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The diacylated lipopeptide FSL-1 enhances phagocytosis of bacteria by macrophages through Toll-like receptor 2-mediated signaling pathway

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Abstract

Enormous lines of evidence have been accumulated that Toll-like receptors (TLRs) function as sensors for microbial invasion. However, less is known about how signaling triggered by TLRs leads to phagocytosis of pathogens. This study was designed to determine whether stimulation of TLR2 with mainly the lipopeptide FSL-1 plays a role in phagocytosis of pathogens by macrophages. FSL-1 markedly enhanced phagocytosis of *E. coli* more strongly than that of *S. aureus*, but did not enhance phagocytosis of latex beads. FSL-1 stimulation resulted in enhanced phagocytosis of bacteria by macrophages from $TLR2^{+/+}$ mice but not those from $TLR2^{-/-}$ mice. Chinese hamster ovary cells stably expressing TLR2 failed to phagocytose these bacteria, but the cells expressing CD14 did. FSL-1 induced upregulation of the expression of phagocytic receptors including MSR1, CD36, DC-SIGN and Dectin-1 in THP-1 cells. Human embryonic kidney 293 cells transfected with DC-SIGN and MSR1 phagocytosed these bacteria.

These results suggest that the FSL-1-induced enhancement of phagocytosis of bacteria by macrophages may be explained partially by the upregulation of scavenger receptors and the C-type lectins through TLR2-mediated signaling pathways and that TLR2 by itself does not function as a phagocytic receptor.

Introduction

Detection of bacterial invasion and the uptake and killing of bacteria are a key function of the innate immune system. Phagocytes, such as macrophages, neutrophils and dendritic cells, play important roles in combating bacterial infection. These phagocytes are able to detect bacterial invasion through various pattern recognition receptors such as Toll-like receptors (TLRs) before uptake and degradation of bacteria. To date, more than ten TLRs have been identified and have been shown to be critical for signaling by pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), peptidoglycan (PGN), lipoteichoic acid (LTA), and lipoprotein/lipopeptide (LP/LPT) (Takeda *et al.*, 2003). The activation of innate immunity by TLRs also leads to the development of antigen-specific adaptive immunity. Thus,

TLRs also play a key role in bridging between innate immunity and adaptive immunity (Takeda *et al.*, 2003). Therefore, it is thought that TLRs not only detect bacterial invasion but also play essential roles in activating signal transduction pathways leading to the killing and clearance of pathogens, since the events after detection of bacterial invasion are uptake and degradation of pathogens for presentation of antigens in the context of MHC to T cells. There have been only a few reports of TLRs playing roles in phagocytosis of bacteria by phagocytic cells. Doyle *et al.* (Doyle *et al.*, 2004) have recently demonstrated that numerous TLR ligands specifically enhance phagocytosis of bacteria by macrophages. Blander and Medzhitov *et al.* (Blander and Medzhitov, 2004) have also suggested that TLR-mediated signaling regulates bacterial phagocytosis. However, it is not fully understood how signaling triggered by TLRs leads to endocytosis and digestion of pathogens in the endosome.

LP is a bacterial cell wall component involved in the systemic inflammatory response caused by gram-negative and gram-positive bacteria as well as in chronic inflammatory disorders (Zhang *et al.*, 1998; Zhang *et al.*, 1997; Brandt *et al.*, 1990; Chamberlain *et al.*, 1989). Recently, we have purified and characterized mycoplasmal LP44 responsible for activation of macrophages and fibroblasts (Shibata *et al.*, 2000; Dong *et al.*, 1999) and synthesized the diacylated LPT called FSL-1 [S-(2,3-bispalmitoyloxypropyl) CGDPKHPKSF] on the basis of the structure of LP44. In addition, we have found that FSL-1 is recognized by TLR2 and/or TLR6 (Okusawa *et al.*, 2004; Fujita *et al.*, 2003).

Therefore, this study was designed to determine whether stimulation of TLR2 with FSL-1 plays a role in phagocytosis of pathogens by macrophages. *Escherichia coli* LPS and *Staphylococcus aureus* PGN were also used as TLR stimulants for comparative studies.

Materials and methods

Antibodies and Reagents

Polyclonal antibodies (pAbs) to phospho-phosphoinositide 3-kinase (PI3-K) p85 binding motif (#3821), nonphosphorylated PI3-K (#4292) and Akt/PKB kinase (#9272) and monoclonal antibodies (mAbs) to phospho-Akt/PKB (Ser473) kinase (#4051) were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-CD36 mAb was obtained from Abcam (Stockholm, Sweden), and phycoerythrin (PE)-conjugated anti-human TLR2 and PE-conjugated mouse IgG2a isotype control were purchased from eBioscience (San Diego, CA, USA). mAbs to the phosphorylated and nonphosphorylated mitogen-activated protein kinases (MAPKs) ERK1/2, SAPK/JNK and p38 were purchased from BD Pharmingen (San Diego, CA). *E. coli* LPS O55:B5, wortmannin, cytochalasin-D and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). *S. aureus* PGN was obtained from Fluka Chemie GmbH. FSL-1 derived from *Mycoplasma salivarium* was synthesized according to the method described previously (Shibata *et al.*, 2000). SB203580 was purchased from Calbiochem-Novabiochem Co. (La Jolla, CA, USA). pUNO-hDC-SIGN1a (human DC-specific intercellular adhesion molecule-grabbing nonintegrin1a) and pUNO-hDectin1b were purchased from InvivoGen (San Diego, CA, USA).

All of the other chemicals were obtained from commercial sources and were of analytical or reagent grade.

Gene cloning and transfection

The cDNAs of human TLR2, CD36 and macrophage scavenger receptor 1 (MSR1) were obtained by RT-PCR of RNA isolated from THP-1 cells, a human monocytic cell line. The cDNA of TLR2 was cloned into a pEF6/V5-His TOPO vector (hereafter referred to as pEF-TLR2) (Invitrogen Co., Carlsbad, CA, USA) and other cDNAs were cloned into a pcDNA3.1-His-TOPO (pcDNA3.1-CD36 and pcDNA3.1-MSR1) (Invitrogen). CHO K1 cells grown in F-12 medium containing fetal bovine serum (FBS) (Gibco BRL, Rockville, MD, USA), penicillin G (100 units mL^{-1}) and streptomycin (100 μg mL^{-1}) were transfected with pEF-TLR2 by Fugene 6 Transfection Reagent (Roche Molecular Biochemicals,

Indianapolis, IN, USA), and stable transfectants (CHO/TLR2) were selected in the presence of 10 μg mL^{-1} blasticidin (Invitrogen). The expression of TLR2 was detected by polyclonal antibody to TLR2 prepared in our laboratory (Fujita *et al.*, 2003). CHO cells expressing CD14 (CHO/CD14) and CHO/CD14/TLR2 cells were kindly provided by Dr. D. G. Golenbock, Department of Medicine, Division of Infectious Diseases & Immunology, University of Massachusetts Medical School, Boston. Human embryonic kidney (HEK) 293 cells obtained from ATCC (CRL-1573) were maintained in DMEM containing 10% FBS, penicillin G (100 units mL^{-1}) and streptomycin (100 μg mL^{-1}). HEK293 cells were transiently transfected with pUNO-hDC-SIGN1a, pUNO-hDectin1b, pcDNA3.1-CD36 or pcDNA3.1-MSR1 and are referred to as 293/DC-SIGN, 293/Dectin1, 293/CD36 or 293/MSR1, respectively.

