Supporting Information

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SI Materials and Methods

Chemical Procedures. ***Recovery of intracellular shigellae for LPS analysis.*** A total of 2 g of intracellular bacteria was necessary to collect and purify the LPS. To recover the LPS material, cells were kept on ice and were disrupted by sonication by using an Elmasonic sonicator (model S 30/H) with a program consisting of 30 cycles (30 on, 30 off). After disruption of the cells, samples were centrifuged at 8,000 × *g* for 15 min at a constant temperature of 4 °C. In particular, to get rid of any contaminant, the sample underwent a first step of enzyme treatment with RNase, DNase, and proteinase K, and then was placed into dialysis tubes. The freeze-dried material was extracted by the phenol/water method (1), and, after extensive dialyses, the extracted phases were subjected to a second step of enzymatic digestions to remove nucleic acids and proteins contaminants, recovered by ultracentrifugation (60,000 rpm, 4° C, 24 h). Water and phenol fractions were analyzed through SDS/PAGE 13.5%; the LPS fraction was exclusively found in water phase as suggested by the presence of the typical ladder in its migration pattern in the gel. The LPS fraction was further purified by size filtration chromatography [Sephacryl S-500 HR in 50 mM NH_4CO_3 (GE Healthcare); yield, 3% of dried material].

Recovery of shigellae grown in trypticase soy broth for LPS analysis. Dried cells were extracted by the phenol/water method (1); the dialyzed extracted phases were subjected to extensive enzymatic digestions to remove cell contaminants, analyzed with SDS/PAGE 13.5%. LPS was found in the water phase, further purified with size exclusion chromatography (Sephacryl S-500 HR; GE Healthcare).

Isolation of the O-chain fraction. The O-chain moiety was isolated performing a mild acid hydrolysis. An aliquot of purified LPS was dissolved in acetate buffer (pH 4.4), and the hydrolysis was run for 3 h at 100 °C. After lipid A removal by centrifugation, the water-soluble product was then purified on gel filtration chromatography on a Sephacryl S-100 column (GE Healthcare).

Compositional analysis. The dried LPS sample (0.25 mg) was left over a drying agent, under continuous vacuum, for a couple of hours. Then, 1 mL of MeOH/HCl was added to the dried LPS and the sample was left at 85 °C for 16 h, the sample was dried in a stream of air, subsequently acetylated extract with pyridine (200 μL) and Ac_2O (100 μL), 80 °C, 30 min, and analyzed by GLC-MS (2). By this typical analytical procedure, any other residue such as muramic acid or amino acids, eukaryotic fatty acids, and lipids would have been detected. Linkage analysis was carried out by methylation of the complete core region as described. The sample was hydrolyzed with 4 M TFA (100 °C, 4 h), carbonyl-reduced with $NaBD_4$, carboxymethylated, carboxyl-reduced, acetylated, and analyzed by GLC-MS compositional analyses, which revealed no presence of any lipid other than LPS lipid A components (3).

Total fatty acid content was obtained by acid hydrolysis. LPS was first treated with HCl 4 M (4 h, 100 °C) and then with NaOH 5 M (30 min, 100 °C). Fatty acids were then extracted in $CHCl_3$, methylated with diazomethane and analyzed by GLC-MS. The ester-bound fatty acids were selectively released by base-catalyzed hydrolysis with NaOH 0.5 M/MeOH (1:1 vol/vol, 85° C, 2 h) and then the product was acidified, extracted in $CHCl_3$, methylated with diazomethane, and analyzed by GLC-MS (4).

