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Molecular mechanism of action responsible for carrageenan-induced inflammatory response☆☆

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ABSTRACT

Carrageenan–induced inflammation has long been used as an *in vivo* model of local inflammation. We developed an *in vitro* model of inflammation using primary blood cells to characterize gene induction following carrageenan (λ-CGN) stimulation and identify the signal transduction pathway(s) through which λ-CGN worked, using swine whole blood cultures from Yorkshire barrows. Blood samples were divided into stimulated and unstimulated groups. Unstimulated blood was a control for λ-CGN treated cultures to delineate treatment effects from time-in-culture effects. All cultures were collected and separated into two fractions; supernatant for ELISA analyses and white blood cells for mRNA expression. Lambda (λ)-CGN induced MCP-1 at the proteomic and the genomic levels. Lambda-CGN increased IL-8 protein production but had no impact on serum amyloid A protein levels. Alveolar Macrophage-Derived Neutrophil Chemotactic Factor-II (AMCF-II), a swine-specific member of the IL8/GRO family, showed increased gene expression. TNF-α and IL-6 protein levels were not induced by λ-CGN stimulation. Stimulation of HEK-293 cells co-transfected with a single pattern recognition receptor and the secreted embryonic alkaline phosphatase (SEAP) read-out system demonstrated that λ-CGN signals through the TLR-2 and TLR-4 signal transduction pathways. Using silencing RNA to inhibit TLR6 expression in TLR2 transfected HEK-293 cells indicated that λ-CGN works through the TLR2/6 pathway. Silencing TLR6 expression in TLR4 transfected HEK-293 cells showed that λ-CGN stimulation of this cell line worked through a TLR4/6 heterodimer, as lipopolysaccharide (LPS) induced SEAP production through a TLR4 homodimer. These results demonstrate that although carrageenan can stimulate through TLR4 signaling pathways, it initiates an inflammatory response in these cells that differs from a typical endotoxin effect such as LPS stimulation, in terms of the pathways and gene products altered, suggesting that activation of TLR2/6 and TLR4/6 are the predominant pathways through which carrageenan induces inflammatory responses.

1. Introduction

Carrageenans are a family of polysaccharides with a long history of use as food additives, personal healthcare products and local inflammation models. There are three forms of carrageenan; the lambda, iota and kappa forms. Lambda-CGN (CGN) is the form that has historically been used as the stimulant for in vivo model of a local inflammatory response (Thomson and Fowler, 1981). In vivo administration of carrageenan in rats, ruminants (cattle & goats), horses, and swine causes localized edema, infiltration of white blood cells, and increased levels of local PGE2 (Clarke, 1989; Fulgenzi et al., 2005; Higgens and Lees, 1984; Hunter, 2002; Sidhu et al., 2003, 2010; Vazquez et al., 2015). This inflammation model in food animals has typically employed a tissue cage into which the carrageenan is injected to simulate a local inflammatory response. However, this swine inflammation model has not been replicated in other laboratories, suggesting a different model of local inflammation may be needed. Integral to the search for a new model (and possibly a new agent inducing local inflammation) is an understanding of the molecular mechanisms of inflammation initiated by carrageenan.

Several studies have shown that CGN exposure predictably induces an increase in interleukin-8 (IL-8) secretion in cells in tissue culture and interferes with macrophage activity. Mechanisms of CGN-induced IL-8 activation require nuclear localization of nuclear factor κB (NFκB) and

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proceed by at least two different pathways: a toll-like receptor 4 (TLR4)–B-cell lymphoma/leukemia 10-mediated pathway and a reactive oxygen species-mediated pathway (Bhattacharyya et al., 2008). Initiation of a local inflammatory response mediated through the TLR4 pathway would suggest similarities to induction by another well-known model of inflammation; namely E. coli-derived lipopolysaccharide (LPS). However, the absence of direct stimulation of TNFα (Ogata et al., 1999), a TLR4 pathway-initiated event, following CGN exposure suggests other signal pathways may be involved. The goals of this study were to 1) identify genomic and proteomic biomarkers associated with CGN-induced inflammation to determine if there are biomarkers in common with LPS-mediated inflammatory biomarkers, and 2) identify the molecular signal pathway(s) responsible for transducing the CGN stimulus.

