

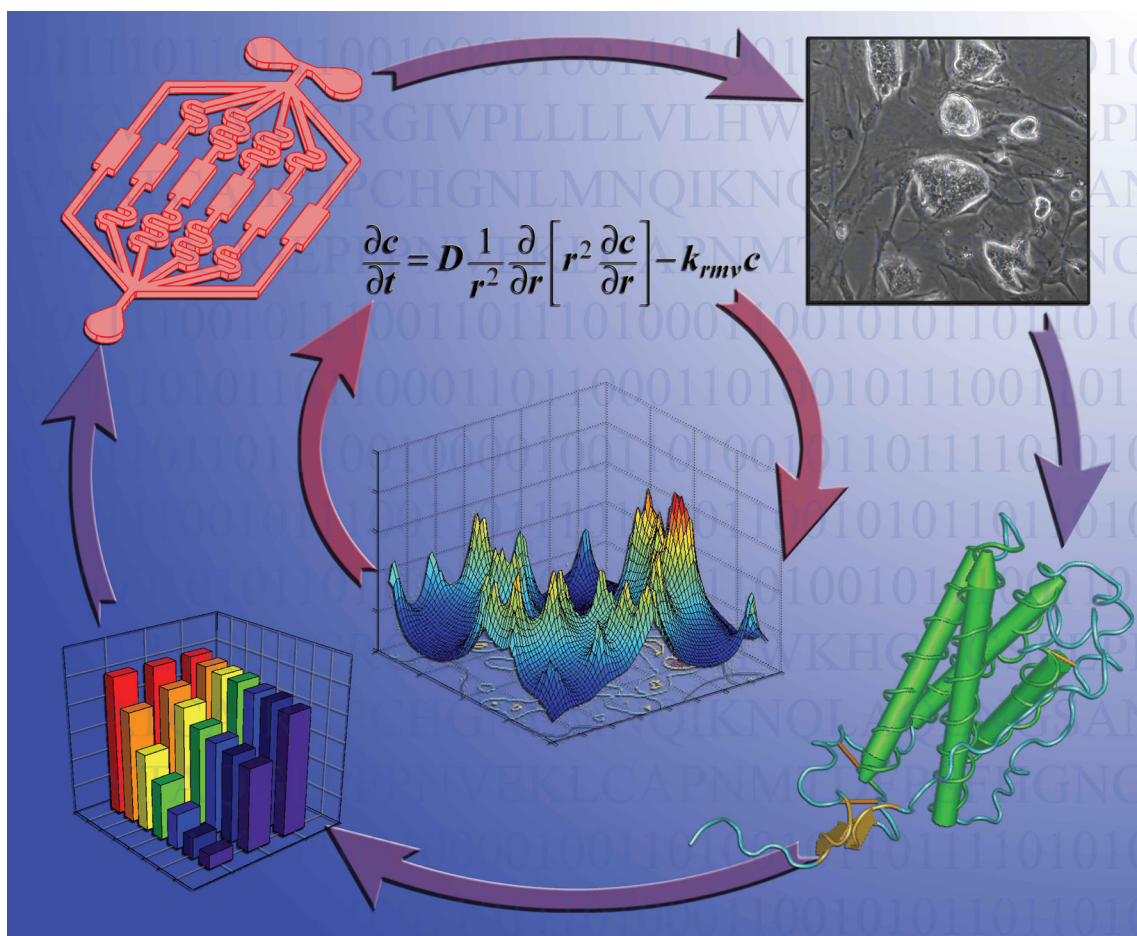
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Construction of biosensor systems for determining the pathophysiological potential of carrageenan variants†

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In vitro systems for monitoring safety of nutritional additives are desirable for high-throughput screenings and as a substitute for animal models. Carrageenan (CGN) is a sulfated polysaccharide widely used as a thickener and texturizer in human nutrition and is intensely discussed regarding its pathophysiological potential. Low molecular weight (lm) variants of CGN are considered to exert more profound pathophysiological effects *in vivo* than high molecular weight (hm) variants. We used a systematic approach to construct reporter systems allowing distinction between CGN-variants with different pathophysiological potential. Reporter systems utilizing segments of the CGN-responsive *DMBT1* promoter did not display substantial activity in SW620 cells of intestinal epithelial origin. Genome-wide profiling revealed stronger qualitative and quantitative changes in global gene activities for hm-CGN than for lm-CGN (824 versus 91 genes; −6.64 to 22.33-fold for hm-CGN versus the range of −2.65 to 2.96-fold for lm-CGN). Reporter systems with segments of the *IL-8* promoter showed a specific activation in response to hm-sulfated polysaccharides with lower pathophysiological potential *in vivo* and provided a better classification of CGN-variants than cytotoxicity assays *in vitro*. *IL-8* reporter systems can be used for discerning between the effects of sulfated polysaccharides *in vivo*. Our data further provide initial insights into the molecular mechanisms that may play a role in the different effects of CGN-variants.

