The Journal of Nutrition. First published ahead of print April 30, 2014 as doi: 10.3945/jn.114.191643.

The Journal of Nutrition
Biochemical, Molecular, and Genetic Mechanisms

Toll-Like Receptor 2 Activation by β2→1-Fructans Protects Barrier Function of T84 Human Intestinal Epithelial Cells in a Chain Length–Dependent Manner[1,2]

Leonie M. Vogt,[4]* Diederick Meyer,[5] Gerdie Pullens,[6] Marijke M. Faas,[4] Koen Venema,[3,7] Uttara Ramasamy,[8] Henk A. Schols,[8] and Paul de Vos[4]

[4]Immunoendocrinology, Division of Medical Biology, Department of Pathology and Medical Biology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands; [5]Sensus, Roosendaal, The Netherlands; [6]Cosun Food Technology Centre, Roosendaal, The Netherlands; [7]TNO Quality of Life, Department of Biosciences, Zeist, The Netherlands; and [8]Laboratory of Food Chemistry, Wageningen University, Wageningen, The Netherlands

Abstract

Dietary fiber intake is associated with lower incidence and mortality from disease, but the underlying mechanisms of these protective effects are unclear. We hypothesized that β2→1-fructan dietary fibers confer protection on intestinal epithelial cell barrier function via Toll-like receptor 2 (TLR2), and we studied whether β2→1-fructan chain-length differences affect this process. T84 human intestinal epithelial cell monolayers were incubated with 4 β2→1-fructan formulations of different chain-length compositions and were stimulated with the proinflammatory phorbol 12-myristate 13-acetate (PMA). Transepithelial electrical resistance (TEER) was analyzed by electric cell substrate impedance sensing (ECIS) as a measure for tight junction–mediated barrier function. To confirm TLR2 involvement in barrier modulation by β2→1-fructans, ECIS experiments were repeated using TLR2 blocking antibody. After preincubation of T84 cells with short-chain β2→1-fructans, the decrease in TEER as induced by PMA (62.3 ± 5.2%, $P < 0.001$) was strongly attenuated (15.2 ± 8.8%, $P < 0.01$). However, when PMA was applied first, no effect on recovery was observed during addition of the fructans. By blocking TLR2 on the T84 cells, the protective effect of short-chain β2→1-fructans was substantially inhibited. Stimulation of human embryonic kidney human TLR2 reporter cells with β2→1-fructans induced activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), confirming that β2→1-fructans are specific ligands for TLR2. To conclude, β2→1-fructans exert time-dependent and chain length–dependent protective effects on the T84 intestinal epithelial cell barrier mediated via TLR2. These results suggest that TLR2 located on intestinal epithelial cells could be a target of β2→1-fructan–mediated health effects. J. Nutr. doi: 10.3945/jn.114.191643.

Introduction

It is becoming more accepted that dietary fiber intake leads to a reduced incidence of disease (1–4) and related mortality (5–8). The mechanisms behind this effect of dietary fibers are still incompletely understood. Factors that were suggested to play an important role are improvement of the gut microbiota composition and the related SCFA profiles in the intestine (9–11). However, direct immune effects by ligand interaction of the fibers with so-called pattern recognition receptors on gut immune cells (12–15) were also suggested.

Another mechanism by which dietary fibers may contribute to health is by modulating the integrity of the intestinal epithelial barrier. A disrupted barrier is considered to play a role in the etiology and pathogenesis of several diseases (16), such as atopic eczema (17,18), asthma (19,20), inflammatory bowel disease (21,22), diabetes (23–25), obesity (26,27), celiac disease (28–30), and diarrhea-predominant irritable bowel syndrome (31,32). A compromised intestinal barrier can be associated with hyperpermeability, also described as leaky gut syndrome (33). Disruption leads to increased translocation of bacteria, endotoxins, and other macromolecules, which can be important triggers for aberrant local or peripheral immune reactions (27,34–36).

A category of widely used dietary fibers is formed by β2→1-fructans. Depending on the polymer chain lengths, these fibers are also described as inulin, inulin-type fructans

[1] Performed within the framework of the Carbohydrate Competence Center. This center has been financially supported by the European Union, the European Regional Development Fund, and the Northern Netherlands Provinces, Course for the North.
[2] Author disclosures: D. Meyer is employed by Sensus. L. M. Vogt, G. Pullens, M. M. Faas, K. Venema, U. Ramasamy, H. A. Schols, and P. de Vos, no conflicts of interest.
[3] Present address: Beneficial Microbes Consultancy, Wageningen, The Netherlands.
* To whom correspondence should be addressed. E-mail: L.M.Vogt@umcg.nl.

Manuscript received January 27, 2014. Initial review completed February 24, 2014. Revision accepted April 11, 2014.
doi: 10.3945/jn.114.191643.

(ITFs)[9], fructooligosaccharides, or oligofructose (10). β2→1-fructans are well studied for their beneficial effects on the gut microbiota and health (10,37–41). Studies into the effects of β2→1-fructans on immune cells suggest that they exert direct effects by receptor–ligand interactions and subsequent cytokine production (15,42). However, direct effects of β2→1-fructans on the barrier integrity of human intestinal epithelial cells are, to the best of our knowledge, not available. Previous studies from our group suggest that chain-length differences of β2→1-fructans should be taken into consideration when studying their signaling effects: shorter-chain β2→1-fructans induce a more anti-inflammatory cytokine profile in isolated human peripheral blood mononuclear cells compared with long-chain β2→1-fructans (15). Differences in chain length of β2→1-fructans may also induce different effects on epithelial cells.

We investigated whether β2→1-fructans exert protective effects on barrier function of human intestinal epithelial cells. This was done by analyzing the effect of β2→1-fructans on transepithelial electrical resistance (TEER) of T84 intestinal epithelial cell monolayers, damaged with the barrier-disruptive agent phorbol 12-myristate 13-acetate (PMA). The dynamics of this process were studied with regard to timing of fiber incubation and fructan chain-length effects. Because β2→1-fructans were identified recently as Toll-like receptor (TLR) ligands (15) and TLR2 is highly important in intestinal barrier regulation (43), the other aim of this study was to investigate whether receptor interactions with TLR2 on the epithelial surface are involved in β2→1-fructan–mediated barrier modulation.

