Supporting information

Carrageenan Activates Monocytes *via* Type-Specific Binding with Interleukin-8: An Implication for Design of Immuno-active Biomaterials†

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Contents

Materials and cell culture

Kappa- (9062-07-1), iota- (11114-20-8) and lambda-type carrageenan (9064-57-7) were purchased from Tokyo Chemical Industry Co. Ltd, Japan. Fetal bovine serum (FBS) (26140-079), human serum (GENIA stock# S0213) and RPMI 1640 medium (22400-105) were obtained from Life Technologies. Sterile Transwell® (6.5mm, with 3.0-μm pore) Polycarbonate Membrane Insert was purchased from Corning (3415). GoTaq 2-Step RT-qPCR system (A6010) was purchased from Promega. Recombinant human CXCL8/IL8 (208-IL-010) and its antibody (MAB208-SP) were purchased from R&D Systems. SiMAG-Hydrazide (1404-1) was purchased from Chemicell GmbH. Red blood cell lysis buffer (C3702) was purchased from Beyotime. Anti-Human/Mouse MyD88 (14622381) was purchased from Ebioscience. Anti-beta actin (4967S), anti-rabbit IgG, HRP-linked (7074S), anti-pAkt (4060P) and anti-Akt (9272) were obtained from Cell Signaling Technology. Goat anti-mouse IgG-HRP (sc-2005) was purchased from Santa Cruz. Anti TLR4 (76B357.1) was purchased from Abcam. Interleukin-8-inhibiting peptide Ac-RRWWCR-NH_2 (AS-62401) was synthesized by ANASPEC. TLR-4 inhibitor CLI-095 was purchased from InvivoGen. SuperSignal™ West Pico Chemiluminescent Substrate (34080) was purchased from Thermo Fisher Scientific. All the primers for real-time quantitative polymerase chain reaction (PCR) were synthesized by Life Technologies and their sequences are listed in **Table S1**.

THP-1 (ATCC® TIB-202™), the human monocytic cell line, was purchased from American Type Culture Collection (ATCC). The cells were maintained in RPMI 1640 medium supplemented with 10% FBS and incubated in 37℃ incubator, 5% CO_2.

Particle size measurement

The sizes of CGN particles (suspended in dH_2O, 100 μg mL^{-1}) were analyzed by Nanoparticle Tracking Analysis system (NS500, Nanosight, UK) at 25℃.

HPSEC-MALLS-RID analysis

The molecular weight (Mw) and polydispersity (Mw/Mn) were determined by HPSEC-MALLS-RID. The measurements were carried out on a multi-angle laser light scattering (DAWN HELEOS, Wyatt Technology Co., Santa Barbara, CA, USA) with an Agilent 1100 series LC/DAD system (Agilent Technologies, Palo Alto, CA, USA), which was equipped with columns of TSK-Gel $G6000PW_{xL}$ (300 mm x 7.8 mm, i.d.) in series at 35℃. Moreover, a refractive index detector (RID, Optilab rEX refractometer, DAWN EOS, Wyatt Technology Co., Santa Barbara, CA, USA) was connected and the MALLS instrument was equipped with a He-Ne laser ($\lambda = 685\ nm$). The Mw was calculated by the Zimm method. The mobile phase was 0.9% NaCl aqueous solution and the flow rate was 0.5 mL min^{-1}. The final concentration of each sample was about 2 mg mL^{-1} and the injection volume was 50 μL. The Astra software (Version 6.0.2, Wyatt Tech. Corp., Santa Barbara, CA, USA) was applied for data acquisition and analysis.

Isolation of murine bone marrow derived monocytes and cell adhesion assay

Protocols for animal experiments were reviewed and approved by both the Animal Ethics Committee at the Institute of Chinese Medical Sciences, University of Macau, and the Animal Care and Use Committee of Nanjing University. All the procedures were performed in

compliance with these institutional guidelines. We isolated bone marrow derived monocytes as described below[1]. Briefly, C57BL/6 mouse was sacrificed by cervical dislocation. After removal of the muscle from the femurs and tibias, both ends of the bone were cut with scissors. Using the PBS to flush the marrow out. The suspend cells (10^6 cells per mL) were seeded on 10 cm^2 ultra-low-attachment surface plates to prevent permanent adhesion to the bottom of the plate. Supplemented the suspension with 20 ng mL^{-1} M-CSF to promote cell differentiation. Then cultured the cells for 5 days at 37 °C and 5% carbon dioxide. After 5 days, we harvested the supernatant with the cells in suspension and characterized the cells by using flow cytometry , which predominantly contain monocytes (**Fig. S2A**). In addition, the rest monocytes were used for adhesion assay, that was, stimulated the cells with 100 μg mL^{-1} CGN or 100 ng mL^{-1} PMA for 24 hours. Then detected the adherent cells viability using MTT assay (**Fig. S2B**).