NMR spectroscopy. NMR spectra of the O-chain fraction were recorded in D_2O at 298 K, at pD 7, on a Bruker 600 DRX spectrometer equipped with a cryoprobe. Rotating-frame Overhauser effect spectroscopy (ROESY) and nuclear Overhauser effect spectroscopy (NOESY) experiments were recorded using data sets (*t*1 × *t*2) of 4,096 × 256 points with mixing times between 100 ms and 400 ms. Double quantum-filtered phase-sensitive correlation spectroscopy (DQF-COSY) experiments were performed using data sets of 4,096 × 512 points. Total correlation spectroscopy (TOCSY) experiments were performed with spinlock times of 100 ms, using data sets (*t*1 × *t*2) of 4,096 × 256 points. In all homonuclear experiments, the data matrix was zero-filled in both dimensions to give a matrix of 4,096 × 2,048 points and was resolution-enhanced in both dimensions by a cosine-bell function before Fourier transformation. Coupling constants were determined by 2D phase-sensitive DQF-COSY (5, 6). Heteronuclear single quantum coherence spectroscopy (HSQC) and heteronuclear multiple bond correlation spectroscopy (HMBC) experiments were measured in the ^{1}H-detected mode via single quantum coherence with proton decoupling in the ^{13}C domain, using data sets of 2,048 × 256 points. Experiments were carried out in the phase-sensitive mode (7). A 60-ms delay was used for the evolution of long-range connectivities in the HMBC experiment. In all heteronuclear experiments, the data matrix was extended to 2,048 × 1,024 points by using forward linear prediction extrapolation.

MALDI-TOF MS. MALDI-TOF mass spectra of the intact LPS were recorded in the negative polarity on a Perseptive Voyager STR equipped with delayed extraction technology. Ions formed by a pulsed UV laser beam (nitrogen laser, λ = 337 nm) were accelerated by 24 kV. Reflectron MALDI-TOF MS and MALDI-TOF/TOF MS2 of lipid A were performed on a 4800 Proteomic analyzer (Applied Biosystems) supplied with a Nd:YAG laser at the wavelength of 355 nm. External calibration was performed and mass accuracy was better than 100 ppm. Mass resolution of the spectra obtained in reflector mode was ∼13,000.

Biological Procedures. ***RNA extraction and quantitative real-time PCR analysis of Pro–IL-1β and IFN-β expression.*** Total RNA was extracted from unstimulated, LPS-treated, or LPS-primed and infected C57BL/6, Myd88$^{-/-}$, and *trif*$^{-/-}$ bone marrow-derived macrophages (BMDMs) through TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was amplified by using Power SYBR Green PCR Master Mix (Applied Biosystem). The $2^{-\Delta\Delta Ct}$ method was applied to analyze the relative changes in expression profiling of interest genes, as already reported (8). Values were normalized to the internal *gapdh* gene control. Gene specific primers used in the experiments of quantitative real-time PCR (qPCR) are as follows:

Primer/direction	Primer sequence (5′–3′)
IFNβ	
Forward	TCCGAGCAGAGATCTTCAGGAA
Reverse	TGCAACCACCACTCATTCTGAG
Il-1β	
Forward	GCAGGCAGTATCACTCATTG
Reverse	CGTTGCTTGGTTCTCCTTGT
GADPH	
Forward	TCCAGTATGACTCCACTC
Reverse	ATTTCTCGTGGTTCACAC

ELISA. Cytokine and chemokine levels in cell culture supernatants were measured using DuoSet (IL-1β, TNF-α, IL-6, IL-8, KC, CCL5, CXCL-10, IL-10; R&D Systems) or an ELISA kit from PBL Biomedical Laboratories (IFN-β) according to the manufacturers' instructions.

1. Westphal O, Jann K (1965) Bacterial lipopolysaccharides: Extraction with phenol-water and further applications of the procedure. *Methods Carbohydr Chem* 5:83–91.
2. Leontein K, Lindberg B, Lonngren J (1978) Assignment of absolute configuration of sugars by g.l.c. of their acetylated glyco-sides formed from chiral alcohols. *Carbohydr Res* 62:359–362.
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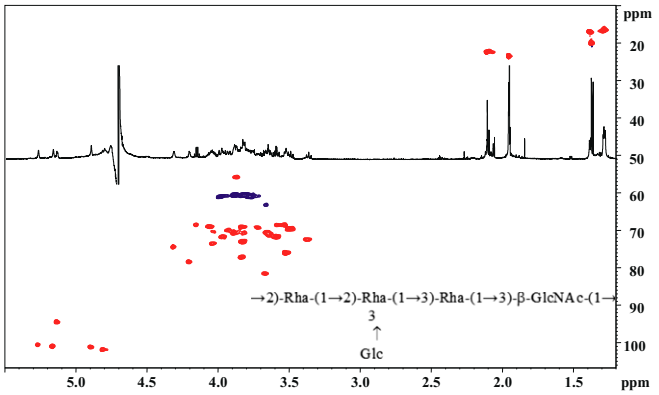

Fig. S1. NMR data for *Shigella* iLPS. HSQC spectrum of the O-polysaccharide. The distortionless enhancement by polarization transfer HSQC spectrum of the O-polysaccharide produced by LPS of intracellular bacteria (iLPS) of *Shigella flexneri* in which all C-H correlations are shown; the usual primary structure as deduced by the NMR data are also shown.