Materials and Methods

Experimental design. T84 intestinal epithelial cells were grown to differentiated monolayer stage, and resistance across the monolayer was continuously measured at multiple frequencies after different challenges. Measurements performed at 500 Hz specifically represent the tight junction–mediated resistance, and these were used to calculate the AUC. To establish whether β2→1-fructans exert protective or recovery effects, a damage model was introduced based on challenge of the T84 cells with a known barrier-disrupting agent, PMA (44). The rationale for the first experiment was to study whether β2→1-fructans protect T84 cells against PMA-induced loss of TEER and whether the β2→1-fructan chain-length profile is important in TEER modulation. T84 cells were incubated for 24 h, with 4 different formulations of β2→1-fructans of different mean degree of polymerization (DP) and DP profile (ITF I–ITF IV) provided by Sensus, followed by addition of PMA (10 nM; Sigma-Aldrich Chemie). The second experiment was designed to investigate the possible time dependency of the effect of β2→1-fructan incubation. Cells were stimulated in different orders with different compounds: *1*) protocol I: incubation with PMA for 6 h, followed by removal of the medium and addition of β2→1-fructans in culture medium at a final concentration of 100 mg/L; or *2*) protocol II: incubation for 24 h with β2→1-fructans, followed by addition of PMA. This stimulation medium was left on the cells for at least 6 h to allow the PMA to take effect. As the interval for recovery in protocol I, a fixed period of time was taken (12 h) after removal of PMA medium. This period was based on the mean recovery time of the cells treated with PMA, followed by control medium, represented by TEER values returning to the initial value (100%). As a measure for induced recovery, the AUC was plotted for this timeframe relative to the AUC of untreated controls (without PMA), which was set to 100%. For protocol II, the interval for protection against the reduction of TEER was based on the timeframe starting at the addition of PMA until the maximal decrease in TEER was reached (after 6 h), and this fixed timeframe was taken to calculate the AUC relative to untreated controls. With the third experiment, we aimed to study the role of TLR2 in β2→1-fructan–mediated effects on T84 cells. To this end, T84 cells were incubated with culture medium or TLR2 blocking antibody (catalog no. pab-hstlr2, 5 mg/L; InvivoGen) for 10 min before the addition of β2→1-fructans. Cells remained in this medium for 24 h, followed by addition of PMA. The 6 h timeframe after PMA addition was used for AUC calculations. Finally, to confirm that β2→1-fructans signal through TLR2, human embryonic kidney (HEK) human TLR2 (hTLR2) reporter cells were stimulated with ITF I–ITF IV.

Investigational compounds. To study the effects of β2→1-fructans on intestinal epithelial cell barrier function, 4 different formulations were applied based on the chemical characterization of their chain-length profiles by high-performance anion-exchange chromatography and high-pressure size exclusion chromatography (15). Endotoxin concentrations in the formulations were analyzed by Toxikon, and all fell below 0.3×10^{-3} endotoxin units μg^{-1}. Briefly, the mean chain length of these formulations is as ITF I (Frutalose OFP; Sensus) < ITF II (Frutafit CLR; Sensus) < ITF III (Frutafit HD; Sensus) < ITF IV (Frutafit TEX; Sensus). ITF I is a fructooligosaccharide compound, with chain lengths of ≤DP10. ITF II is an inulin, enriched with fructooligosaccharides, with most chains ≤DP10, but also containing chains with DPs up to 60. ITF III is "native" inulin, and ITF IV can be described as a long-chain enriched inulin. Both ITF III and ITF IV consist predominantly of chains ranging from DP10 to DP60. Chain-length profiles of the applied formulations are summarized in **Figure 1**.

T84 cell culture. T84 human colon carcinoma cells (Sigma-Aldrich Chemie) were grown to ~80% confluency at 37°C, 5% CO_2 in culture medium consisting of 1:1 Ham's F-12 medium:DMEM (acquired premade from Sigma-Aldrich Chemie), supplemented with 10% HyClone FBS (Thermo Fisher Scientific) and gentamicin (50 mg/L; Life Technologies Europe). Cells were maintained as described previously (45). Trypsin was acquired from MP Biomedicals, and EDTA (Titriplex III) was from Merck Millipore.

TEER measurements. Multiple electrode gold-plated 8-well chamber slides (8W10E; IBIDI via Applied Biophysics) were coated with

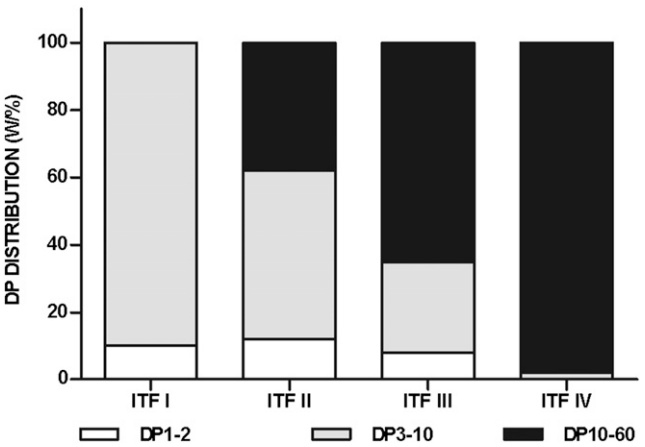

FIGURE 1 Chain-length distribution of the applied fructan formulations. ITF I consists predominantly of fructan chains of DP3–DP10 and a small portion of monomers. ITF II consists mainly of fructan chains of DP3–DP10 but also contains chain lengths up to DP60 and a small portion of monomers. ITF III consists mainly of fructan chains of DP10–DP60, and a smaller portion is made up of fructan chains of DP3–DP10 and monomers. ITF IV comprises mostly chains longer than DP10, a small portion of DP3–DP10, and no monomers or dimers. DP, degree of polymerization; ITF, inulin-type fructan; W, weight.

[9] Abbreviations used: DP, degree of polymerization; HEK, human embryonic kidney; hTLR2, human Toll-like receptor 2; ITF, inulin-type fructan; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TEER, transepithelial electrical resistance; TLR, Toll-like receptor.

400 μL/well of a 0.2% L-cysteine (Sigma-Aldrich Chemie) solution in PBS for 30 min at room temperature. Wells were washed twice with PBS and coated overnight at room temperature with 400 μL/well 1% PureCol bovine tail collagen (Nutacon) and 0.1% BSA (Sigma-Aldrich Chemie) in PBS. Wells were then washed twice with culture medium, and cells were seeded at a density of 2×10^4 cells per well in a final culture volume of 400 μL/well. Before stimulation, the cells were maintained in the wells for 14 d to reach a stable TEER. Medium was changed twice a week. The chamber slides were put into an electric cell substrate impedance sensing incubator (Z-Theta model; Applied Biophysics), and resistance was measured continuously at multiple frequencies (46).