Cell viability of pre-activated monocytes under CGN treatment

Ten thousand THP-1 cells were seeded in 96-well plate in maintained medium with 100 ng mL^{-1} phorbol 12-myristate 13-acetate (PMA). Twenty-four hours later, the medium was replaced by RPMI 1640 medium with different concentrate CGNs, supplemented with 0.5% FBS. After 24 hours incubation, 10 μL of 5 mg mL^{-1}3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added into each well. Four hours later, the supernatant was discarded and dimethyl sulfoxide (DMSO) was used to dissolve the crystals formed in the bottom of the plate. Then, the absorbance at 590 nm was read by VICTOR 3V Multilabel Reader. The results are shown in **Fig. S3**.

Cell adhesion assay and Calcein-AM staining

Ten thousand THP-1 cells were seeded in 96-well plate. Kappa, iota and lambda CGNs were prepared into 10, 50 or 100 μg mL^{-1} in the medium. The supernatant was carefully discarded after 24 hours incubation. Five mg mL^{-1} MTT was diluted in RPMI 1640 supplemented with 0.5% FBS, following the ratio 1:10. 100 μL of the mixture was added into each well. Four hours later the supernatant was discarded. DMSO was used to dissolve the crystals formed in the bottom of the plate, and the absorbance at 590 nm was read by VICTOR 3V Multilabel Reader. A parallel experiment was done in RPMI 1640 medium supplemented with 0.5% FBS. In addition, given the heterogeneity, we repeated the above experiments with 5% or 0.5% human serum in **Fig. S4**. For inhibitor tests, Ac-RRWWCR-NH2 and CLI-095 was prepared to 10^{-4} M and 100 nM respectively in the medium. As for Calcein-AM staining, THP-1 was seeded in the plate and treated as mentioned above. After treated with CGNs for 24 hours, the supernatant was discarded and 1 μg mL^{-1} Calcein-AM was added. Cells were incubated for 20 minutes at 37℃ before the observation by fluorescent microscope. The cell number of adhesion THP-1 cells was calculated with ImageJ 1.50i.

Carrageenan hydrolysis

1 mg mL^{-1} lambda CGN was prepared in 0.1 M HCl and incubated for 0.5, 1 and 2 hours at room temperature. The acid was neutralized and gone through dialysis for 48 hours. The mixture was then lyophilized and its particle size was detected by NTA.

Carbohydrate-magnetic beads preparation

Kappa, iota, lambda, and 2-hour-degraded lambda CGN (2 mg mL^{-1}) were oxidized with Sodium periodate (10 mg mL^{-1}) in 0.1 M Sodium Acetate-Acetic acid buffer, pH 5.0, for 2 hours in dark. The reaction was terminated with addition of excess glycerol and standing for half an hour. The polysaccharide was then precipitated by adding excess ethanol and re-suspended with distilled water before lyophilization. 200 µl SiMAG-Hydrazide beads were washed several times with PBS. 2 mg mL^{-1} dried oxidized polysaccharides were prepared with PBS and reacted with the beads for 6 hours at room temperature. The beads were washed with PBS for several times. 200 µL of blocking buffer (0.1 M D-glyceraldehyde in ten times PBS) was added to each sample. After 2 hours incubation, the beads were again washed with PBS. Finally, the beads were resuspended in 1 mL storage buffer (0.1% BSA, 0.05% sodium azide in PBS) and kept in 4℃.