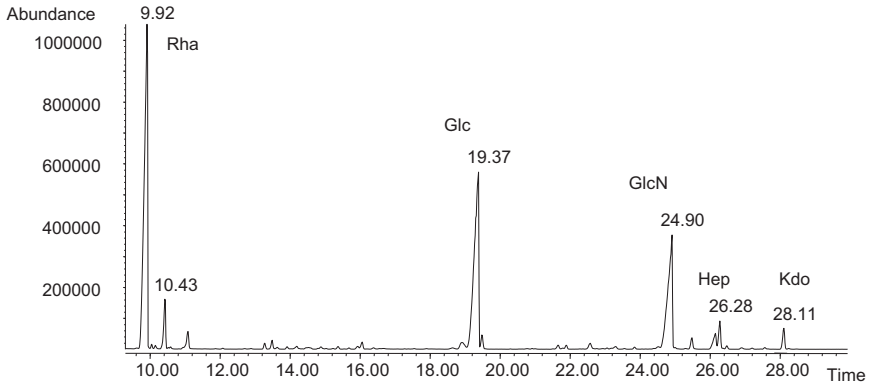

Fig. S2. GLC-MS analysis of *Shigella* iLPS. GLC chromatogram of *Shigella* iLPS in which all of the carbohydrate peaks are assigned. Minor peaks are belonging to same monosaccharides in the alternative anomeric configuration. An identical procedure was carried out for the isolation and purification of LPS of bacteria grown in laboratory medium (aLPS).

