Supporting Information

A GalNAc/Gal-specific lectin modulates immune responses via Toll-like receptor 4 independently of carbohydrate-binding ability

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Experimental material and methods

Materials

LPS (Escherichia coli 0111:B4) were purchased from Sigma-Aldrich (St. Louis, MO). Phospho-specific antibodies for ERK, JNK and p38 were purchased from Cell Signaling Technology (Danvers, MA). HRP-conjugated mouse monoclonal antibodies against beta actin and 6*His-tag were purchased from Proteintech (Rosemont, IL). HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PE/Cy7 anti-TLR4/MD2 complex (clone: MTS510) and APC anti-TLR4 (clone: SA15-21) were purchased from BioLegend (San Diego, CA). Bis(sulfosuccinimidyl)suberate (BS3), TNF-α, IL-6 and IL-8 ELISA kits were purchased from Thermo Fisher Scientific (Rockford, IL). Recombinant human TLR4/MD2 complex was purchased from R&D Systems (Minneapolis, MN).

Protein expression and purification

Recombinant CGL was prepared as described in our previous study.[1 2] In brief, 6*His-tagged CGL was produced in endotoxin-free ClearColi BL21(DE3) strain (Lucigen, Euromedex, France), and purified by Ni-NTA affinity chromatography. The purified proteins were tested for endotoxin levels by gel-clot LAL assay (Pyrotel gel-clot, Cape Cod). The constructs for CGL mutants (N26S, D34S, D35A, R39A, K61A, R87A, N141S and D143A) were generated using a QuikChange site-directed mutagenesis protocol (Stratagene) and verified by DNA sequencing. The CGL mutant (CGL-HA) was constructed by mutating carbohydrate binding residues (H16A, H64A, and H108A) to eliminate the carbohydrate binding ability of CGL. [2]

The C-terminal His-tagged ectodomain of mouse TLR4 (amino acids 26–627) and C-terminal protein A tagged mouse MD2 (amino acids 19–160) were co-expressed in High Five insect cells (Invitrogen, Carlsbad, CA) and purified by IgG Sepharose and then Ni-NTA affinity chromatography (GE Healthcare Life Sciences, Chicago, IL). The His-tag and Protein A tag were removed with TEV digestion at 4°C overnight. The TLR4/MD2 complex was further purified by gel filtration chromatography with Superdex 200 column (GE Healthcare Life Sciences).

Cell culture

The murine macrophage cell line J774A.1 and HEK293 were obtained from American Type Culture Collection (Rockville, MD). HEK293-mTLR4/MD2/CD14 was purchased from InvivoGen (San Diego, CA). Primary bone marrow derived macrophages (BMDM) were derived from bone marrow of C57BL/6 or TLR4−/− mice.

Bone marrow cells from mice femur and tibia were incubated for 7 days in RPMI-1640 medium containing M-CSF (Peprotech, London, UK). Bone marrow isolation experiments were performed with the approval of the Institutional Animal Care and Use Committee of the National Ilan University (approval number: No. 106-13), according to the NIH Guide for the Care and Use of Laboratory Animals. J774A.1, HEK 293 and HEK 293-mTLR4/MD2/CD14 cells were cultured in RPMI-1640 medium with 10% FBS. All cells were maintained at 37 °C under a humidified atmosphere of 5% CO2 in an incubator.

Proteinase K treatment of CGL

30 μg of CGL were incubated with 3 μg of proteinase K in a total volume of 30 μl for 3 hours at 55 ˚C, and then the sample was incubated for 15 minutes at 95 ˚C to denature the proteinase K and CGL.

Cytokine measurement

Cells (1×10^5/well) were seeded in 24-well plates and treated as indicated. TNF-α and IL-6 release were determined by ELISA according to the manufacturer instruction (Thermo Fisher Scientific, Rockford, IL).