2. Materials & methods

2.1. Blood collection and treatment

Blood from nine Yorkshire barrows was obtained from Bioreclamation Inc. (Hicksville, NY). Blood was diluted at a 1:1 ratio of Ultraculture media (Lonza, Walkersville, MD) supplemented with L-glutamine (20 mM), gentamicin (50ug/ml), HEPES (10 mM), sodium bicarbonate (0.075%), and amphotericin B (0.25ug/mL). Approximately 15 ml of blood was added to each flask (Nalgene-Nunc). Each blood sample was separated into two fractions; one for serum collection for ELISA analyses, and one for RNA extraction. The whole blood cultures were stimulated with lambda carrageenan (Sigma-Aldrich) at a concentration of 1 μg/ml, with samples collected at 0, 0.5, 1, 3, 6, and 24 h after initiation of culture.

2.2. RNA isolation

RNA was extracted from swine blood using the LeukoLock™ Total RNA Isolation kit (Ambion). Isolation was performed according to the manufacturer's protocol with minor modifications. Briefly, approximately 15 ml of the whole blood cultures were passed through the LeukoLock filter using a 10cc syringe connected directly to the filter. Each filter was flushed with 3 ml of PBS, followed by 3 ml of RNAlater, and then flushed with 2.5 ml of pH-adjusted Lysis/Binding Solution. The lysate was collected and treated with 25 ul of proteinase K. The RNA was isolated via RNA binding beads. Following incubation and centrifugation, the beads were transferred to a fresh microcentrifuge tube and underwent a series of washes. RNA was recovered in 40ul of elution buffer. RNA quality was assessed using an Agilent Bioanalyzer (Santa Clara, CA) and quantified using a NanoDrop (Thermo Fisher Scientific, Wilmington, DE). Equal amounts of RNA from each time point were pooled together for gene expression analysis. RNA for qRT-PCR analysis was normalized to 50 ng.

2.3. Real-time RT-PCR

Quantitative real-time RT-PCR (qRT-PCR) was performed using custom primers designed using Allele ID (PREMIER Biosoft International, Palo Alto, CA) and synthesized by Invitrogen (Carlsbad, CA). Total RNA was reverse transcribed using the SuperScript III Platinum Sybr Green One-Step qRT-PCR Kit (Invitrogen). Briefly, 25ul of 2X Sybr Green Reaction Mix, 1ul of Platinum Taq Mix, 21ul of DEPC-treated water, and 1ul each of a 10 u M concentration of forward and reverse primers were combined to compose the master mix. Twenty-five microliters of master mix were aliquotted into SmartTubes followed by the addition of 50 ng of RNA. Real-time detection was carried out using Cepheid's Smart Cycler (Sunnyvale, CA) with the following parameters: cDNA synthesis at 50 °C for 3 min, 95 °C for 5 min, 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 60 °C for 30 s. Cycle threshold (Ct) values were recorded and utilized for data analysis.

2.4. ELISA assessment

Collected culture supernatant was used for measurement of protein production using commercial ELISA kits for swine IL-8, swine MCP-1, TNFα (Kingfisher Biotech, Inc., St. Paul, MN) and SAA (R&D systems, St. Paul, MN) following the manufacturer's instructions.