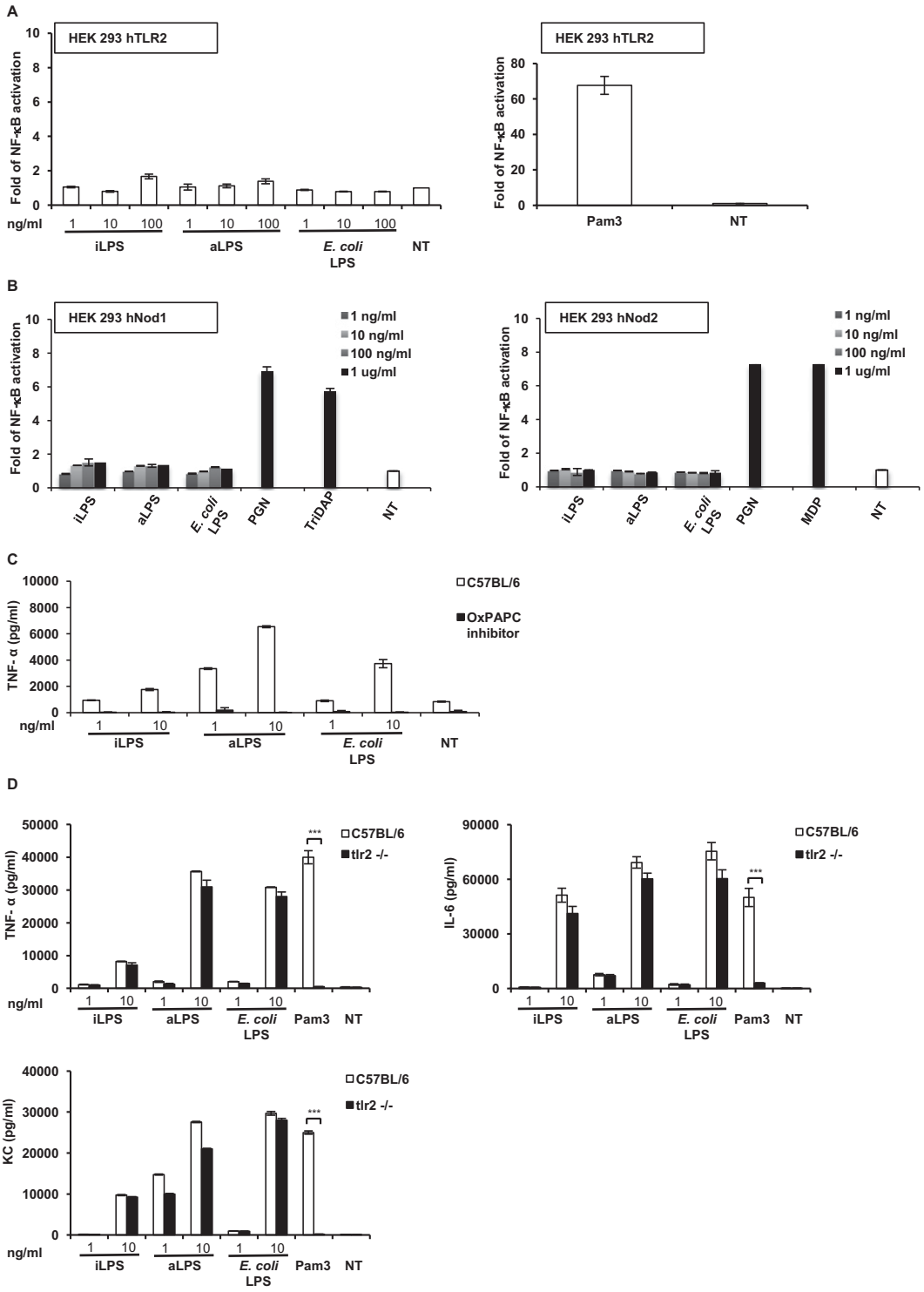

Fig. S3. Assessment of contaminations in LPS. (*A*) Fold of NF-κB activation upon stimulation of HEK 293 Toll-like receptor (TLR) 2 [human (h)TLR2] with 1,10, and 100 ng/mL of iLPS and aLPS for 6 h. Hexaacylated *Escherichia coli* LPS and Pam3CSK4 were used as controls. (*B*) Fold of activation of NF-κB in HEK 293 cells expressing hNod1 (*Left*) or hNod2 (*Right*) upon transfection with iLPS or aLPS or *E. coli* LPS. HEK hNod1 or HEK hNod2 (1) were transfected with 1 ng/mL, 10 ng/mL, or 100 ng/mL of aLPS, iLPS, or *E. coli* LPS. Internal stimulation was maintained during 18 h as described previously (1). *E. coli* sonicated peptidoglycan (PGN; 1 μg/mL) and triDAP (1 μg/mL) were used as controls. (*C*) TNF-α release in BMDMs stimulated with 1 ng/mL or 10 ng/mL of aLPS, iLPS, or *E. coli* LPS and treated with OxPAPC. OxPAPC is a mixture of oxidized phospholipids that inhibits the signaling induced by bacterial lipopeptide and LPS. It acts by competing with CD14, bacterial lipoprotein, and MD2, thus blocking the signaling of TLR2 and TLR4 (2). BMDMs stimulation was performed in the presence or absence of OxPAPC (40 μg/mL), and TNF-α release was measured after 6 h of incubation. Significant difference between iLPS-generated values and the corresponding iLPS plus OxPAPC inhibitor values are indicated (LPS vs. OxPAPC plus LPS). (*D*) Cytokine release (TNF-α, IL-6, KC) in WT or *tlr2*$^{-/-}$ BMDMs stimulated with *Shigella* iLPS or aLPS at 18 h. *E. coli* LPS (LPS-EB Ultrapure; InvivoGen) and Pam3CSK4 (InvivoGen) were used as controls. Mean values (±SEM) of three representative experiments are shown. Significant difference between values of WT cells and corresponding values in defective cells are indicated (WT vs. defective: $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$).