Protein-carbohydrate interaction

200 µL of the polysaccharide-modified beads were incubated with 2 µg interleukin-8 in PBS for 2 hours at room temperature. The magnetic beads were separated from the solvent with the magnet. 30 µL of PBS was added into each sample to remove the loading dye. The samples were boiled for 10 minutes. Samples were stacked in 5% polyacrylamide gel and then separated in 10% gel. A denatured 1 µg interleukin-8 sample was run as a positive control. Proteins were transferred to methanol-activated PVDF membrane and underwent an immunoblotting procedure.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

One million THP-1 cells were treated with 100 ng m^{-1} PMA, 50 μg mL^{-1} lambda CGN or 20 ng mL^{-1} interleukin-8 for 72 hours. The RNA was extracted with TRIzol reagent. The process was suggested by the instruction of the reagent. Collected RNA was reverse transcribed into cDNA for the sake of qPCR analysis, following the guidance of GoTaq 2-Step RT-qPCR system.

Western blotting

One million THP-1 cells were incubated with 100 ng mL^{-1} PMA, 50 μg mL^{-1} kappa, iota or lambda CGN. 15 minutes or 24 hours later, cells were washed 3 times with PBS and lysed with lysis buffer (RIPA: Cocktail: PMSF=100:1:1). The mixture were centrifuged at 15,000 g for 15 minutes at 4℃. Supernatant was collected and the protein concentration was determined with Pierce™ BCA Protein Assay Kit following its manual. Denature loading-dye was added to one-fifth volume of the samples and then boiled for 10 minutes. 25 μg of total proteins were loaded to a 5% stacking- then 10% separation poly-acrylamide gel. Proteins on the gel were transferred to PVDF membrane at 250 mA for 90 minutes. The membrane was blocked with 5% non-fat milk for an hour then different parts were incubated in 1st antibodies prepared in 2.5% non-fat milk overnight. Dilute ratio: anti-beta-actin and anti-TLR4: 1:1000, anti-p-Akt: 1:2000, anti-Akt: 1:2000, anti-MyD88:1:500. Membranes were then washed with TBST and incubated in anti-rabbit-HRP (1:5000) or anti-mouse (1:5000) for 2 hours at room temperature. Then the membranes were washed again and developed with

SuperSignal™ West Pico Chemiluminescent Substrate, following the instruction. The results are shown in **Fig. S7**.

Mouse bone marrow neutrophils isolation and characterization

We extracted neutrophils by following the modified protocol described by Swamydas and Lionakis[2]. Briefly, mouse was enthanized and the body surface was sterized with 75% ethanol. Femur and tibia were picked out. Then bone marrow was flushed with PBS and traveled through a 100 μm filter. And the extract was centrifuged at 1200 rpm for 5 minutes at 4 °C. After that, red blood cells were lysed by red blood cell lysis buffer and wash three times of cells with RPMI 1640 supplemented with 10% FBS and 2mM EDTA. Cells were resuspended in 1 ml cool PBS. Histopaque 1119 was laid in a conical tube and them Histopaque 1077 and bone marrow cells. The tube was centrifuged for 30 minutes at 25 °C without brake. Neutrophils were collected at the interface of 1119 and 1077 layers and characterized with CD11b and Ly-6G. The results are shown in **Fig. S8A**.

Neutrophil migration assay

Three hundred thousand neutrophils extracted from mouse bone marrow were seeded on the upper chamber of Corning® Transwell® PC membrane cell culture inserts (hole size: 0.3 μm). In the lower chamber, CGNs were prepared to 50 or 100 μg mL^{-1} in 5% FBS supplemented 1640. Interleukin-8 (200 ng mL^{-1}) was prepared as a positive control. After 2 hours, neutrophils in lower chamber were counted three times on hemocytometer under microscope. In addition, in order to investigate whether the cytokines or chemokine

produced from macrophages could stimulate neutrophil migration, the supernatant of THP-1 treated with PMA and lambda carrageenan was prepared, which was added in the lower chamber. The results are shown in **Fig. S8B**.

Statistical analysis

Data were expressed as mean ± SD, unless otherwise indicated, at least three independent experiments. Comparisons were performed by means of a two-tailed Student t test. Values of $P < 0.05$ was considered significant.

