

Suppression of allergic reaction by λ -carrageenan: Toll-like receptor 4/MyD88-dependent and -independent modulation of immunity

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Summary

Background Recognition of foreign substances by innate immunity through pattern recognition receptors (PRRs) regulates acquired immunity such as allergic reaction. Because PRRs recognize heterogeneous ligands, daily food intake can potentially regulate immune allergic reaction.

Objective Elucidation of the effect of λ -carrageenan on allergic reactions was aimed.

Method IFN- γ and IL-4 was measured in *in vitro* T cell-stimulated culture. Cytokine production from macrophages in response to λ -carrageenan was measured as indicator for innate immunity activation. Mice were immunized with OVA in alum to induce specific IgE, and then histamine release was induced by systemic injection of OVA.

Results Activation of innate immunity by λ -carrageenan is dependent on Toll-like receptor-4 (TLR4) and MyD88, in which induction of pro-inflammatory cytokines such as TNF- α and IL-6 was largely impaired in macrophages from TLR4- and MyD88-deficient mice. Footpad oedema, a model for *in vivo* inflammatory reactions, was significantly reduced in these mice. Similar to recent evidence showing a preference for the stimulation of Th1 via TLR/MyD88 signalling, λ -carrageenan showed enhanced IFN- γ and decreased IL-4 in stimulated T cell cultures. Interestingly, increased IFN- γ production was still seen in TLR4- and MyD88-deficient splenocytes. Oral administration of λ -carrageenan to immunized mice successfully decreased OVA-specific IgE, and λ -carrageenan was also effective in previously immunized mice. Further, serum histamine release upon systemic challenge of OVA was significantly inhibited. Neither OVA-specific IgG1/IgG2a nor cytokine secretion from *in vitro* cultures were altered, suggesting the involvement of multiple PRRs as demonstrated by TLR4/MyD88-independent IFN- γ up-regulation. The simultaneous feeding of OVA with lipopolysaccharide abrogated oral tolerance, but λ -carrageenan was not only devoid of such an effect but was also found to promote oral tolerance in the absence of TLR4.

Conclusion λ -Carrageenan was suggested to be a useful dietary supplement to ameliorate allergic reactions while maintaining oral tolerance-dependent intestinal homeostasis.

Keywords λ -carrageenan, histamine, IgE, innate immunity, MyD88, Toll-like receptor-4

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Introduction

Carrageenans are the generic name for high molecular weight poly galactans isolated from certain species of red algae, and they comprise three basic subtypes, κ , ι and λ , which have different colloidal properties [1]. Carrageenans are widely used as additives in food processing and are recognized as safe by the US Food and Drug Administration (FDA). Because carrageenan has been known to activate macrophages, it is of particular interest to investigate the efficacy of carrageenan with respect to immune modulation, toward the goal of developing

protocols for preventing immune disorders via dietary supplements.

Carrageenan has been used as a reagent to induce non-specific inflammatory reactions, which may be a consequence of activation of innate immunity through pattern recognition receptors (PRRs). Thus, footpad oedema and pleurisy are commonly used model systems induced by either subcutaneous (s.c.) or intrapleural administration of carrageenan. Several reports describe the modulation of acquired immunity by carrageenan, where both adjuvant and suppressive effects are observed. Adjuvant effects are usually obtained by systemic administration of carrageenan, resulting in augmentation of antibody responses [2, 3]. On the contrary, several studies demonstrate that both systemic and oral administration of carrageenans suppress antibody production and mitogen-induced T cell proliferation [4–7]. Further, some studies suggest that prostaglandin E2 is produced by macrophages in response to

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carrageenans and is responsible for immunosuppressive activities [4], while others show that down-regulatory CD8⁺ T cells are induced to mediate active suppression [7]. These various properties reported for carrageenans most likely reflect different dosages and routes of administration as well as heterogeneity in carrageenan preparations. In addition, such diversity of action may imply that multiple PRRs are involved in recognition of heterogeneous carrageenan structures.

PRRs act as pathogen sensors of the immune system, which confers effective and protective immune responses. To this end, innate immune components are located in the physiological 'front line' in order to recognize and discriminate noxious from innocuous foreign substances. These immune components may be activated through interaction between PRRs and pathogen-associated molecular patterns (PAMPs) [8]. PAMPs can be quite heterogeneous, and include lipids, glycans and nucleotides that are essential for pathogen survival. The recent discovery of a mammalian homologue of *Drosophila* Toll, Toll-like receptor (TLR) [9], led to a re-evaluation of the fundamental roles of innate immunity and PRRs. Toll was first identified as a decisive factor for the development of the dorsal-ventral axis in the fruit fly, and was later found to be a protective agent against fungal infection [10]. In mammals, many TLR subtypes recognize microbial components. TLR4 was first to be reported as a lipopolysaccharide (LPS) receptor [11], and later TLR2 was found to be a receptor for peptidoglycan and lipopeptide by dimerizing with TLR6 [12]. Therefore, it was postulated that recognition of Gram-negative and Gram-positive bacteria was differentially mediated by TLR4 and TLR2, respectively [13]. TLR9 and TLR5 were subsequently identified as receptors for the unmethylated bacterial DNA motif CpG and bacterial flagellin, respectively [14, 15]. Very recently, double-stranded viral RNA (poly I:C) was found to bind TLR3 [16].

It has been suggested that activation of innate immunity by these TLRs regulates acquired immune responses [8, 17]. Overexpression of TLR4 was originally shown to augment acquired immune responses [9]. LPS [18] and CpG [19–21] were shown to stimulate dendritic cells (to induce Th1 differentiation) via TLR-4 [11] and TLR-9 [14], respectively. Recently, Schnare et al. [22] showed that signals from TLR and MyD88 (a common adaptor protein for TLRs) play crucial roles in inducing Th1 immunity. Similarly, *Listeria* and lactic acid bacteria promote Th1 immunity by inducing IL-12 production from macrophages [23, 24], suggesting the involvement of TLRs. The Th1-specific nature of TLR ligands led to their possible use as preventatives of allergic disorders via regulation of Th1/Th2 balance. Observations in germ-free mice also suggest a role for innate immunity in the regulation of immune homeostasis. Oral tolerance induction and IgA production were largely impaired in these mice, but these processes were restored by re-colonization of microflora prior to 3 weeks of age [25]. These observations strongly suggest that microbial components recognized by PRRs can stimulate the intestinal immune system. Because PRRs recognize a wide variety of molecular determinants, the possibility that PRRs may recognize materials from daily food might contribute to homeostasis of immunity. Furthermore, it is also probable that such natural substances like food components are capable of regulating immune responses via interaction between PRRs such as TLR.

In this study, we investigated the potential for PRRs to recognize carrageenan and thereby activate innate immunity.

We found that TLR4/MyD88 plays a dominant role in carrageenan recognition and the induction of pro-inflammatory cytokines. These results suggest that carrageenan may prove efficacious for the induction of Th1 immunity. Our data also demonstrate TLR4/MyD88-independent effects of λ -carrageenan on stimulated T cell cultures. We discuss the effects of λ -carrageenan on *in vivo* inflammation and allergic reactions.

