM (*p* < 0.005) (Fig. 4.D).

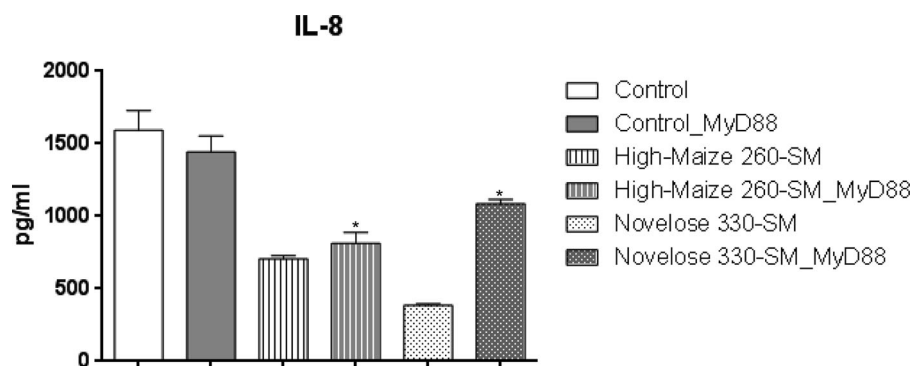### 3.7 MyD88 blockade attenuates the proinflammatory responses

As MyD88 is a key adaptor of TLR signaling pathway, except for the TLR3 pathway, we investigated the impact of MyD88

blockade in the production of pro- and anti-inflammatory cytokines by DCs. To this end, 1×10^6 DCs were exposed either to starch-SM or to Caco-SM in the presence and absence of a MyD88 inhibitor. Overall, the effect of Caco-SM was diminished in the presence of MyD88 inhibitor (data not shown) but most pronounced for IL-8 production, which was statistically significantly enhanced when the inhibitor was applied (Fig. 5).

3.8 High-maize® 260 stimulated DCs induce Th1-cell polarization

As resistant starch stimulates typical Th1-type cytokines such as IL-12 and TNF-α, we investigated the biological relevance of

IL-8

Control
Control_MyD88
High-Maize 260-SM
High-Maize 260-SM_MyD88
Novelose 330-SM
Novelose 330-SM_MyD88

Figure 5. MyD88 blockade attenuates the proinflammatory responses. The production of IL-8 by 1×10^6 DCs treated with MyD88 inhibitor and fibers stimulated IECs supernatant (Caco-SM). Statistical significance was determined with a nonparametric Mann–Whitney *U*-test for unpaired observations (two-tailed). The data shown are the medians and SD of five different experiments. * *p*-values < 0.05.

the upregulated cytokines by studying T-cell cytokine skewing under the influence of High-maize® 260. To this end High-maize® 260 stimulated DCs were cocultured with naïve autologous T cells. We analyzed the production of the Th1 cytokines IL-2, IFN-γ, TNF-α (Fig. 5A); the Th2 cytokines IL-4, IL-6 (Fig. 5B); the Th17 cytokine IL-17 (Fig. 5C); and the Treg cytokine IL-10 (Fig. 5D).

Analysis of the supernatant showed an increase of typical Th1 cytokines (Fig. 5A) such as IFN-γ ($p < 0.05$) and TNF-α ($p < 0.005$) (Fig. 5A). However, also the Th2 cytokine, IL-6, was induced by High-maize® 260 (Fig. 5B). The fiber had virtually no effect on IL-17 production (Fig. 5C). High-maize® 260 slightly increased the Treg IL-10 production, but this effect never reached statistical significant differences (Fig. 5D).