Mice and peritoneal macrophages (PM)

Sex-matched C57BL/6 mice ($TLR2^{+/+}$ mice) were purchased from Japan Clea (Tokyo, Japan). TLR2-deficient mice ($TLR2^{-/-}$ mice) generated by gene targeting on the same genetic background were kindly provided by Dr. Shizuo Akira, Department of Host Defense, Research Institute for Microbial Diseases, Osaka University (Osaka, Japan). All mice were maintained in specific pathogen-free conditions at the animal facility at Hokkaido University, and all experiments were approved by the regulations of Hokkaido University Animal Care and Use Committee.

Peritoneal exudate cells were harvested by lavage with 6 ml of cold Hank's balanced salt solution. They were washed twice in RPMI 1640 medium without serum and antibiotics (RPMI 1640 base medium) and then resuspended in RPMI 1640 medium containing 10% (vol/vol) FBS, penicillin G (100 units mL^{-1}) and streptomycin (100 μg mL^{-1}) (RPMI 1640 complete medium). The cells were incubated for 2 h in a 10-cm dish in a humidified 5% CO_2 atmosphere. The cells adhered to the bottom of the dish were collected and used as peritoneal macrophages (PM). PM (1×10^6 cells per well) were cultured for 24 h at 37 ^oC alone or with 10 nM FSL-1 in a 24-well flat-bottomed plate and used for the phagocytosis assay described below.

Reverse transcriptase (RT)-polymerase chain reaction (PCR)

Total RNA isolated from 1 x 10^6 each of THP-1 cells was prepared by using an RNeasy kit (Qiagen Inc., Chatsworth, CA, USA.) according to the manufacturer's instructions. RNA was reverse-transcribed to cDNA in a 20-μl reaction volume containing a 1 μM concentration of each of the antisense primers using an RNA PCR kit (Takara Biochemicals, Shiga, Japan) according to the manufacturer's instructions. The PCRs were performed in 40-μl final volumes containing 10 μl of cDNA, 2.5 mM $MgCl_2$, and 20 pmol of each sense primer. After initial denaturation at 94 °C for 2 min, amplifications were carried out for 25, 30 or 35 cycles as follows: denaturation at 94 °C for 30 s, annealing at primer-specific temperatures (see below) for 30 s, and extension at 72 °C for 30s. After the final PCR cycle, extension was allowed to proceed at 70 °C for 2 min. The annealing temperatures were 54 °C for β-actin, 59 °C for CD36 and DC-SIGN, 46 °C for MSR1, and 55°C for Dectin-1. The primer sequences was as follows: for β-actin, 5'-atgtttgagaccttcaacac-3' (sense) and 5'-caggtcacacttcatgatgg-3' (antisense); for MSR1, 5'-caaaatttcagcatgacaactg -3' (sense) and 5'- ttcatctattgcattcc -3' (antisense); for CD36, 5'-ctggctgtgtttggaggtattct -3' (sense) and 5'- agcgtcctgggttacattttcc -3' (antisense); for DC-SIGN, 5'-gccacccctgtccctgggaatg-3'(sense) and 5'- taaaggtcgaaggatggagagaag-3' (antisense); and for Dectin-1, 5'-gggaatcctatgcttggtaat-3' (sense) and 5'- tggagatgggttttcttggg-3'(antisense). The PCR products were separated on 2% gel of NuSieve 3:1 agarose (FMC, Rockland, ME, USA) in 0.5 × Tris-borate-EDTA (TBE) buffer containing ethidium bromide (5 μg mL^{-1}).

Western blotting

In order to assess the activation of MAPKs and Akt, THP-1 cells (2 x 10^6 cells) grown in RPMI 1640 complete medium were washed twice with ice-cold phosphate-buffered saline (PBS) and then incubated for various periods of time with 1 μM FSL-1. The cells were lysed in 100 μl of a lysis buffer (pH 7.5) containing 0.4% (vol/vol) Triton X-100, 0.3% (wt/vol) NP-40, 20 mM HEPES, 150 mM NaCl, 1 mM EGTA, 1.5 mM $MgCl_2$, 10 mM NaF, 1 mM Na_3VO_4, 12.5 mM β-glycerophosphate and protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) and boiled for 10 min. The lysates were centrifuged at 8,000 x g for 10 min, and the resulting supernatants containing cytosolic and membrane

proteins were collected and mixed 1:1 with a SDS sample buffer. Proteins in 20 μl of the supernatant were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated at 4 ^{o}C overnight with Abs to phosphorylated or nonphosphorylated MAPKs, PI3-K p85 or Akt and then stained with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG Ab. Immunoreactive proteins were detected by using ECL detection reagents (Amersham Biosciences, Buckinghamshire, UK).

Surface expression of TLR2 and TNF-α production in THP-1 cells

THP-1 cells were cultured in 6-well plates at 1 x 10^6 cells per well in the absence or presence of 20 nM PMA and then incubated for 24 h. The cells were stained with PE-conjugated mAb against TLR2 and appropriate isotype controls, fixed in PBS containing 0.5% formaldehyde, and then analyzed by a flow cytometer (FCM) (a FACS Caliber®, BD Biosciences, Sunnyvale, CA, USA). Data for 10,000 cells falling within appropriate forward and side light scatter gates were collected from each sample using FCM and CellQuest software (BD Biosciences).

THP-1 cells were incubated at 37 °C for 24 h in the absence or presence of 20 nM PMA. After a 0.2-ml volume of cell suspension of THP-1 (5 x 10^5 cells) in each well of a 96-well tissue culture plate had been incubated at 37 °C for 16 h with various concentrations of FSL-1 in culture medium supplemented with 0.1% (vol/vol) human serum, the culture supernatant was collected by centrifugation at 400 x *g* for 10 min. The amount of TNF-α in the supernatant was determined by using an HU TNF-α Flexia (Biosource, San Diego, CA, USA).