Materials and methods

Mice

Specific pathogen-free female BALB/c, C3H/HeN, C57BL/6 mice (6–8 weeks old) were obtained from Charles River Japan, Inc. (Tokyo, Japan), and C3H/HeJ was from JAPAN SLC, Inc. (Shizuoka, Japan). Toll-like receptor 2- and 4-deficient mice (TLR2^{-/-} and TLR4^{-/-}), and MyD88-deficient (MyD88^{-/-}) mice on a C57BL/6 background were established and maintained in filtered microisolators as previously described [11, 13, 26]. Experiments were performed according to the guidelines of the Prime Minister's Office in Japan (no. 6, March 27, 1980).

Reagents

Concanavalin A (Con A) and *Salmonella minnesota* Re-595 LPS were from Sigma Chemical Co. (St Louis, MO, USA) and *Staphylococcus enterotoxin B* (SEB) was from Toxin Technology Inc. (Sarasota, FL, USA). Anti-CD3 mAb (2C11) was from Pharmingen (San Diego, CA, USA). OVA, was from Nacalai Tesque Inc. (Kyoto, Japan) and contained less than 15 pg/mg endotoxin. Synthetic lipopeptide MALP was prepared as previously described [27]. Carrageenan type IV was from Sigma as a source of λ -carrageenan, and was depleted of any contaminants such as κ and ι -carrageenans by selective precipitation with 1 M KCl. Soluble fraction was dialysed against water for 24 h to exclude possible low molecular weight contaminants and KCl. Polymixin-B sepharose column was then employed to deplete endotoxin, followed by acetone-precipitation. Precipitates were dissolved in water and then lyophilized to obtain white powder. LPS was measured by limulus assay (Wako Chemical Co., Osaka, Japan) and found to contain less than 1.5 pg/mg λ -carrageenan. Purity of λ -carrageenan was also confirmed by infrared spectroscopy and ¹³C-NMR analysis which detected only λ -carrageenan specific signals (data not shown). Water used throughout this study was exclusively prepared from Milli-Q biocel A-10 (Millipore Corp., Bedford, MA, USA) which is capable of excluding endotoxin contaminants. A clinical grade of saline solution (Otsuka Pharmaceuticals Inc., Tokyo, Japan), with undetectable levels of endotoxin, was used for administration of λ -carrageenan.

Cell culture

A single-cell suspension of splenocytes (depleted of red blood cells by hypotonic treatment) was prepared under aseptic conditions. Splenocytes (1 or 5×10^6 cells/mL) were then stimulated with Con A (2 μ g/mL) or SEB (0.1 μ g/mL), respectively, in the presence of varying concentrations of λ -carrageenan in 0.2 mL RPMI-1640 containing 100 U/mL penicillin, 100 μ g/mL streptomycin, 2 mM L-glutamine, 25 mM HEPES, 5×10^{-5} M 2-mercaptoethanol, and 10% fetal bovine serum (FBS). In the case of anti-CD3 stimulation, a 96-well plate was coated

overnight at 4 °C with 1 μ g/mL anti-CD3 mAb dissolved in 0.1 M NaHCO₃ (pH 8.2). The wells were washed prior to seeding of splenocytes at 5×10^6 cells/mL. Supernatants were harvested 48 h later and stored at -80 °C for subsequent ELISA assay. For antigen-specific responses of splenocytes from immunized mice, 100 μ g/mL OVA was used instead of Con A or SEB. Macrophages were collected as peritoneal exudated cells 3 or 4 days after intraperitoneal (i.p.) injection of thioglycolate. Macrophages (5×10^5 or 1×10^6 cells/mL) were incubated with varying concentrations of λ -carrageenan for 24 h and the supernatant harvested for cytokine determination by ELISA.

Quantitative sandwich ELISA for cytokines and mouse IgM

For mouse IFN- γ and TNF- α ELISA, two specific capture and biotinylated detection mAbs (Pharmingen) for each cytokine were employed: R4-6A2, XMG1.2 for IFN- γ , and G281-2626, MP6-XT3 for TNF- α , respectively [28]. We also used a TNF- α ELISA kit from Genzyme/Techne (Boston, MA, USA). For mouse IgM ELISA, 2 μ g/mL capture anti-IgM mAb (R6-60.2) (Pharmingen) and biotinylated polyclonal anti-IgM μ (Cappel Organon Teknika Corp., West Chester, PA, USA) were used. Briefly, wells were coated overnight at 4 °C with capture antibody in 0.1 M NaHCO₃ (pH 8.3). Following blocking with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBS-Tween) at 25 °C for 2 h, samples and recombinant mouse IFN- γ (Genzyme, Cambridge, MA, USA) or IgM (Pharmingen) were added and incubated for 1 h at room temperature. Detection antibody, and subsequently 1:3000 diluted horseradish peroxidase (HRP)-conjugated streptavidin (Vector, Burlingame, CA, USA), were then added for 1 h or 30 min, respectively. Tetramethylbenzidine (TMB), peroxidase substrate and 0.5 N HCl stop solution were used for colour development at 450 nm. OptEIA kits (Pharmingen) were employed for determination of IL-4, IL-6, IL-10 and IL-12.

Anti-OVA IgE, IgG1 and IgG2a ELISA

Wells were coated overnight at 4 °C with 50 μ g/mL OVA in 0.1 M NaHCO₃ (pH 8.3). Following blocking with 1% BSA in PBS-Tween at 25 °C for 2 h, serially diluted serum samples with blocking buffer were added and incubated for 1 h at room temperature. Detection antibodies (1 μ g/mL) biotinylated anti-mouse IgE (R35-72), IgG1 (A85-1) and IgG2a (R19-15, Pharmingen), were added for 1 h, followed by addition of 1:3000 diluted HRP-conjugated streptavidin (Vector). TMB, peroxidase substrate and 0.5 N HCl stop solution were used for colour development at 450 nm. Anti-mouse IgE ELISA was simultaneously performed using a capture mAb (R35-92, Pharmingen) and biotinylated detection mAb (R35-72, Pharmingen). Anti-OVA IgE in serum was expressed in U/mL relative to standard IgE ELISA. Standard immune serum was obtained by i.p. immunization of mice with 50 μ g OVA, which was absorbed onto 2 mg alum, this was repeated three times with 2-week intervals. Blood was then collected and the serum extracted. Anti-OVA IgG1 and IgG2a titres were calculated by comparing to standard immune serum. Concentrations of anti-OVA IgG1 and IgG2a in the standard immune serum were arbitrarily assigned to 100 U/mL.

