Supporting Information

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SI Materials and Methods

Cells and Reagents. RAW 264.7 murine cell line and mouse L cell line expressing Wnt5a or Wnt3a were purchased from ATCC. DMEM–high glucose, RPMI-1640, heat-inactivated FBS, Penicillin, Streptomycin, L-Glutamine, and 1× Trypsin-EDTA were purchased from Gibco. Recombinant mouse Wnt5a and TNF-α, monoclonal anti-mouse TNF-α antibody raised in mouse, anti-mouse Wnt5a antibody raised in goat were purchased from R&D System, and anti-Fz5 antiserum raised in rabbit was generated according to published procedures (1). Monoclonal anti–β-actin antibody (mouse host), anti-rabbit IgG-HRP conjugate, anti-mouse IgG-HRP conjugate, and anti-goat IgG-HRP conjugate were purchased from Sigma. Soluble 3,3′,5,5′-Tetramethylbenzidine (TMB), LY294002, Rac1 Inhibitor, BAY 11–7082, Nystatin, MCD, and OGP were purchased from Calbiochem. Super-signal westpico chemiluminescent substrate for immunoblotting was purchased from Pierce, and PVDF membrane was purchased from PALL Life Science. Cytochalasin D, L-α-phosphatidylcholine, octadecylamine, FITC dextran, and LPS from *E. coli* O55:B5 were purchased from Sigma. IWP-2 was purchased from Santa Cruz Biotechnology. IL-10 and IL-6 ELISA kits (BD OptiEIA ELISA kit) were purchased from BD Biosciences. LysoTracker Red DND-99 and Alexa Fluor 594-Cholera Toxin Subunit B was from Molecular Probes, Invitrogen. Lipofectamine, TRIzol, and cDNA synthesis kits were purchased from Invitrogen and Biobharati. Mouse Wnt5a siRNA, Fz5 siRNA, and Dharmafect transfection reagent were from Dharmacon. GFP–*E. coli* was obtained from S. Roy (IICB).

Mice Infection. About 3-mo-old C57BL/6 mice were obtained from the Central Drug Research Institute, Lucknow, India. The animals were housed four to five in a cage at 23 °C in a 12-h light/dark cycle. Mice were injected intraperitoneally (i.p.) first with either 200 μL of liposome-IWP2 (LI) or liposome (L) and then after 2 h with 1×10^8 or 2×10^8 CFU *E. coli* in 200 μL of sterile PBS. After 2 h or 24 h mice were killed, and the peritoneal cavity was washed with 5 mL of sterile ice-cold PBS. The peritoneal lavage fluid was centrifuged at 300 × *g* for 5 min, the cell pellet was resuspended in RPMI 1640 complete medium, and the supernatant was used for cytokine assay. For ex vivo experiments, peritoneal phagocytes were isolated as above from normal mice, and equal numbers of cells were plated in medium overnight at 37 °C in 5% CO_2 before performing further experiments.

Preparation of L-Wnt5a and L-Wnt3a–Conditioned Medium. As per ATCC direction, mouse L cells expressing Wnt5a or Wnt3a were grown in high-glucose complete DMEM supplemented with 0.1% G418 sulfate at 5% CO_2 in a 37 °C incubator up to confluence (2–3 d). The cells were split 1:10 and further grown in DMEM without G418 sulfate for 4 d, after which the culture supernatant was collected and filtered through a 0.2 μm filter. Cells were grown for another 3 d in high-glucose DMEM, subsequent to which culture supernatant was again collected and filtered through 0.2 μm filter. Both sets of culture supernatants were mixed 1:1 and used as conditioned medium. Mouse L-cell-conditioned medium was prepared following a similar procedure.

RNA Isolation and RT-PCR. RNA was isolated from transfected cells using TRIzol. cDNA was generated using the cDNA synthesis kit following instructions provided by the manufacturer. The following primer pairs were used for reverse transcription PCR on the cDNA generated: GAPDH: 5′-accacagtccatgccatcac-3′ (forward); 5′-tccaccaccctgttgctgta-3′ (reverse); mouse Fz5: 5′–ctgggtgctcatgctcaagtac-3′ (forward); 5′-cgacagggacacttgcttgtg-3′ (reverse).