HEK hTLR2 reporter cell culture and reporter assay. HEK hTLR2 reporter cells were cultured, and NF-κB activity was determined as described previously (15). As positive control, the additional TLR2 agonist heat-killed *Listeria monocytogenes* was applied by adding 20 μL of solution to 180 μL of cell suspension (1×10^8 bacteria/mL; InvivoGen).

Statistical analysis. Statistical analysis was performed using Prism 5.0 software (GraphPad Software). D'Agostino and Pearson omnibus normality test was used to test for normal data distribution. Statistical significance levels were determined by 1-factor ANOVA and Tukey's multiple comparison test or by Dunnett's multiple comparison test to compare treatment with controls. Results are expressed as means ± SDs. P values < 0.05 were considered statistically significant.

Results

β2→1-fructans exert chain length–dependent protection against PMA-induced loss of barrier function in T84 intestinal epithelial cells. To study whether β2→1-fructans protect T84 cells against PMA-induced loss of TEER and whether the β2→1-fructan chain-length profile is important in TEER modulation, T84 cells were incubated with 4 different formulations of β2→1-fructans of different mean DP and DP profile (ITF I–ITF IV). The AUC for the 6-h time period after PMA addition was plotted for the different ITF treatments, as a percentage of the AUC of untreated controls, which was set to 100% (**Fig. 2**). The damage model of PMA treatment induced a decrease in TEER, resulting in an AUC of 61.5 ± 5.8% (P < 0.001) compared with controls. Strikingly, 24 h of preincubation of T84 cells with 100 mg/L ITF I or ITF II β2→1-fructans conferred a protective effect against PMA-induced loss of resistance (P < 0.001 and P < 0.01, respectively). ITF I conferred the strongest protection, with an AUC of 91.0 ± 6.6% of the control AUC, followed by ITF II, which rendered an AUC of 75.4 ± 3.2% of the control AUC. TEER values for treatment with the longer-chain compounds (ITF III and ITF IV) were not statistically different from TEER values as induced by PMA treatment, demonstrating that these compounds did not exert a protective effect. These results indicate that the protective effect of β2→1-fructans is a chain length–dependent phenomenon, which is only conferred by the short-chain formulations ITF I and ITF II.

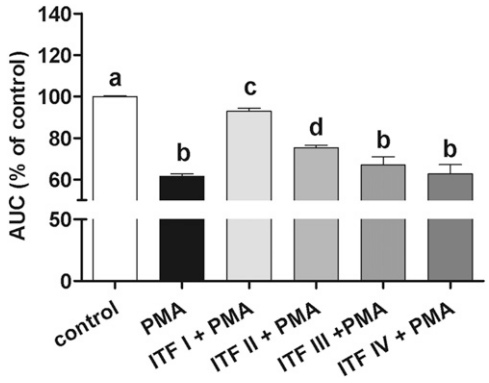

FIGURE 2 Chain length–dependent protection of transepithelial electrical resistance across T84 epithelial cell monolayers treated with different ITF formulations and PMA. AUC was plotted for the time range starting at the addition of PMA and after a 6-h time period. Values are means ± SDs, n = 3. Data are representative of 3 individual experiments. Statistical significance levels were determined with 1-factor ANOVA and Tukey's multiple comparison test. Labeled means without a common letter differ, P < 0.05. ITF, inulin-type fructan; PMA, phorbol 12-myristate 13-acetate.

The protective effect of short-chain β2→1-fructans against PMA-induced T84 barrier loss is time dependent. To establish whether timing of short-chain β2→1-fructan incubation is important in their functionality, 2 protocols for incubation were applied: *1*) protocol I: preincubation of T84 cells with PMA for 6 h, followed by removal of the medium and addition of short-chain β2→1-fructans (ITF I) in culture medium; and *2*) protocol II: preincubation of T84 cells for 24 h with ITF I, followed by addition of PMA (**Fig. 3**). Figure 3*A* shows an example representative of relative TEER values obtained with protocol I and the 12-h interval used to calculate the AUC, which was subsequently plotted in Figure 3*B*. Figure 3, *A* and *B*, shows that protocol I did not induce TEER recovery effects compared with PMA treatment. A representative example of relative TEER values obtained with protocol II and the 6-h interval used to calculate the AUC are plotted in Figure 3*C*. Here, a substantial protection was established, minimizing the decrease in TEER at 6 h after PMA addition to 15.2 ± 8.8% (P < 0.01), whereas PMA treatment alone induced a reduction of 62.3 ± 5.2% (P < 0.001) of the initial TEER values. This protective effect was also observed when calculating the AUC (Fig. 3*D*). Over the 6-h period, the AUC of PMA treatment was 69.5 ± 2.3% (P < 0.001) of the AUC of untreated controls, whereas the AUC of cells pretreated with ITF I followed by PMA was 91.0 ± 6.6% (P < 0.01) of the AUC of untreated controls. These results indicate that timing of incubation with β2→1-fructans is an important factor for protection of the T84 barrier function.

Blocking of TLR2 inhibits short-chain β2→1-fructan–mediated protection of TEER. Because β2→1-fructans were identified recently as TLR ligands (15) and TLR2 is highly important in intestinal barrier regulation (43), we hypothesized that the protective effect of short-chain β2→1-fructans on epithelial cells against PMA might be mediated through TLR2. T84 cells were preincubated with normal culture medium or with TLR2 blocking antibody before the 24-h incubation with short-chain β2→1-fructans, followed by PMA challenge (**Fig. 4**). Because the effects of longer-chain β2→1-fructans did not induce protective effects, ITF III and ITF IV were not included in the TLR2 blocking experiments. Figure 4, *A* and *B*, shows representative examples of TEER values as induced by PMA, preincubation with IFT I and ITF II, respectively, and pretreatment with the blocking antibody. AUC for a 6-h timeframe after PMA addition was plotted in Figure 4*C*, showing that pretreatment with TLR2 blocking antibody substantially reduced the short-chain β2→1-fructan–mediated protection for both short-chain formulations, with a TEER curve approaching the PMA curve. These results indicate that, in T84 cells, TLR2 is involved in the protective mechanism of short-chain β2→1-fructans against PMA.