Extracellular crosslinking and LC-MS/MS

J774A.1 cells were grown in a 10 cm dish and used when 80% confluent. Cells were washed three times with ice-cold PBS and then treated with CGL (10 μg/ml) in ice-cold PBS for 1 hour at 4 ˚C. Cells were then resuspended at 2.5 x 10^7 /ml in ice-cold PBS (pH 8.0), to which BS3 solution was added to a final concentration of 5 mM, and incubated for 1 hour at 4 ˚C. After crosslinking, the reaction was quenched in 20 mM Tris for 15 minutes at 4 ˚C. The membrane proteins of crosslinked cells were extracted with ProteoExtract transmembrane protein extraction Kit (Merck Millipore, Billerica, MA) according to the manufacturer's protocol. The extracted membrane proteins were resolved on SDS-PAGE and the position of the CGL-crosslinked proteins was visualized via western blot stained with HRP conjugated 6*His-tag antibody. The gels containing crosslinked CGL were excised and digested with MS grade chymotrypsin (Promega, Madison, WI) according to standard in-gel digestion protocol. The digested peptides were lyophilized and subjected to NanoLC-nanoESI-MS/MS (LTQ-Orbitrap Elite, Thermo Fisher, Waltham, MA). The crosslinked peptides were identified by MassMatrix[3] software against the Swiss-Prot mouse inflammation-related receptor (TLRs, G Protein-Coupled Receptors, and receptor for advanced glycation endproducts) database. The quality of a match peptide was evaluated based on their PP, PP2 and PPtag scores. The minimum scores for PP and PP2 were 5.3, and the minimum

PPtag score was 1.3. The search results were viewed using by XMAP (version 0.5.1; MassMatrix).

Microscale Thermophoresis

Binding affinities of CGL for mouse or human TLR4/MD-2 were measured using Monolith NT.115 Pico (NanoTemper Technologies, Germany). The CGL protein was purified as described above, and labeled using Alexa Fluor 647 NHS dye at 3:1 molar ratio for 30 minutes in the dark at room temperature. The un-conjugated dye was removed by passing the solution over a Thermo Dye removal column. The unlabeled mice or human TLR4/MD2 was serially diluted in 16 reactions, then the fluorescently labeled CGL was added into each reaction to a final concentration of 2 nM. After 30 minutes incubation at room temperature, the reactions were transferred to monolith NT.115 premium capillaries. The capillaries were loaded to the Monolith NT.115 Pico, and the measurements were performed at 30% LED and 40% MST power. Data were analyzed using MO Affinity Analysis software (NanoTemper).

TLR4 dimerization and Endocytosis

Analyses of TLR4 dimerization and endocytosis were performed as described.[4-6] J774A.1 macrophages were seeded in 24-well plate (1 x 10^5 per well) overnight and then stimulated with LPS (10 μg/ml) or CGL (50 μg/ml) for indicated times at 37 ˚C. Cells were then washed twice with ice-cold PBS and stained with PE/Cy7 labeled TLR4 antibody (Clone MTS510) or APC labeled TLR4 antibody (Clone SA15-21) on ice for 30 minutes. The stained cells were then washed twice with ice-cold PBS and resuspended in 500 μl ice-cold PBC. The staining of receptors was analyzed with Beckman CytoFLEX. The mean fluorescence intensity was recorded and the percentage of TLR4 dimerization and endocytosis was determined as described.[4-6]

Generation of ternary model of the mTLR4–MD2–CGL complex

To generate the initial pose of the ternary model, the CGL structure (PDB ID: 5F90) was manually docked onto the structure of the mTLR4–MD2 complex (PDB ID: 3VQ2), with the program *Coot*, on the basis of the cross linking and mass spectrometry results and the assumption that one CGL dimer binds to one mTLR4–MD2 complex. The initial ternary model was optimized by energy minimization with the program *Discovery Studio* (Accelrys Software Inc.), and the resulting model with the lowest value of potential energy was selected. The stereochemical quality of the model was further checked with the programs *3D-profile* and *PROCHECK*. The structure figures were generated with the program *PyMOL* (Schrödinger, New York, NY, USA).