1. Nigro G, et al. (2008) Muramylpeptide shedding modulates cell sensing of *Shigella flexneri*. *Cell Microbiol* 10(3):682–695.
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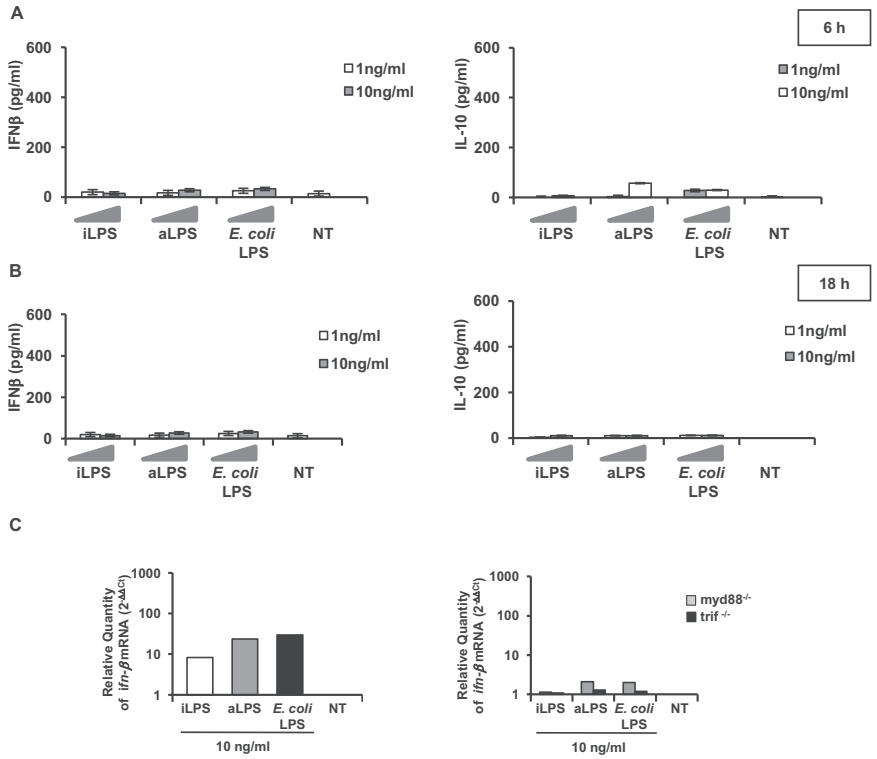

Fig. S4. IFN-β and IL-10 release and expression of *ifn-β* mRNA in BMDMs stimulated with *Shigella* iLPS or aLPS. (*A* and *B*) IFN-β and IL-10 release in BMDMs stimulated with *Shigella* iLPS, aLPS, and *E. coli* LPS. IFN-β and IL-10 released by BMDMs after stimulation with 1 and 10 ng/mL of iLPS, aLPS, and *E. coli* LPS measured by ELISA at 6 h (*A*) and 18 h (*B*). Mean values (±SEM) of three representative experiments are shown. (*C*) qPCR1 of *ifn-β* mRNA following 10 ng of LPS stimulation at 3 h in WT BMDMs (*Left*) and BMDMs from *myd88*$^{-/-}$ or *trif*$^{-/-}$ mice (*Right*). Results are normalized to the internal *gapdh* gene control and are presented on a logarithmic scale as the ratio of gene expression between stimulated and unstimulated BMDMs.

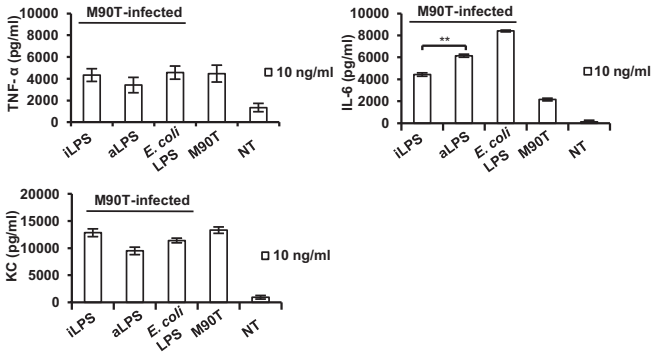

Fig. S5. Cytokine release in M90T-infected BMDMs previously stimulated with iLPS or aLPS. TNF-α, IL-6, and KC release measured through ELISA in BMDMs unstimulated or pretreated with 10 ng/mL of iLPS or aLPS or *E. coli* LPS (4 h) and infected with M90T at a multiplicity of infection (MOI) of 10 (3 h). Mean values (±SEM) of three representative experiments are shown. Significant difference between iLPS-generated values and the corresponding aLPS values are indicated (iLPS vs. aLPS: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001).