DNA array

Total RNA was isolated with Invisorb Spin Cell RNA Mini Kit (Invitek, Berlin, Germany) from C3H/HeN or C3H/HeJ-macrophages 16 h after λ -carrageenan stimulation. Each individual mRNA was then purified from 30 μ g of total RNA with MicroPoly(A)Purist (Ambion, Austin, TX, USA). Probes were labelled with Panorama Mouse Cytokine cDNA Labeling Kit (Sigma-Genosys, Woodlands, TX, USA). Briefly, 15 μ L of a mixture containing 0.5 μ g mRNA, 4 μ L mouse cytokine cDNA labelling primers was incubated at 90 °C for 2 min. After cooling to 42 °C for 20 min, these reagents were added: 6 μ L of 5 $\times$ reverse transcriptase buffer, 333 μ M dNTPs (lacking dCTP), 1.67 μ M dCTP, 2 μ L [³²P]dCTP (10 m Ci/mL; Amersham Pharmacia Biotech, Piscataway, NJ, USA), 20 U RNase inhibitor (Takara, Tokyo, Japan), 50 U of AMV reverse transcriptase, and ddH₂O to a final volume of 30 μ L. The reaction was performed at 42 °C for 3 h and then purified by Sephadex G-25 spin columns. Panorama mouse cytokine gene array membranes (Sigma-Genosys) were rinsed with 2 $\times$ SSPE (360 mM NaCl, 20 mM sodium phosphate buffer pH 7.7, 2 mM EDTA) at room temperature for 5 min, and pre-hybridized in 7 mL hybridization solution (Sigma) at 65 °C for 4 h in a sealed plastic bag. Hybridization was then carried out overnight in 6 mL of hybridization solution mixed with denatured cDNA probes. Washing was performed as follows: three times with solution I [0.5 $\times$ SSPE, 1% sodium dodecyl sulphate (SDS)] at room temperature for 2–3 min, then twice at 65 °C for 20 min; and once with solution II (0.1 $\times$ SSPE, 1% SDS) at 65 °C for 20 min. Membranes were exposed to BAS-III imaging plates overnight and then analysed with BAS-1000 system (Fuji Film Co., Tokyo, Japan). The relative intensity for each gene was quantified after background correction. The expression was considered as negative in cases where a negative value was recorded. The signal of a cyclophilin (a housekeeping gene) was chosen as reference for normalization. The ratio of each gene's signal to that of cyclophilin on the same filter was chosen to compare the relative expression levels between the two different conditions.

Footpad oedema

λ -Carrageenan-induced footpad oedema was performed as described previously [29]. Briefly, 40 μ L of 2 mg/mL λ -carrageenan dissolved in saline was injected into s.c. tissue of both hind footpads. The footpad thickness was measured with a dial caliper (Ozaki, Tokyo, Japan) and the increase was calculated by comparing footpad thickness before injection and at 4 h.

Immunization and histamine release

BALB/c mice were immunized i.p. with 50 μ g OVA absorbed onto 2 mg alum in 0.1 mL sterile endotoxin-free saline on days 0 and 7. On day 28 or 35, blood was drawn from the retro-orbital plexus to prepare serum samples. For the systemic induction of histamine release, the mice were challenged with 100 μ g OVA in 200 μ L PBS which was injected i.p., and blood was collected 10 min later as previously described [30]. Serum level of histamine was measured by reversed-phase HPLC with simultaneous fluorometric detection by post-column method as described [31]. The indicated concentration of λ -carrageenan (0.5 mL) was orally administered by intragastric flexible tube. Oral administration commenced on day 0, and continued for

6 consecutive days per week until the termination of experiments on day 28 or 35.

Oral tolerance induction and abrogation

For oral tolerance induction, OVA (5 mg/mouse) was administered for five consecutive days by intragastric instillation using a flexible plastic needle. Systemic and i.p. immunizations with OVA in alum were done twice, on days 7 and 14 (3 and 10 days after last feeding). Serum samples were collected 7 and 14 days after last immunization. Anti-OVA-specific antibodies in serum were measured by ELISA. For oral tolerance abrogation, OVA was administered in the presence of λ -carrageenan or LPS (Difco: Becton Dickinson, Franklin Lakes, NJ, USA).

Statistics

Statistics were performed using the paired two-tailed Student's *t*-test, and $P < 0.05$ was taken as the level of significance.

Results

TLR4/MyD88-dependent activation of macrophages by λ -carrageenan

Due to the potent ability of λ -carrageenan to stimulate macrophages, this substance has been used extensively to induce non-specific inflammation. Thus, we first investigated the pathway responsible for λ -carrageenan-mediated induction of pro-inflammatory cytokines from macrophages. TNF- α production in response to λ -carrageenan and LPS was normal in macrophages from TLR2 $^{-/-}$ mice but absent in TLR4 $^{-/-}$ macrophages (Fig. 1a). As a control, Lipopeptide MALP, which was previously shown to stimulate innate immunity in TLR2-dependent fashion [27], induced TNF- α in macrophages from TLR4 $^{-/-}$ mice but not from TLR2 $^{-/-}$ mice (Fig. 1a). We confirmed these results using C3H/HeJ mice that are hyporesponsive to LPS due to a point mutation (P712H) in TLR4 [11]. Again, both TNF- α and IL-6 production were strongly inhibited in C3H/HeJ-derived macrophages in response to λ -carrageenan relative to normal TLR4 macrophages (C3H/HeN; Fig. 1b). MyD88 is a common adaptor protein for all subtypes of TLRs, and macrophages from MyD88 $^{-/-}$ mice demonstrated that the production of TNF- α and IL-6 was largely dependent on MyD88 signalling (Fig. 1c). We also verified that the production of inflammatory cytokines was impaired at the mRNA level using a DNA-array method (results not shown). Macrophage mRNA was prepared 16 h after λ -carrageenan stimulation. Consistent with protein determination by ELISA, IL-6 mRNA in C3H/HeN macrophages was induced (0.08 compared to cyclophilin, a control housekeeping gene). In contrast, no IL-6 mRNA was detected in C3H/HeJ macrophages. Further experiments showed that IL-1 α and IL-1 β transcripts were also reduced in C3H/HeJ macrophages (IL-1 α = 0.52, IL-1 β = 0.30) compared with C3H/HeN (IL-1 α = 1.03, IL-1 β = 0.77). Collectively, these data suggest that TLR4 is the dominant PRR responsible for recognition of λ -carrageenan, resulting in pro-inflammatory cytokine production.