ELISA and Immunoblot. Cell lysate was made using lysis buffer (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 1% Triton-X100, 1 mM EDTA, 20 mM PMSF). The amount of IL-6 and IL-10 in the culture medium of macrophages and peritoneal lavage was quantified following the standard protocol of the kit. For TNF-α, coated antigens were probed with the appropriate antibodies (R&D Systems). After overnight incubation with primary antibody, wells were washed with 1% BSA in Tris-buffered saline. Bound antibody was detected with the appropriate IgG-HRP and developed with TMB. Absorbance was measured at 450 nm. For immunoblot analysis, similarly prepared lysates were analyzed using appropriate antibodies.

Cell Transfection. The transfection procedure using Lipofectamine was the same as described previously (1). For siRNA knockdown of Wnt5a or Fz5 in RAW cells, 50%–60% of confluent cells were transfected with 100 nM Wnt5a siRNA or 25 nM Fz5 siRNA using Dharmafect transfection reagent. Transfected cells were treated as described earlier for lipofectamine-mediated transfection and analyzed ∼60 h posttransfection.

Estimation of Phagocytosis. Latex beads, either blue dye filled (0.8 μm) or fluorescent red (2 μm), were added to transfected RAW cells (about 10 beads per cell), about 60 h after transfection, and the plates were incubated at 37 °C in 5% CO_2 for 6 h. Extracellular particles were removed by washing the cells extensively with ice-cold PBS several times. In the case of fluorescent beads, cells were treated for 1 min with trypan blue after the required incubation to quench extracellular fluorescence. Cells were subsequently harvested for assay. Fluorescence trapped by cells was measured by fluorometer using excitation and emission wavelengths of 575 nm and 610 nm, respectively. Uptake of blue-dye-filled beads was estimated by measuring absorbance at 595 nm. Uptake of red fluorescent beads by RAW cells was assessed separately by confocal microscopy. To assess uptake of GFP expressing *E. coli* [*E. coli* DH5α bacteria (avirulent) transformed with Green Fluorescent Protein plasmid], RAW cells pretreated with L-Wnt5a or L-Wnt3a conditioned medium (150 μL in 1 mL of culture medium) or recombinant Wnt5a (50 ng/mL, dissolved in PBS with 0.1% BSA) for 6 h were incubated with GFP–*E. coli* at a multiplicity of infection (MOI) of 50 for 120 min at 37 °C in 5% CO_2. Unbound bacteria were removed by extensive washing several times with cold PBS. Infected RAW cells were subsequently lysed by adding distilled water. The diluted aliquots were then spread on LB agar plates, and colony -forming units (CFUs) were counted after incubating the plates overnight at 37 °C. As control experiments, PBS with 0.1% BSA was used instead of recombinant Wnt5a. Both peritoneal macrophages and RAW cells pretreated with either 0.05 μM IWP-2 or the liposome formulation for 48 h were processed similarly to assess phagocytosis. For estimating inhibition by designated inhibitors, specific concentrations as noted in the figure legends were added for the last 2 h of Wnt5a or PBS incubation before addition of GFP–*E. coli* or latex bead.

Estimation of Bacterial Killing. An equal number of *E. coli*–infected cells, RAW 264.7, or mouse peritoneal macrophages were extensively washed with PBS and treated for estimating internalized bacteria, as explained above, at different time points.

Preparation of Liposome-IWP2. Liposome-IWP2 was prepared with L-α-phosphatidylcholine, octadecylamine, and IWP-2 in a 20:2:0.1 ratio (100 μg of IWP-2 was used). The lipid mixture was dissolved in 1 mL of chloroform, and the solvent was evaporated under low pressure by a rotatory evaporator. The thin dry film was dispersed in 1 mL PBS, and the suspension was sonicated for 30 s twice in an ultrasonicator. Liposome with entrapped IWP2 was separated from excess free drug by two successive washings in PBS with ultracentrifugation (100,000 × *g*, 30 min, and 4 °C). Control liposome was prepared similarly without adding IWP-2 (2).