Introduction

Supplementation of human nutritional products with synthetic and natural additives has steadily increased over the past decades. Negative effects on human health of additives primarily regarded as safe may occur with a lag of several decades and commonly are difficult to trace back. The intestinal epithelium is particularly affected by harmful nutritional compounds. Immediate exposure to detrimental agents affects the healthy state equilibrium in the regulation of a delicate balance in regenerative processes and between pathogen defence and a functioning endogenous micro flora.

Carrageenan (CGN), is a sulfated polygalactan extracted from red seaweed, which is widely used as a thickener,

stabilizer and texturizer in human nutritional products and well known for its ability to substitute fat in dietary products and solubilize milk proteins in dairy products. In general, *in vivo* studies have demonstrated that low molecular weight carrageenan (lm-CGN; $M_r < 100$ kDa) exerts more profound effects than high molecular weight carrageenan (hm-CGN; $M_r > 100$ kDa), resulting in more severe epithelial damage of the intestines resulting in ulcerations, inflammation, and the formation of colorectal cancer.¹

In vitro, CGN may confer activation of NF- κ B-signalling through interaction with the pattern recognition receptor Toll-like receptor 4 (TLR4) in intestinal epithelial cells.² *In vivo* CGN closely mimics the effects of a related sulfated polysaccharide, e.g. dextran sodium sulfate (DSS). Administration of DSS to mice is one of the most commonly used model systems to investigate intestinal acute and chronic inflammation and to study inflammation-associated carcinogenesis.^{3,4} DSS in low molecular weight form (lm-DSS) is also known to exert a greater pathophysiological potential *in vivo* than its high molecular weight variants (hm-DSS).⁵

We recently likewise gained hints that CGN may interfere with several central molecular processes. The human scavenger receptor cysteine-rich (SRCR) protein deleted in malignant brain tumours 1 (DMBT1) regulates aspects of the inflammatory cascade at the intestinal epithelium. DMBT1 functions as a negative regulator of TLR4 and nucleotide oligomerization

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domain 2 (NOD2)-mediated NF- κ B-activation by interaction with bacterial lipopolysaccharide and inhibition of bacterial invasion into intestinal epithelial cells.⁶ Genetic polymorphisms reducing the number of bacterial binding sites within DMBT1 confer an increased risk of the chronic inflammatory disorder Crohn's disease.⁷ DMBT1 functions as a pattern recognition receptor for polysulfated and polyphosphorylated structures and CGN as well as DSS inhibit its bacterial binding activity through direct interaction with the pathogen-binding sites.⁸ Beyond that, DMBT1 has been shown to be responsive to a broad spectrum of pathophysiological stimuli *in vitro* and *in vivo*, including exposure to bacterial lipopolysaccharide (LPS), colonization of germ-free mice by the endogenous micro flora, DSS, heme, and copper ions.^{6,7,9,10,11} We hypothesized that the *DMBT1* promoter or promoters of genes with similar response profiles can be used to construct reporter systems to monitor the pathophysiological potential of certain agents. Here, we evaluated this approach using hm- and lm-CGN with low and high pathophysiological potential *in vivo* as a test system. While the *DMBT1* promoter lacked corresponding activity when dissected out of the genomic context, *IL-8* promoter segments displayed an activity profile that corresponded well with the known *in vivo* effects of lm- and hm-CGN and DSS.

Results

Identification of carrageenan responsive cells

Utilization of a reporter system with incorporated *DMBT1*-promoter requires that the basal transcriptional machinery and the signalling cascades are active within the cellular background of choice. We initially tested, by qRT-PCR, the responsiveness of a panel of cell lines of intestinal epithelial origin for *DMBT1* up regulation after challenge with CGN. Of the cell lines tested, (HCT-116, HT29, SW480, and SW620) only SW620 cells showed a substantial response to lm- and hm-CGN (Fig. 1) The *DMBT1* promoter is responsive to lipopolysaccharide (LPS)-triggered TLR4-mediated NF- κ B activation.⁶ Bhattacharyya and co-workers proposed that carrageenan triggers NF- κ B-activation *via* TLR4.² In the case of undetectable or low *TLR4* expression, supplementation of the cells with a *TLR4* expression plasmid could have been a conceivable strategy for increasing the responsiveness. Hence, we next quantified the *TLR4* expression levels by qRT-PCR in selected cell lines. However, these data showed that the non-responsive HT29 and SW480 cells displayed substantially higher *TLR4* mRNA levels compared to the responsive SW620 cells (Fig. 1B). Thus, CGN responsiveness seemed to be inversely correlated with *TLR4* expression levels. Time-course studies of *DMBT1* expression levels in the responsive SW620 cells revealed strong induction at 24 h post incubation with 100 $\mu\text{g ml}^{-1}$ hm-CGN. At this point, lm-CGN also resulted in a detectable *DMBT1* up regulation at a concentration of both 10 and 100 $\mu\text{g ml}^{-1}$, although the response was lower compared to hm-CGN (Fig. 1C). In summary, SW620 cells could be used for construction of a cellular reporter system.