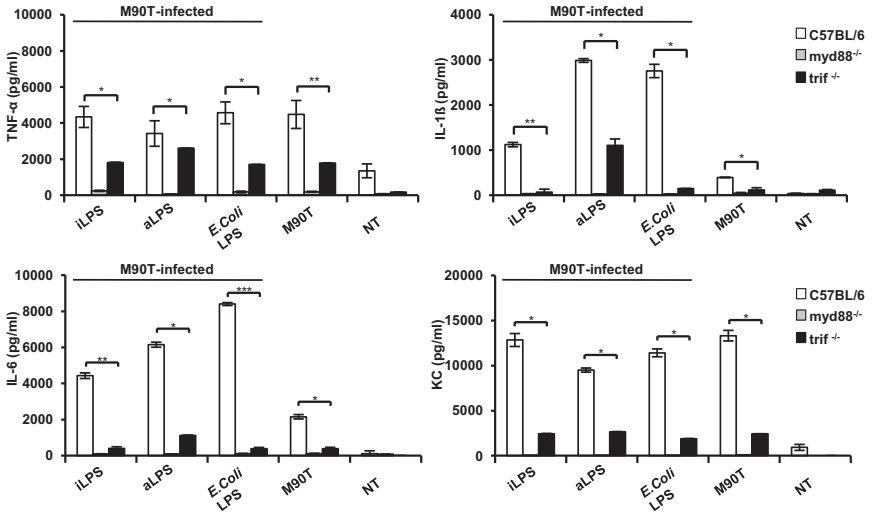

Fig. S6. Cytokine release in M90T-infected *myd88*$^{-/-}$ or *trif*$^{-/-}$ BMDMs previously stimulated with iLPS or aLPS. TNF-α, IL-1β, IL-6, and KC release measured through ELISA in *myd88*$^{-/-}$ and *trif*$^{-/-}$ BMDMs unstimulated or pretreated with 10 ng/mL of iLPS or aLPS or *E. coli* LPS (4 h) and infected with M90T at an MOI of 10 (3 h). Mean values (±SEM) of three representative experiments are shown. Significant difference between values generated in WT cells and the corresponding conditions in *trif*$^{-/-}$ BMDMs are indicated (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$). $P < 0.001$ for values of WT BMDMs vs. *myd88*$^{-/-}$ BMDMs.