Measurement of Reactive Oxygen Species (ROS). The formation of ROS was measured by using cell permeable dye 2′, 7′- DCFDH-DA. The cells were incubated with 10 μM of the dye for 30 min at 37 °C, after which ROS was determined by fluorometric analysis (3). To determine diphenyleneiodonium chloride (DPI)-inhibitable ROS, cells were treated with 10 μM of DPI at the time of infection.

Confocal Microscopy. For confocal microscopy, RAW cells were grown on coverslips overnight and incubated either with fluorescent red latex beads (five beads per cell) or GFP–*E. coli* (MOI-50) for 2 h at 37 °C in 5% CO_2. Cells were washed several times with PBS and fixed with methanol for 10 min at room temperature. Coverslips were mounted with 70% glycerol and examined using either Nikon A1R confocal imaging system (Fig. 1*A*) or Andor Revolution XD Spinning Disk Microscope with Andor ixon 897 EMCCD camera (Fig. S2; Fig. 2 *H–J*) at 600× magnification. Additionally, RAW cells were grown on coverslips overnight, treated with LysoTracker Red DND-99 to a final concentration of 50 nM for 15 min, and infected with GFP–*E. coli* at 37 °C in 5% CO_2. Coverslips were subsequently processed for confocal microscopy as mentioned above. DAPI (1 μM) was used to stain nucleus. To stain lipid rafts, RAW cells were grown on chamber slide overnight and treated with either recombinant Wnt5a or PBS control for 6 h. Cells were then incubated with GM1-specific (1 μg/mL) Alexa594–Cholera Toxin B subunit conjugate (Alexa594-CTB) for 10 min at 37 °C and washed thrice with PBS. The cells were then fixed with 3% paraformaldehyde and mounted for confocal microscopy.

Statistical Analysis. Statistical analysis was done using GraphPad Prism 4 software. Comparisons were made using unpaired Student *t* test. Bar graphs are expressed as mean ± SE. *P* values < 0.05 were considered significant.

1. Sen M, Chamorro M, Reifert J, Corr M, Carson DA (2001) Blockade of Wnt-5A/frizzled 5 signaling inhibits rheumatoid synoviocyte activation. *Arthritis Rheum* 44:772–781.
2. Delemarre FGA, Kors N, Kraal G, van Rooijen N (1990) Repopulation of macrophages in popliteal lymph nodes of mice after liposome-mediated depletion. *J Leukoc Biol* 47: 251–257.
3. Scarfi S, et al. (2009) Ascorbic acid pre-treated quartz stimulates TNF-α release in RAW 264.7 murine macrophages through ROS production and membrane lipid peroxidation. *Respir Res* 10:25.

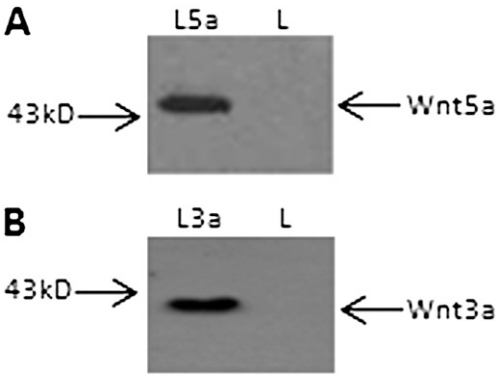

Fig. S1. Presence of Wnt5a and Wnt3a in L-Wnt5a and L-W3a cell-conditioned medium. Immunoblot demonstrating the presence of Wnt5a (*A*) and Wnt3a (*B*) in L-Wnt5a- and L-Wnt3a-conditioned medium but not in L-conditioned medium (control).