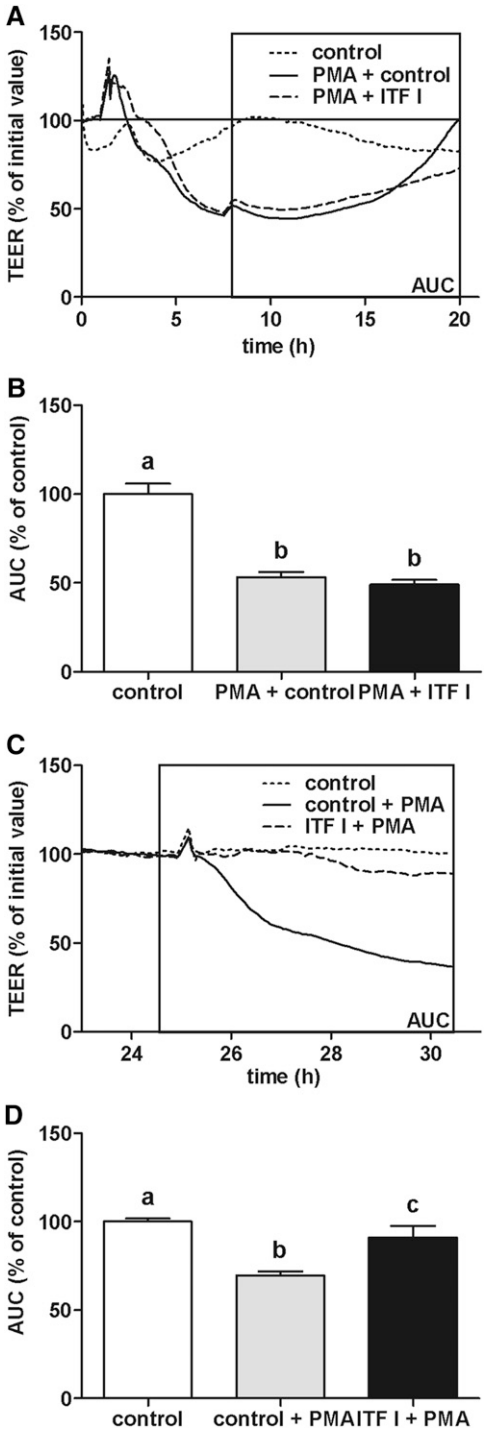

FIGURE 3 Time-dependent impact of different incubation protocols on TEER across T84 epithelial cell monolayers. Example of TEER as induced by protocol I (*A*). AUC for 12 h from the time point of PMA addition, representing the period of recovery for PMA-treated cells to 100% of the initial value for protocol I (*B*). Values are means ± SDs, $n = 6$. Example of TEER as induced by protocol II (*C*). AUC for 6 subsequent hours after addition of PMA plotted as percentage of the AUC of untreated controls (*D*). Values are means ± SDs, $n = 6$. Data are representative of 3 individual experiments, and data for ITF I are shown for $n = 6$. Statistical significance levels were determined with 1-factor ANOVA and Tukey's multiple comparison test. Labeled means without a common letter differ, $P < 0.05$. ITF, inulin-type fructan; PMA, phorbol 12-myristate 13-acetate; TEER, transepithelial electrical resistance.

β2→1-fructans exert TLR2-mediated NF-κB activation in HEK hTLR2 reporter cells. TLR2 was suggested as a mediator of β2→1-fructan signaling in immune cells (15), and in previous studies, an important role was attributed to TLR2 in modulating barrier function (43,47–49). To confirm the role of TLR2 in β2→1-fructan signaling, HEK hTLR2 reporter cells were incubated with a concentration series of β2→1-fructans of different chain lengths. β2→1-fructans induced TLR2-mediated NF-κB activation in the reporter cell line (**Fig. 5**). Short-chain β2→1-fructans (ITF I) induced a 2.8 ± 1.2-fold induction ($P < 0.05$) of NF-κB/activator protein 1 activation compared with control, and with increasing mean fructan chain length, the

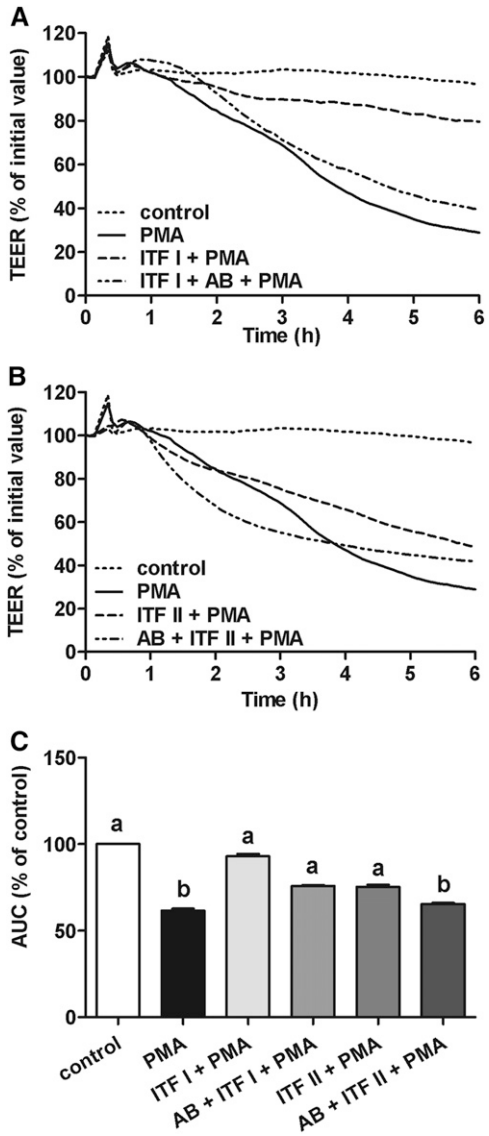

FIGURE 4 Effects of blocking TLR2 on β2→1-fructan–mediated protection of T84 TEER. TEER of T84 cells with or without TLR2 blocking antibody (AB), incubation with ITF I for 24 h, and 10 nmol/L PMA (*A*). TEER of T84 cells with or without TLR2 blocking antibody (AB), incubated with ITF II for 24 h, and 10 nmol/L PMA (*B*). The AUC for 6 subsequent hours of each treatment (*C*). Data are representative of 3 individual experiments, and data for ITF I are shown for $n = 9$. Labeled means without a common letter differ, $P < 0.05$. Statistical significance levels were determined with 1-factor ANOVA and Tukey's multiple comparison test. AB, antibody; ITF, inulin-type fructan; PMA, phorbol 12-myristate 13-acetate; TEER, transepithelial electrical resistance; TLR2, Toll-like receptor 2.

