Resveratrol modulates phagocytosis of bacteria through an NF-κB-dependent gene program

Running title: Inhibition of bacterial phagocytosis by resveratrol

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ABSTRACT

Many studies have shown that the pharmacological effects of resveratrol, a phytoalexin polyphenolic compound, include protective effects against cancer and inflammation as well as enhancement of stress resistance. In this study, we examined whether resveratrol affected phagocytosis of bacteria by macrophages and activation of the transcription factor NF-κB after stimulation with or without the ligand FSL-1 for Toll-like receptor 2 (TLR2). Both phagocytosis of *E. coli* or *S. aureus* by THP-1 cells or RAW264.7 cells was inhibited by resveratrol in a dose-dependent manner regardless of stimulation with FSL-1. The NF-κB activity in HEK293 cells stably expressing TLR2 was also inhibited by resveratrol after stimulation with FSL-1. Resveratrol also inhibited both the translocation of p65 of NF-κB into nuclei in the transfectant and TNF-α production by THP-1 cells or RAW264.7 cells. It has recently been reported that TLR-mediated signaling pathways lead to upregulation of mRNAs of phagocytic receptors, including scavenger receptors and C-type lectin receptors. This study also demonstrated that FSL-1 induced upregulation of mRNAs of phagocytic receptors such as macrophage scavenger receptor-1, CD36, DC-SIGN and Dectin-1, and the FSL-1-induced upregulation of their mRNAs was inhibited by resveratrol. In addition, it was found that the expression of DC-SIGN in HEK293 cells stably expressing DC-SIGN was reduced by resveratrol and the phagocytic activity was significantly inhibited by resveratrol.

Thus, this study suggested that resveratrol inhibited the bacterial phagocytosis by macrophages by downregulating the expression of phagocytic receptors and the NF-κB activity.
INTRODUCTION

Resveratrol (trans-3,4',5-trihydroxystilbene) is a phytoalexin polyphenolic compound found in various plants, including grapes, berries and peanuts (2, 4). Dozen of studies have shown that the pharmacological effects of resveratrol include protective effects against cancer, cardiovascular diseases and ischemic injuries as well as enhancement of stress resistance and extension of the lifespans of various organisms from yeast to vertebrates (2). Of the diverse effects of resveratrol, it has been suggested that biological activities of resveratrol involve downregulation of the expression of proinflammatory markers, including inducible nitric oxide synthase and cyclooxygenase-2, by reducing the activities of nuclear factor κB (NF-κB) or the activator protein-1 (4). Although inhibitory effects of resveratrol on NF-κB activity has been clearly demonstrated (4), there are few reports about effects of resveratrol on TLRs signaling, which plays important roles in the recognition of bacterial invasion and in bridging between innate and acquired immunity (19). Moreover, little is known about effects of resveratrol on bacterial phagocytosis, which is also essential in activating signal transduction pathways leading to the killing and clearance of pathogens after detection of bacterial invasion (22). Therefore, we have a great interest in effects of resveratrol on bacterial phagocytosis because phagocytosis plays a vital role in host antibacterial responses.

Phagocytosis is an evolutionarily ancient host cell endocytic response to stimulation of microbes in both innate and acquired immunity (1, 22). Phagocytes, such as monocytes, macrophages and neutrophils, detect bacterial invasion through various germline-encoded pattern recognition receptors (PRRs) such as TLRs before internalization of bacteria and killing them (19, 21, 22). Thus, recognition of bacterial invasion by TLRs and bacterial clearance by phagocytosis play key roles in innate immunity. Nevertheless, little is known about the crosstalk between TLRs and phagocytic receptors. Recently, several studies have shown that TLR-mediated signaling upregulates bacterial phagocytosis by macrophages.
and dendritic cells (5, 7). We have also found that the diacylated lipopeptide FSL-1 promotes phagocytosis of bacteria, possibly through up-regulation of a phagocytic gene subset (13).

The present study, therefore, was designed to determine the effects of resveratrol on bacterial phagocytosis and NF-κB activity, which are mediated by TLRs, especially TLR2. We demonstrated that resveratrol downregulates the MyD88-mediated bacterial phagocytosis as well as NF-κB activity in macrophages.