Circular dichroism spectroscopy

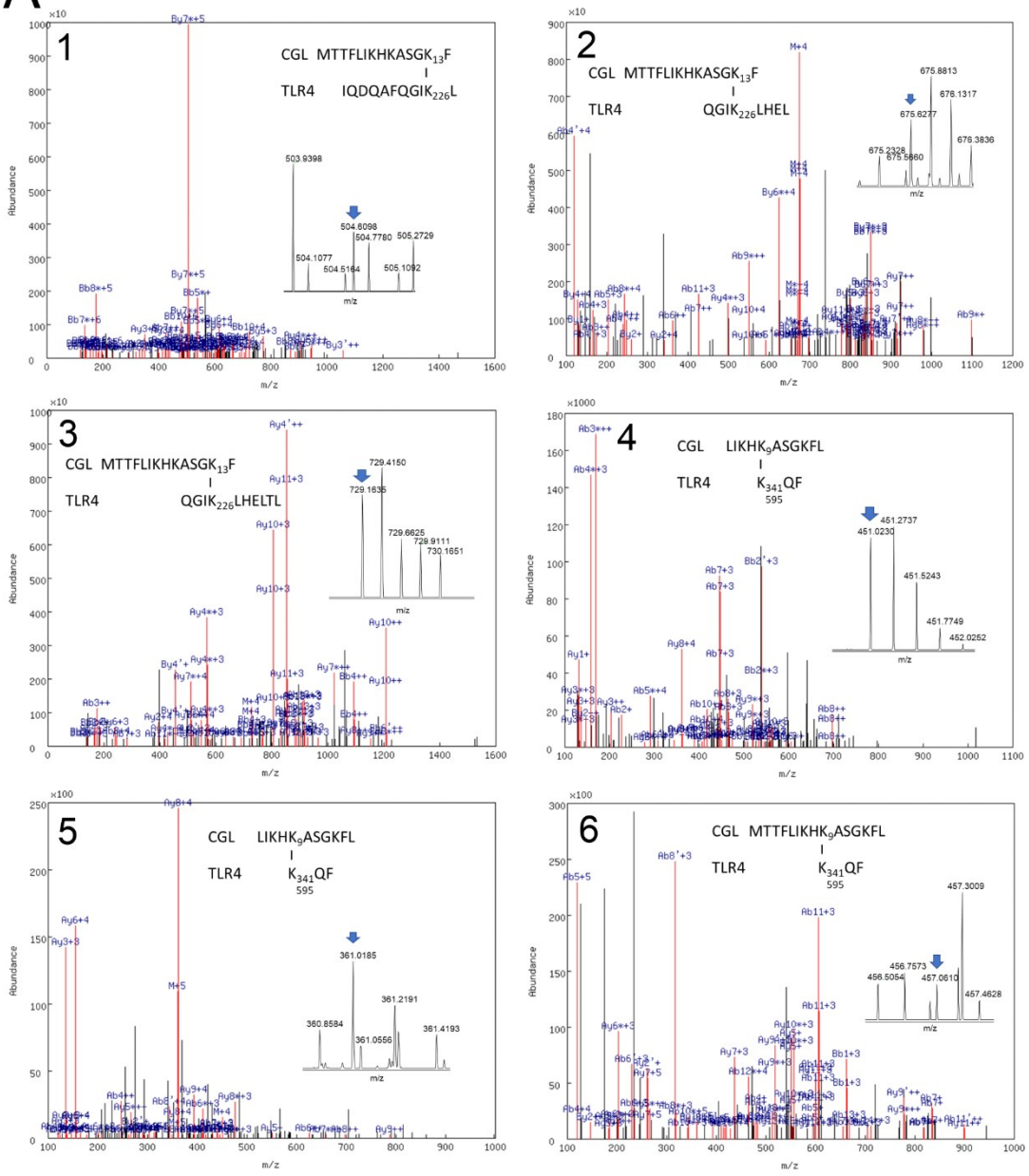
CD spectra were obtained on a JASCO J-815 spectrometer (Jasco, Tokyo, Japan) using a concentration of 0.4 mg/ml for wild type CGL protein and its mutants. Experiments were performed at 25 ˚C with 1 mm path length quartz glass cell. Spectra were recorded from 195~260 nm under the conditions of 1 nm bandwidth and 0.5 nm data pitch at a scan speed of 20 nm/min. The CD signals were corrected by subtracting the buffer spectrum and collected by transforming the CD signal into mean residue molar ellipticity.

Statistical analysis

All values are represented as mean ± SD. The data were analyzed by one-way ANOVA with a subsequent Scheffé test.