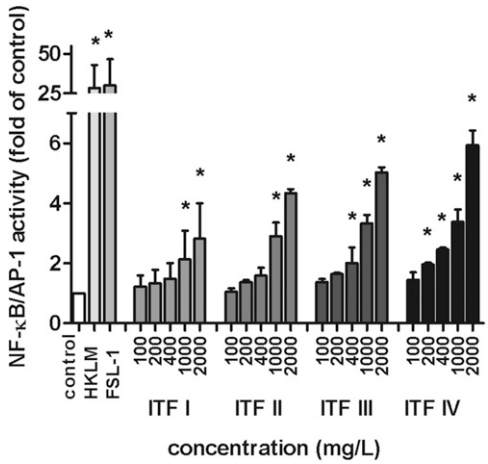

FIGURE 5 NF-κB/AP-1 activity of HEK human TLR2 reporter cells. Fold induction for positive controls represented by 10^8 cells/mL heat-killed *Listeria monocytogenes*, and TLR agonist FSL-1, and a concentration range of β2→1-fructans (milligrams per liter) was plotted as compared to unstimulated control (medium, set to 1). Values are means ± SDs, n = 3. Statistical significance levels were determined with Dunnett's multiple comparison test. *Labeled means are different from control, $P < 0.05$. AP-1, activator protein 1; FSL-1, Pam2CGDPKHPKSF, synthetic lipoprotein derived from *Mycoplasma salivarium*; HEK, human embryonic kidney; HKLM, heat-killed *Listeria monocytogenes*; ITF, inulin-type fructan; TLR2, Toll-like receptor 2.

fructans conferred stronger activation, up to 5.9 ± 0.5-fold induction ($P < 0.05$) for the longest mean fructan chain formulation (ITF IV) compared with control. These results confirm the role of TLR2 in β2→1-fructan signaling and indicate that fructan chain length is an important factor in determining the strength of the TLR2 response. Short-chain β2→1-fructans conferred a moderate TLR2 activation, whereas the longer-chain β2→1-fructans induced a relatively strong TLR2 response.

Discussion

To the best of our knowledge, this is the first time β2→1-fructans have been identified in their capacity as modulators of human intestinal epithelial cell barrier function through TLR2. Proof of principle was established that β2→1-fructan dietary fibers can protect the integrity of intestinal epithelial cell monolayers. This effect was observed for the shorter-chain ITFs (ITF I and ITF II) but not for the longer-chain formulations (ITF III and ITF IV). This is in accordance with a previous study in our group, in which chain-length differences of the applied β2→1-fructans were important factors in inducing different effects on human immune cells with regard to cytokine profiles (15). In this study, the short-chain β2→1-fructans induced a more anti-inflammatory cytokine pattern compared with the longer-chain formulations, indicating chain length–dependent differences in downstream effects in immune cells. In addition, 2 studies by Ito et al. (50,51) performed in rats corroborated chain length–dependent effects on immune cells and intestinal barrier function in vivo. The studies by Ito et al. demonstrated that β2→1-fructans stimulated intestinal immune variables in a chain length–dependent manner (50) and that short-chain β2→1-fructans reduced translocation of endotoxins and bacteria in a trinitrobenzene sulfonic acid-induced colitis model (51).

By using different timing protocols regarding short-chain β2→1-fructan or PMA treatment, we observed a protective effect of the fructans on TEER, provided that the cells were incubated with the fructans for 24 h, before stimulation with PMA. Treatment with PMA followed by incubation with β2→1-fructans did not induce recovery effects, indicating that, in this model, fructans exerted protective effects rather than effects on repair processes. This conclusion was based on the described model using low-dose treatment. The rationale behind treatment with low-dose fructans (100 mg/L) was based on our previous results in peripheral blood mononuclear cells (15), in which this dose already induced substantial cell activation in the form of production of several cytokines during 24 h of stimulation. In addition, several studies showed that preincubating cells of different tissue types with TLR2 agonists can protect against detrimental effects of barrier-disruptive agents or ischemia-reperfusion injury (47–49,52,53). In these studies, protection is often established under low-dose stimulatory conditions.

With the current study, novel proof of principle was demonstrated for time-dependent protective effects of β2→1-fructans on barrier function of human intestinal epithelial cells. Whether the critical timeframe of preincubation with β2→1-fructans can be reduced compared with the 24-h preincubation protocol while retaining a protective effect remains to be studied. This timeframe may give an indication of which sort of cellular processes are affected, such as receptor/adapter molecule assembly at the cell membrane, modulation of kinase activity, or further downstream effects, such as nuclear translocation of messenger molecules, gene transcription, and protein expression. These results prompt additional studies into the exact mechanisms behind the observed protective effects of the fructans on the epithelium.

Considering epithelial cells of the intestine specifically, TLR2 is an important player in regulating permeability and thus barrier function (43). The role of TLR2 in the protective action of β2→1-fructans on intestinal epithelial cells was confirmed with blocking experiments. In addition, the specific dynamics of β2→1-fructan–mediated TLR2 activation were demonstrated. Besides possible fructan chain length–dependent or dose-dependent effects on the strength of TLR2 activation in intestinal epithelial cells, differences in TEER modulation may be due to the ability of TLR2 to heterodimerize with TLR1 (54), TLR6 (54), or TLR10 (55), depending on the stimulus (43,54), and the ability to signal together with a spectrum of different coreceptors (56). These features of TLR2 diversify the downstream effects of TLR2 and may provide an explanation for chain length–induced differences between the different fructan formulations.

The discrepancy between the longer chains inducing strong TLR2 activation but not exerting a protective effect on barrier function could be due to mechanistic differences in receptor interactions at the cellular surface (15). This type of mechanism was described previously for TLR4 by Visintin et al. (57). They suggest that differences in agonist clustering mechanisms for TLRs culminate in enhanced signal transduction and different downstream reactions. This is in accordance with our previous observations that ITFs have different TLR activation patterns (15). We suggest that, by activating different numbers of receptors at a time and at different distance from each other on the cell membrane, long-chain β2→1-fructans may induce a cellular response different from the short-chain β2→1-fructans. However, an in-depth discussion of these pathways is beyond the scope of the present study, and the role of TLR2 dynamics in

barrier function is the subject of additional research in our laboratory.