MATERIALS AND METHODS

Reagents and antibodies. Resveratrol (trans-3,4′,5-trihydroxystilbene) was purchased from Sigma-Aldrich (St. Louis, MO) and was dissolved in dimethyl sulfoxide (DMSO). FSL-1 derived from *Mycoplasma salivarium* was synthesized according to the method described previously (17). pUNO-DC-SIGN1a (human DC-specific intercellular adhesion molecule-grabbing nonintegrin1a) was purchased from InvivoGen (San Diego, CA). A rabbit polyclonal antibody (pAb) against human-p65 of NF-κB was obtained from Immuno-Biological Laboratories Co., Ltd. (Gunma, Japan). Alexa Fluor 594-conjugated anti-rabbit IgG Ab was purchased from Molecular Probes (Eugene, OR). A mouse monoclonal Ab (mAb) against human-DC-SIGN (MAB161) was purchased from R&D Systems, Inc. (Minneapolis, MN). A mouse mAb against human-β-actin (AC-15) was purchased from Abcam (Stockholm, Sweden). A horseradish peroxidase (HRP)-conjugated anti-mouse IgG Ab was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). All other reagents were purchased from commercial sources and were of analytical or reagent grade.

Cell cultures. THP-1 cells (ATCC TIB-202) and RAW264.7 cells (ATCC TIB-71) were grown at 37°C and in 5% CO₂ in RPMI1640 medium (Sigma) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Gibco BRL, Rockville, MD), 100 units/ml
penicillin (Sigma) and 100 µg/ml streptomycin (Sigma) (complete medium). Human embryonic kidney (HEK) 293 cells (ATCC CRL-1573) were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma) complete medium.

**Stable transfectants.** The cDNA of human TLR2 obtained by RT-PCR of total RNA isolated from THP-1 cells was cloned into a pEF6/V5-His TOPO vector (Invitrogen Co., Carlsbad, CA) (hereafter referred to as pEF-TLR2). pEF-TLR2 or pUNO-hDC-SIGN1a was transfected into HEK293 cells using METAFACTENETM Transfection Reagent (Biontex Laboratories GmbH, München, Germany) according to the manufacturer’s instructions. The transfectants were selected in the presence of 50 µg/ml blasticidin S (Invitrogen). The expression of TLR2 or DC-SIGN was confirmed by immunoblot analysis using Abs to TLR2 or DC-SIGN.

**Fluorescein isothiocyanate (FITC)-conjugated bacteria.** *Esherichia coli* K12 and *Staphylococcus aureus* 209P were cultured in brain-heart infusion medium (Eiken, Tokyo, Japan) at 37°C to reach a concentration of approximately 1 x 10^9 /ml. Bacteria were washed and resuspended in phosphate-buffered saline (PBS) and then inactivated at 95°C for 5 min. Heat-killed bacteria were incubated at 37°C for 1 h with a 0.5 mg/ml solution of FITC (Sigma) in 0.1 M carbonate buffer (pH 9.5). The FITC-conjugated bacteria or heat-killed bacteria were washed three times with PBS and resuspended with PBS at a concentration of 1 x 10^{10} /ml.

**Phagocytosis assay.** A 0.5-ml suspension of THP-1 cells (1 x 10^6 /ml) or RAW264.7 cells (1 x 10^6 /ml) was added to each well of a 24-well plate and incubated at 37°C for 24 h with various concentrations (0, 1, 10, 100 nM) of FSL-1. In the case of HEK293 transfectant expressing DC-SIGN (293/DC-SIGN cells), a 1.0-ml suspension of the cells (5 x 10^5 /ml) was added to each well of a 12-well plate and then incubated at 37°C on the day before the assay. After the cells had been washed three times with base medium warmed at 37°C, they were
treated at 37°C for 1 h with various concentrations (10, 50, 100 µM) of resveratrol. The cells were then incubated for 1 h with 5 x 10^7 particles of FITC-conjugated *E. coli* or *S. aureus*. After the cells had been washed three times with cold PBS, they were 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. Flow cytometry (FCM) was conducted using a FACS Caliber® machine (BD Biosciences, San Diego, CA) and CellQuest software (BD Biosciences). Phagocytic activity was expressed as the mean fluorescence intensity (MFI) obtained by CellQuest software.