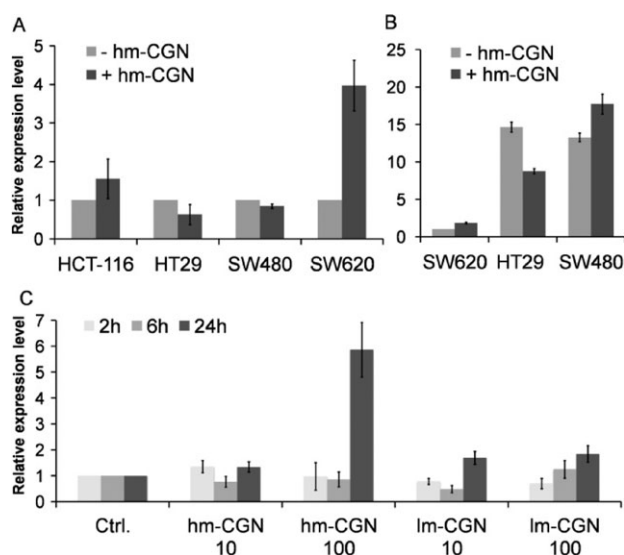


Fig. 1 Identification of CGN-responsive cells. (A) Relative *DMBT1* expression levels in intestinal epithelial cell lines exposed to hm-CGN (+hm-CGN; 24 h; $c = 100 \mu\text{g ml}^{-1}$ hm-CGN) or to an equivalent volume of the solvent in the medium (H_2O ; -hm-CGN). (B) *TLR4* expression levels in selected cell lines. Expression levels were calculated to the lowest expression measured in SW620 (-hm-CGN). Responsive SW620 cells display the lowest levels of *TLR4* mRNA. (C) Relative expression levels of *DMBT1* in SW620 cells at 2, 6 and 24 h after exposure to different concentrations of hm- and lm-CGN. Cells incubated with water served as controls (Ctrl). Highest levels can be observed after 24 h exposure to 100 $\mu\text{g ml}^{-1}$ hm-CGN, while only a moderate response is obtained when using lm-CGN.

Carrageenan responsiveness of the *DMBT1* promoter requires the genomic context

We next cloned *DMBT1* promoter segments of 1, 2, and 3 kb upstream of the TATA-box into the luciferase vector system pGL4.23[*luc2*/minP] (Promega) to identify regions that could be responsive to carrageenan. The co-transfected reporter pGL4.73[*hRluc*/SV40] was used to normalize for variable transfection efficacies. In SW620 cells none of the *DMBT1* promoter constructs displayed evidence for activation either upon hm- or upon lm-CGN exposure (both at 100 $\mu\text{g ml}^{-1}$; data not shown). This suggested that the *DMBT1* promoter activity may require further elements present within the genomic context and is thus not covered by the segments used for construction of the promoter constructs.

Systematic identification of carrageenan-responsive genes

In order to systematically identify CGN-responsive genes, of which the promoters could alternatively be used, we performed genome-wide expression profiling of SW620 cells incubated for 24 h with 100 $\mu\text{g ml}^{-1}$ hm- or lm-CGN *versus* non-treated control cells using Sentrix Human-6 BeadChips (Illumina). We considered genes with > 1.5 -fold changes and with $P \leq 0.01$ for further analyses. Remarkably, the lm-CGN resulted in a substantially lower number of significantly deregulated genes (91 *versus* 824 for hm-CGN) and the fold changes of significantly deregulated genes were substantially lower than for hm-CGN (range -2.65 to 2.96-fold for lm-CGN *versus* -6.64 to 22.33-fold for hm-CGN; the top