2.5. TLR/NLR/CLR ligand screening

The pathogen-associated molecular pattern (PAMP) receptor responsible for transducing the carrageenan-mediated signal was determined by measuring NF-κβ mediated production of secreted embryonic alkaline phosphatase (SEAP) using HEK-293 cells transfected with one PAMP receptor (Invivogen; San Diego, CA). These cells had also been co-transfected with a plasmid that expressed SEAP under the control of 5 NF-κβ promoter regions. The PAMP receptors examined for responsiveness to carrageenan were human Toll-Like Receptors (2, 3, 4, 5, 7, 8, 9), two human NOD-like Receptors (NLR; NOD1 & NOD2), and two human C-type Lectin Receptors (CLR; Dectin-1a and Dectin-1b). Carrageenan was used at a final concentration of 1 μg/ml. The positive controls for the TLR expressing cells were heat-killed *Listeria monocytogenes* (TLR2), poly I:C (TRL3), *E. coli* LPS (TLR4), S. typhimurium flagellin (TLR5, CL097 (TLR7), CL075 (TLR8), and CpG ODN (TLR9). The positive controls for the NLR expressing cells were C12-iE-DAP (acetylated dipeptide iE-DAP; NOD1) and L18-MDP (muramyldipeptide with a C18 fatty acid chain; NOD2). The positive controls for CLR expressing cells were β-glucan from *S. cerevisiae*, β-1,3-glucan from *A. faecalis*, and hot alkali treated zymosan. All positive controls demonstrated their respective PAMP receptor was fully functional, with O.D. values for the positive controls ranging between 2.4 to 2.9, depending upon the control being used.

2.6. RNA silencing

HEK-293 cells co-transfected with either a TLR2-expressing plasmid or a TLR-4-expressing plasmid were used (Novus Bio Littleton, CO; cat# NBP2-26276 and cat# NBP2-26274). Both cell lines had a second plasmid expressing SEAP under NF-κβ control; both lines were used in the RNA silencing studies. Expression of TLR6 was silenced by transfection with siRNA (Invivogen, San Diego, CA; cat# psirna43-htlr6) using Lyovec (Invivogen, San Diego, CA; cat# lyec-12). Silencing of TLR6 was confirmed by PCR or qRT-PCR. The PCR analyses of the RNA silenced TLR6-/-HEK/TLR2 cells and the TLR6-/-HEK/TLR4 cells demonstrated a complete absence of TLR6 gene transcripts (data not shown). Cells were seeded at 5×10^5 in a 6-well plate. TLR6-/-HEK/TLR2 cells were stimulated with λ-CGN (5.0 μg/ml) and PAM3CSK4 (Invivogen, San Diego, CA; cat# tlrl-rpms) (10.0, 50.0, 100.0 ng/mL) for 24 h. TLR6-/-HEK/TLR4 cells were stimulated with λ-CGN (5ug/mL) or LPS (10, 50, 100 ng/ml). SEAP release was measured at 30 and 60 min using Novus Bio's SEAP Release Kit (cat# NBP2-25285). Resulting activity was determined using a SPECTRAmax plate reader with Softmax Pro software.

2.7. Statistical analysis

Fold changes were assessed using the delta-delta Ct method, with GAPDH utilized as the normalizing gene. Data are presented as mean ± SEM. A One-Way ANOVA followed by Dunnett's multi-range test was used to compare qRT-PCR data across samples to the 0 h values. Student's *t*-test was used to compare the 24 h λ-CGN stimulated r and 24 h control data. A P value $\leq$ 0.05 was considered statistically significant.