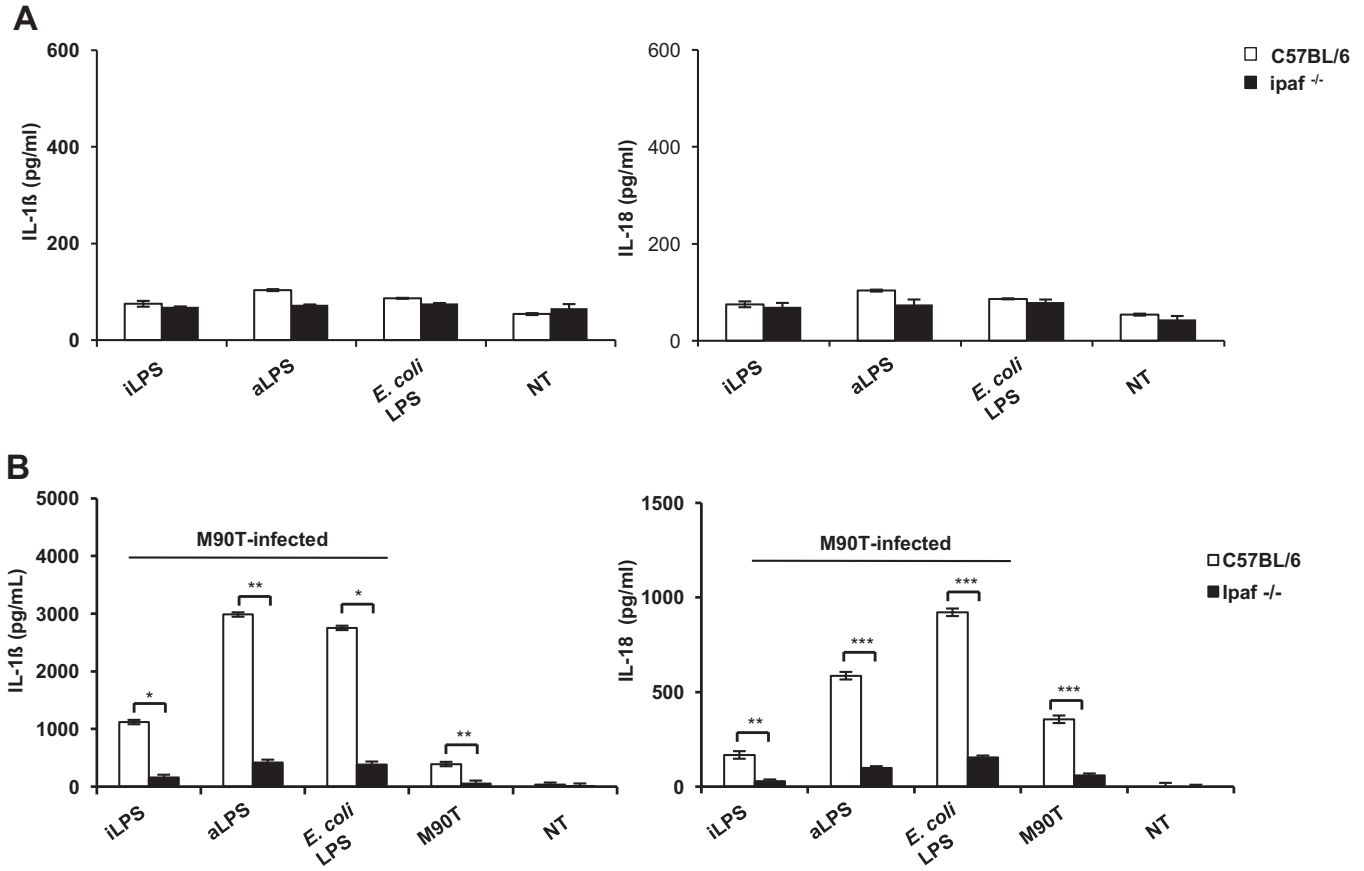

Fig. S7. IL-1β and IL-18 release in *ipaf* (*nlrc4*)$^{-/-}$ BMDMs stimulated with iLPS or aLPS and in M90T-infected *ipaf* (*nlrc4*)$^{-/-}$ BMDMs prestimulated with iLPS or aLPS. IL-1β and IL-18 release measured through ELISA in *ipaf* (*nlrc4*)$^{-/-}$ BMDMs unstimulated or treated with 10 ng/mL of iLPS or aLPS or *E. coli* LPS (4 h) (*A*) and in treated cells as described earlier and infected with M90T at an MOI of 10 (3 h) (*B*). Mean values (±SEM) of three representative experiments are shown. Significant difference between values generated in WT cells and the corresponding conditions in *ipaf* (*nlrc4*)$^{-/-}$ BMDMs are indicated (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