Next, we took the High-maize® 260-IEC spent medium (High-maize® 260-SM) from the experiments presented in Fig. 4, and added to cocultures of DCs and naïve TCs to investigate the damping effect of High-maize® 260-SM on T-cell skewing. IEC medium not exposed to High-maize® 260 (Caco SM) served as control. As illustrated in Fig. 6, the TNF-α production was tenfold lower than in Fig. 5, illustrating the attenuating effect of epithelial cells on Th1 cytokines. The production of the other Th1 cytokine IFN-γ, however, was still increased in both High-maize® 260-SM and the control (Fig. 6A). Overall there was still a typical Th1 skewing as the IFN-γ increase was accompanied by a decrease of the typical Th2 cytokine IL-4 ($p < 0.05$ for both High-maize® 260-SM and Caco SM) (Fig. 6B). The Th17 cytokine, IL-17, was diminished in DC-TC cultures exposed to High-maize® 260-SM and control Caco SM ($p < 0.05$) (Fig. 6C). Finally, Caco SM and High-maize® 260-SM induced the production of the Treg cytokine IL-10 ($p < 0.05$) (Fig. 6D), illustrating also on T-cell level a regulatory effect of IECs.

4 Discussion

Certain fibers have been shown to interact with receptors on immune cells and to modulate the host immune system directly [8, 10]. Here, we show that different α-glucan-based dietary fibers can also directly stimulate immune cells by binding to the sensors of the human immune system [7, 10, 20], the pattern recognition receptors (PRRs) TLR2 and TLR5. In-

terestingly, TLR-2 stimulation by dietary fibers has been implicated in prevention of rupture of the intestinal barrier and various immune responses [20]. Moreover, TLR2 has been implicated in the induction of regulatory T-cell responses, which further emphasizes the immunosuppressive potential of TLR2 signaling [21].

TLR-5 stimulation by Novelose® 330 is also very interesting as TLR-5 stimulation is not only involved in immune regulation but also in maintaining metabolic control [22, 23] and could play a role in the reported reduction in postprandial glycemia and increased insulin sensitivity of some types of starches [2]. Vijay-Kumar et al. [22] demonstrated that mice deficient in TLR5 develop obesity, hyperphagia, dyslipidaemia, hypertension, and insulin resistance. Furthermore, Uematsu et al. [23] proposes that microbiota induce IgA-production through a mechanism involving TLR5. The role of IgA is to maintain the balance between the host and microbiota by binding and eliminating abundances or undesired bacteria. Altogether our results suggest that Novelose® 330 may be an interesting compound for prevention of obesity and related diseases.

High-Maize® 260 and Novelose® 330 are the results of different production processes resulting in differences in particle size and morphology. Notably both starch preparations are composed not only of RS but also of digestible starch. It cannot be excluded that this digestible starch has had some effect on the signaling. This however does not influence the findings on particle size and morphology that might be responsible for the different binding patterns and the different mode of TLR-dependent NF-**k**B/AP-1. Particle size has also been implicated in efficacy of beta-glucan binding to the pattern recognition receptor Dectin-1 [24], and is the most likely cause for the difference in binding ability to TLR-5. To confirm this, more knowledge has to become available about the 3D structure of Novelose® 330 to allow modeling of the interaction with TLR-5.