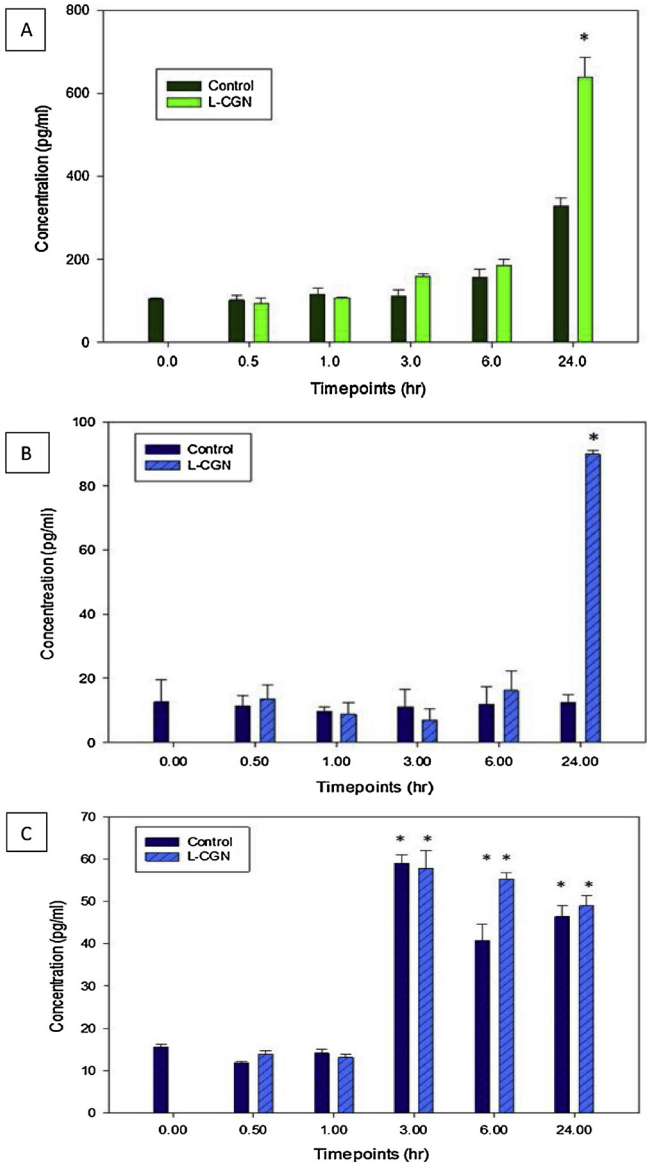

Fig. 1. Production of IL-8 (A), MCP1 (B) or TNFα (C) protein after λ-CGN stimulation of swine whole blood cultures. The cultures were stimulated with nothing (unstimulated control) or λ-CGN, with samples collected at various times through 24 h, with the levels of IL8, MCP-1 or TNFα protein levels from λ-CGN cultures compared to unstimulated control swine whole blood cultures over the same time periods. n = 9 animals. * $p < 0.05$ compared to 0 h values.

3. Results

3.1. λ-CGN-induced changes in gene expression and protein production

The potential for λ-CGN to induce changes in inflammatory mediates was initially assessed using a screening approach to determine if λ-CGN could also affect expression of a subset of genes previously identified as biomarkers of lipopolysaccharide (LPS)-induced inflammation in swine (Peters et al., 2010 & 2012), along with two genes known to be induced during an LPS-induced inflammation (TNFα & IL-6). These results demonstrated that λ-CGN stimulation could induce the expression of MCP-1and AMCF-II but had no effect on the mRNA expression of TNFα, IL-6, or SAA (data not shown).

AMCF-II is a swine-specific member of the IL8/GRO family; the absence of detection antibodies makes it difficult to measure protein production. Therefore, production of IL-8 was evaluated as a surrogate for AMCF-II, reasoning that they might be co-regulated. λ-CGN

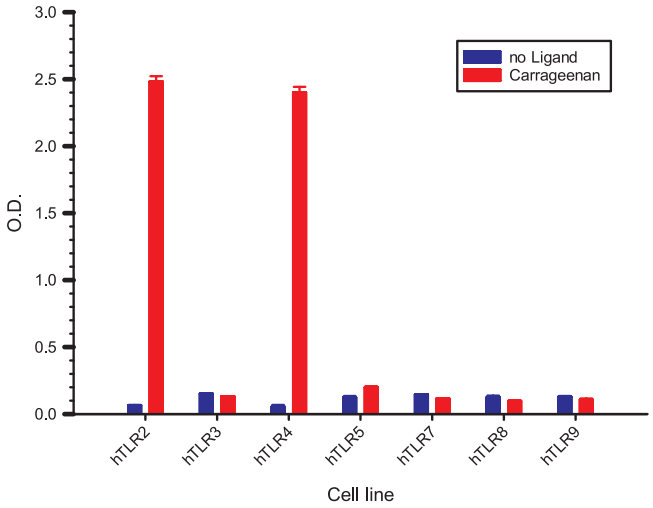

Fig. 2. Ligand screening in HEK293 cells stimulated with λ-CGN. HEK293 cells expressing a single PAMP receptor were stimulated with λ-CGN, and the resulting SEAP production was determined. Results of triplicate analyses per experimental condition.

stimulation increased IL-8 and MCP-1 protein levels, in agreement with changes in mRNA expression (Fig. 1A & B). The increases in IL-8 and MCP-1 protein levels at 24 h were due to λ-CGN stimulation and not time in culture. λ-carrageenan had no effect on protein production for IL-6 or SAA (data not shown) while there was an increase in TNFα protein levels due to time in culture only (Fig. 1C). There was no change in the TNF-α mRNA levels (data not shown).