Table 1 Selected genes with differential expression after CGN exposure

Top 20 genes with highest positive fold change				lm-CGN specific genes		
hm-CGN			lm-CGN			
	Gene	FC	Gene	FC	Gene	FC
1	<i>PLAU</i>	22.33	<i>IL8</i>	2.96	<i>INHBE</i>	1.82
2	<i>IL8</i>	19.95	<i>DUSP5</i>	2.53	<i>RGS16</i>	1.78
3	<i>PLAUR</i>	14.39	<i>PLAU</i>	2.46	<i>LARP2</i>	1.73
4	<i>DUSP5</i>	11.82	<i>GDF15</i>	2.04	<i>NFXL1</i>	1.72
5	<i>ISG20</i>	11.50	<i>ERRFI1</i>	2.03	<i>MOCOS</i>	1.68
6	<i>CDKN1A</i>	10.65	<i>AKAP12</i>	2.00	<i>HIST2H2BE</i>	1.67
7	<i>AKAP12</i>	10.50	<i>MAP1LC3B</i>	1.98	<i>ZC3H8</i>	1.58
8	<i>LAMC2</i>	9.87	<i>TRIB3</i>	1.98	<i>ZNF569</i>	1.58
9	<i>IL23A</i>	8.48	<i>EMP1</i>	1.91	<i>L3MBTL3</i>	1.55
10	<i>KRT81</i>	7.96	<i>LDLR</i>	1.89	<i>BXDC1</i>	1.54
11	<i>SDC4</i>	6.99	<i>TNFRSF12A</i>	1.88	<i>ARL5B</i>	1.53
12	<i>ETS1</i>	6.91	<i>PLAUR</i>	1.86	<i>WDR33</i>	1.51
13	<i>CSF2</i>	6.74	<i>RBCK1</i>	1.86	<i>FKBP14</i>	1.51
14	<i>GJB3</i>	6.69	<i>BHLHB2</i>	1.86	<i>PHLDB1</i>	−1.50
15	<i>SDCBP2</i>	6.60	<i>KRT81</i>	1.85	<i>BRD8</i>	−1.56
16	<i>CCL20</i>	6.58	<i>HBEGF</i>	1.85	<i>FAM79A</i>	−1.57
17	<i>LAMA3</i>	6.11	<i>IL20RB</i>	1.84	<i>C1orf85</i>	−1.73
18	<i>EMP1</i>	5.99	<i>JAG1</i>	1.83	<i>CD24</i>	−1.93
19	<i>KRT17</i>	5.54	<i>MAG1</i>	1.83	<i>SPANXC</i>	−2.16
20	<i>C13orf15</i>	5.27	<i>INHBE</i>	1.82		

20 genes with highest positive fold changes are presented in Table 1). 72 of the 91 genes (79%) responsive to lm-CGN were also found among the hm-CGN responsive genes. Of the 19 genes that were uniquely deregulated in response to lm-CGN, *INHBE* (inhibin) displayed the highest positive fold change (1.82-fold; Table 1).

A comparative GO annotation of genes found to be differentially regulated in response to hm-CGN versus lm-CGN was performed (see Experimental section). Significantly different prominent categories (e.g. genes scoring in a given category or not at all) were extracted for biological process annotation. Roughly 20% of the hm-CGN-responsive genes fell into the two categories “Cell cycle progression” and “DNA-repair”, with no significant number scoring (below 1%) in processes related to cell death. By contrast, 20% of lm-CGN responsive genes fell into the biological process categories “Cell death” and “Angiogenesis”. Of the 70 genes overlapping between the two datasets, 83% were annotated to be associated with NF- κ B signalling. Likewise, 53% of the genes found to be unique for lm-CGN were also annotated to be associated with NF- κ B signalling.

Continuing with *IL-8* as one of the genes with most pronounced up regulation, we identified SW620 cells as the cell line with the strongest responsiveness at the level of *IL-8* induction (Fig. 2A). Induction peaked at 24 h and was substantially higher when using hm-CGN compared to lm-CGN (Fig. 2B), which was in accordance with the data obtained from the expression profiling.

Construction of a carrageenan-responsive reporter system

We consecutively used promoter segments of 1, 2, and 3 kb upstream of the *IL-8* TATA-box to construct a new set of reporter constructs in the luciferase vector system. After transient transfection into SW620 cells, all three constructs displayed equivalent inducibility, more pronounced with