Figures

A



B

Crosslinked Lysine residues	PP / PP2 / PPtag score	Assigned peptide sequence		Calculated primary MS peak
		CGL peptide	TLR4 peptide	
CGL K13 – TLR4 K226	10/5.9/1.6	MTTFLIKHKASGK$_{13}$F	IQDQAFQGIK$_{226}$L	504.6133, +6
	9.6/7.9/1.4	MTTFLIKHKASGK$_{13}$F	QGIK$_{226}$LHEL	675.6305, +4
	6.1/8.5/1.5	MTTFLIKHKASGK$_{13}$F	QGIK$_{226}$LHELTL	729.1630, +4
CGL K9 – TLR4 K341 CGL K9 – TLR4 K595	7.7/9.6/1.3	LIKHK$_9$ASGKFL	K$_{341,595}$QF	451.0238, +4
	9.7/6.3/2.7	LIKHK$_9$ASGKFL	K$_{341,595}$QF	361.0205, +5
	8.8/6.4/1.3	MTTFLIKHK$_9$ASGKFL	K$_{341,595}$QF	457.0614, +5

Figure S1. MS of crosslinked peptides. (A) CGL K13 crosslinks to TLR4 K226 (linkage 1 - 3). CGL K9 crosslinks to TLR4 K341 and CGL K9 crosslinks to TLR4 K595 (linkage 4 - 6). The peaks of the b and y ions are highlighted in red and labeled

with their charge stages. The letter A and B are assigned to CGL and TLR4 peptides, respectively. Insets are the respective primary MS peaks. The spectra of the crosslinked peptide fragments in Figure 1 are presented in linkage 1 and 4. (B) The identified crosslinks and their assigned peptides are summarized in the table.

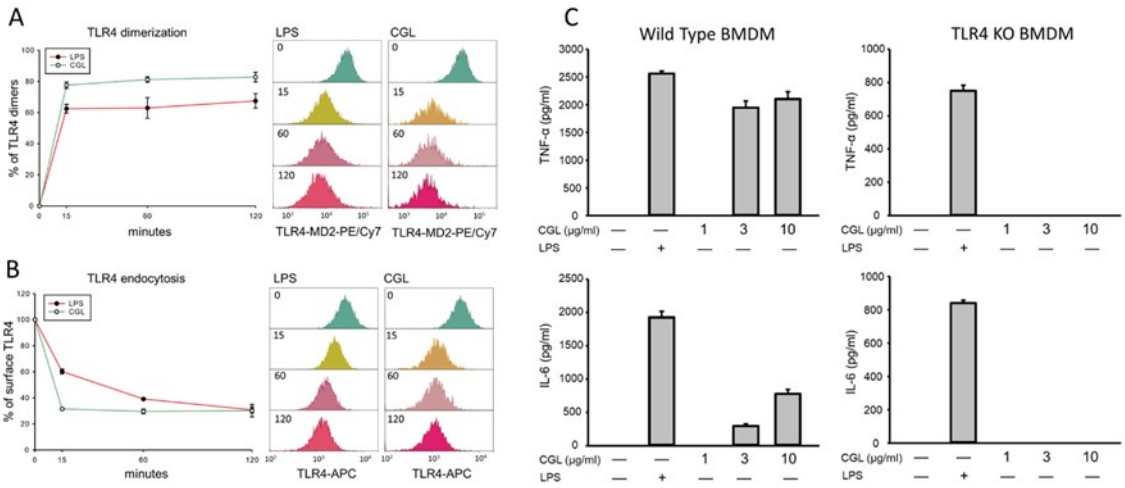


Figure S2. TLR4 mediates CGL-induced immune responses. (A and B) J774.A1 macrophages were treated with LPS (10 μg/ml) or CGL (50 μg/ml) for the indicated times. TLR4 dimerization (A) and endocytosis (B) were measured by flow cytometry. Line graphs represent the mean ± SD from three biological replicates. (C) WT BMDM and TLR4-/- BMDM were treated with LPS (0.1 μg/ml) or CGL at indicated concentration for 24 hours and the cytokines production were measured by ELISA. Bar graphs represent means ± SD of three biological replicates.