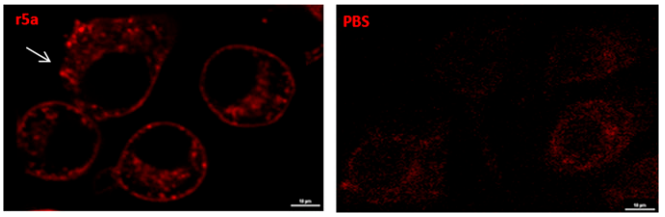

Fig. S2. Confocal microscopic representation of lipid raft clustering in RAW 264.7 macrophages. RAW cells pretreated with recombinant Wnt5a (r5a) or PBS (control) were stained with Alexa 594-cholera toxin B (CTB) to estimate lipid raft clustering and processed for microscopy as described in *Materials and Methods*. (Scale bar: 10 μm.)

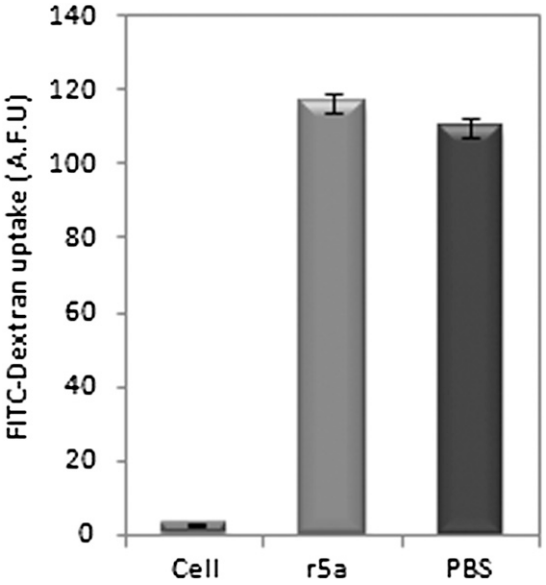

Fig. S3. Estimation of pinocytosis by recombinant wnt5a (r5a). Fluorometric analysis of r5a (50 ng/mL) or PBS pre-exposed RAW 264.7 macrophages treated for 2 h with 150 μg/mL FITC dextran for half an hour, demonstrating that r5a does not have any significant effect on pinocytosis. Background fluorescence from the cell was used as reference.

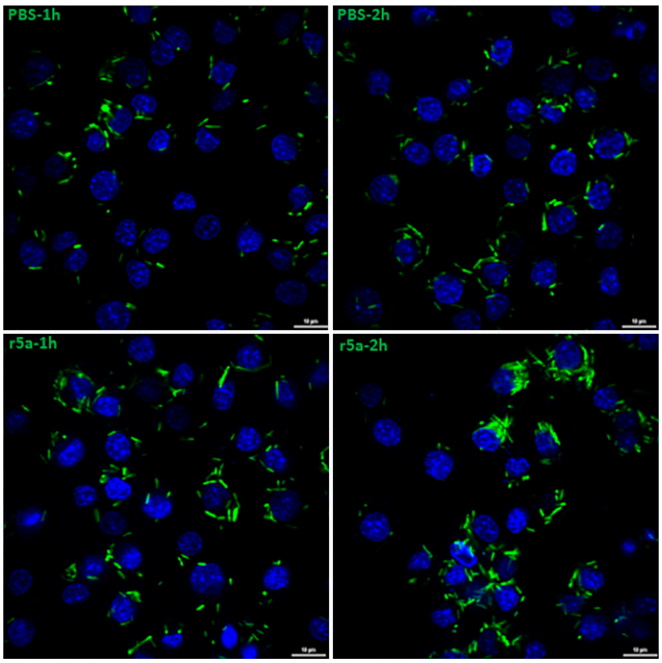

Fig. S4. Depiction of GFP–*E. coli* uptake by RAW 264.7 macrophages. RAW cells pretreated with r5a or PBS, infected with *E. coli* (MOI:50), and processed for confocal microscopy after DAPI staining (600× magnification) at different time points. Cell processing for microscopy is described in *Materials and Methods*.