TLR4/MyD88-dependency of λ -carrageenan-induced footpad oedema

Footpad oedema was induced by λ -carrageenan to investigate the role of TLR4/MyD88 during *in vivo* inflammatory reactions

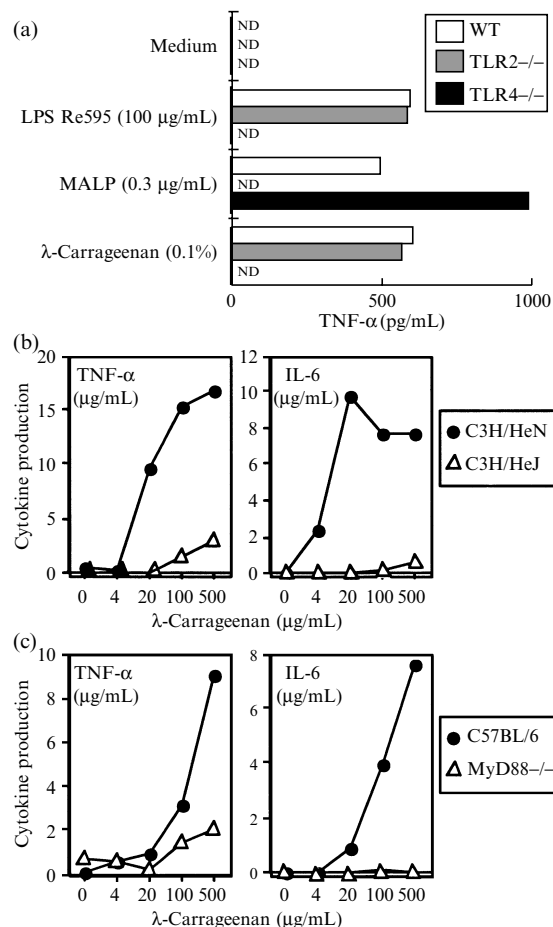


Fig. 1. TLR4/MyD88-dependent activation of macrophages by λ -carrageenan. (a) Peritoneal macrophages from wild-type, TLR2 $^{-/-}$ and TLR4 $^{-/-}$ mice were isolated 3 days after intraperitoneal thioglycolate injection. Cells (5×10^5 cells/mL) were cultured with the indicated concentrations of *Salmonella minnesota* Re-595 LPS, synthetic lipopeptide MALP, or λ -carrageenan for 24 h. Concentrations of TNF- α in the culture supernatants were measured by ELISA. Peritoneal macrophages were obtained from (b) TLR4-normal (●, C3H/HeN) and TLR4-mutant (Δ, C3H/HeJ) mice or from (c) C57BL/6 (●) and MyD88 $^{-/-}$ mice (Δ). λ -carrageenan was added to the cells (1×10^6 cells/mL). Supernatants were collected and assayed for cytokine determination by ELISA. ND, not detected. Each experiment was done at least twice and similar results were obtained.

(Fig. 2). The increase in footpad thickness comparing to thickness before injection was measured 4 h after s.c. injection of λ -carrageenan, and oedema was significantly impaired in TLR4 $^{-/-}$ mice compared to wild-type and TLR2 $^{-/-}$ mice. Similarly, λ -carrageenan-induced oedema was MyD88-dependent since a much lower increase in footpad thickness was observed in MyD88 $^{-/-}$ mice compared to C57BL/6 mice (Fig. 2). If correction is made for the increase in footpad thickness due to injection of 40 μ L of viscous solution, the net inhibition in TLR4 $^{-/-}$ and MyD88 $^{-/-}$ is calculated to be at least 80% compared to wild-type mice. These results indicate an important role for TLR4/MyD88 during *in vivo* inflammatory reactions induced by λ -carrageenan.

Modulation Th1/Th2 balance by λ -carrageenan

The TLR4-dependent activities of λ -carrageenan promoted us to investigate whether Th1/Th2 balance could be shunted

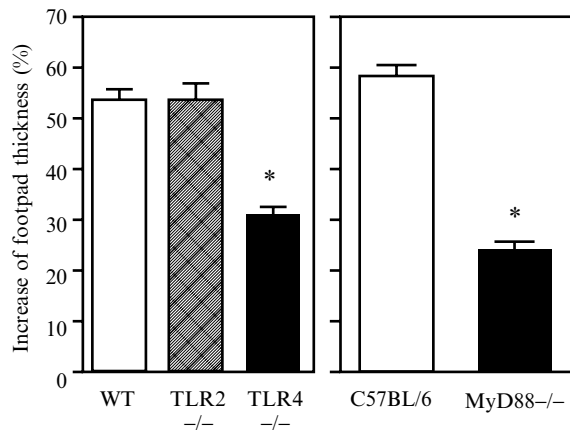


Fig. 2. Impaired footpad oedema in TLR4^{-/-} and MyD88^{-/-} mice. Footpad oedema was induced by s.c. injection of 40 μ L of λ -carrageenan (2 mg/mL) into both hind footpads. Prior to and 4 h after injection, footpad thickness was measured and the percentage increase in thickness calculated. An asterisk denotes statistical significance compared to wild-type (WT) for TLR4^{-/-} or C57BL/6 for MyD88^{-/-} mice. Five mice were used per group.

toward Th1 as the TLRs/MyD88 pathway is known to favour Th1 stimulation [19–22]. When splenocytes were stimulated by polyclonal activators of T cells such as Con A, SEB superantigen and anti-CD3 mAb, both IFN- γ and IL-4 were produced (Fig. 3). Addition of 20 μ g/mL λ -carrageenan increased IFN- γ production > threefold in Con A-stimulated splenocytes (Fig. 3, left, upper graph), and a similar threefold increase was observed in SEB-stimulated splenocytes (Fig. 3, middle, upper graph). In a reciprocal manner, IL-4 production was inhibited 40–60% after stimulation with either Con A or SEB (Fig. 3, middle graphs). In response to anti-CD3, lower doses of λ -carrageenan (4 and 20 μ g/mL) elicited only a slight increase in IFN- γ production, while that of IL-4 was significantly diminished. However, at higher λ -carrageenan doses, both IFN- γ and IL-4 were strongly inhibited while IL-10 production increased markedly (Fig. 3, lower graphs). These data suggest that Th1 responses are enhanced at lower doses of λ -carrageenan while higher doses may preferentially down-regulate the immune response.

We next asked if the IFN- γ -promoting and IL-4-inhibiting activity of λ -carrageenan at lower doses also depends on TLR4/MyD88 in SEB-stimulated splenocyte cultures. The enhanced production of IFN- γ from SEB-stimulated TLR4^{-/-} splenocytes by λ -carrageenan was comparable to wild-type splenocytes (Fig. 4a), while IL-4 was not detected in SEB-stimulated splenocytes. (data not shown). We next compared responses between MyD88^{-/-} and wild-type C57BL/6 splenocytes, and found substantially lower IFN- γ production in MyD88^{-/-} versus C57BL/6 cells at all λ -carrageenan concentrations tested (Fig. 4b). However, IFN- γ production from MyD88^{-/-} splenocytes in response to SEB alone was almost undetectable (0.04 ± 0.72 ng/mL, compared to 33.5 ± 4.2 ng/mL for C57BL/6; Fig. 4b). Still, even though IFN- γ production was unresponsive, λ -carrageenan was able to significantly induce IFN- γ to 8.41 ± 0.51 ng/mL (Fig. 4b, insert). Further, κ -carrageenan, a sulphated polygalactan that lacks the ability to induce pro-inflammatory cytokines (data not shown), was able to enhance IFN- γ production from C57BL/6 splenocytes, although with less efficiency than λ -carrageenan (Fig. 4c, left). However, it is interesting to note that, in MyD88^{-/-} splenocytes, κ - and