Although, we show that High-maize® 260 and Novelose® 330 can stimulate TLR2 and or TLR5 and modulate the immune response of DCs, we do not claim that these are the only PRRs involved. There are numerous PRRs that also may be influenced and modulate DCs [25]. However, when we applied the MyD88 inhibitor Pepinh-myd88, the High-maize® 260-SM, and Novelose® 330-SM enhanced immune activation in
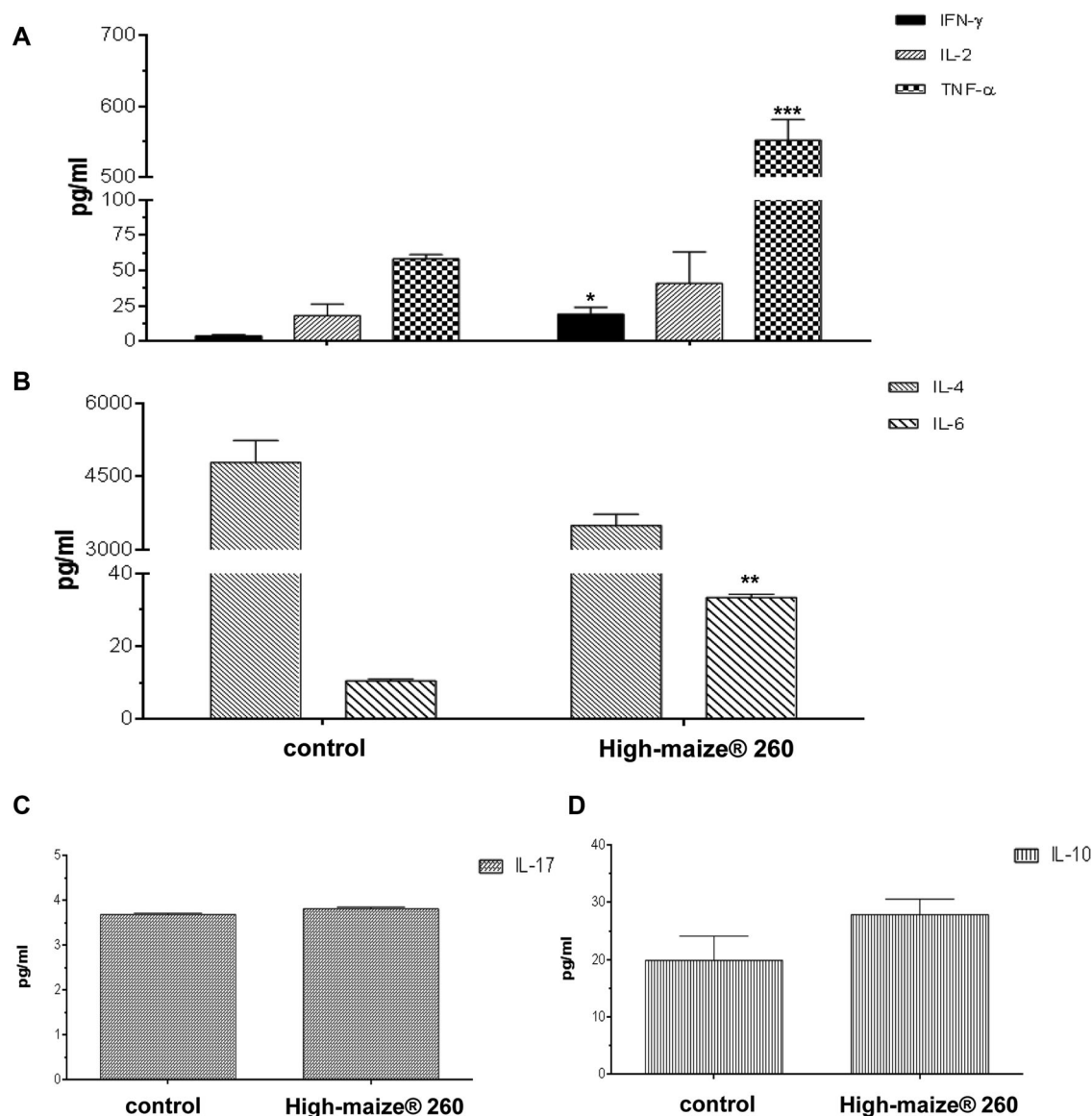
Figure 6. High-maize® 260 induced Th1-cell polarization. DCs were cocultured with TCs and stimulated with starch (400 μg/mL) for 24 h. The production of the Th1 cytokines IL-2, IFN-γ, TNF-α (Panel A); the Th2 cytokines IL-4, IL-6 (Panel B); the Th17 cytokine IL-17 (Panel C); and the Treg cytokine IL-10 (Panel D) was measured. Statistical significance was determined with the Kruskal–Wallis test followed by the Bonferroni multiple comparison test. The data shown are the median and range of three different experiments, in duplicates. p-values < 0.05 are denoted with * p-values < 0.01 are denoted with ** and p-values < 0.005 are denoted with ***.

DCs but it was not zero illustrating the involvement of TLR independent pathways. As shown in the present study direct incubation with DCs induced a proinflammatory phenotype in the DCs. This was more pronounced with High-maize® 260 than with Novelose® 330 despite the strong effects of Novelose® 330 on TLR5.