As an analogue of the endogenous second-messenger diacylglycerol, PMA induces activation and translocation of protein kinase C (PKC), leading to elevated intracellular Ca^{2+} and modulation of TEER (44). PMA challenge of T84 cells can be viewed as a simplified model for dietary stimuli (58) or intestinal pathogens (59) involved in modulating PKC signaling and affecting the gut barrier. The readout for TLR2 activation in the HEK reporter cell line was analyzed by the production of secreted embryonic alkaline phosphatase during NF-κB activation. In intestinal epithelial cells, TLR2 activation was shown to act as an NF-κB inhibitor (43). Because PMA can also target NF-κB, downstream of PKC-β1 via stabilization of NF-κB inhibitor α (60,61), inhibition of NF-κB could be 1 of the downstream mechanisms by which the epithelial barrier is partially protected by the fructans.

Although PKC was not studied in our experiments, results from our model suggest that short-chain β2→1-fructans may interfere in this pathway, by partly inhibiting the cellular response to PMA. Previous studies with inulin showed that their signaling can induce PKC activation in RAW 264.7 cells (60) and in rat distal colonic mucosa (62). However, the typical PKC dynamics are greatly dependent on the type of tissue and the organism that is studied (44). Moreover, the PKC family consists of different subclasses and several isoforms, each having distinct dynamics for activation and downstream effects (63–67). A study in T84 cells by Song et al. (68) showed that PMA induced PKCα translocation to the apical surface, which was correlated with a decreased TEER. Interestingly, PKCα activation was linked previously to TLR2 signaling cascades in mouse and human dendritic cells (69).

In conclusion, β2→1-fructans may protect the integrity of the intestinal barrier from damage by directly binding to TLR2. Because they also have immunomodulating effects and prebiotic effects, they form a promising category of dietary fibers with regard to intestinal health effects, and additional studies into their effects after ingestion on physiologic, cellular, and molecular levels are warranted.

Acknowledgments

P.d.V. and L.M.V. designed the research; L.M.V. conducted the research and analyzed the data; L.M.V., D.M., G.P., M.M.F., K.V., U.R., H.A.S., and P.d.V. wrote the paper; and L.M.V. had primary responsibility for the final content. All authors read and approved the final manuscript.

References

1. Aune D, Chan DS, Lau R, Vieira R, Greenwood DC, Kampman E, Norat T. Dietary fibre, whole grains, and risk of colorectal cancer: systematic review and dose-response meta-analysis of prospective studies. BMJ 2011;343:d6617.
2. Fardet A. New hypotheses for the health-protective mechanisms of whole-grain cereals: what is beyond fibre? Nutr Res Rev 2010;23:65–134.
3. Pereira MA, O'Reilly E, Augustsson K, Fraser GE, Goldbourt U, Heitmann BL, Hallmans G, Knekt P, Liu S, Pietinen P, et al. Dietary fiber and risk of coronary heart disease: a pooled analysis of cohort studies. Arch Intern Med 2004;164:370–6.
4. Schulze MB, Schulz M, Heidemann C, Schienkiewitz A, Hoffmann K, Boeing H. Fiber and magnesium intake and incidence of type 2 diabetes: a prospective study and meta-analysis. Arch Intern Med 2007;167:956–65.
5. Chuang SC, Norat T, Murphy N, Olsen A, Tjonneland A, Overvad K, Boutron-Ruault MC, Perquier F, Dartois L, Kaaks R, et al. Fiber intake and total and cause-specific mortality in the European Prospective Investigation into Cancer and Nutrition cohort. Am J Clin Nutr 2012;96:164–74.
6. Jacobs DR Jr, Andersen LF, Blomhoff R. Whole-grain consumption is associated with a reduced risk of noncardiovascular, noncancer death attributed to inflammatory diseases in the Iowa Women's Health Study. Am J Clin Nutr 2007;85:1606–14.
7. Landberg R. Dietary fiber and mortality: convincing observations that call for mechanistic investigations. Am J Clin Nutr 2012;96:3–4.
8. Park Y, Subar AF, Hollenbeck A, Schatzkin A. Dietary fiber intake and mortality in the NIH-AARP diet and health study. Arch Intern Med 2011;171:1061–8.
9. Meijer K, de Vos P, Priebe MG. Butyrate and other short-chain fatty acids as modulators of immunity: what relevance for health? Curr Opin Clin Nutr Metab Care 2010;13:715–21.
10. Roberfroid M, Gibson GR, Hoyles L, McCartney AL, Rastall R, Rowland I, Wolvers D, Watzl B, Szajewska H, Stahl B, et al. Prebiotic effects: metabolic and health benefits. Br J Nutr 2010;104(Suppl 2):S1–63.
11. Vinolo MA, Rodrigues HG, Nachbar RT, Curi R. Regulation of inflammation by short chain fatty acids. Nutrients. 2011;3:858–76.
12. Brown GD, Taylor PR, Reid DM, Willment JA, Williams DL, Martinez-Pomares L, Wong SY, Gordon S. Dectin-1 is a major beta-glucan receptor on macrophages. J Exp Med 2002;196:407–12.
13. Underhill DM, Ozinsky A, Hajjar AM, Stevens A, Wilson CB, Bassetti M, Aderem A. The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. Nature 1999;401:811–5.
14. Underhill DM, Rossnagle E, Lowell CA, Simmons RM. Dectin-1 activates Syk tyrosine kinase in a dynamic subset of macrophages for reactive oxygen production. Blood 2005;106:2543–50.
15. Vogt L, Ramasamy U, Meyer D, Pullens G, Venema K, Faas MM, Schols HA, de Vos P. Immune modulation by different types of beta →1-fructans is toll-like receptor dependent. PLoS ONE. 2013;8:e68367.
16. Brenchley JM, Douek DC. Microbial translocation across the GI tract. Annu Rev Immunol 2012;30:149–73.
17. Iemoli E, Trabattoni D, Parisotto S, Borgonovo L, Toscano M, Rizzardini G, Clerici M, Ricci E, Fusi A, De Vecchi E, et al. Probiotics reduce gut microbial translocation and improve adult atopic dermatitis. J Clin Gastroenterol 2012;46: Suppl:S33–40.
18. Penders J, Stobberingh EE, van den Brandt PA, Thijs C. The role of the intestinal microbiota in the development of atopic disorders. Allergy 2007;62:1223–36.
19. Benard A, Desreumeaux P, Huglo D, Hoorelbeke A, Tonnel AB, Wallaert B. Increased intestinal permeability in bronchial asthma. J Allergy Clin Immunol 1996;97:1173–8.
20. Hijazi Z, Molla AM, Al-Habashi H, Muawad WM, Molla AM, Sharma PN. Intestinal permeability is increased in bronchial asthma. Arch Dis Child 2004;89:227–9.
21. Gassler N, Rohr C, Schneider A, Kartenbeck J, Bach A, Obermuller N, Otto HF, Autschbach F. Inflammatory bowel disease is associated with changes of enterocytic junctions. Am J Physiol Gastrointest Liver Physiol 2001;281:G216–28.
22. Söderholm JD, Peterson KH, Olaison G, Franzen LE, Westrom B, Magnusson KE, Sjodahl R. Epithelial permeability to proteins in the noninflamed ileum of Crohn's disease? Gastroenterology 1999;117:65–72.
23. Bosi E, Molteni L, Radaelli MG, Folini L, Fermo I, Bazzigaluppi E, Piemonti L, Pastore MR, Paroni R. Increased intestinal permeability precedes clinical onset of type 1 diabetes. Diabetologia 2006;49:2824–7.
24. Sapone A, de Magistris L, Pietzak M, Clemente MG, Tripathi A, Cucca F, Lampis R, Kryszak D, Carteni M, Generoso M, et al. Zonulin upregulation is associated with increased gut permeability in subjects with type 1 diabetes and their relatives. Diabetes 2006;55:1443–9.
25. Vaarala O, Atkinson MA, Neu J. The "perfect storm" for type 1 diabetes: the complex interplay between intestinal microbiota, gut permeability, and mucosal immunity. Diabetes 2008;57:2555–62.
26. Boroni Moreira AP, Fiche Salles Teixeira T, do C Gouveia Peluzio M, de Cassia Goncalves Alfenas R. Gut microbiota and the development of obesity. Nutr Hosp 2012;27:1408–14.
27. Fasano A. Leaky gut and autoimmune diseases. Clin Rev Allergy Immunol 2012;42:71–8.