Table S1 Primers for RT-qPCR

Human beta-actin (Forward)	CCTGGCACCCAGCACAAT
Human beta-actin (Reverse)	GGGCCGGACTCGTCATACT
Human CD206 (Forward)	CGAGGAAGAGGTTCGGTTCACC
Human CD206 (Reverse)	GCAATCCCGGTTCTCATGGC
Human Dectin-1 (Forward)	TCTTTCCAGCCCTTGTCCTC
Human Dectin-1 (Reverse)	CCAGTTGCCAGCATTGTCTT
Human IL-8 (Forward)	ATGACTTCCAAGCTGGCCTGGCT
Human IL-8 (Reverse)	TCTCAGCCCTCTTCAAAAACTTCTC
Human TNFα (Forward)	CCTCTCTCTAATCAGCCCTCTG
Human TNFα (Reverse)	GAGGACCTGGGAGTAGATGAG
Human IL1β (Forward)	ATGATGGCTTATTACAGTGGCAA
Human IL1β (Reverse)	GTCGGAGATTCGTAGCTGGA
Human OSM (Forward)	AGTACCGCGTGCTCCTTG
Human OSM (Reverse)	CCCTGCAGTGCTCTCTCAGT
Human COX2 (Forward)	CAAAAGCTGGGAAGCCTTCT
Human COX2 (Reverse)	CCATCCTTGAAAAGGCGCAG
Human VEGF (Forward)	AGGGCAGAATCATCACGAAGT
Human VEGF (Reverse)	AGGGTCTCGATTGGATGGCA
Human PDGFB (Forward)	CTCGATCCGCTCCTTTGATGA
Human PDGFB (Reverse)	CGTTGGTGCGGTCTATGAG

Table S2 Motif that found to bind different Carrageenan (L=Lambda, I=Iota, K=Kappa, C=Control).

Accession	Description	MW [kDa]	calc. pI	Sample: L	Sample: K	Sample: I	Sample: C
P02769	Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4	69.248	6.18	Y	Y	Y	Y
P12763	Alpha-2-HS-glycoprotein OS=Bos taurus GN=AHSG PE=1 SV=2	38.394	5.5	Y	N	Y	Y
P34955	Alpha-1-antiproteinase OS=Bos taurus GN=SERPINA1 PE=1 SV=1	46.075	6.52	Y	Y	Y	Y
F1MSZ6	Antithrombin-III OS=Bos taurus GN=SERPINC1 PE=3 SV=1	52.407	6.8	Y	N	N	Y
P41361	Antithrombin-III OS=Bos taurus GN=SERPINC1 PE=1 SV=2	52.314	7.33	Y	N	N	Y
F1MD83	C-X-C motif chemokine OS=Bos taurus GN=PF4 PE=3 SV=1	12.559	9.1	Y	N	N	N
Q3ZBS7	Uncharacterized protein OS=Bos taurus GN=VTN PE=2 SV=1	53.541	6.28	N	N	Y	N
Q9N2I2	Plasma serine protease inhibitor OS=Bos taurus GN=SERPINA5 PE=1 SV=1	45.268	9.36	Y	N	N	N
Q5GN72	Alpha-1 acid glycoprotein OS=Bos taurus GN=agp PE=2 SV=2	23.144	5.67	Y	N	N	N
Q3SZR3	Alpha-1-acid glycoprotein OS=Bos taurus GN=ORM1 PE=2 SV=1	23.168	5.87	Y	N	N	N
G5E604	Uncharacterized protein (Fragment) OS=Bos taurus PE=4 SV=1	11.051	7.99	N	N	Y	N
F1N3A1	Thrombospondin-1 OS=Bos taurus GN=THBS1 PE=4 SV=1	129.309	4.94	N	N	N	Y
Q28178	Thrombospondin-1 OS=Bos taurus GN=THBS1 PE=2 SV=2	129.451	4.97	N	N	N	Y