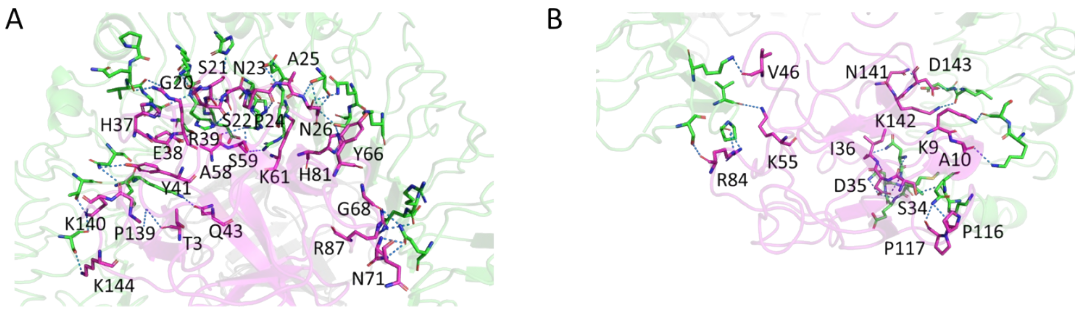


Figure S3. The predicted protein interface between CGL and TLR4 consists of hydrogen bonds. (A, B) The dashed blue lines show the hydrogen bonds between CGL (magenta) and TLR4 receptor (green).

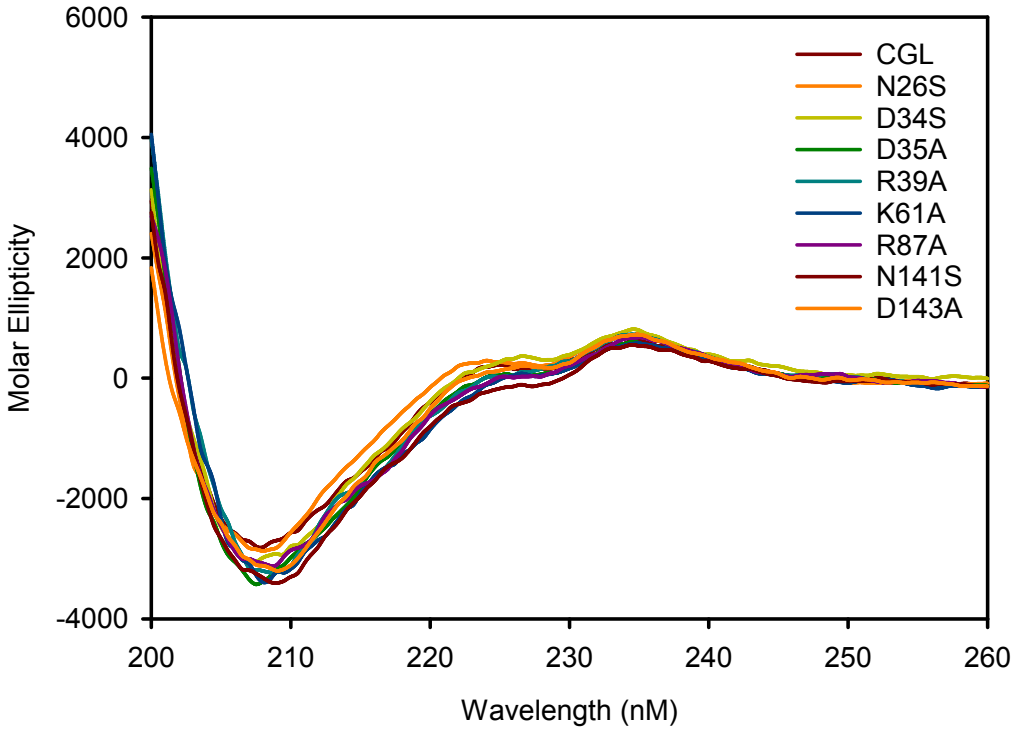


Figure S4. CD spectra of wild type CGL and its eight point mutants (N26S, S34A, D35A, R39A, K61A, R87A, N141S AND D143A). The CD spectra for the mutants were comparable to that of the wild type.