28. Montalto M, Cuoco L, Ricci R, Maggiano N, Vecchio FM, Gasbarrini G. Immunohistochemical analysis of ZO-1 in the duodenal mucosa of patients with untreated and treated celiac disease. Digestion 2002;65: 227–33.
29. Schulzke JD, Bentzel CJ, Schulzke I, Riecken EO, Fromm M. Epithelial tight junction structure in the jejunum of children with acute and treated celiac sprue. Pediatr Res 1998;43:435–41.
30. Vogelsang H, Wyatt J, Penner E, Lochs H. Screening for celiac disease in first-degree relatives of patients with celiac disease by lactulose/mannitol test. Am J Gastroenterol 1995;90:1838–42.
31. Dunlop SP, Hebden J, Campbell E, Naesdal J, Olbe L, Perkins AC, Spiller RC. Abnormal intestinal permeability in subgroups of diarrhea-predominant irritable bowel syndromes. Am J Gastroenterol 2006;101:1288–94.
32. Lee H, Park JH, Park DI, Kim HJ, Cho YK, Sohn CI, Jeon WK, Kim BI, Chae SW. Mucosal mast cell count is associated with intestinal permeability in patients with diarrhea predominant irritable bowel syndrome. J Neurogastroenterol Motil 2013;19:244–50.
33. Liu Z, Li N, Neu J. Tight junctions, leaky intestines, and pediatric diseases. Acta Paediatr 2005;94:386–93.
34. Geurts L, Neyrinck AM, Delzenne NM, Knauf C, Cani PD. Gut microbiota controls adipose tissue expansion, gut barrier and glucose metabolism: novel insights into molecular targets and interventions using prebiotics. Benef Microbes 2014;5:3–17.
35. Ruutu M, Thomas G, Steck R, Degli-Esposti MA, Zinkernagel MS, Alexander K, Velasco J, Strutton G, Tran A, Benham H, et al. beta-Glucan triggers spondylarthritis and Crohn's disease-like ileitis in SKG mice. Arthritis Rheum 2012;64:2211–22.
36. Seo YS, Shah VH. The role of gut-liver axis in the pathogenesis of liver cirrhosis and portal hypertension. Clin Mol Hepatol 2012;18:337–46.
37. Albers R, Antoine JM, Bourdet-Sicard R, Calder PC, Gleeson M, Lesourd B, Samartin S, Sanderson IR, Van Loo J, Vas Dias FW, et al. Markers to measure immunomodulation in human nutrition intervention studies. Br J Nutr 2005;94:452–81.
38. de Vrese M, Schrezenmeir J. Probiotics, prebiotics, and synbiotics. Adv Biochem Eng Biotechnol 2008;111:1–66.
39. Kelly G. Inulin-type prebiotics: a review. (Part 2). Altern Med Rev 2009;14:36–55.
40. Rijnierse A, Jeurink PV, Garssen J, Knippels LM. Food-derived oligosaccharides exhibit pharmaceutical properties. Eur J Pharmacol 2011;688(Suppl 1):S117–23.
41. Vogt LM, Meyer D, Pullens G, Faas MM, Smelt MJ, Venema K, Ramasamy U, Schols HA, de Vos P. Immunological properties of inulin-type fructans. Crit Rev Food Sci Nutr 2013;Aug 25 (Epub ahead of print; DOI:10.1080/10408398.2012.656772).
42. Eiwegger T, Stahl B, Haidl P, Schmitt J, Boehm G, Dehlink E, Urbanek R, Szepfalusi Z. Prebiotic oligosaccharides: in vitro evidence for gastrointestinal epithelial transfer and immunomodulatory properties. Pediatr Allergy Immunol 2010;21:1179–88.
43. Cario E. Barrier-protective function of intestinal epithelial Toll-like receptor 2. Mucosal Immunol 2008;1(Suppl 1):S62–6.
44. Tai YH, Flick J, Levine SA, Madara JL, Sharp GW, Donowitz M. Regulation of tight junction resistance in T84 monolayers by elevation in intracellular Ca^{2+}: a protein kinase C effect. J Membr Biol 1996;149:71–9.
45. Marcon MA, McCool D, Forstner J, Forstner G. Inhibition of mucin secretion in a colonic adenocarcinoma cell line by DIDS and potassium channel blockers. Biochim Biophys Acta 1990;1052:17–23.
46. Lo CM, Keese CR, Giaever I. Impedance analysis of MDCK cells measured by electric cell-substrate impedance sensing. Biophys J 1995;69:2800–7.
47. Karczewski J, Troost FJ, Konings I, Dekker J, Kleerebezem M, Brummer RJ, Wells JM. Regulation of human epithelial tight junction proteins by *Lactobacillus plantarum* in vivo and protective effects on the epithelial barrier. Am J Physiol Gastrointest Liver Physiol 2010;298:G851–9.
48. Lu C, Liu L, Chen Y, Ha T, Kelley J, Schweitzer J, Kalbfleisch JH, Kao RL, Williams DL, Li C. TLR2 ligand induces protection against cerebral ischemia/reperfusion injury via activation of phosphoinositide 3-kinase/Akt signaling. J Immunol 2011;187:1458–66.
49. Yuki T, Yoshida H, Akazawa Y, Komiya A, Sugiyama Y, Inoue S. Activation of TLR2 enhances tight junction barrier in epidermal keratinocytes. J Immunol 2011;187:3230–7.