A2I7M9	Serpin A3-2 OS=Bos taurus GN=SERPINA3-2 PE=3 SV=1	46.208	5.99	Y	N	N	N
Q9TTE1	Serpin A3-1 OS=Bos taurus GN=SERPINA3-1 PE=1 SV=3	46.208	5.99	Y	N	N	N
P00978	Protein AMBP OS=Bos taurus GN=AMBP PE=1 SV=2	39.209	7.62	Y	N	N	N
F1MMK9	Protein AMBP OS=Bos taurus GN=AMBP PE=4 SV=2	39.268	7.78	Y	N	N	N
Q7SIH1	Alpha-2-macroglobulin OS=Bos taurus GN=A2M PE=1 SV=2	167.47	6.02	N	N	N	Y
F1N427	Septin-12 OS=Bos taurus GN=SEPT12 PE=3 SV=2	41.108	7.72	N	N	N	Y
A5D7Q3	Septin-12 OS=Bos taurus GN=SEPT12 PE=2 SV=1	41.184	7.72	N	N	N	Y
Q5E971	Transmembrane emp24 domain-containing protein 10 OS=Bos taurus GN=TMED10 PE=2 SV=1	24.812	6.68	Y	N	N	N
Q2KJF1	Alpha-1B-glycoprotein OS=Bos taurus GN=A1BG PE=1 SV=1	53.52	5.43	Y	N	N	N
E1BP14	Uncharacterized protein OS=Bos taurus GN=ARHGEF17 PE=4 SV=2	220.56	6.25	N	N	Y	N
F1N619	Cadherin-1 (Fragment) OS=Bos taurus GN=CDH1 PE=4 SV=2	91.803	4.79	N	N	N	Y
G3X6N3	Serotransferrin OS=Bos taurus GN=TF PE=3 SV=1	77.616	7.27	N	N	N	Y
E1BBI1	Uncharacterized protein OS=Bos taurus PE=4 SV=1	3.306	10.9	N	N	N	Y
F1N0A6	Uncharacterized protein (Fragment) OS=Bos taurus GN=GPR98 PE=4 SV=2	598.59	4.59	Y	N	N	N
F1N3K8	Transmembrane emp24 domain-containing protein 10 OS=Bos taurus GN=TMED10 PE=3 SV=2	25.058	6.55	Y	N	N	N
Q6R8F2	Cadherin-1 OS=Bos taurus	97.877	4.89	N	N	N	Y

	GN=CDH1 PE=2 SV=1						
Q29443	Serotransferrin OS=Bos taurus GN=TF PE=2 SV=1	77.703	7.08	N	N	N	Y
Q0VCT9	Cbp/p300-interacting transactivator 2 OS=Bos taurus GN=CITED2 PE=2 SV=1	28.691	6.95	N	N	Y	N
A5D791	EIF2AK3 protein OS=Bos taurus GN=EIF2AK3 PE=2 SV=1	124.517	5.52	Y	N	N	N

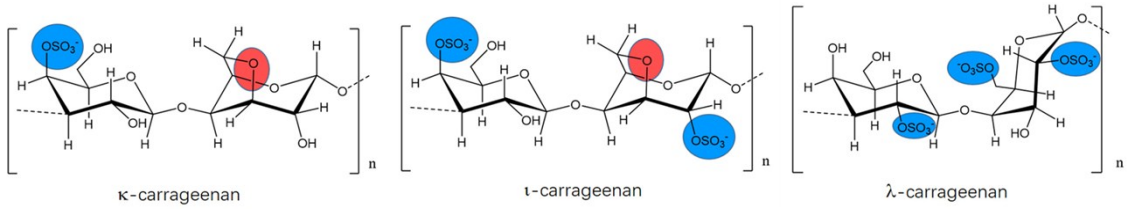

Fig. S1 The structure of three types of Carrageenan.