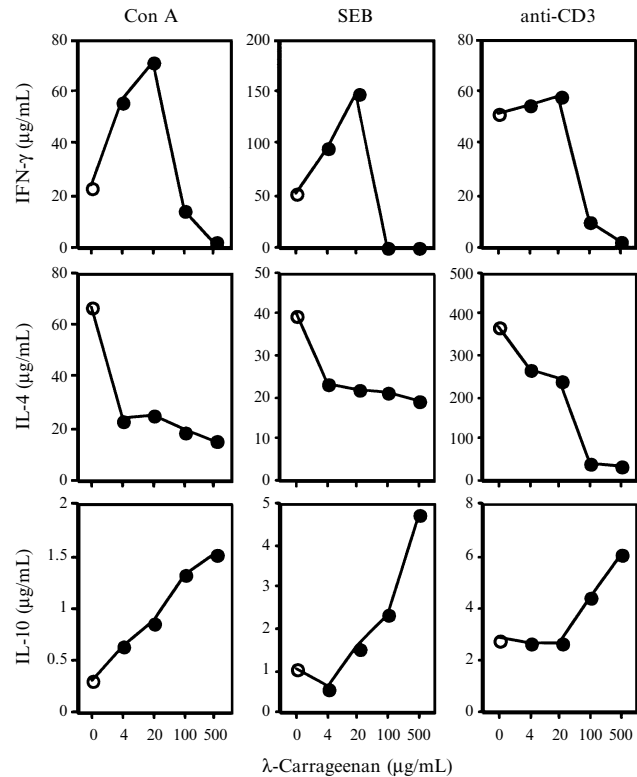


Fig. 3. Effect of λ -carrageenan on production of IFN- γ , IL-4 and IL-10 in splenocyte culture. Splenocytes were stimulated *in vitro* with Con A (2 μ g/mL) or SEB (0.1 μ g/mL) and coated anti-CD3 mAb for 48 h. The indicated concentrations of λ -carrageenan were added to culture wells in triplicate. Supernatants were collected for determination of IFN- γ , IL-4 and IL-10 by ELISA. A typical cytokine profile from three independent experiments is shown.

λ -carrageenan induced IFN- γ with equal efficiency (Fig. 4c, right), suggesting that the TLR4/MyD88-independent pathway can regulate Th1/Th2 balance. These observations suggest that λ -carrageenan promotes Th1 immunity *in vitro* via both TLR4/MyD88-dependent and -independent pathways. Because IL-12 has been shown critical for Th1 promotion, we analysed IL-12 production from macrophages, which was found to be impaired in C3H/HeJ macrophages in response to λ -carrageenan, compared to C3H/HeN (Fig. 5).

Oral administration of λ -carrageenan inhibits allergic reactions

To test the efficacy of λ -carrageenan for the purpose of inhibiting Th2 responses *in vivo*, we first evaluated the pattern of specific antibody production by immunizing mice with OVA absorbed onto alum. This immunizing protocol is known to induce Th2 immunity, with concomitant IgE production. Frequent oral administration of λ -carrageenan from day 0 for 6 consecutive days per week inhibited anti-OVA-specific IgE on day 28 (Fig. 6a, top) and on day 35 (data not shown). Because it is clinically important to reverse established Th2 immunity, we found that λ -carrageenan administration from on day 14 was also able to inhibit anti-OVA specific IgE on day 28 (Fig. 6b).

Unexpectedly, neither Th2-associated specific IgG1 (Fig. 6a, middle) nor Th1-related specific IgG2a production (Fig. 6a, bottom) was altered significantly. Therefore, we evaluated

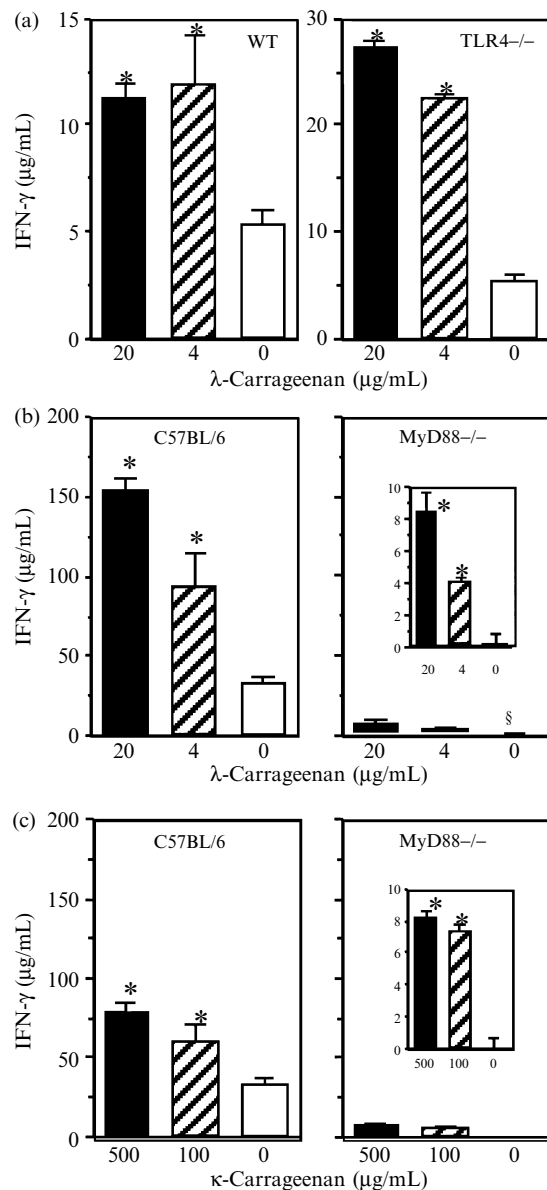


Fig. 4. Effect of TLR4 and MyD88 on λ-carrageenan-mediated augmentation of IFN-γ in splenocyte culture. Splenocytes, prepared from wild-type (WT), TLR4^{-/-}, C57BL/6 or MyD88^{-/-} mice, were stimulated *in vitro* with SEB (0.1 μg/mL) for 48 h. The indicated concentrations of λ-carrageenan (a and b) or κ-carrageenan (c) were added to triplicate culture wells. Supernatants were collected for determination of IFN-γ by ELISA. Asterisk denotes statistical significance comparing to control wells in the absence of carrageenan. Inserts shows the same results with enlarged scale for Y-axis.

the *in vitro* profile of cytokine secretion in OVA-stimulated splenocyte cultures. In accord with the *in vivo* observation, oral administration of λ-carrageenan did not alter antigen-specific IFN-γ and IL-4 production from splenocytes (data not shown). It is possible that the ability of λ-carrageenan to specifically inhibit IgE *in vivo* is due to general suppressive properties of carrageenan which may impair responses against T cell mitogens [4, 6]. To exclude this possibility, splenocytes from λ-carrageenan-fed mice were stimulated with Con A *in vitro*. There was no impairment of IFN-γ production in splenocytes from λ-carrageenan-fed mice in response to Con A (Fig. 7a). Further, LPS was used to induce IgM production