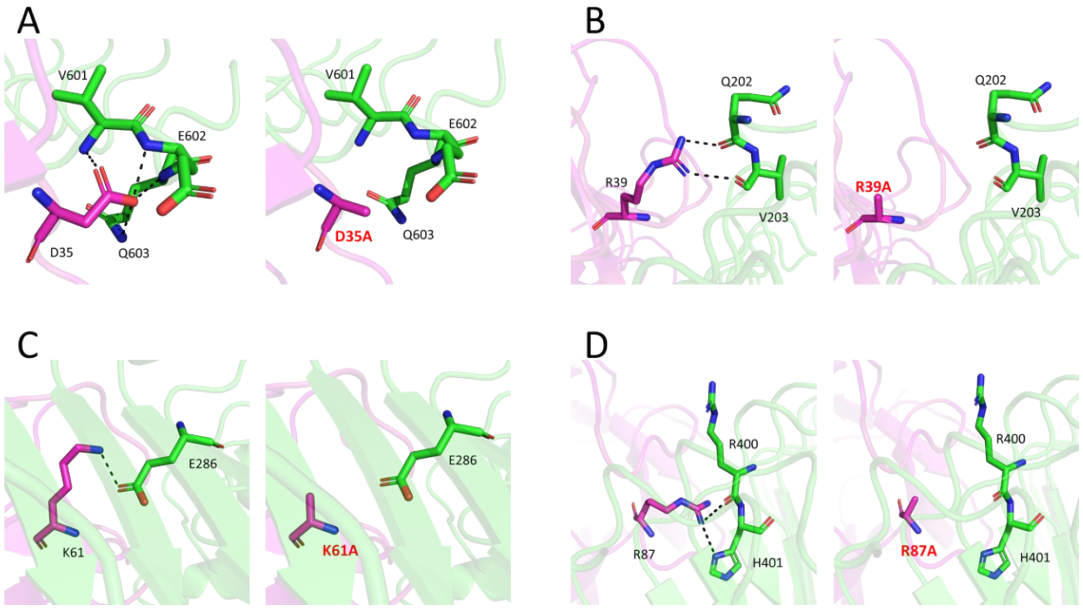


Figure S5. Interactions between the TLR4 and CGL / CGL mutants. D35A, R39A, K61A and R87A mutations on CGL (magenta) are predicted to diminish the interaction with TLR4 (green) (A, B, C, D).

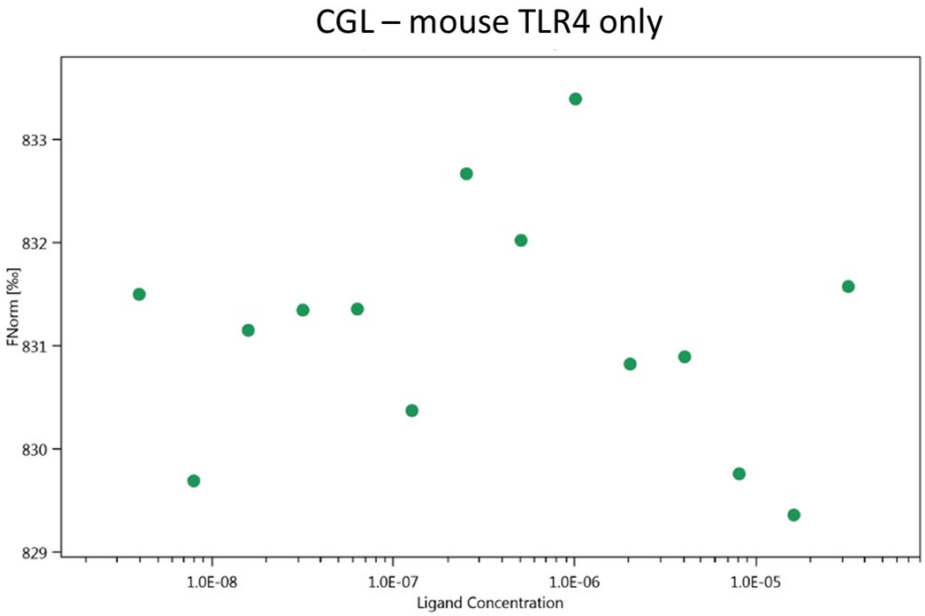


Figure S6. CGL doesn't bind to mouse TLR4 only.