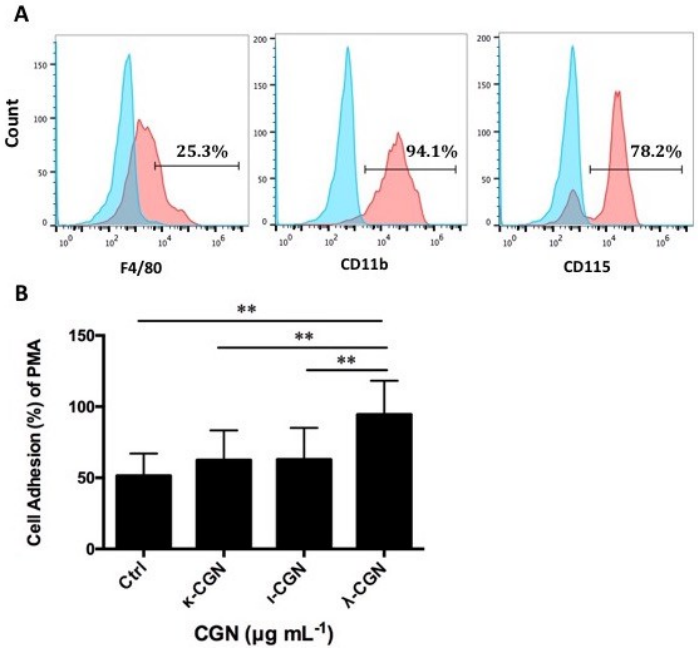

Fig. S2 (A) Phenotype characterization of murine bone marrow derived monocytes. (B) The relative adherent density of bone marrow derived monocytes under different CGN treatment in medium supplemented with 10% FBS（** p<0.01）.

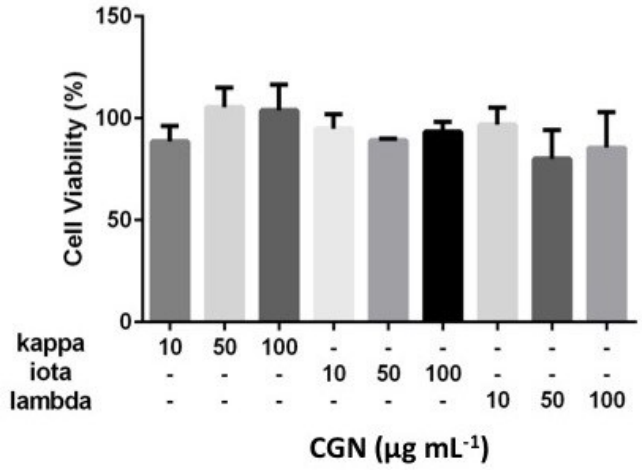

Fig. S3 Cell Viability of macrophages under CGN treatment.

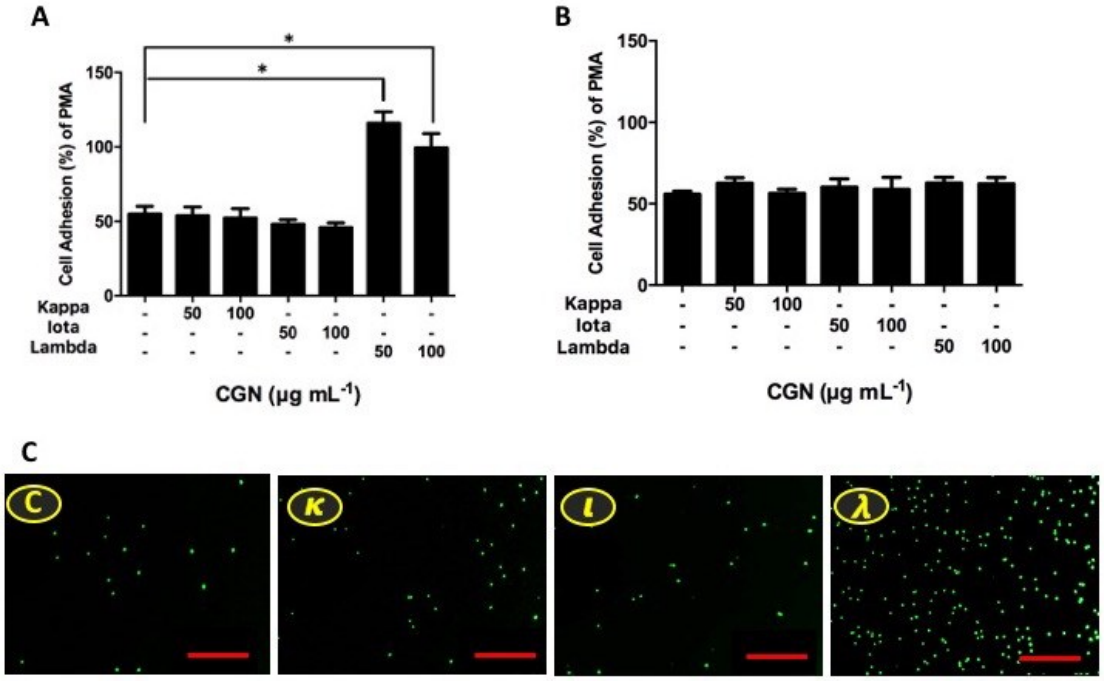

Fig. S4 The relative adherent density of THP-1 under different CGN treatment in medium supplemented with (A) 5% or (B) 0.5% human serum （* p<0.05）. The adhesion can also be observed with (C) Calcein-AM staining of the cells with no treatment or treated with κ-, ι-, or λ-CGN (Scale bar: 500 μm).