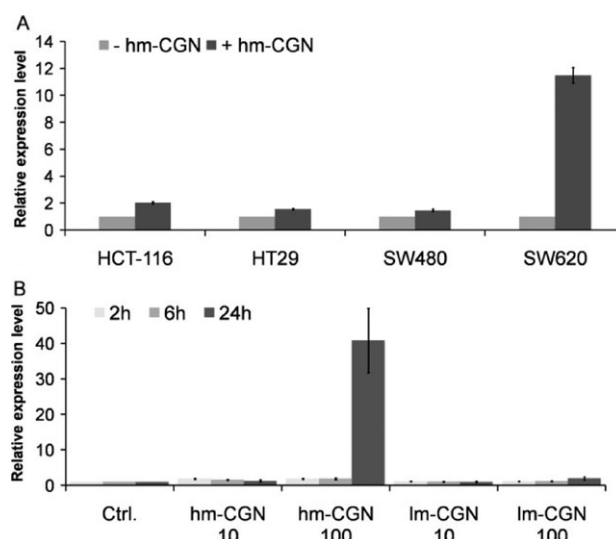


Fig. 2 *IL-8* expression in response to CGN exposure. (A) relative expression level of *IL-8* in different cell lines after 24 h of stimulation with 100 $\mu\text{g ml}^{-1}$ hm-CGN in the medium (+hm-CGN) versus matched controls (−hm-CGN; incubated with equivalent volume of H_2O in the medium). (B) Relative expression level of *IL-8* in SW620 cells, at 2, 6 and 24 h after exposure to different concentrations of hm- and lm-CGN in the medium. Cells incubated with water served as controls (Ctrl).

hm- compared to lm-CGN (Fig. 3), as observed for the natural *IL-8* promoter.

The *IL-8* reporter system is specifically responsive to high molecular weight sulfated polysaccharides

We next tested the responsiveness of the reporter system that was based on the *IL-8* 1kb-promoter segment to a broader range of agents and compared the readout to a commercially available, optimized NF- κ B reporter system. The agents that

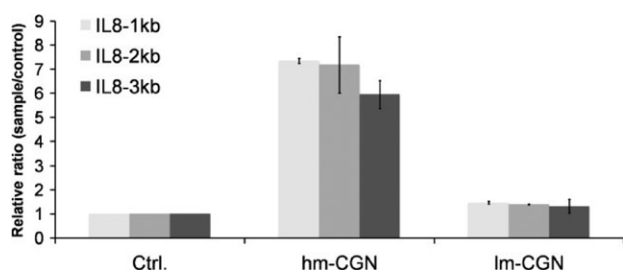


Fig. 3 Relative activation of the *IL-8* promoter luciferase reporter constructs after exposure of SW620 cells to 100 $\mu\text{g ml}^{-1}$ hm- and lm-CGN.

were tested included hm- and lm-dextran sodium sulfate (DSS), which are structurally similar to hm- and lm-CGN and exert similar effects in mouse models, as well as dextran, which is the non-sulfated counterpart of DSS. We further included several agents that represent natural food components (heme, fucose, amylopectin), nutritional additives (alginate) and/or which have been suspected to exert unfavourable effects on health and shown to result in molecular changes in the intestinal epithelium *in vivo* (heme, copper ions).^{10,11} Both reporter systems displayed a similar qualitative induction profile, responding specifically to high molecular weight sulfated carbohydrates but not to other agents. The *IL-8* reporter system, however, delivered two-fold higher readouts in response to hm-CGN and hm-DSS (> 100 kDa) compared to the commercially available optimized NF- κ B reporter system (Fig. 4).

The responsiveness of the *IL-8* reporter system is superior to cytotoxicity assays *in vitro*

CGN and DSS have previously been shown to affect cell viability *in vitro*,^{8,12} raising the possibility that a cytotoxicity assay could be used to test the pathophysiological potential of such agents. We finally tested whether a cytotoxicity assay in SW620 cells *in vitro* would be informative with regard to the pathophysiological potential of the sulfated polysaccharides

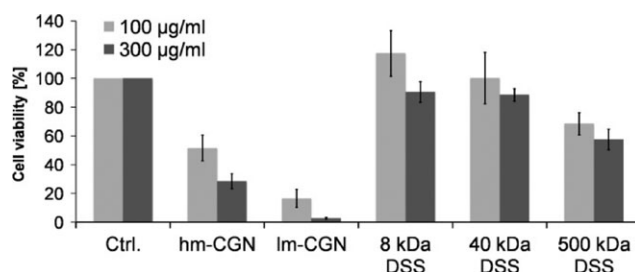


Fig. 5 Viability of SW620 cells after exposure to different concentrations (100 $\mu\text{g ml}^{-1}$ and 300 $\mu\text{g ml}^{-1}$) of hm- and lm-CGN and -DSS after 5 days of incubation.

in vivo. Cell viability was measured after 5 days exposure to hm- and lm-CGN and different variants of DSS at concentrations of 100 and 300 $\mu\text{g ml}^{-1}$. These data indicated that lm-CGN resulted in a more pronounced cytotoxicity compared to hm-CGN (Fig. 5), which is in accordance with observed *in vivo* effects. By contrast, hm-DSS exerted stronger effects on cell viability than lm-DSS, inverse to what is observed *in vivo* and DSS had an overall smaller effect on cell viability compared to CGN. Thus, the *IL-8* reporter system delivers a readout that is superior to a classical cytotoxicity assay, because it resembles more closely the *in vivo* situation and allows for a sharper discrimination between variants with low and high pathophysiological potential.