50. Ito H, Takemura N, Sonoyama K, Kawagishi H, Topping DL, Conlon MA, Morita T. Degree of polymerization of inulin-type fructans differentially affects number of lactic acid bacteria, intestinal immune functions, and immunoglobulin A secretion in the rat cecum. J Agric Food Chem 2011;59:5771–8.
51. Ito H, Tanabe H, Kawagishi H, Tadashi W, Yasuhiko T, Sugiyama K, Kiriyama S, Morita T. Short-chain inulin-like fructans reduce endotoxin and bacterial translocations and attenuate development of TNBS-induced colitis in rats. Dig Dis Sci 2009;54:2100–8.
52. Hua F, Ma J, Ha T, Kelley J, Williams DL, Kao RL, Kalbfleisch JH, Browder IW, Li C. Preconditioning with a TLR2 specific ligand increases resistance to cerebral ischemia/reperfusion injury. J Neuroimmunol 2008;199:75–82.
53. Cario E, Gerken G, Podolsky DK. Toll-like receptor 2 controls mucosal inflammation by regulating epithelial barrier function. Gastroenterology 2007;132:1359–74.
54. Ozinsky A, Underhill DM, Fontenot JD, Hajjar AM, Smith KD, Wilson CB, Schroeder L, Aderem A. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. Proc Natl Acad Sci USA 2000;97:13766–71.
55. Hasan U, Chaffois C, Gaillard C, Saulnier V, Merck E, Tancredi S, Guiet C, Briere F, Vlach J, Lebecque S, et al. Human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells, which activates gene transcription through MyD88. J Immunol 2005;174: 2942–50.
56. van Bergenhenegouwen J, Plantinga TS, Joosten LA, Netea MG, Folkerts G, Kraneveld AD, Garssen J, Vos AP. TLR2 & Co: a critical analysis of the complex interactions between TLR2 and coreceptors. J Leukoc Biol 2013;94:885–902.
57. Visintin A, Latz E, Monks BG, Espevik T, Golenbock DT. Lysines 128 and 132 enable lipopolysaccharide binding to MD-2, leading to Toll-like receptor-4 aggregation and signal transduction. J Biol Chem 2003;278:48313–20.
58. Murray NR, Davidson LA, Chapkin RS, Clay Gustafson W, Schattenberg DG, Fields AP. Overexpression of protein kinase C betaII induces colonic hyperproliferation and increased sensitivity to colon carcinogenesis. J Cell Biol 1999;145:699–711.
59. Agbor TA, Demma ZC, Mumy KL, Bien JD, McCormick BA. The ERM protein, ezrin, regulates neutrophil transmigration by modulating the apical localization of MRP2 in response to the SipA effector protein during *Salmonella typhimurium* infection. Cell Microbiol 2011; 13:2007–21.
60. Koo HN, Hong SH, Seo HG, Yoo TS, Lee KN, Kim NS, Kim CH, Kim HM. Inulin stimulates NO synthesis via activation of PKC-alpha and protein tyrosine kinase, resulting in the activation of NF-kappaB by IFN-gamma-primed RAW 264.7 cells. J Nutr Biochem 2003;14:598–605.
61. Farhadi A, Keshavarzian A, Ranjbaran Z, Fields JZ, Banan A. The role of protein kinase C isoforms in modulating injury and repair of the intestinal barrier. J Pharmacol Exp Ther 2006;316:1–7.
62. Pajari AM, Oikarinen S, Grasten S, Mutanen M. Diets enriched with cereal brans or inulin modulate protein kinase C activity and isozyme expression in rat colonic mucosa. Br J Nutr 2000;84:635–43.
63. Mochly-Rosen D, Das K, Grimes KV. Protein kinase C, an elusive therapeutic target? Nat Rev Drug Discov 2012;11:937–57.
64. Coussens L, Parker PJ, Rhee L, Yang-Feng TL, Chen E, Waterfield MD, Francke U, Ullrich A. Multiple, distinct forms of bovine and human protein kinase C suggest diversity in cellular signaling pathways. Science 1986;233:859–66.
65. Ono Y, Fujii T, Ogita K, Kikkawa U, Igarashi K, Nishizuka Y. The structure, expression, and properties of additional members of the protein kinase C family. J Biol Chem 1988;263:6927–32.
66. Ono Y, Kikkawa U, Ogita K, Fujii T, Kurokawa T, Asaoka Y, Sekiguchi K, Ase K, Igarashi K, Nishizuka Y. Expression and properties of two types of protein kinase C: alternative splicing from a single gene. Science 1987;236:1116–20.
67. Parker PJ, Coussens L, Totty N, Rhee L, Young S, Chen E, Stabel S, Waterfield MD, Ullrich A. The complete primary structure of protein kinase C–the major phorbol ester receptor. Science 1986;233:853–9.
68. Song JC, Hanson CM, Tsai V, Farokhzad OC, Lotz M, Matthews JB. Regulation of epithelial transport and barrier function by distinct protein kinase C isoforms. Am J Physiol Cell Physiol 2001;281:C649–61.
69. Langlet C, Springael C, Johnson J, Thomas S, Flamand V, Leitges M, Goldman M, Aksoy E, Willems F. PKC-alpha controls MYD88-dependent TLR/IL-1R signaling and cytokine production in mouse and human dendritic cells. Eur J Immunol 2010;40:505–15.