3.2. Identification of λ-CGN signal pathways

Identification of the pathogen recognition receptor(s) responsible for transducing the carrageenan-induced inflammatory signal was determined using a panel of cells transfected with just one PAMP receptor, using production of secreted embryonic alkaline phosphatase (SEAP) as the read-out system. λ-carrageenan stimulation resulted in SEAP production in cells expressing either TLR4 or TLR2 (Fig. 2). No other single TLR expressing cell was capable of responding to stimulation by λ-CGN. Likewise, cells expressing NLR (NOD 1 or NOD2) or CLR (Dectin-1a or Dectin-1b) were not capable of responding to λ-CGN stimulation (data not shown).

As TLR2 is a heterodimer consisting of either TLR2 & TLR1 or TLR2 & TLR6, it was important to determine which TLR2 complex was responsible for transducing the carrageenan-induced response. TLR2 expressing HEK-293 cells were transfected with silencing TLR6 RNA, then stimulated with λ-CGN. The results also demonstrate that these cells constitutively express TLR1 and TLR6 (Fig. 3). Silencing of TLR6 expression was confirmed by PCR (Fig data now shown). HEK-TLR2+/TLR6- cells, when stimulated with λ-CGN, did not produce SEAP (Fig. 3). However, stimulation of these same cells with Pam3CSK4, a TLR2/1 ligand, did result in the production of SEAP (Fig. 3).

While λ-CGN stimulated TLR2 transfected HEK-293 reporter cells and TLR4 transfected HEK-293 reporter cells (Fig. 2), the absence of TNFα production by white blood cells following λ-CGN (Fig. 1C) was inconsistent with those results. These incongruous results suggested another pathway may exist that is capable of transducing the λ-CGN stimulus in the TLR4 transfected HEK-293 reporter cells. TLR4 has been reported to form a heterodimer with TLR6 (Stewart et al., 2010). Since TLR6 is constitutively expressed in HEK-293 cells, it was possible that it was the key signal transduction molecule responsible for transmitting the λ-CGN stimulus. Therefore, the contribution of TLR6 to λ-CGN stimulation of TLR4 transfected HEK-293 reporter cells was examined by silencing the expression of TLR6. These results demonstrated that λ-

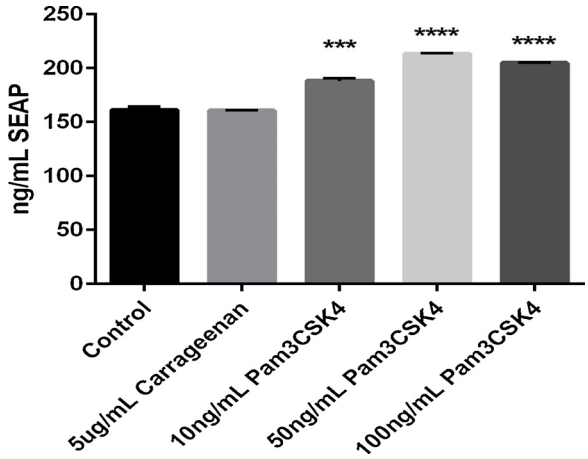

Fig. 3. Results of λ-CGN stimulation of HEK/TLR2 + /TLR6-/- compared to HEK/TLR2 + /TLR6-/- stimulation using the TLR1/2 agonist Pam3CSK4 for 24 h, measuring production of secreted embryonic alkaline phosphatase (SEAP). Representative experiment; all conditions performed in duplicate. ***p < 0.001; ****p < 0.0001.