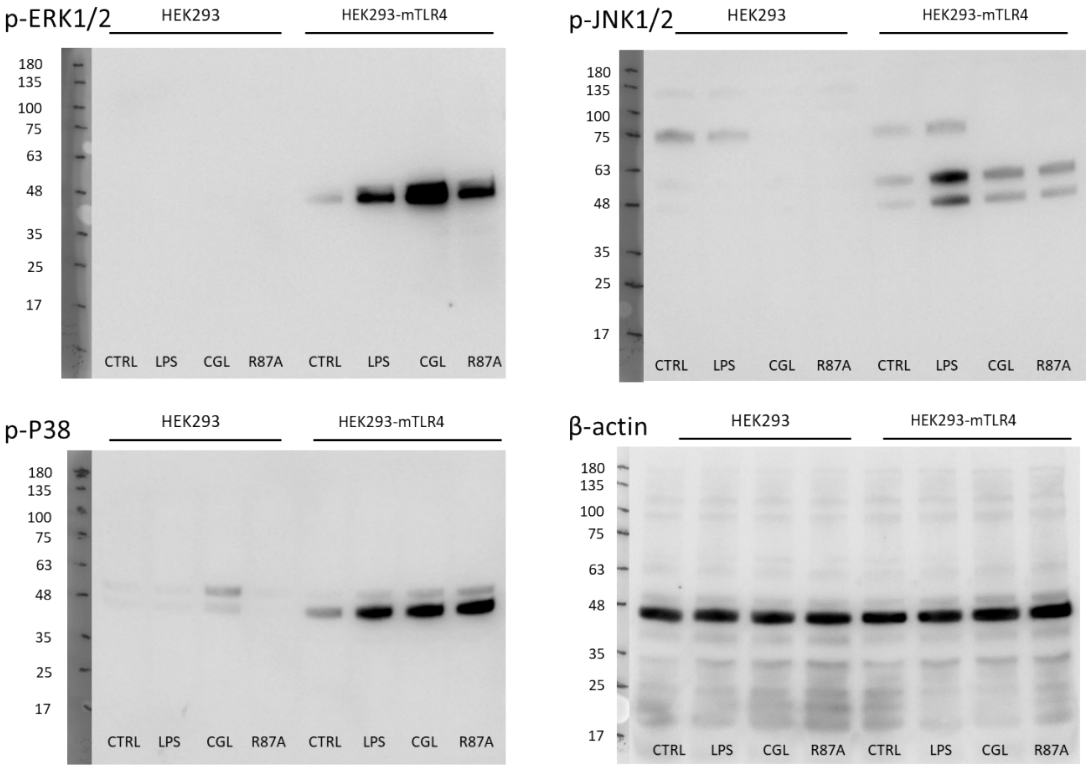


Figure S7. CGL induces MAPK activation via TLR4 receptor. HEK293 and HEK293-mTLR4 cells were stimulated with LPS (0.1 μg/ml), CGL (10 μg/ml) or CGL R87A mutant (10 μg/ml) for 30 minutes. Total cell lysates were analyzed by Western blotting for the levels of phosphorylated ERK1/2, JNK1/2 and P38.