Discussion

Recently, studies utilizing well-defined *in vivo* models showed that nutritional compounds considered to exert pathophysiological potential may result in differential regulation of large gene sets within intestinal lining cells. This raises the question as to whether the regulatory elements of such genes can be utilized for the construction of biosensors allowing for the identification of potentially harmful nutritional compounds *ex vivo*. Carrageenan (CGN) was offered as a test compound, since it is widely used in human nutritional products, and

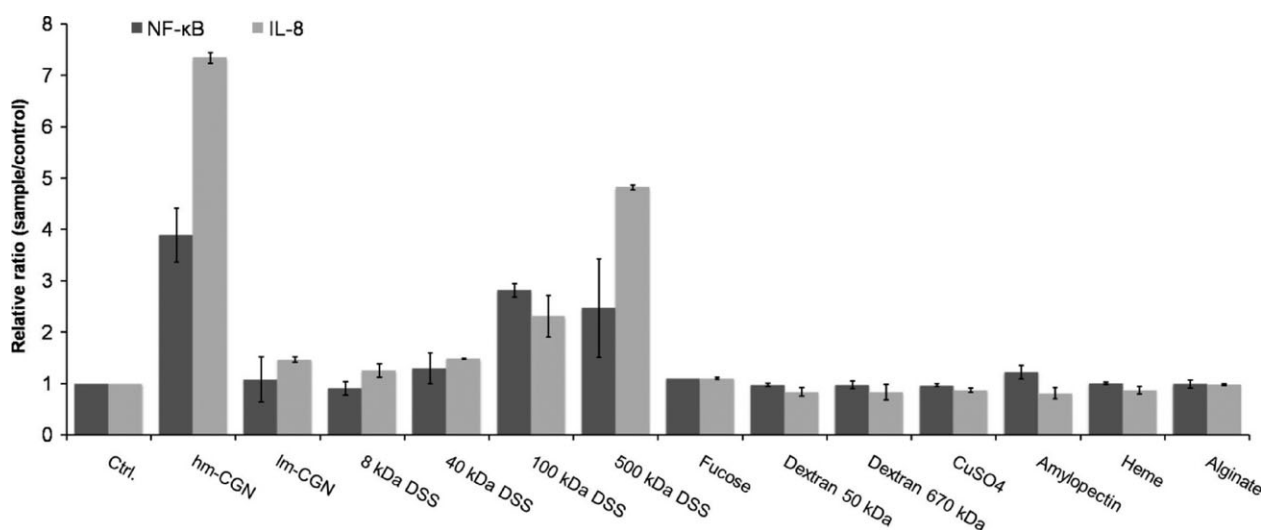


Fig. 4 Relative activation of the NF- κ B and *IL-8* promoter luciferase reporter constructs after exposure of SW620 cells to different typical food substances.

comprehensive *in vivo* data from rodent models exist, which indicate that the pathophysiological potential of CGN increases with decreasing molecular weight.

DMBT1 was reported to be up regulated in the intestinal epithelium in response to various stimuli, including exposure to DSS in mice and to heme in rats.^{7,10} Feeding a copper-deficient diet to rats resulted in down regulation of *dmbl1* expression levels in the intestine,¹¹ suggesting *vice versa* that copper ions up regulate the *dmbl1* levels. We found that *DMBT1* expression is up regulated in the human cell line SW620, which is of intestinal epithelial origin, in response to CGN exposure. With a 4- to 6-fold up regulation, SW620 cells manifested the order of magnitude observed within *in vivo* models for the related DSS.^{7,8}

Among the cell lines tested, only SW620 was responsive to CGN with regards to both *DMBT1* and *IL-8* up regulation. Bhattacharyya and co-workers provided evidence that in normal intestinal epithelial cells TLR4 is involved in recognition of CGN in the cells, which consecutively results in NF- κ B-activation.² Our study indicated that the *TLR4* expression levels were inversely correlated with responsiveness to CGN. HT29 and SW480 cells with higher *TLR4* mRNA levels were not responsive, while the responsive SW620 cells displayed the lowest expression levels. A difference is that we used cancer cell lines, hence it cannot be ruled out that certain components of the signalling cascade downstream of *TLR4* are inactive or that other receptors for CGN are expressed. Nonetheless, carrageenan exposure finally resulted, in SW620 cells, in an activation of NF- κ B signalling as demonstrated by the readouts obtained from the NF- κ B reporter system and by the expression profiling data.