Phagocytosis assay

A 0.5-ml suspension of THP-1 cells (1 x 10^6 mL^{-1}) grown in RPMI 1640 complete medium was added to wells of a 24-well plate and incubated at 37 ^{o}C for 24 h with various concentrations of FSL-1. In the case of CHO transfectants, 1.0 ml of CHO-K1, CHO/TLR2 or CHO/CD14 cells (5 x 10^5 mL^{-1}) grown in F-12 complete medium was added to wells of a 12-well plate and then incubated at 37 ^{o}C for 24 h. In the case of HEK293 transfectants, 1.0 ml of 293/DC-SIGN, 293/Dectin-1, 293/MSR1 and 293/CD36 cells (5 x 10^5 mL^{-1}) grown in DME complete medium was added to wells of a 12-well plate and then incubated at 37 ^{o}C for 24 h after transient transfection. After the cells had been washed three times with base medium warmed at 37 ^{o}C, they were incubated for various times of intervals (0, 20, 40, 60 and 80 min) with 5 x 10^6 bioparticles of Alexa Fluor 488-conjugated *E. coli* K12 and *S. aureus* Wood (Molecular Probes, Eugene, OR, USA) or Latex Beads (Sigma-Aldorich). After being incubated at 37 ^{o}C, the cells were washed three times with cold PBS and suspended in PBS containing 0.2% (wt/vol) trypan blue to quench fluorescence caused by binding of bacteria to the surface of the cells and 1% (wt/vol) paraformaldehyde to fix the cells. Analysis by FCM was conducted using FCM and CellQuest software (BD Biosciences). Phagocytosis (%) was expressed as [number of cells taking up particles]/[total cell number analyzed (10000)] x 100. In addition, phagocytic activity was expressed as the mean fluorescence intensity obtained by CellQuest software.

For phagocytosis assay by a confocal laser scanning microscope (CLSM), a 1.0-ml suspension of THP-1 cells (1 x 10^6 mL^{-1}) was added to wells of a 24-well plate and incubated at 37 ^{o}C for 24 h with various concentrations of FSL-1, LPS or PGN. After the cells had been washed three times with RPMI 1640 base medium warmed at 37 ^{o}C, they were incubated for 1 h with 1 x 10^7 bioparticles of Alexa Fluor 488-conjugated *E. coli* or *S. aureus*. The cells were then washed with PBS and reacted for 15 min with rhodamine-conjugated concanavalin A, followed by fixation with PBS containing 1% paraformaldehyde. The cells were finally sealed in the presence of mounting medium (Thermo Electron Co., Pittsburgh, PA, USA) and observed using a CLSM (LSM510, Carl Zeiss, Tokyo, Japan). Digital images were acquired and processed using Adobe Photoshop, version 5.0 (Mountain View, CA, USA). Phagocytosis (%) in one field containing 50 to 100 cells is expressed as [the number of cells taking up particles]/[total number (50-100) of cells in one field] x 100. Data are expressed as mean ± SD of phagocytosis (%) obtained by five fields.

Results

FSL-1-induced enhancement of phagocytosis of bacteria by THP-1 cells

In order to determine whether FSL-1 stimulation affects phagocytosis of bacteria by THP-1 cells, a human monocytic cell line, were treated with FSL-1 and then examined for the uptake of Alexa 488-labeled bioparticles of *E. coli* and *S. aureus* by FCM analysis. It was found that FSL-1 treatment markedly enhanced both % of phagocytosis and phagocytic activity of the gram-positive bacterium *S. aureus* or the gram-negative bacterium *E. coli* by THP-1cells in time- and dose-dependent manners (Fig. 1A, B and C) and also enhanced phagocytosis of *E. coli* more strongly than that of *S. aureus*. Latex beads were also phagocytosed by THP-1 cells, but FSL-1 did not enhance the phagocytosis (Fig. 1A). In addition, we tried to assay for the phagocytosis by using a CLSM. Observation by a CLSM also showed that FSL-1 enhanced phagocytosis of these bacteria by THP-1 cells in a dose-dependent manner (Fig. 2A and 2D). Therefore, we investigated whether other TLR ligands, LPS and PGN, enhanced the phagocytosis. Both LPS and PGN also significantly enhanced the % of phagocytosis of *S. aureus*, whereas they slightly, but not significantly, enhanced that of *E. coli* (Fig. 2B and 2C). .

Effects of inhibitors on the FSL-1-induced enhancement of phagocytosis

It is well known that rearrangement of the actin cytoskeleton and PI3-K play important roles in phagocytosis (Underhill and Ozinsky, 2002; Gottlieb *et al.*, 1993). In addition, Doyle *et al.* (Doyle *et al.*, 2004) have demonstrated that TLRs induce a phagocytic gene through p38 MAPK. Therefore, experiments were carried out to determine whether rearrangement of the actin cytoskeleton, PI3-K and p38 MAPK are responsible for the FSL-1-induced enhancement of phagocytosis of these bacteria by THP-1 cells. It was found that the p38 MAPK inhibitor SB203580 and the PI3-K inhibitor wortmannin significantly reduced the FSL-1-induced enhancement of phagocytosis of *E. coli* and *S. aureus* and, to a lesser extent, cytochalasin-D, an inhibitor of actin microfilament (Gottlieb *et al.*, 1993), also reduced the phagocytosis (Fig. 3). These results suggest that actin microfilaments, PI3-K and p38 MAPK play critical roles in the FSL-1-induced enhancement of phagocytosis. In order to further confirm these findings, experiments were carried out to determine whether PI3-K and p38 MAPK were activated in THP-1 cells in response to FSL-1. It was found that PI3-K p85 and its downstream Akt kinase were activated 15 min after stimulation with 1 μM FSL-1 (Fig. 4A). In addition, MAPKs, p38 and JNK were activated 15 min after stimulation with FSL-1 (Fig. 4B). ERK was slightly activated 15 min after stimulation and then the activation decreased in a time-dependent manner. However, ERK was activated even in the absence of FSL-1 stimulation (Fig. 4B). Although it remains exactly unknown why ERK was activated, it is speculated that ERK activation is attributed to FBS components added to the RPMI1640 complete medium used for cultivation of THP-1 cells.

Taken together, these results suggest that rearrangement of the actin cytoskeleton, PI3-K and p38 MAPK play key roles in the FSL-1-induced enhancement of phagocytosis of these bacteria by THP-1 cells.