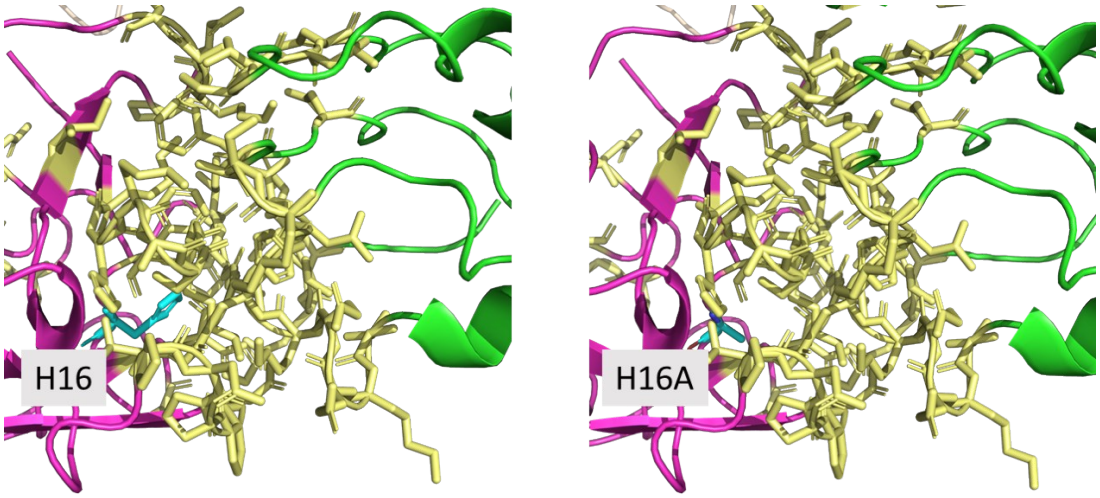


Figure S8. On CGL (magenta)-TLR4 (green) structure, the mutation of CGL H16 (cyan) to Alanine loses the interaction at the CGL-TLR4 interface. The yellow sticks present the residues at the CGL and TLR4 interface.

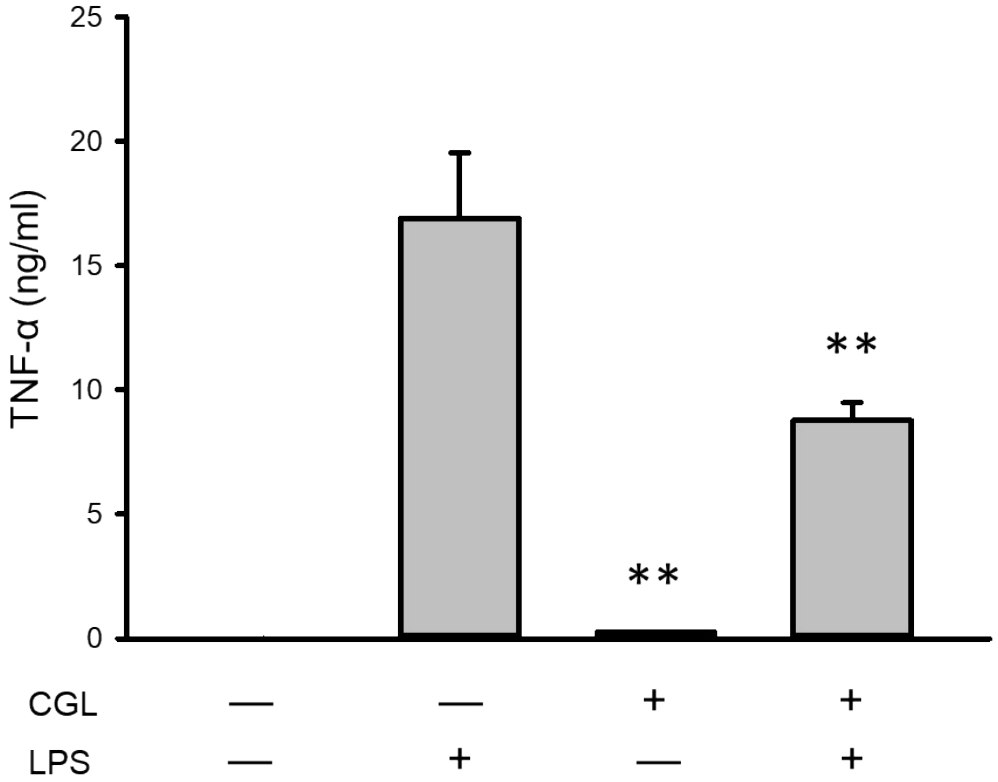


Figure S9. CGL modulates LPS-induced immune response. J774.A1 macrophages were incubated with or without CGL (10 μg/ml) for 30 minutes, and then treated with or without LPS (1 μg/ml) for 6 hours. The levels of TNF-α were measured by ELISA. The data are expressed as the mean ± SD of three replicates. ** indicate significant differences at the level of $p<0.01$ compared to LPS-treated cells.

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