Many studies have been performed demonstrating the direct immunomodulatory effects of bioactive components such as probiotics and fibers on DCs [26–28]. This direct exposure of bioactive components to DCs might be relevant for dietary fibers as it has been reported that some dietary fibers may pass the intestinal barrier and directly interact with DCs

[29–31]. However in many cases bioactive food components will not ever encounter DCs but only IECs. Also dietary fibers will in vivo first encounter epithelial cells in the intestine. Epithelial cells are regulatory cell types and might modulate the response of starch-exposed DCs [8]. This is why we performed the presented step-wise analysis. And, as shown, the immune activation of DCs was strongly attenuated when DCs were cocultured with IECs previously exposed to starch. Our data therefore suggest that it is advisable to apply cocultures of epithelial-cells, fibers, and DCs rather than incubation with single cell types such as dendritic cells, to gain insight in the immunomodulatory effects of bioactive food components.
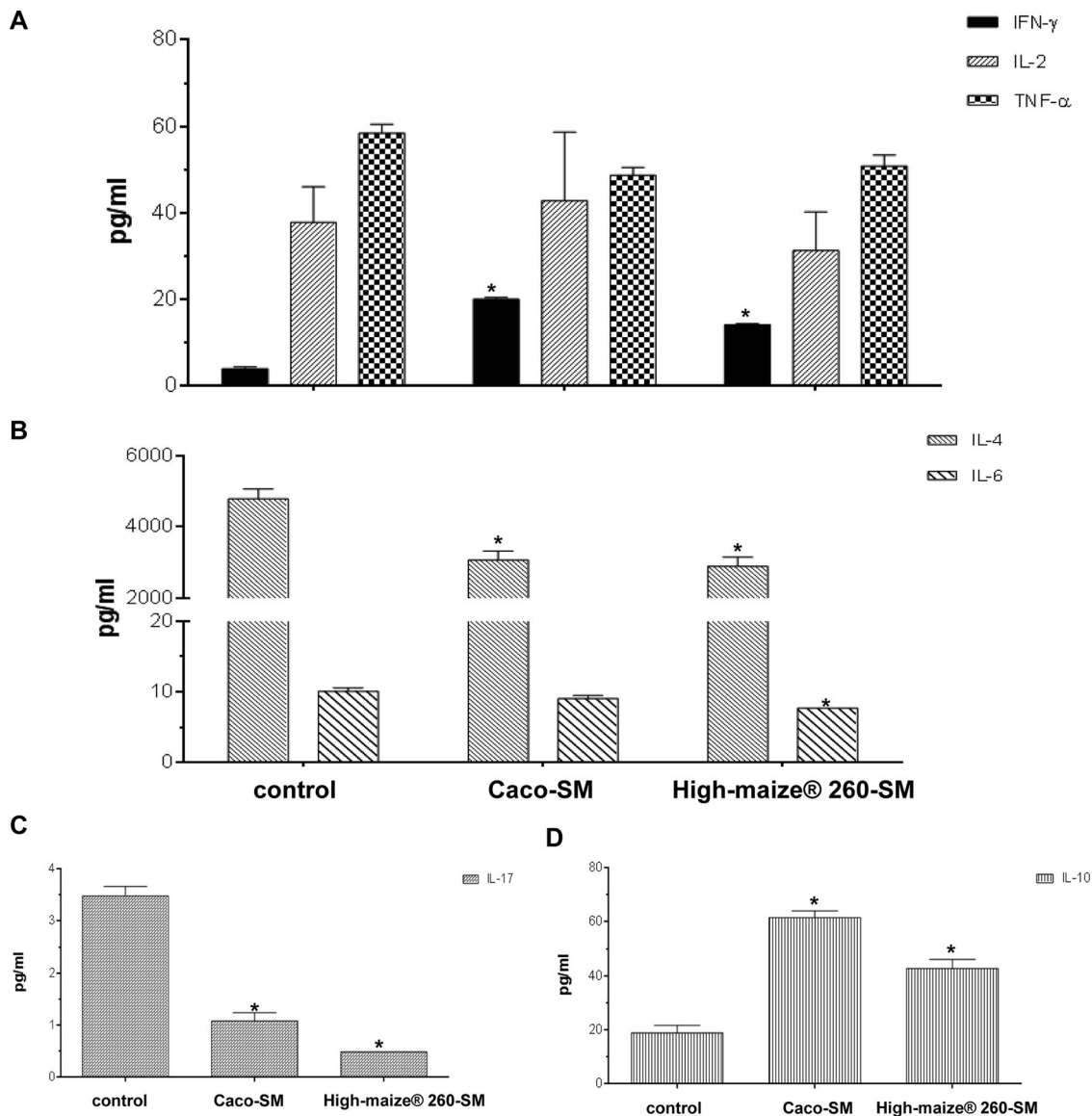
Figure 7. The action of IECs modulated High-maize® 260 effects in DCs and TCs responses. The production of Th1 cytokines (IL-2, IFN-γ, TNF-α) (Panel A); Th2 cytokines (IL-4, IL-6) (Panel B); Th17 cytokine (IL-17); and Treg cytokine (IL-10) by TCs and DCs incubated with the supernatant of IECs pretreated with High-maize® 260 (High-maize® 260-SM). Statistical significance was determined with the Kruskal–Wallis Test followed by the Bonferroni multiple comparison test. The data shown are the median and range of three different experiments, in duplicates. *p*-values < 0.05 are denoted with * *p*-values < 0.01 are denoted with ** and *p*-values < 0.005 are denoted with ***.