We used three different segments of the *DMBT1* promoter to construct a reporter system, because both activatory and inhibitory elements have been identified in the *DMBT1* promoter.¹³ All three segments contained an NF- κ B responsive site that locates close to the TATA box and confers LPS-inducibility of the *DMBT1* promoter in HT29 cells.⁶ However, none of these segments displayed substantial activity towards CGN exposure, indicating that further regulatory elements outside the region covered by our constructs may play an important role for this particular promoter.

Because such regulatory regions may be located up to several hundreds of kb apart from the promoter, we performed a systematic screen to identify alternative CGN-inducible genes. According to the microarray data, *IL-8* was one of most strongly up regulated genes in response to both hm- and lm-CGN, which is in accordance with previous data pointing to an up regulation of *IL-8* after carrageenan exposure in normal intestinal epithelial cells.¹⁴ *IL-8* promoter-reporter gene constructs retained the ability to respond to hm-CGN, but also responded to DSS variants with 100 and 500 kDa, while no response was observed upon exposure of the cells to lm-CGN, lm-DSS, non-sulfated high molecular weight polysaccharides or other agents. With this response profile, the *IL-8* reporter system showed an improved correlation with *in vivo* effects of sulfated polysaccharides compared to a cytotoxicity assay in SW620 cells, displaying less pronounced discrimination and—for the DSS variants—an inverse pattern. While lm-CGN is considered to have a

stronger pathophysiological potential *in vivo* than hm-CGN, it resulted not only in less substantial up regulation of both *IL-8* and *DMBT1* in SW620 cells, but also induced fewer global changes in the gene expression patterns. In total, 91 genes were found to be significantly deregulated after exposure to lm-CGN compared to 824 genes after exposure to hm-CGN. In addition, fold change ranges were clearly more modest than compared to hm-CGN, and 79% of the genes displayed overlapping deregulation in both experiments. Remarkably, a substantial set of both the overlapping genes and the genes specifically deregulated in response to lm-CGN were annotated bioinformatically to associate with NF- κ B signalling. This raises the possibility that the differential pathophysiological potential of CGN variants could rely on a combination of two molecular mechanisms. Low molecular weight variants could exert more deleterious effects by triggering a less pronounced protective response and by unfavourable modulation of NF- κ B signalling in intestinal epithelial cells.

In summary, we have demonstrated that an *IL-8* promoter-reporter system in SW620 cells can distinguish between lm- and hm-variants of CGN as well as another sulfated polysaccharide, *e.g.* DSS, which exerts differential pathophysiological effects *in vivo*. The reporter system specifically responds to hm-variants with less pathophysiological potential *in vivo* and displays the same response profile as a commercially available NF- κ B reporter system, but delivers higher readouts with hm-variants. A more precise definition of the responsive sites may deliver starting points to further improve the sensitivity and signal strength of the *IL-8* reporter constructs. Beyond that our data may deliver novel insight into the molecular mechanisms underlying the differential effects of CGN on intestinal health and disease.

Experimental

Cell culture and carrageenan exposure

Cell lines HT29, HCT-116, SW480 and SW620 were obtained from the American Type Culture Collection (ATCC). HT29 and HCT-116 cells were cultivated in McCoy 5A medium. SW480 and SW620 cells in RPMI 1640. All cell lines were cultured with 10% FCS, 2 mM L-glutamine, 100 U ml⁻¹ penicillin and 100 μ g ml⁻¹ streptomycin at 37 °C in a humidified, 5% CO₂ environment. Cells were seeded in 12-well plates at 1 $\times$ 10⁶ cells per well, then treated the following day with CGN for 24 h in standard settings and for 2, 6, and 24 h in time course studies. High molecular weight λ -CGN (1–8 $\times$ 10⁵ Da) was obtained from Fluka. Low molecular weight λ -CGN (lm-CGN) was obtained by mild acid hydrolysis of hm-CGN by the method for sulfated fucans (incubation with 0.01 M HCl at 60 °C for 3 h), which yields carrageenan of an approximate molecular weight of 2 $\times$ 10⁴–1 $\times$ 10⁵ Da.¹⁵ After neutralization with 0.1 M NaOH the lm-CGN was dissolved in sterile ddH₂O to a final concentration of 5 mg ml⁻¹ and the molecular weight was monitored by PAGE. The sulfated polysaccharides were stained with 0.1% toluidine blue in 1% acetic acid and washed for 4 h in 1% acetic acid. The molecular weight was then

confirmed by comparison with the 100 kDa high molecular weight band of non-hydrolyzed λ -CGN and the 8 kDa band of low molecular weight DSS. Untreated control samples of each cell line were treated with the same volume of double-distilled water (ddH₂O) in the culture medium.