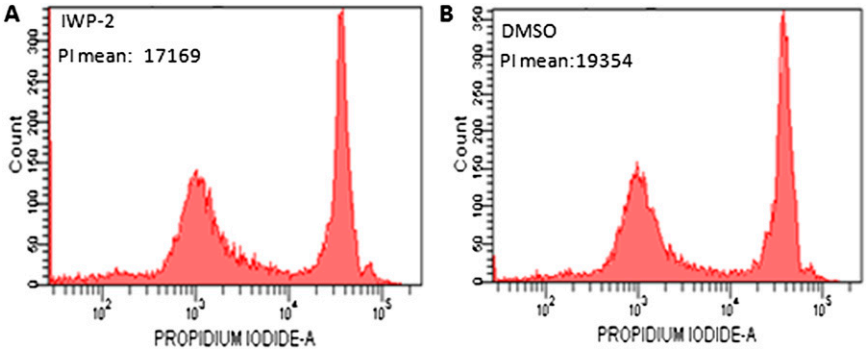

Fig. S5. IWP-2 does not exert toxic effect in vitro. Flow cytometry analysis demonstrating less propidium iodide (PI) incorporation in peritoneal macrophages treated with IWP-2 compared with DMSO (control). Similar results were obtained with liposome-IWP-2 vs. liposome treatment.