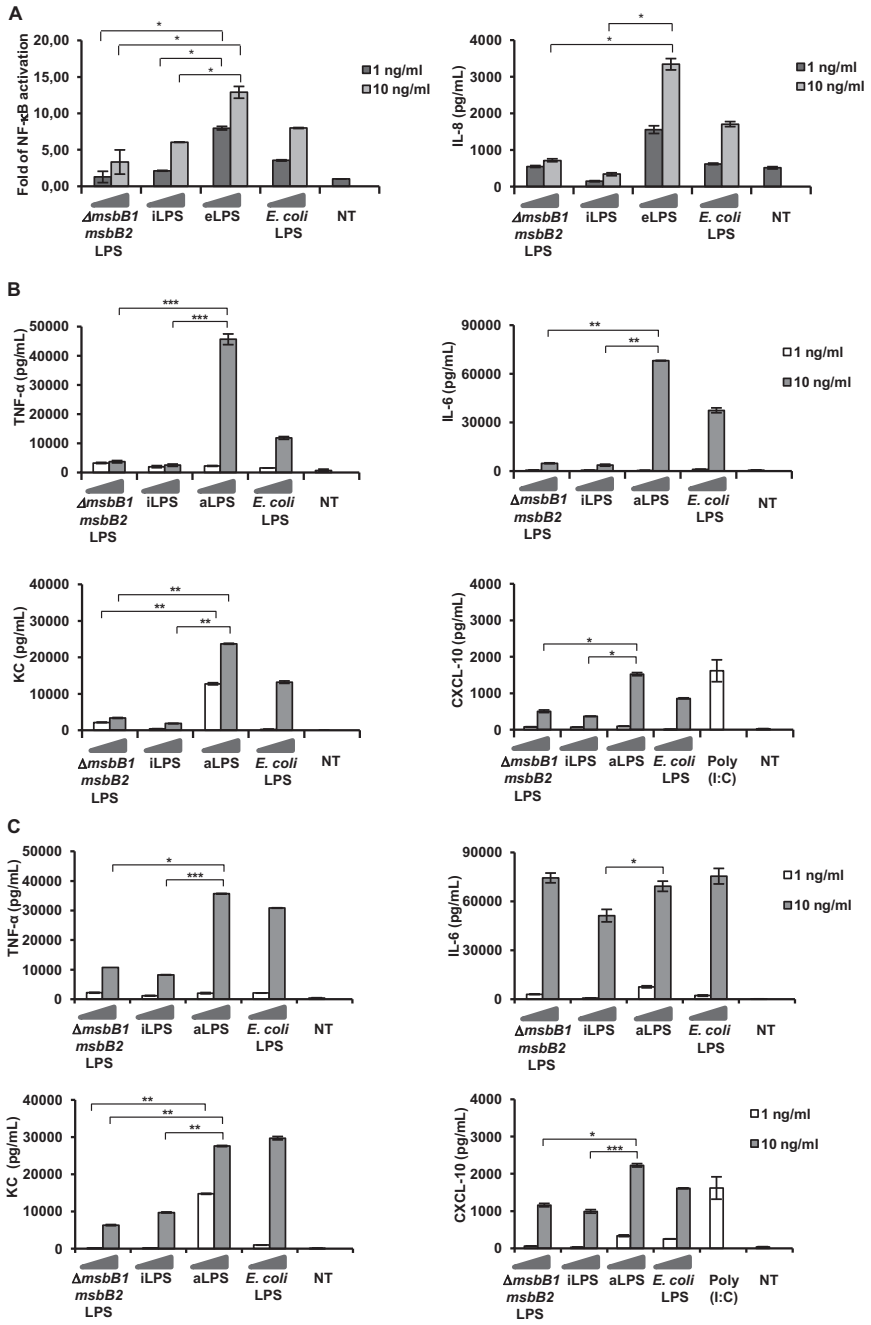

Fig. S8. Immunopotential of LPS purified from the *S. flexneri* Δ*msbB1msbB2* mutant. (*A*) activation of TLR4 in HEK293 cells and (*B* and *C*) production of cytokines in BMDMs. (*A*) Fold of NF-κB activation upon stimulation of HEK 293-hTLR4/MD2-CD14 during 4 h with 1 and 10 ng/mL of LPS derived from M90T Δ*msbB1msbB2* (1), from intracellular shigellae (i.e., iLPS), and from shigellae grown in laboratory medium (i.e., aLPS). Commercial hexaacylated *E. coli* LPS was used as a control in parallel; (*Right*) IL-8 release measured through ELISA at 18 h under conditions as described earlier. (*B* and *C*) Cytokine release in BMDMs stimulated with LPS of M90T Δ*msbB1msbB2*, iLPS, aLPS, and *E. coli* LPS: TNF-α, IL-6, KC, and CXCL-10 released by BMDMs after stimulation with 1 and 10 ng/mL and measured by ELISA at 6 h (*B*) and 18 h (*C*). Mean values (±SEM) of three representative experiments are shown. Significant difference between LPS of M90T Δ*msbB1msbB2*-generated values and the corresponding aLPS values and between iLPS-generated values and the corresponding ones of aLPS are indicated (*msbB* vs. aLPS and iLPS vs. aLPS; $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$).

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Table S1. ^{1}H, ^{13}C NMR chemical shifts of the polysaccharide derived from acid treatment of the LPS from *Shigella*

Unit	1	2	3	4	5	6
A	5.27	4.31	4.03	3.66	3.82	1.38
2,3-α-Rha	100.5	74.4	73.4	70.7	69.0	17.0
B	5.16	4.20	3.93	3.35	3.72	1.28
2-α-Rha	100.89	78.3	69.9	72.3	69.2	16.5
C	5.14	3.63	3.82	3.49	3.82	3.96/3.76
t-α-Glc	94.4	70.9	69.0	69.2	72.9	60.8
D	4.90	3.89	3.83	3.60	4.05	1.28
3-α-Rha	101.1	70.7	76.9	71.7	69.0	16.5
E	4.81	3.86	3.66	3.52	3.53	3.86/3.81
3-β-GlcN	101.74	55.7	81.6	68.7	76.1	60.6