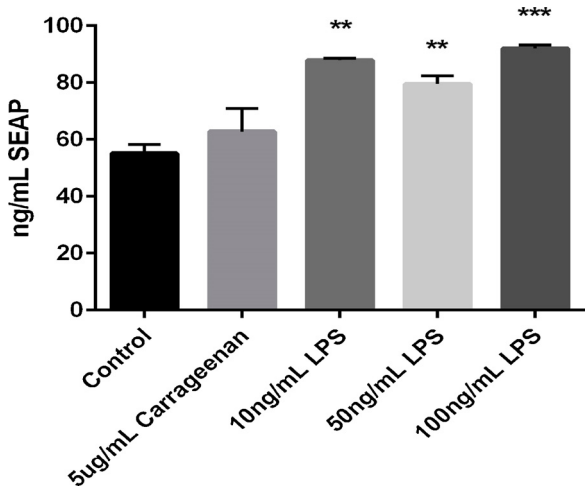

Fig. 4. TLR6 silencing in HEK-TLR4 cells. Results of λ-CGN stimulation of HEK/TLR4 + /TLR6-/- compared to stimulation of HEK/TLR4 + /TLR6-/- using the TLR4/4 agonist E. coli LPS for 24 h, measuring production of secreted embryonic alkaline phosphatase (SEAP). Representative experiment; all conditions performed in duplicate. **p < 0.01; ***p < 0.001.

CGN stimulation of SEAP production in HEK-TLR4 + /TLR6- cells was eliminated, while LPS-induced production was retained (Fig. 4).

4. Discussion

λ-carrageenan has long been used to induce a local inflammation in a variety of laboratory species and domestic animals. While many aspects of the physiological inflammatory responses induced by λ-CGN have been characterized, the precise molecular mechanisms responsible for the responses elicited by λ-CGN have not previously been identified. The identification of TLR2/6 and TLR4 as the receptors responsible for the transmission of the λ-CGN-induced signaling provides insight into how λ-CGN stimulation leads to an inflammatory response. However, the patterns of gene expression and protein production observed following λ-CGN stimulation of whole blood cultures suggests differences between λ-CGN stimulation of TLR4 and that induced by other TLR4 ligands such as E. coli lipopolysaccharide (LPS).

Carrageenan stimulation of swine whole blood cultures resulted in the expression of IL-8, AMCF-II, and MCP1. The induction of these molecules is consistent with the known tendency of λ-CGN to cause the migration of neutrophils and monocytes to the site of administration. These three genes are also induced by LPS. AMCF-II is intercrine-α protein unique to swine, but with similarities to GRO (Goodman et al., 1992). The lack of a λ-CGN stimulation increase in TNFα protein levels, in conjunction with no change in TNFα mRNA levels, suggest that changes in TNF-α protein levels were culture-related artifacts. However, the lack of induction of TNFα after in vitro exposure to λ-CGN is different from both the in vitro models of LPS induced inflammation (Peters et al., 2011) and the in vivo models of LPS and λ-CGN induced inflammation (Myers et al., 1999; Peters et al., 2012; Zhang et al., 2013; Butturini et al., 2014; Sukegawa et al., 2014), all of which resulted in production of TNFα. These differences in the patterns of TNFα production suggested that in this in vitro model, λ-CGN is not binding to the TLR4 homodimer as postulated by others (Bhattacharyya et al., 2008), but rather to a different receptor or receptors.

The results from this study using cell lines expressing a single PAMP receptor demonstrated that λ-CGN was capable of binding to either TLR2 or TLR4. As the HEK-293 cell line constitutively expresses TLR1 and TLR6, gene silencing studies were used to demonstrate that λ-CGN preferentially bound to the TLR2/6 heterodimer. The results showing TLR6 was involved in λ-CGN signal transduction also suggested a means to explain its capacity to bind to TLR4 expressing HEK-293 cells in the absence of its ability to induce TNFα production in white blood cells. Gene silencing studies with TLR4 expressing HEK-293 cells demonstrated the absence of λ-CGN signal transduction, whereas LPS signal transduction was maintained. These results indicated that λ-CGN can stimulate through TLR4/6 and TLR2/6 heterodimers, with TNFα production being an apparent function of TLR4/4 homodimers.