Quantitative RT-PCR

Total RNA was isolated using the RNeasy kit from QIAGEN, according to manufacturer's protocol. The RNA (500 ng total RNA) was then reversely transcribed into cDNA using oligo-dT priming. For qRT-PCR we used 10 ng cDNA and TaqMan assays on-demand for the respective genes (Applied Biosystems) following the supplier's instructions. All TaqMan reactions were done in triplicate and signal detection was performed by an ABI Prism 7900 HT sequence detection system (Applied Biosystems). The cycle thresholds (C_t) were normalized against the C_t values obtained for the human house keeping genes β -actin and GAPDH. The $\Delta\Delta C_t$ value was calculated based on the ΔC_t values of the control. The expression level was then calculated as X_L (expression level) = $2^{-\Delta\Delta C_t}$. Analyses were made by the SDS 2.2 program and the RT-PCR-script for R (provided by Markus Ruschhaupt, Molecular Genome Analyses, DKFZ Heidelberg, Germany).

Cloning and analysis of reporter constructs

For promoter studies we amplified 1, 2, and 3 kb products comprising the respective promoter sequences upstream of the TATA-box from DNA isolated from the SW620 cell line. The PCR-product was cloned into the pGL4.23 plasmid (Promega) in front of a firefly luciferase reporter gene. For NF- κ B analyses the plasmid NF- κ B Luc (Stratagene) was used. Firefly luciferase activity was normalized against *Renilla* activity under control of a SV40 constitutive promoter on a separate plasmid (pGL4.73, Promega). Transfection was performed using Effectene transfection reagent (QIAGEN) according to the manufacturer's protocol. We seeded SW620 cells into 12-well plates at a density of 0.5×10^6 cells per well. After 24 h, cells were transfected with 0.75 μ g DNA, which was a 1 : 10 mixture of control to experimental plasmid. After 72 h, the medium was replaced by a medium containing 100 μ g ml⁻¹ of hm-CGN (Fluka), lm-CGN, 8 kDa DSS (Sigma), 40 kDa DSS (MP-Biomedicals), 100 kDa DSS (Sigma), 500 kDa DSS (Sigma), fucose (Sigma), dextran 50 kDa (Fluka), dextran 670 kDa (Fluka), amylopectin (Fluka), or alginate (Sigma) or CuSO₄ (161 μ g ml⁻¹; Sigma) or heme (49 mg ml⁻¹, hemin, Sigma). After 96 h, the fluorescent Dual-Glo luciferase assay (Promega) was performed according to the provider's instructions. Relative luciferase activities were measured by the Fluorometer Fusion (Packard Biosciences). Relative ratios were calculated by dividing the RLUs (relative light units), after normalization, of the experimental conditions by the RLUs of the control conditions. Experiments were made in duplicate and standard deviations were calculated.

Cell viability assays

The cell viability was measured by using a commercially available CellTiter-Blue Cell viability assay (Promega) according to the manufacturer's instructions. Cells were

seeded in 96-well plates at a density of 4000 cells per well, in triplicate. Carrageenan was added at final concentrations of 100 μ g ml⁻¹ and 300 μ g ml⁻¹. Control cells were treated with the same volume of water. After 5 days 20 μ l of CellTiter-Blue reagent was added and the fluorescence measured after 3 h of incorporation.

Expression profiling

Total RNA of treated and control cells, from three independent experiments was reversely transcribed into cDNA. Tagged cRNA was then transcribed *in vitro* in the presence of biotin-labelled uracil bases. This cRNA was hybridized on an Illumina human Sentrix-6 V2 Bead Chip. After several washing steps the chip was incubated with fluorescent streptavidin molecules and the fluorescent signal was quantified. Measured values were analyzed through a variance stabilization transformation method.^{16,17} The data were then normalized with the rsn (robust spline normalization).^{16,17} For the two groups (hm-CGN and lm-CGN) with three independent replicates each a modified t-test was performed with the LIMMA software to identify genes with significant deregulation compared to the control cells.^{18,19} For the resulting *p*-values a multiple testing correction after Benjamini and Hochberg, as it is integrated in LIMMA, was performed. Genes with a *p*-value smaller than 0.01 and more than +1.5 and -1.5 fold changes were chosen for further analyses.

Bioinformatics

We used the Babelomics application at <http://bioinfo.cipf.es> to annotate genes to biological processes and transcription factor association. The two-list comparison was undertaken applying comparative FatiGO-annotation scans. The species chosen for parsing was *Homo sapiens*. The search setting for removal of duplicates in the lists was engaged and a two tailed test (Fischer exact test) was employed. An assignment to a particular category was accepted only at *p*-values lower than 0.0001.

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