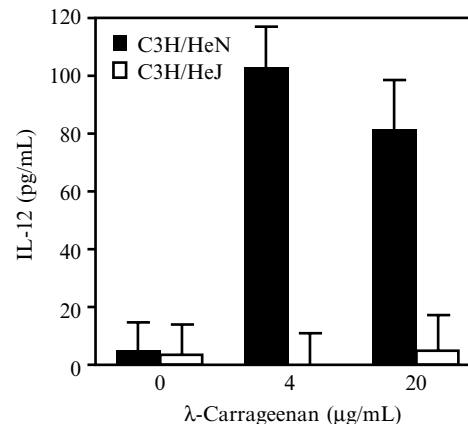


Fig. 5. TLR4-dependent production of IL-12 by λ-carrageenan in macrophage culture. Peritoneal macrophages were obtained from TLR4-normal C3H/HeN (■) and TLR4-mutant C3H/HeJ (□) mice, and treated with λ-carrageenan as described in Fig. 1. Supernatants were collected and assayed for IL-12 determination by ELISA.

in these splenocytes, and the responses of λ-carrageenan-fed mice were comparable to that of saline-fed controls (Fig. 7b). The fact that normal responses were observed for anti-OVA IgG1 and IgG2a *in vivo*, OVA-specific cytokine secretion *in vitro*, and mitogen responses *in vitro* indicated that oral administration of λ-carrageenan specifically inhibited IgE production *in vivo* without inducing non-specific immune suppression.

Next, we asked if specific IgE inhibition via oral administration of λ-carrageenan leads to reduced histamine release. Similar to our previous experiment, mice were immunized with OVA and fed carrageenan, and were then challenged i.p. with OVA on day 35 to induce systemic anaphylaxis. Blood was collected 10 min after challenge (for serum preparation), and the level of histamine was determined. As expected, histamine was increased upon OVA challenge (56.8 ± 8.8 ng/mL in naive mice to 493.3 ± 112.2 ng/mL in immunized mice), but this increase was significantly negated by 0.005% and 0.001% λ-carrageenan (Fig. 8). Feeding at low concentration, 0.0002%, was not effective. Simultaneously, a blinded observer graded the induction of anaphylaxis. The anaphylaxis scores in 0.005% and 0.001% λ-carrageenan-fed groups were significantly reduced compared to the saline-fed control group (data not shown).

Collectively, these results indicate that λ-carrageenan may prove useful as a dietary substance for improving allergic symptoms, due partly to its effect on Th1/Th2 balance.

The effect of λ-carrageenan on oral tolerance is distinct from that of LPS

Because activators of TLRs/MyD88 may induce active immune responses [8], it was important to address whether they have any influence on oral tolerance, which is thought to be crucial for homeostasis in the intestine for the prevention of food allergies. LPS was used as a TLR4/MyD88-activator, and was fed to C3H/HeN mice in concert with OVA to induce oral tolerance. At doses of 50 and 10 μg, LPS abrogated OVA-specific IgG and IgG1 levels in serum (Fig. 9a, left). However, no such abrogation was evident in TLR4-mutant C3H/HeJ mice (Fig. 9a, right). On the contrary, tolerized specific IgG and IgG1 were not altered by simultaneous feeding of 0.01% λ-carrageenan along with OVA in TLR4-normal C3H/HeN mice (Fig. 9b, left). Moreover, oral

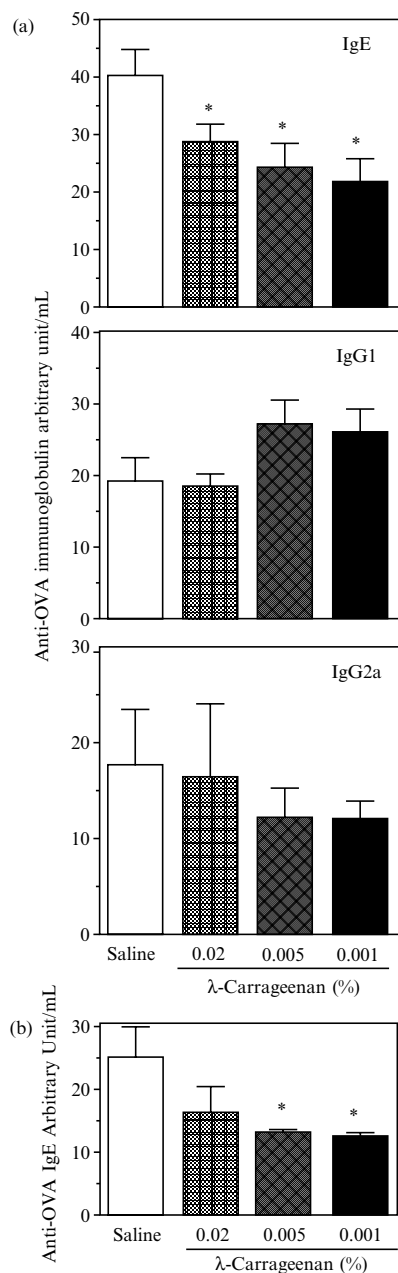


Fig. 6. Effect of oral administration of λ -carrageenan on OVA-specific antibody production. BALB/c mice were immunized i.p. with OVA in alum on days 0 and 7. λ -Carrageenan was orally administered for 6 consecutive days per week from day 0 for 5 weeks (a) or starting from on day 14 with same dose regimen (b). Blood was collected on day 28 and arbitrary antibody units in serum were calculated relative to anti-mouse IgE ELISA, or control hyperimmune serum for IgG1 and IgG2a, as described in Materials and methods. Asterisks denote statistical significance between saline-fed group and λ -carrageenan-fed group. Ten mice were used for each group.

tolerance was promoted in TLR4-mutant C3H/HeJ mice (Fig. 9b, right), suggesting that PRRs other than TLR4 are stimulated by λ -carrageenan to prevent the abrogation of TLR4-mediated oral tolerance. Thus, other PRRs may promote oral tolerance induction which can be revealed only in the absence of TLR4. These data imply that λ -carrageenan and LPS use distinct mechanisms (and perhaps different PRRs) to affect innate immunity and thereby modulate the immune system.