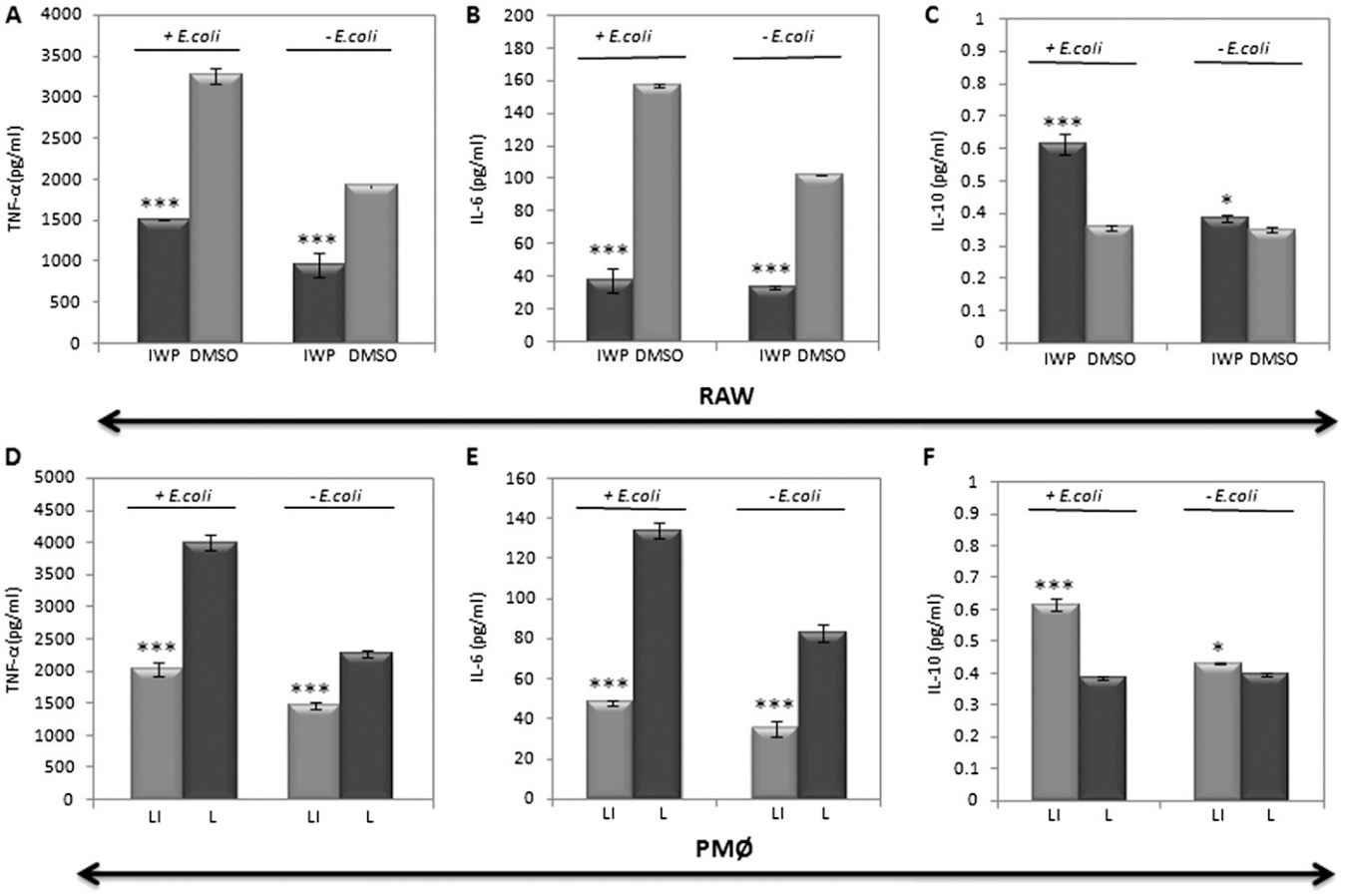

Fig. S6. Influence of IWP-2 and IWP-Liposome on cytokine profile in vitro. (*A*–*C*) TNF-α (*A*), IL6 (*B*), and IL10 (*C*) level in culture supernatant of equal number of RAW cells pretreated with IWP-2 or DMSO in the presence or absence of *E. coli* as determined by ELISA. (*D*–*F*) TNF-α (*D*), IL6 (*E*), and IL10 (*F*) level in culture supernatant of equal number of peritoneal macrophages isolated from C57BL/6 mice pretreated with IWP-liposome (LI) or liposome control (L) in the presence or absence of *E. coli*. $*P < 0.05$; $***P < 0.001$; $n = 3$.