TLR2 dependency of the FSL-1-induced enhancement of phagocytosis

We have demonstrated that the diacylated LPT FSL-1 is recognized by TLR2 (Okusawa *et al.*, 2004; Fujita *et al.*, 2003). First of all, we examined whether THP-1 cells treated with or not treated with PMA and FSL-1 expressed TLR2 on the cell surface. It was found that THP-1 cells expressed TLR2 and that the expression was upregulated by treatment with PMA (Fig. 5A and B). FSL-1did not upregulate the expression of TLR2 (Fig. 5B), but activated the cells to induce production of TNF-α even without stimulation with PMA (Fig. 5C). Thus, THP-1 cells used for this study express TLR2 on the cell surface and are activated by the TLR2 ligand FSL-1. On the basis of these results, we speculated that TLR2 played a key role in the FSL-1-induced enhancement of phagocytosis of these bacteria by THP-1 cells. In order to further confirm this, experiments were carried out to determine whether the FSL-1-induced enhancement of phagocytosis was mediated by TLR2. PM prepared from both $TLR2^{+/+}$ and $TLR2^{-/-}$ mice were examined for phagocytosis of particles of *E. coli* and *S. aureus*. Percentages of phagocytosis of *E. coli* and *S. aureus* by PM were 89% and 90%, respectively. This is because PM are potent phagocytic cells. Thus, there was no differences in percentages of PM taking up bacterial particles, but ratios of MFI obtained by PM from $TLR2^{+/+}$ mice stimulated with FSL-1 to that by nonstimulated PM were 1.3 in *E.*

coli and 1.4 in *S. aureus*, whereas those by PM from TLR2$^{-/-}$ mice were 0.9 and 1.1, respectively (Fig. 6). These results suggest that FSL-1 stimulation results in an increase in the number of bacteria internalized into PM, which is equivalent to the phagocytic activity.

On the basis of these results, it is thought that the FSL-1-induced enhancement of phagocytosis of bacteria by macrophages is mediated by TLR2.

TLR2 does not function as a phagocytic receptor

THP-1 cells stimulated with FSL-1 as a TLR2 ligand internalized the gram-positive *S. aureus* more strongly than the gram-negative *E. coli* (Fig. 1 and 2). *S. aureus* contains PGN and LTA as TLR2 ligands, but not LPS as a TLR4 ligand, whereas *E. coli* contains LPS. In addition, as described above, THP-1 cells express TLR2 on the surface (Fig. 5) and TLR2 plays a key role in the FSL-1-induced enhancement of phagocytosis (Fig. 6). On the basis of these results, we speculated the possibility that TLR2 by itself functioned as a phagocytic receptor in the FSL-1-induced enhancement of bacterial phagocytosis by THP-1 cells. In order to confirm this, CHO-TLR2 cells were examined for phagocytosis of these *E. coli* and *S. aureus*. This is because CHO-TLR2 cells are thought to take up these bacteria if TLR2 functions as a phagocytic receptor. Although CHO-TLR2 cells expressed a TLR2 protein (Fig. 7A), CHO/TLR2 as well as CHO-K1 cells failed to take up these bacterial particles (Fig. 7B). However, uptake of these bacteria was clearly observed in CHO cells transfected with CD14, which is known to be a phagocytic receptor (Underhill and Ozinsky, 2002). In addition, it was found that CHO/TLR2 cells did not uptake these bacteria even after FSL-1 stimulation (Fig. 7C). These results suggest that TLR2 by itself does not function as a phagocytic receptor.

Involvement of phagocytic receptors in the FSL-1-induced enhancement of phagocytosis

As one of the molecular mechanisms underlying the FSL-1-induced enhancement of phagocytosis of bacteria by THP-1 cells, we thought that FSL-1 stimulation upregulated surface expression of phagocytic receptors capable of binding to bacteria such as MSR1, CD36, Dectin-1 and DC-SIGN in THP-1 cells. We examined the expression of these surface molecules on THP-1 cells stimulated with FSL-1, because we thought that FSL-1 induced upregulation of expression of these molecules, which in turn enhances bacterial phagocytosis by THP-1 cells. It was found that the expression of mRNAs of MSR1, CD36, Dectin-1 and DC-SIGN was upregulated in response to FSL-1, suggesting that these molecules play important roles in the FSL-1-induced enhancement of phagocytosis by THP-1 cells (Fig. 8A). In order to further confirm this, HEK293 cells were transiently transfected with genes of MSR1, CD36, Dectin-1 and DC-SIGN and examined for phagocytosis of these *E. coli* and *S. aureus*. It was found that 293/DC-SIGN preferentially phagocytosed *E. coli*, whereas 293/MSR1 incorporated bioparticles of both bacteria (Fig. 8B).

Discussion

There has been an accumulation of evidence showing how TLRs detect microbial invasion (Takeda *et al.*, 2003). In the innate immune system, microbes are taken up and digested in endosomes of phagocytes for presentation of antigens in the context of MHC to T cells after detection of microbial invasion. Nevertheless, there are only a few reports on how signaling triggered by TLRs leads to endocytosis and digestion of pathogens in the endosome. Recently, Doyle *et al.* (Doyle *et al.*, 2004) have demonstrated that numerous TLR ligands specifically enhance phagocytosis of bacteria, such as *E. coli* and *S. aureus*, while exhibiting minimal effects on nonbacterial targets such as latex beads. Various TLRs differentially promote phagocytosis through induction of a phagocytic gene program. Blander and Medzhitov (Blander and Medzhitov, 2004) have also demonstrated that MyD88$^{-/-}$ and TLR2x4$^{-/-}$ macrophages take up *E. coli* at significantly reduced levels compared with wild-type mice-derived macrophages. However, it still remains unknown how TLR-mediated signaling pathways lead to uptake and killing of microbes by phagocytes.

In this study, we tried to clarify the roles of TLR2 in the killing and clearance of pathogens, since we

have studied the mechanisms by which TLR2 recognizes its ligands, including the diacylated LPT FSL-1 (Okusawa *et al.*, 2004; Fujita *et al.*, 2003; Shibata *et al.*, 2000). The present study demonstrated that FSL-1 specifically enhanced phagocytosis of bioparticles of *E. coli* and *S. aureus*, but not latex beads, by THP-1 cells (Fig. 1) and, to a lesser extent, both *E. coli* LPS and *S. aureus* PGN also enhanced the phagocytosis (Fig. 2). These results support the findings of Doyle *et al.* (Doyle *et al.*, 2004) and Blander and Medzhitov (Blander and Medzhitov, 2004) described above. We speculated that TLR2 played a key role in the FSL-1-induced enhancement of phagocytosis, since FSL-1 as a TLR2 ligand enhanced phagocytosis of these bacteria by THP-1 cells, which express TLR2 on the cell surface (Fig. 5A). In order to confirm this, PM prepared from $TLR2^{+/+}$ and $TLR2^{-/-}$ mice were examined for phagocytosis of *E. coli* and *S. aureus*. However, it was very hard to detect differences in levels of phagocytosis by PM derived from both types of mice because of extremely high % of phagocytosis more than 89%. However, in $TLR2^{+/+}$ mice, ratios of MFI obtained by PM stimulated with FSL-1 to nonstimulated PM were 1.3 in *E. coli* and 1.4 in *S. aureus*, whereas those by PM were 0.9 and 1.1 in $TLR2^{-/-}$ mice, respectively (Fig.6). That is, FSL-1 stimulation resulted in an increase in the number of bacterial particles incorporated into THP-1 cells. These results suggest that the FSL-1-induced enhancement of phagocytosis of bacteria is mediated by TLR2 and also the possibility that TLR2 by itself functions as a phagocytic receptor. In order to determine this, CHO cells stably expressing TLR2 were established and examined for phagocytosis of these bacteria. For comparative studies, CHO cells stably expressing CD14 (CHO/CD14), which is known to be a phagocytic receptor (Underhill and Ozinsky, 2002; Devitt *et al.*, 1998; Onozuka *et al.*, 1997; Schiff *et al.*, 1997), were also used. However, CHO/TLR2 cells as well as wild-type CHO-K1 cells failed to take up these bacteria (Fig. 7), whereas uptake of these bacteria was clearly observed in CHO/CD14 cells. These results suggest that TLR2 by itself does not function as a phagocytic receptor. Recently, Underhill and Gantner (Underhill and Gantner, 2004) have described four principle steps in which TLR signaling affects phagocytosis. First, TLRs may function directly as phagocytic receptors. Second, TLR signaling may modulate the efficiency of phagosome formation. Third, TLR signaling may affect maturation of newly formed phagosomes. Fourth, TLR-mediated transcriptional responses may affect genes involved in all steps of phagocytosis. As described above, the present study demonstrated that TLR2 was not able to function as a phagocytic receptor. This is supported by previous findings that targeted deletion of TLR2 or MyD88 has no effect on the phagocytosis of zymosan by macrophages and that expression of dominant-negative forms of TLR2 and MyD88 does not affect phagocytosis (Gantner *et al.*, 2003; Underhill *et al.*, 1999). On the basis of these results, the first possibility that TLRs function as phagocytic receptors is denied, although other three hypotheses still remain to be evaluated.