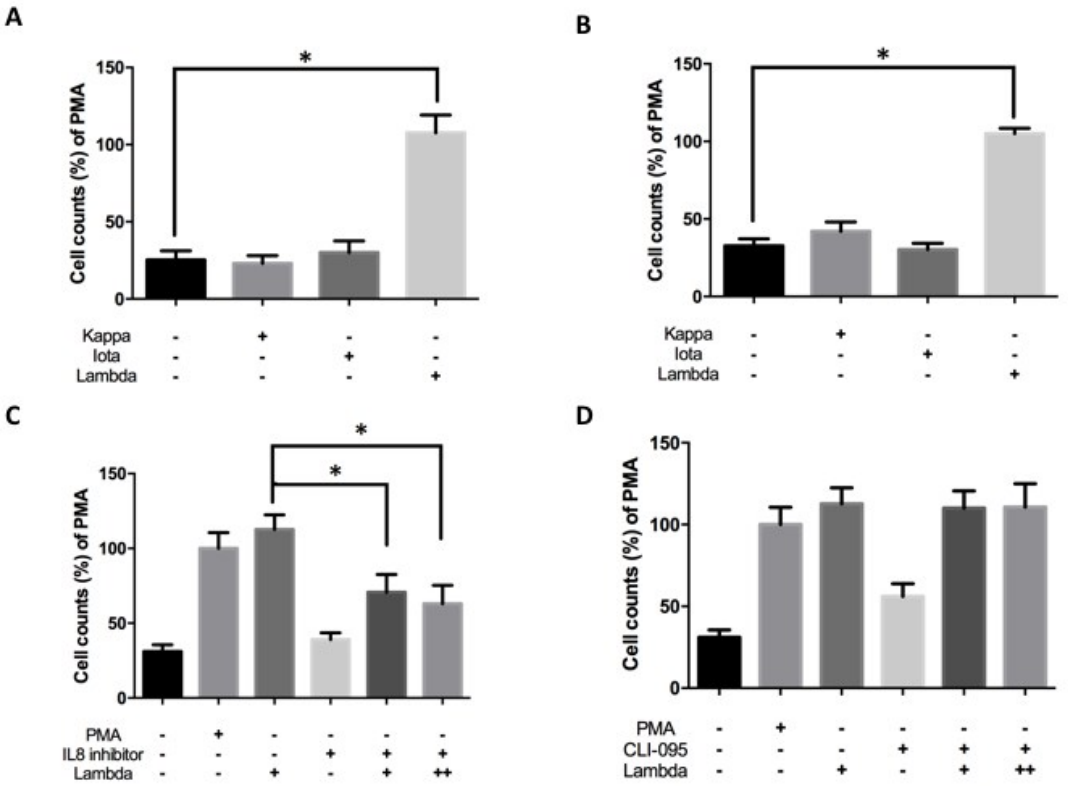

Fig. S5 The relative number of adherent THP-1 under different CGN (100 μg mL^{-1}) treatment in medium supplemented with (A) 10% FBS or (B) 5% human serum. The relative number of adherent THP-1 treated with lambda-CGN (50 or 100 μg mL^{-1}) in the presence or absence of IL-8 (C) or TLR4 (D) inhibitor in 10% FBS medium. The cell number was calculated with ImageJ 1.50i （* p<0.05）.