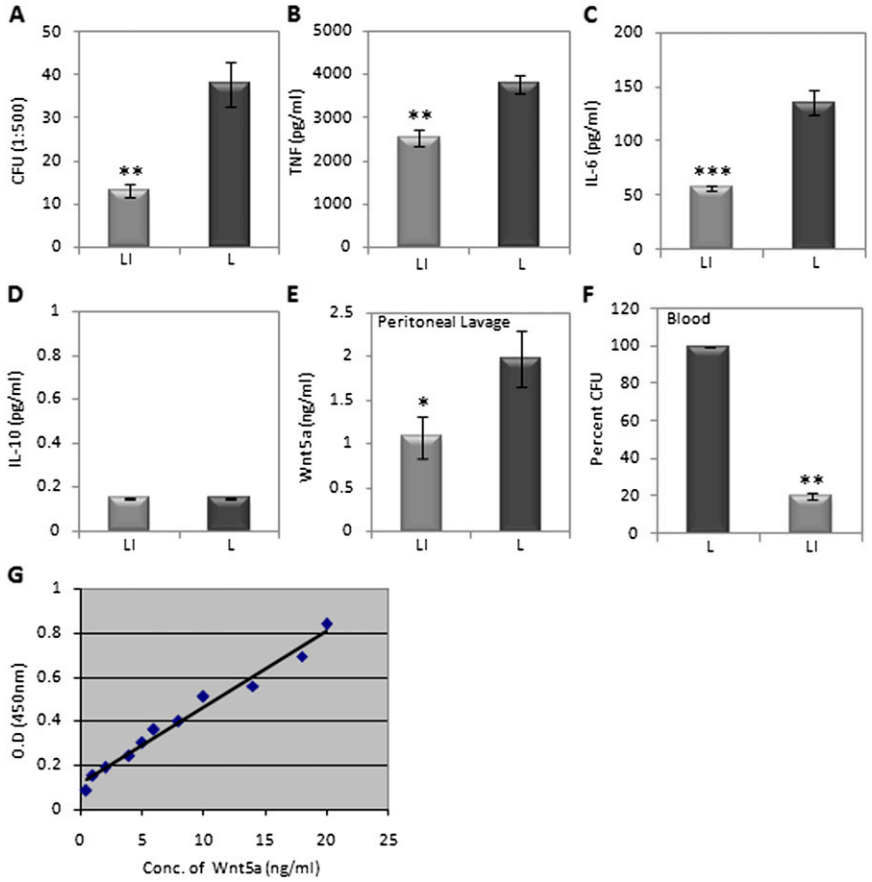

Fig. S7. Influence of IWP-2 on Wnt5a production and sustenance of bacterial infection in vivo. (*A*) Quantitation of *E. coli* in peritoneal cells of C57BL/6 mice injected i.p. with LI or L before i.p., injection with 2×10^6 *E. coli* CFU (10^5 CFU/g body weight) for 3 consecutive days. Peritoneal lavage and cells were harvested after sacrificing mice. (*B–E*) Differential levels of TNF-α, IL6, IL10, and Wnt5a (LI vs. L) in the peritoneal lavage of the corresponding mice. (*F*) Quantitation of *E. coli* in the cardiac blood (LI vs. L) of the corresponding mice. (*G*) Depiction of standard curve for recombinant Wnt5a protein. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $n = 3$ mice per group. No colonies were detected in peritoneal lavage or cardiac blood from normal mice.

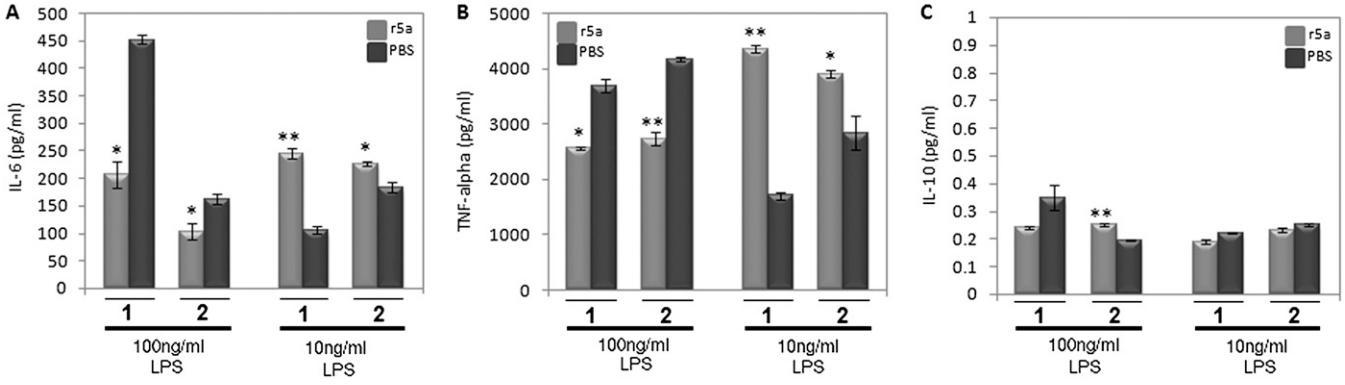

Fig. S8. Immunostimulation by Wnt5a and LPS. (*A–C*) Differential cytokine profiles produced by RAW cells treated with r5a and LPS (1). RAW cells pretreated with r5a or PBS for 6 h and then incubated with 10 ng/mL or 100 ng/mL LPS for 2 h (2). RAW cells pretreated with LPS for 2 h and then with r5a/PBS for 6 h. $*P < 0.05$; $**P < 0.01$.

Table S1. Percent cell death in r5a vs. PBS

	Ti (%)	Ti+4 (%)
r5a	14.58 ± 2.08	33.75 ± 3.75
PBS	12.69 ± 1.58	30.3 ± 3.03

RAW cells pretreated with r5a or PBS for 6 h, infected with *E. coli* for 2 h, and stained with trypan blue. T_i, Internalization for 2 h; T_{i+4}, 4 h after internalization.