Phagocytosis of bacteria requires activation of a number of signaling pathways that regulate rearrangement of the actin cytoskeleton, extension of the plasma membrane, and fusion to form a phagolysosome (Underhill and Ozinsky, 2002). PI3-K catalyzed the phosphorylation of PI(4,5)P2 to PI(3,4,5)P3, a phospholipid important in recruiting signaling molecules such as the kinase Akt/PKB to specific regions of the membrane. PI3K is not required for particle binding or initial actin polymerization but is required for membrane extension and fusion behind the bound particle (Underhill and Ozinsky, 2002). Inhibition of PI3-K blocks phagocytosis of a broad spectrum of particles, including IgG- and complement-opsonized particles, unopsonized zymosan and bacteria (Aderem, 2003; Underhill and Ozinsky, 2002). The present study also demonstrated that TLR2 stimulation with FSL-1 induced activation of PI3-K and Akt and that the PI3-K inhibitor wortmanin downregulated the FSL-1-induced enhancement of phagocytosis of bacteria by macrophages (Figs. 3 and 4), suggesting a universal role of PI3-K activation in internalization of bacterial particles.

Doyle *et al.* (Doyle *et al.*, 2004) also demonstrated that TLR-mediated induction of scavenger receptors (SRs) such as SR-A, MARCO and LOX-1 in murine bone-marrow-derived macrophges occurs through MyD88, IRAK4, and p38 and that activation of this pathway is essential for TLR promotion of phagocytosis. The present study also demonstrated that TLR2 stimulation with FSL-1 induced activation of p38 a human macrophage cell line, THP-1 cells and that the p38 inhibitor SB203580 suppressed the FSL-1-induced enhancement of phagocytosis by human macrophages (Fig. 3 and 4). In addition, the present study demonstrated that stimulation of human macrophages with FSL-1 induced upregulation of

expression of SRs such as MSR1 and CD36, and C-type lectin such as Dectin-1 and DC-SIGN. Although it is not known what happens downstream of p38 activation, there is a possibility that the signal triggered by p38 activation leads to expression of MSR1, CD36, Dectin-1 or DC-SIGN, which are then involved in the FSL-1-induced enhancement of bacterial phagocytosis. On the basis of the findings by Doyle *et al.* (Doyle *et al.*, 2004), we also thought that C-type lectins, Dectin-1 and DC-SIGN as well as SRs might be involved in the FSL-1-induced enhancement of bacterial phagocytosis by macrophages. CD36, which is known as a thrombospondin receptor or a member of class B SRs, is involved in the clearance of apoptotic cells (Silverstein and Febbraio, 2000) and is known to be a sensor of diacylglyceride (Hoebe *et al.*, 2005), which forms the N-terminal part of the diacylated LPTs such as FSL-1. MSR1, also known as a member of class B SRs, functions as an receptor for modified low density lipoprotein (LDL), acetylated LDL and polyanions including lipid A (Taylor *et al.*, 2005). Peiser *et al.* (Peiser *et al.*, 2002) showed that bacteria were taken up via MSR1. Dectin-1, a member of the NK cell-receptor-like C-type lectin family, was originally thought to be a DC-specific receptor, but the receptor is now known to be expressed by many other cell types, including macrophages (Brown, 2006; Underhill and Ozinsky, 2002). Brown and Gordon (Brown and Gordon, 2001) have recently defined dectin-1 as a β-glucan binding lectin capable of mediating phagocytosis of zymosan when the receptor is expressed in normally nonphagocytic cells. The C-type lectin DC-SIGN is also known to interact with various bacteria as well as viruses (van Kooyk and Geijtenbeek, 2003). We found that the expression of mRNAs of MSR1, CD36, Dectin-1 and DC-SIGN was upregulated in response to FSL-1, suggesting that these molecules play important roles in the FSL-1-induced enhancement of phagocytosis by THP-1 cells (Fig. 8A). Therefore, HEK293 cells expressing these molecules were examined for phagocytosis of these bacteria. As a result, it was found that 293/DC-SIGN preferentially phagocytosed *E. coli* particles, whereas 293/MSR1 incorporated bioparticles of both bacteria (Fig. 8B). However, 293/Dectin-1 and 293/CD36 failed to incorporate these bacteria.

On the basis of these findings, it is considered that FSL-1 induces upregulation of expression of CD36, Dectin-1 and DC-SIGN, which is essential for theFSL-1-induced enhancement of bacterial phagocytosis by THP-1 cells.

As shown in Fig. 1 and 2A, FSL-1 enhanced phagocytosis of *S. aureus* and *E. coli* by THP-1 cells. However, PGN and LPS enhanced phagocytosis of *S. aureus*, but not *E. coli* (Fig. 2B and 2C). The difference in phagocytic pattern by THP-1 cells stimulated by FSL-1 and LPS may be explained by the difference in their recognition receptors, TLR2 and TLR4. That is, it is speculated that phagocytic receptors induced by TLR2-mediated signaling pathway have higher affinity for surface substances of *E. coli* including LPS than those by TLR4-mediated signaling pathway. However, the difference between FSL-1 and PGN is not explainable, because both PAMPs are recognized by TLR2.

Studies are in progress to further characterize phagocytic receptors induced by these PAMPs on the cell surfaces of macrophages.