To our best knowledge this is the first study addressing the effects of cytokines induced by starch on skewing of T-cell responses. To our opinion there has been a too biased view on classical pro- and anti-inflammatory cytokines in nutritional science. In fact all DC-derived cytokines serve a function in T-cell activation. Classical proinflammatory cytokines such as IFN-γ and TNF-α can stimulate Th1 responses and suppress Th1-Th2 toward less pronounced Th2 responses. This is beneficial for prevention of allergy [26, 32]. As shown in the present study High-maize® 260 and to a lesser extent Novelose® 330 might have such an effect. Other

beneficial effects might be stimulation of Th1 driven vaccination protocols [33].

Up to now it was assumed that resistant starch mainly contributes to anti-inflammatory effects by enhancing production of short chain fatty acids by the microbiota [34–36]. Here, we show that resistant starch by direct interaction with IECs-DCs can induce a regulatory immune phenotype independent of effects on microbiota. This effect is to our opinion profound as DCs strongly decrease CD83 and CD86 expression. Interestingly, similar effects have been reported for highly *O*-glycosylated mucin that is secreted by goblet cells

in the epithelium [37–39] [40, 41]. Recently, Shan et al. [38] demonstrated that Muc2 provides anti-inflammatory signals to DCs in the lamina propria. This results in more tolerogenic CD103+CD11b+CX3CR1–DCs that finally prevents inflammatory responses.

In Conclusion, our findings clearly demonstrate that starch can directly stimulate DCs by interaction with PRRs on immune cells. The interaction with PRRs might depend on the difference in particle size, particle morphology, or insolubility of RS, as High-maize® 260 predominantly binds to TLR2 while Novelose® 330 binds to TLR2 and TLR5. The strong immune-stimulating effects of High-maize® 260 were attenuated by starch-exposed IECs. Despite these attenuating effects, DCs kept producing Th1 cytokines. Cocultures of High-maize® 260 stimulated DCs and naïve autologous T cells with or without Caco SM demonstrate that indeed the final effect of High-maize® 260 is a skewing of Th1–cytokines and attenuation of Th2-cytokines. Our data demonstrate that starch modulate human intestinal immune cells in a physical-morphology-dependent manner. Our data also stresses the importance of taking into account the cross-talk between IECs, DCs, and T cells when interpreting effects of dietary fibers on DCs. Finally, our data demonstrate that in vitro High-maize® 260 can balance T-cell immunity in favor of a more regulatory status, via stimulating Th1 cytokine skewing, attenuating Th2 cytokines and by stimulating regulatory T-cell cytokine production.

There are no patents, products in development or marketed products to declare. This does not alter the authors' adherence to all the Molecular Research & Food Nutrition policies on sharing data and materials.

The authors have declared no conflict of interest.

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