The results from the gene knock out cell line experiments are consistent with published in vivo studies with TLR4 KO mice. The major difference between those *in vivo* studies and the present work is the target gene of the knock-out work and the resulting interpretation. The TLR4 in vivo KO studies concluded that the results indicated that TLR4 homodimers were responsible for transducing the λ-CGN stimulation. In contrast, by knocking out TLR6 instead, our results demonstrate that a TLR4 homodimer is not involved. Rather it is a TLR4/TLR6 heterodimer that is responsible for transducing the λ-CGN. The cells in which TLR6 was knocked out lost all ability to respond to λ-CGN while retaining their ability to respond to LPS, a TLR4 homodimer ligand. The difference in cytokine production produced by WBC compared to cytokine production patterns following in vivo administration suggests that tissue-specific differences may exist following systemic λ-CGN administration. Whether systemic λ-CGN administration produces TNFα mediated by direct signal transduction or through the induction of other cytokines cannot be addressed at this time.

Tissue specific differences in responsiveness to λ-CGN administration would also explain why systemic administration of λ-CGN results in an inflammatory response while oral consumption of λ-CGN as a food additive does not. Indeed, carrageenan is a generally recognized as safe (or GRAS) food additive (Anonymous, 2018) for a wide range of species, including pigs (Weiner et al., 2015), suggesting that oral administration of λ-CGN does not stimulate TLR2/6 or TLR4/6 receptors in the gastrointestinal (GI) tract following exposure. Similar difference between systemic and GI responsiveness has been reported for *Yersinia enterocolitica* (DePaolo et al., 2012). While both TLR2/1 and TLR2/6 initiated immune response to Yersinia, oral exposure initiated a Th17 response mediated by TLR2/1 signaling, leading to the induction of tolerance and intestinal colonization. In contrast, systemic administration of Yersinia induced a Th1 response.

The results of this study using the HEK-293 transfected cell lines differ from the results of McKim et al. (2015). The latter study was unable to demonstrate carrageenans (kappa, lambda, or iota) were capable of stimulating HEK-293 cells transfected to express TLR4. We cannot at present explain the different outcomes between our study and the McKim et al. study. However, we should note that the earlier results

are expressed as percent of the maximum LPS positive control response, which tends to minimize the CGN responses. Other possible differences include their use of media containing fetal bovine serum and the use of twice the number of reporter cells as used in this study. It should be noted that an inability to cause stimulation through TLR4 is also in contrast to in vivo and in vitro results demonstrating that functional TLR4 was required for CGN initiated responses (Tsuji et al., 2003; Bhattacharyya et al., 2008).

The importance of the no- λ-CGN treatment control cultures cannot be over emphasized. While most of the proteins evaluated in this study were unaffected by time in culture, this was not the case for TNFα. Mere time in culture demonstrated an increase in total TNFα protein levels, which began 3 h after culture initiation and remained elevated through 24 h of culture. TNFα protein levels were elevated to the same extent in both the no- λ-CGN treatment control cultures and in the λ-CGN treated cultures. If the time in culture controls had not been included, we would have incorrectly concluded that λ-CGN stimulation increased TNFα protein levels in the absence of an increase in TNFα mRNA. This highlights that another difference between the *in vitro* results from this study compared to systemic administration of λ-CGN is the production of TNFα after systemic λ-CGN exposure. These results suggest there may be tissue specific regulation of gene expression following exposure to λ-CGN.

The results of this study suggest there may be a set of inflammation-related genes that are shared between different models/stimulating agents. This in turn suggests that these genes may provide suitable candidates to be developed as a standardized set of biomarkers that can be used to measure the induction of an inflammatory response regardless of the stimulating agent.

5. Conclusion

The results demonstrated that the pattern of inflammatory cytokines elicited following carrageenan stimulation differs from that induced by lipopolysaccharide, the most commonly used agent to elicit an inflammatory response. Befittingly, the TLR4/6 and TLR2/6 pattern recognition receptors responsible for transducing carrageenan's response were also unique. The identification of a unique mechanism of action provides the molecular basis by which carrageenan induces a unique set of inflammatory cytokines.

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