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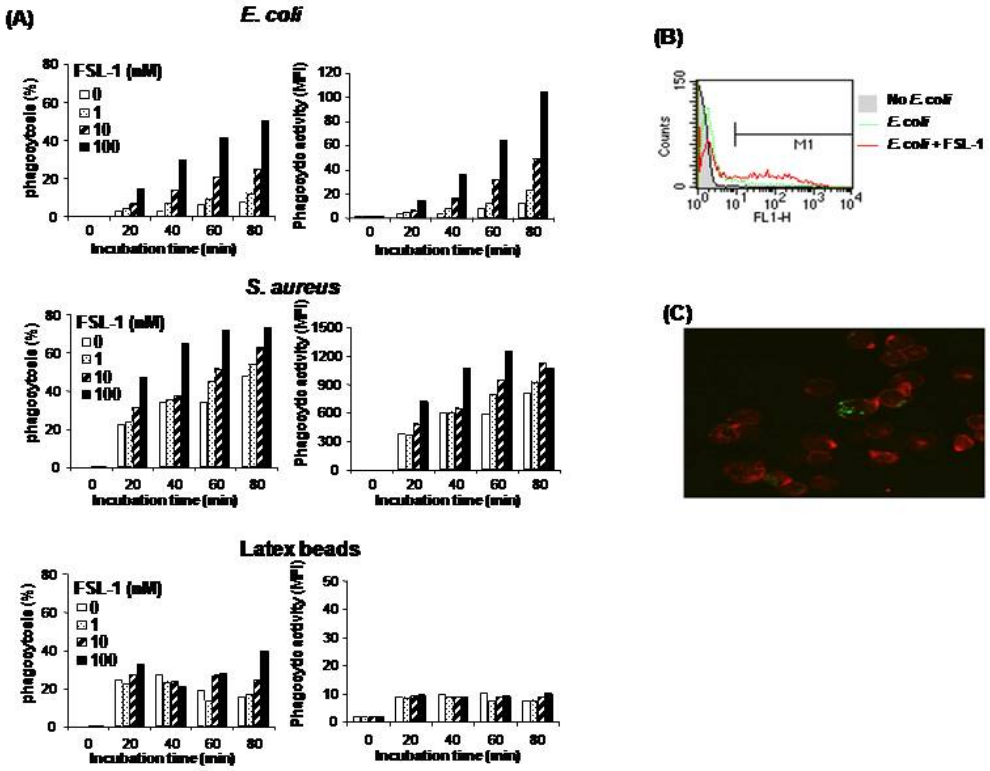


Fig. 1

Fig. 1. FSL-1-induced enhancement of phagocytosis of bioparticles of *E. coli* and *S. aureus* by THP-1 cells.

THP-1 cells were incubated at 37 ^{o}C for 24 h with various concentrations (1, 10, 100 nM) of FSL-1. After the cells were washed, they were incubated for 0, 20, 40, 60 or 80 min with bioparticles of Alexa Fluor 488-conjugated *E. coli* and *S. aureus* or Latex Beads (A). After being incubated, the cells were washed and suspended in PBS containing trypan blue to quench fluorescence caused by binding of bacteria to the surface of the cells and paraformaldehyde to fix the cells. Analysis of phagocytosis was performed by flow cytometry. Phagocytosis (%) is expressed as [number of cells taking up particles]/[total cell number analyzed (10000)] x 100. Phagocytic activity (MFI), which indicates the number of bacteria internalized into THP-1 cells, was expressed as the mean fluorescence intensity in the histogram (B) obtained by flow cytometry. Internalization of *E. coli* into THP-1 cells (C) was observed by a confocal laser scanning microscope. See text for details.

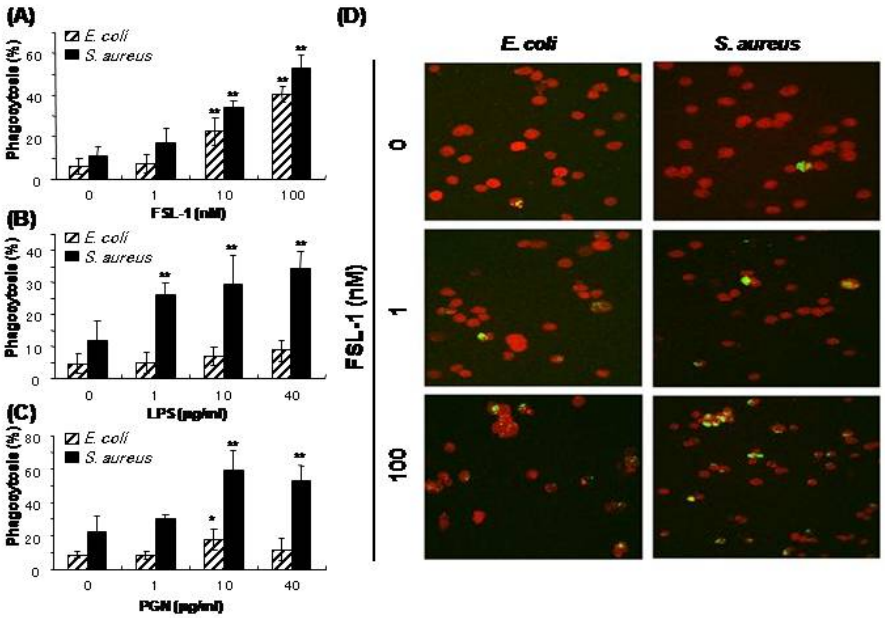


Fig. 2

Fig. 2. Enhancement by THP-1 cells by FSL-1, LPS or PGN of phagocytosis of bioparticles of *E. coli* and *S. aureus*.

THP-1 cells were incubated at 37 ^{o}C for 24 h with various concentrations of FSL-1 (A), LPS (B) or PGN (C). After the cells were washed, they were incubated for 1 h with bioparticles of Alexa Fluor 488-conjugated *E. coli* or *S. aureus*. The cells were then washed with PBS and reacted for 15 min with rhodamine-conjugated concanavalin A, followed by fixation with paraformaldehyde and trypan blue. The cells were finally sealed in the presence of mounting medium, and observed using a confocal laser scanning microscope. Digital images were acquired and processed using Adobe Photoshop, version 5.0. Phagocytosis (%) in one field containing 50 to 100 cells is expressed as [the number of cells taking up particles]/[total number (50–100) of cells in one field] x 100. Each value is the mean ± SD of phagocytosis (%) obtained by five fields. Representative photos of phagocytosis of bioparticles of *E. coli* and *S. aureus* by THP-1 cells stimulated with FSL-1 were shown in (D). See text for details. The statistical difference is assessed by the Student's t-test: *, $p<0.05$; and **, $p<0.01$ for the cells stimulated with FSL-1 versus unstimulated.