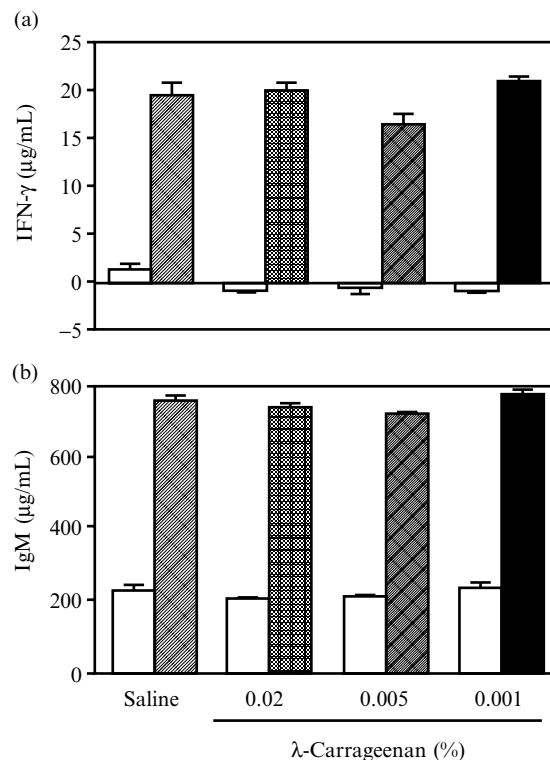


Fig. 7. Effect of oral administration of λ -carrageenan on responses against mitogens in splenocyte culture. BALB/c mice were immunized i.p. with OVA in alum on days 0 and 7. λ -Carrageenan was orally administered for 6 consecutive days per week from day 0 for 5 weeks. Single cell suspensions, prepared from these mice on day 35, were stimulated *in vitro* with 2 μ g/mL of Con A (a) or 2 μ g/mL of LPS (b) for 48 h. Supernatants were collected to determine IFN- γ (a) or total IgM (b) by ELISA. Open bars represent the basal level of IFN- γ or IgM in the absence of mitogenic stimulation.

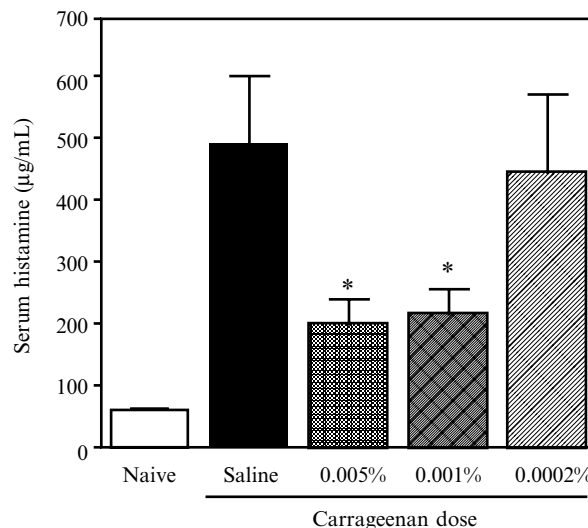


Fig. 8. Effect of oral administration of λ -carrageenan on serum histamine release. BALB/c mice were immunized i.p. with OVA in alum on days 0 and 7. λ -carrageenan was orally administered as in Fig. 5. On day 35, mice were challenged i.p. with 100 μ g/mL OVA in PBS. Blood was collected 10 min after OVA challenge and serum was prepared. Histamine levels in serum were measured by HPLC as described in Materials and methods. Asterisks denotes statistical significance relative to saline-fed group. Number of mice used was 10 per group.

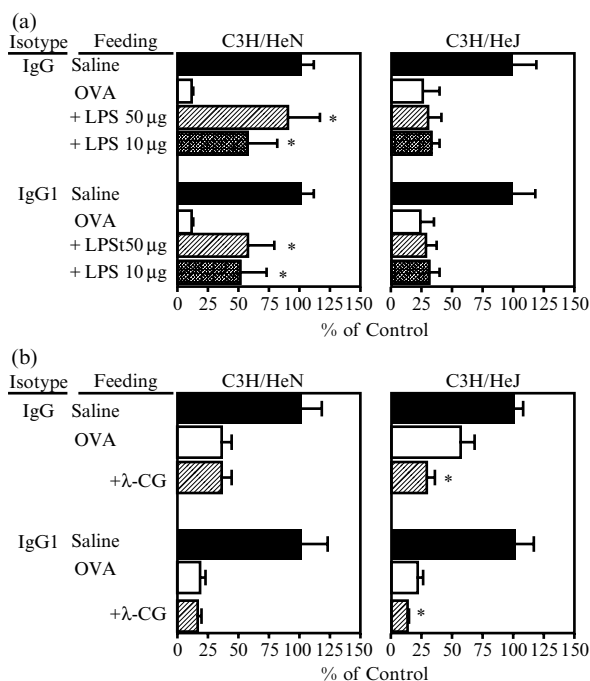


Fig. 9. λ -Carrageenan's effect on oral tolerance is distinct from LPS. BALB/c mice were fed 5 mg OVA five times from day 0 to day 4, with or without the indicated amount of LPS (a) or 0.01% λ -carrageenan (λ -CG, b) to induce oral tolerance. As a control, saline was fed instead of OVA. These mice were immunized i.p. with OVA in alum on days 7 and 14, and blood was collected to prepare serum on day 28. OVA-specific IgG and IgG1 were determined by ELISA relative to IgG and IgG1 standards. Units were computed and shown in percentage of titre in the saline-fed control group. Asterisks denote statistical significance between OVA- and saline-fed groups. Six mice were used per group.

Discussion

Recent appreciation for the role of innate immunity in instructing acquired immune responses suggests that foreign substances like food components utilize basic immunological machinery to modulate the immune system. Thus, the initial interaction of these substances with components of innate immunity appear to play a decisive role in determining whether immunity should be induced and, if so, what type. In this context, the nature of PRRs is of great importance in the modulation of immunity. As PRRs may interact with heterogeneous substances contained in food, it is of interest to investigate a possible dietary manipulation of the immune system. Carrageenan is widely used by the food industry due to its gelling and stabilizing properties, and it has been extensively used as a reagent to induce non-specific inflammation, possibly through activation of innate immune responses. Carrageenan has also been ascribed the property of modulating acquired immunity, and both adjuvant and suppressive effects have been reported [2–7]. Such inconsistent outcomes may be due to differences in the route of administration, dosage and heterogeneity of carrageenan preparations. Therefore, in this study, we re-purified commercially available λ -carrageenan which itself is thought relatively homogenous. We confirmed the homogeneity of our re-purified preparation using infrared spectroscopy and ^{13}C -NMR. In order to investigate TLR-involvement, endotoxin-contamination was rigorously excluded through the use of a polymyxin-B column followed by a limulus assay.

Thus, our purified λ -carrageenan contained < 1.5 pg endotoxin per milligram of solid.

We first sought to characterize possible PRRs that recognize λ -carrageenan, thus leading to activation of macrophages. Both IL-6 and TNF- α have been described as pro-inflammatory cytokines responsible for λ -carrageenan-induced inflammatory reactions. Indeed, λ -carrageenan-induced pleurisy is largely attenuated in IL-6-deficient mice as well as in wild-type mice treated with an IL-6 neutralizing antibody (both mice exhibited concomitant reduction in TNF- α) [32]. Furthermore, neutralization of TNF- α itself leads to reduced pleurisy [33]. Here, we demonstrated that production of these pro-inflammatory cytokines from macrophages *in vitro* is dependent on TLR4 and MyD88. These data are further supported by decreased mRNA levels as shown by our DNA-array. To extend our *in vitro* findings, footpad oedema was induced, and a significant reduction of footpad swelling was observed specifically in TLR4-/- and MyD88-/- mice. Studies of pleurisy in TLR4-/- mice also revealed marked reduction of pleural TNF- α as well as a lack of tissue destruction (unpublished data).