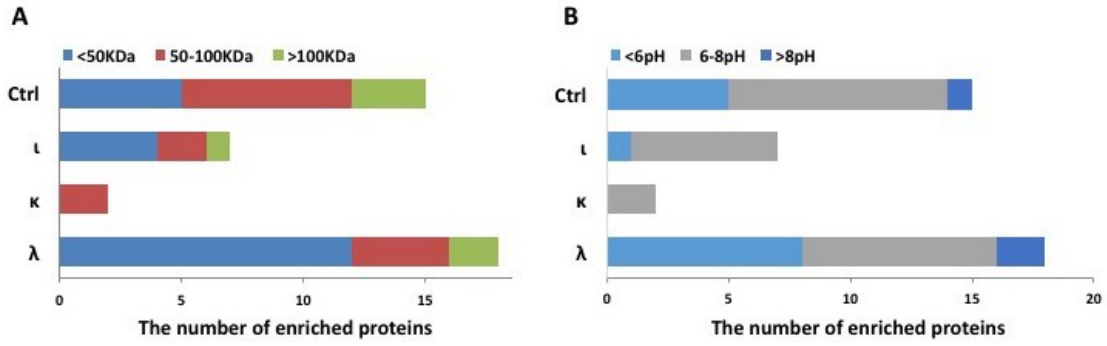

Fig. S6 (A) Molecular weight distribution of enriched proteins. (B) Isoelectric point distribution of enriched proteins.

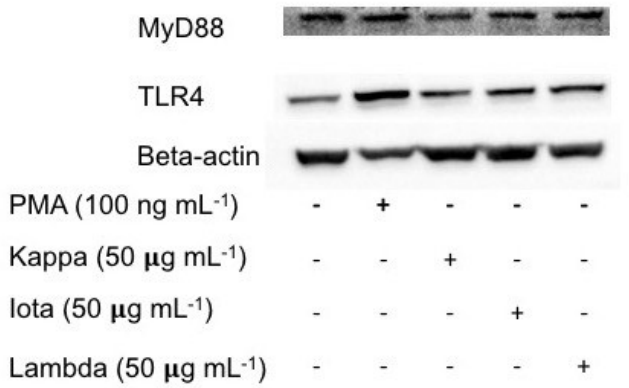

Fig. S7 TLR4 and MyD88 expression of THP-1 cells treated with PMA, kappa, iota or lambda CGN were determined with Western blot.

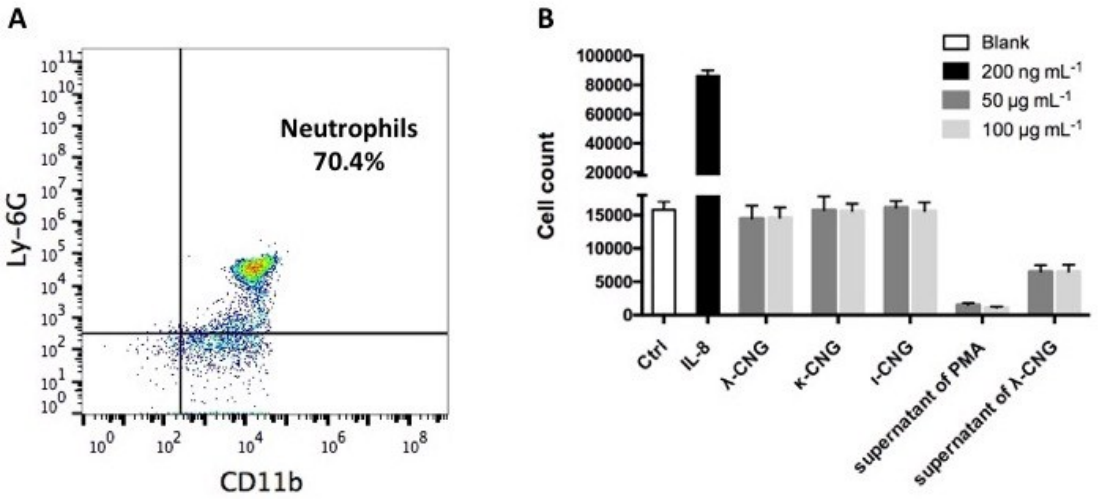

Fig. S8 (A) Phenotype of mouse bone marrow neutrophils. As shown, neutrophils with Ly-6G$^+$ CD11b$^+$ are >70% pure. (B) Neutrophil migration assay. Blank group is 1640 with 5% FBS and IL-8 group is as positive control.

Notes and references

1. M. Wagner, H. Koester, C. Deffge, S. Weinert, J. Lauf, A. Francke, J. Lee, R. Braun-Dullaeus and J. Herold, *JoVE (Journal of Visualized Experiments)*, 2014, e52347-e52347.
2. M. Swamydas and M. S. Lionakis, *JoVE (Journal of Visualized Experiments)*, 2013, e50586-e50586.