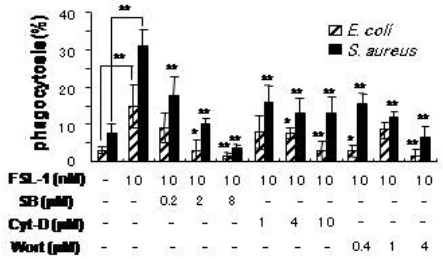


Fig. 3

Fig. 3. Roles of actin cytoskeleton, PI3-K and p38 MAPK in the FSL-1-induced enhancement of phagocytosis of bioparticles of *E. coli* and *S. aureus* by THP-1 cells.

THP-1 cells were incubated at 37 ^{o}C for 24 h with 10 nM FSL-1. After the cells were washed, they were incubated at 37 ^{o}C for 1 h with various concentrations of cytochalasin-D (Cyt-D), SB203580 (SB) or wortmannin (Wort) and then incubated for 1 h with bioparticles of Alexa Fluor 488-conjugated *E. coli* or *S. aureus*. After the cells were washed, they were reacted for 15 min with rhodamine-conjugated concanavalin A, followed by fixation with paraformaldehyde and trypan blue. The cells were finally sealed in the presence of mounting medium and observed using a confocal laser scanning microscope. Digital images were acquired and processed using Adobe Photoshop, version 5.0. Phagocytosis (%) in one field containing 50 to 100 cells is expressed as [the number of cells taking up particles]/[total number (50-100)of cells in one field] x 100. Each value is the mean ± SD of phagocytosis (%) obtained by five fields. See text for details. The statistical difference of is assessed by the Student's t-test: *, $p<0.05$; and **, $p<0.01$ for the cells treated with inhibitor versus untreated but stimulated with FSL-1. For the cells stimulated with FSL-1 versus unstimulated: +, $p<0.01$.

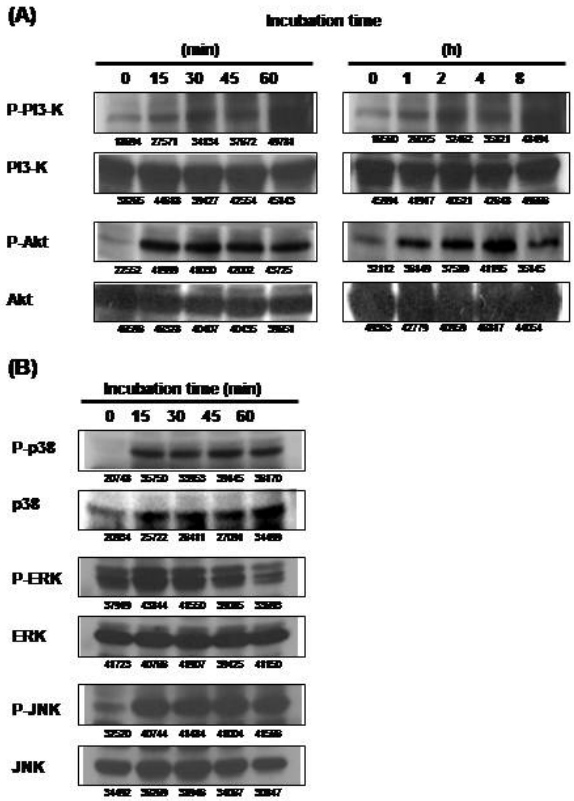


Fig. 4

Fig. 4. Activation of PI3-K, Akt and MAPKs in THP-1 cells by FSL-1 stimulation.

THP-1 cells (2 x 10^6) were washed and then incubated for various periods of time with 1 μM FSL-1 in RPMI1640 base medium. The cells were lysed in 100 μl of a lysis buffer and boiled for 10 min. The lysates were centrifuged, and the resulting supernatants containing cytosolic and membrane proteins were collected and mixed 1:1 with a SDS sample buffer. Proteins in 20-μl of the mixture were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated with Abs to phosphorylated or nonphosphorylated MAPKs, PI3-K p85 or Akt and then stained with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG Ab. Immunoreactive proteins were detected by using ECL detection reagents. See text for details.

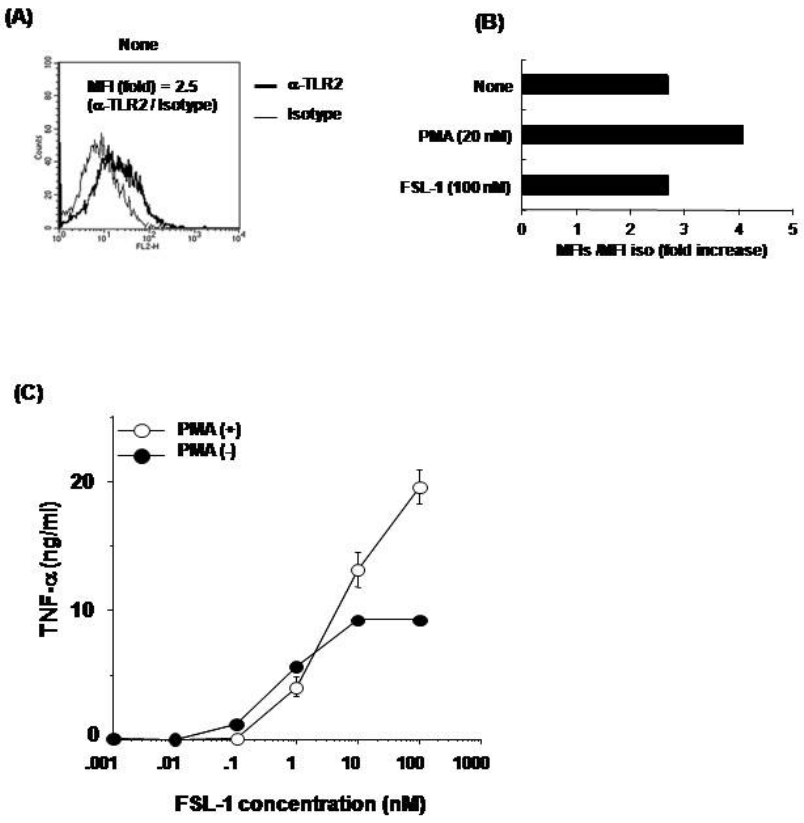


Fig. 5

Fig. 5. Expression of cell surface TLR2 in THP-1 cells and FSL-1-induced TNF-α production by the cells.

THP-1 cells were cultured in the absence or presence of 20 nM PMA or 100 nM FSL-1 and then incubated for 24 h. The cells were stained with PE-conjugated mAb against TLR2 and appropriate isotype controls, fixed in formaldehyde, and then analyzed by flow cytometry (A and B). Data for 10,000 cells falling within appropriate forward and side light scatter gates were collected from each sample using a FACS Caliber® machine. The expression of TLR2 after stimulation with FSL-1 or PMA was also measured and the data (MFI s/MFI iso) was expressed as ratios of the mean fluorescent intensity (MFI s) of the stimulated cells to that (MFI iso)of the cells stained with isotype control (B). THP-1 cells were incubated at 37 °C for 24 h in the absence (●) or presence (○) of 20 nM PMA. After THP-1 cell sus were incubated at 37 °C for 16 h with various concentrations of FSL-1, the culture supernatant was collected. The amount of TNF-α in the supernatant (C) was determined by using an ELISA kit. See text for details.