Collectively, our observations indicate that activation of innate immunity by λ -carrageenan is TLR4/MyD88-dependent. To our knowledge, this study is the first to identify a polysaccharide ligand for TLR4. Although it has been proposed that LPS binds directly to TLR4 and the MD2 complex with the aid of CD14 [34], it is currently unknown whether λ -carrageenan is recognized by TLR4 or whether additional PRRs are involved. However, the types of ligands that bind TLR4, such as LPS (lipid A), taxol and λ -carrageenan, are highly variable and therefore TLR4 may require unique accessory components in order to recognize such a broad spectrum of ligands.

Given the recent reports that demonstrate preferential induction of Th1 immunity by TLR/MyD88 [18–22], we investigated the efficacy of λ -carrageenan for the treatment of allergic reactions. Our data support these findings, in that increased IFN- γ production was observed in concert with a reduction in IL-4 in T cell cultures stimulated with Con A or SEB or anti-CD3 (Fig. 3). However, we were surprised to find TLR4/MyD88-independent promotion of Th1 immunity given our finding that the λ -carrageenan-induced alteration of IFN- γ /IL-4 secretion patterns was comparable between TLR4-/- and wild-type splenocytes. Due to its intrinsic characteristic of Th2-dominant immunity [22], the absolute amount of IFN- γ produced by MyD88-/- splenocytes was significantly smaller than that produced by C57BL/6, although the fold increase in IFN- γ production in response to λ -carrageenan was even higher for MyD88-/- . As IL-12 is critical for promoting Th1 immunity [35, 36], IL-12 production from macrophages of C3H/HeN and C3H/HeJ mice were evaluated. IL-12 also requires TLR4 in order to be induced by λ -carrageenan at doses of 4 and 20 $\mu\text{g}/\text{mL}$ (Fig. 5). These data suggest that IL-12 cannot account for TLR4/MyD88-independent Th1 promotion. This observation is further strengthened by the fact that κ -carrageenan is unable to activate innate immunity (e.g. pro-inflammatory cytokine production; unpublished data) yet promotes Th1-associated IFN- γ production. Interestingly, although the level of increase in IFN- γ by κ -carrageenan in C57BL/6 was significantly lower than that for λ -carrageenan in wild-type mice, it was comparable in MyD88-/- mice suggesting that both TLR4/MyD88-dependent and -independent pathways are used. Presumably, the TLR4/MyD88-independent pathway can be stimulated

by both κ - and λ -carrageenan and does not involve IL-12 production. Among polysaccharides, large chitin particles (capable of being phagocytized) can induce Th1 cytokines probably through IL-12 production, but such is not the case for soluble chitin or β -glucan [37]. In this context, the ability of λ -carrageenan to induce Th1 cytokines in an IL-12-dependent as well as-independent manner is unique among soluble polysaccharides.

Oral administration of λ -carrageenan induces non-specific suppression of immunity. Nicklin and Miller [5] conducted experiments in which rats were fed 0.5% carrageenan via drinking water for 90 days. These rats showed impaired haemagglutinating antibody formation. Bash and Vago [4] reported hyporesponsiveness of T cells against mitogens after chronic feeding of rats with 0.01% carrageenan via drinking water. Cochran et al. [6] used $\sim 0.02\%$ carrageenan for intragastric instillation and observed suppression of T cell proliferation in response to mitogens. In our study, we used doses ranging from 0.0002% to 0.02% for oral administration of λ -carrageenan and found that a relatively low dose of 0.001% was optimal for inducing specific decreases in IgE (Figs 5a and b) and systemic histamine release (Fig. 7). When mice were fed 0.02% λ -carrageenan, the decreases in IgE and histamine release were less significant. Further, *in vitro* antigen-stimulation of splenocytes from 0.02%-fed mice revealed a slight suppression of IFN- γ and IL-4 production (data not shown), suggesting that this dosage range might be immunosuppressive. However, mitogenic responses of these splenocytes were found to be comparable to those from saline-fed controls, indicating that decreases in IgE and histamine release were specifically induced without systemic suppression in our studies. These *in vivo* findings were also consistent with our *in vitro* observation that doses higher than 100 $\mu\text{g/mL}$ in stimulated T cell cultures led to diminished IFN- γ and IL-4 production and a significant induction of IL-10. Because IL-10 is a down-regulatory cytokine [38], preferential induction of systemic suppression at relatively high doses may be due to IL-10 production. Importantly, low-dose administration of λ -carrageenan, as shown in this study, may result in specific IgE reduction *in vivo*. It should also be noted that oral administration of λ -carrageenan was effective to reverse established Th2 immunity, as onset of administration 1 week after final immunization was able to inhibit IgE production. Therefore, these data suggest the potential of λ -carrageenan for the treatment of allergy patients. Although there are many foods using λ -carrageenan as an ingredient, in which the usual concentration used is from 0.05% to 0.2%, our current findings suggested that the concentration to promote Th1 activity should be lower. Therefore, another functional food to ameliorate allergy could be tailored by use of optimal concentration of λ -carrageenan.

Given the significant effect of λ -carrageenan on modification of IFN- γ and IL-4 production in T cell stimulation *in vitro*, it is puzzling that λ -carrageenan treatment caused no significant reduction in serum IgG1 yet enhanced serum IgG2a levels (Fig. 5a). Furthermore, we could not detect *in vitro* alteration of IFN- γ and IL-4 (data not shown) from splenocytes, prepared from OVA-immunized mice. These conflicting data may be a consequence of the fact that λ -carrageenan effects a decrease in IL-4 production more potently than it induces IFN- γ . Further, although humoral immunity is more difficult to tolerized than cellular immunity, IgE is quite susceptible to tolerance induction [39, 40]. Such may also be the case in our system.

Because activation of innate immunity via TLR induces the active immune response, the effect of activators of innate immunity on induction of oral tolerance is of great importance. Oral tolerance is the mechanism that maintains the homeostasis of intestinal immunity by silencing active immune responses against food substances and indigenous microflora. It is noteworthy that while simultaneous feeding of LPS and cell wall preparations of Gram-negative bacteria (such as *Escherichia coli* and *Pseudomonas*) with OVA prevent the induction of oral tolerance against OVA (data not shown), λ -carrageenan did not have such an effect. Furthermore, in TLR4-mutant C3H/HeJ mice, λ -carrageenan promoted oral tolerance, suggesting an involvement of putative down-regulatory PRRs that are recognized by λ -carrageenan. This effect on oral tolerance in the absence of TLR4 signalling is consistent with a recent report in which the induction of oral tolerance in C3H/HeJ mice prevented anaphylaxis [41]. However, these experiments were limited by the fact that only C3H/HeJ mice were tested.

We identified λ -carrageenan as a new class of ligand for TLR4/MyD88 that activates innate immunity, and showed that PRRs recognize λ -carrageenan and induce Th1-cytokines and reduce histamine release *in vivo* by suppressing IgE production. Yet, in contrast to LPS, abrogation of oral tolerance does not follow. Thus, λ -carrageenan may represent an effective dietary supplement for the prevention and/or improvement of allergy-related diseases.

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