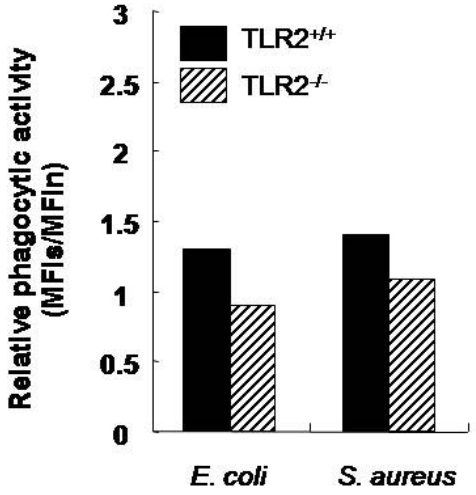


Fig. 6

Fig. 6. Phagocytosis of bioparticles of *E. coli* and *S. aureus* by PM from TLR2$^{+/+}$ and TLR2$^{-/-}$ mice.

PM from TLR2$^{+/+}$ and TLR2$^{-/-}$ mice were harvested by lavage with cold Hank's balanced salt solution. They were washed, and then resuspended in RPMI 1640 complete medium. PM were cultured for 24 h at 37 ^{o}C alone or with 10 nM FSL-1 and used for phagocytosis of bioparticles of *E. coli* and *S. aureus*. Relative phagocytic activity is expressed as ratio of the mean fluorescence intensity of the cells internalizing bacterial particles after stimulation with FSL-1(MFIs) to that of the cells nonstimulated (MFIn). See text for details.

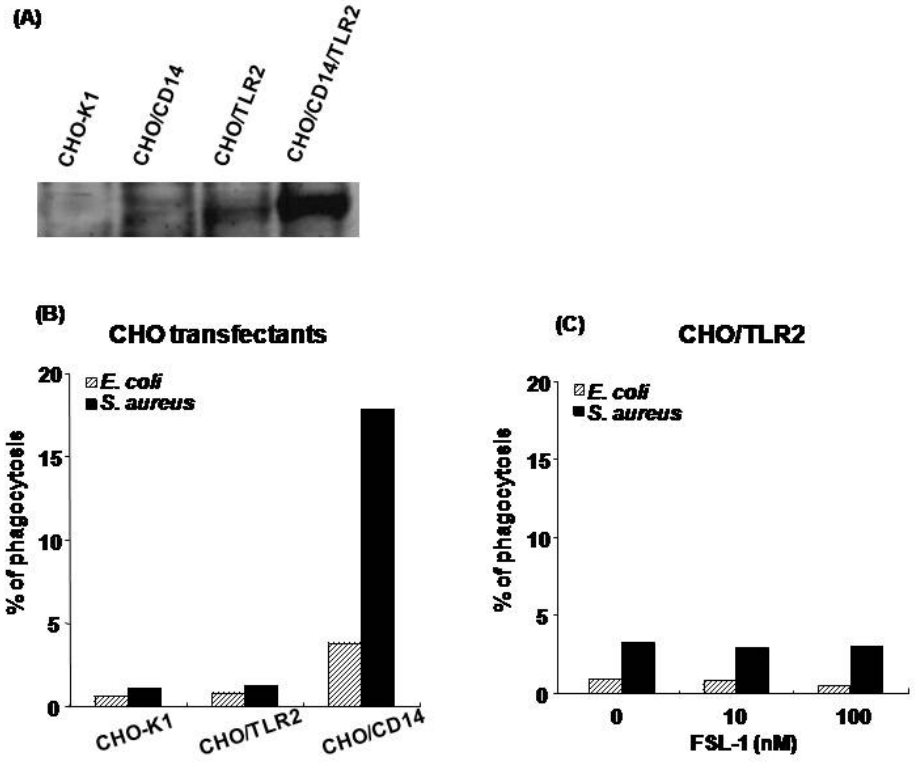


Fig. 7

Fig. 7. Phagocytosis of bioparticles of *E. coli* and *S. aureus* by CHO-K1, CHO-TLR2 and CHO-3E10 cells.

The expression of TLR2 was detected by polyclonal antibody to TLR2 using CHO/CD14/TLR2 cells as a TLR2-positive cell (A). After the cells were washed, they were incubated for 1 h with bioparticles of Alexa Fluor 488-conjugated *E. coli* and *S. aureus* in the absence of FSL-1 stimulation (B). CHO/TLR2 cells were also stimulated with FSL-1 and then examined for phagocytosis (C). After 1-h incubation at 37 ^{o}C, the cells were washed three times with cold PBS, and then suspended in PBS containing trypan blue and paraformaldehyde. Analysis was performed by flow cytometry. See text for details.

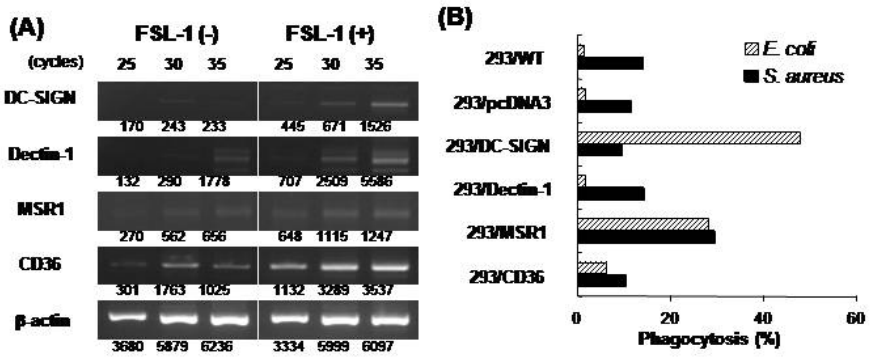


Fig. 8

Fig. 8. FSL-1-induced upregulation of phagocytic receptors in THP-1 cells and bacterial phagocytosis by HEK293 transfectants.

Expression of mRNAs of DC-SIGN, Dectin-1, MSR1, CD36 and β-actin (A) was confirmed by RT-PCR by using total RNA isolated from THP-1 cells. The strength of these signals was expressed as numerical values on the basis of the data obtained by densitometric analysis. HEK293 cells were transiently transfected with pUNO-hDC-SIGN1a, pUNO-hDectin1b, pcDNA3.1-CD36 or pcDNA3.1-MSR1 and examined for phagocytosis of *E. coli and S. aureus* (B) by flow cytometry. See text for details.