For the phagocytosis assay by confocal laser scanning microscopy (CLSM), a 1.0-ml suspension of THP-1 cells (1 x 10^6 /ml) was added to each well of a 24-well plate and incubated at 37°C for 24 h with or without 100 nM FSL-1. After the cells had been washed three times with RPMI 1640 base medium warmed at 37°C, they were treated for 1 h with 100 µM resveratrol or 0.1% (vol/vol) DMSO and then incubated for 1 h with 1 x 10^8 particles of FITC-conjugated *E. coli* or *S. aureus*. The cells were then washed with PBS and reacted for 20 min with 50 µg/ml Alexa Fluor 594-conjugated concanavalin A (Molecular Probes) in PBS, followed by fixation with PBS containing 3% (wt/vol) paraformaldehyde for 20 min. An LSM510 invert Laser Scan Microscope (Carl Zeiss, Tokyo, Japan) using a 63 x objective (Leica Microsystems, Tokyo, Japan) was used for image acquisition.

**Luciferase reporter gene assay.** HEK293 cells or HEK293 transfectant expressing TLR2 (293/TLR2 cells) were plated at 1 x 10^5 cells per well in a 24-well plate on the day before transfection. The cells were transiently transfected by METAFACTENE™ Transfection Reagent with 50 ng of an NF-κB reporter plasmid (pNF-κB-Luc, Stratagene, San Diego, CA) and 5 ng of a construct directing expression of *Renilla* luciferase under the control of a constitutively active thymidine kinase promoter (pRL-TK, Promega, Madison, WI) together with 445 ng of pcDNA3 empty vector (Invitrogen). After a 24-h incubation, the cells were
stimulated at 37°C for 6 h with FSL-1 or heat-killed bacteria in DMEM base medium, and luciferase activity was measured using a Dual-Luciferase reporter assay system (Promega) according to the manufacturer’s instructions.

**Immunostaining for p65 elements of NF-κB.** 293/TLR2 cells were seeded on poly-L-lysine-coated coverslips in wells of a 6-well plate on the day before transfection. The next day, the cells were washed three times with DMEM base medium warmed at 37°C and incubated for 1 h with 100 µM resveratrol or 0.1% (vol/vol) DMSO. After a 6-h stimulation with *E. coli* or *S. aureus*, the cells were incubated with PBS containing Alexa Fluor 594-conjugated concanavalin A (50 µg/ml), followed by methanol fixation for 5 min at –20°C. After the cells had been washed twice with PBS, they were incubated at room temperature for 45 min with a rabbit pAb against p65 of NF-κB (1 µg/ml of PBS) and then for another 45 min with Alexa Fluor 594-conjugated anti-rabbit IgG Ab. The cells were washed three times with PBS and observed using an LSM410.

**ELISA.** THP-1 cells or RAW264.7 cells were plated at 1 x 10^6 cells per well of a 24-well plate in RPMI1640 complete medium and incubated at 37°C for 16 h. The cells were washed three times with RPMI1640 base medium and treated with various concentrations (1, 10, 100 nM) of resveratrol. The cells were stimulated with heat-inactivated *E. coli* or *S. aureus* at a cell:bacterium ratio of 1:100, and 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 ELISA Development Kit Human TNF-alpha (PeproTech, Rocky Hill, NJ) for THP-1 cells or OptEIA™ Set: Mouse TNF-α (BD Pharmingen, San Diego, CA) for RAW264.7 cells.

**Reverse transcriptase (RT)-PCR.** THP-1 cells were incubated at 37°C for 24 h with 100 nM FSL-1 in a well of a 6-well plate and were then incubated for 1 h with 100 µM resveratrol or 0.1% (vol/vol) DMSO. Total RNA isolated from 5 x 10^6 of the cells was prepared by using an RNeasy kit (Qiagen Inc., Chatsworth, CA) according to the manufacturer’s instructions.
The RNA was reverse-transcribed to cDNA in a 20-µl reaction volume containing 2.5 µM of each of anchored-oligo[dT]₁₈ primers. The PCRs were performed in 40-µl final volumes containing 10 µl of cDNA, 2.5 µM MgCl₂, and 20 pmol of each sense primer of macrophage scavenger receptor 1 (MSR1), CD36, DC-SIGN, Dectin-1 and β-actin, sequences of which were described previously (13). After initial denaturation at 94°C for 30 s, amplifications were carried out with 25 cycles for β-actin or 30 cycles for the others. The PCR products were separated of 3% gel of NuSieve 3:1 agarose in Tris-acetate-EDTA buffer containing ethidium bromide (5 µg/ml).

**Western blotting.** 293/DC-SIGN cells were plated at 1 x 10⁷ cells in a 10 cm dish in DMEM complete medium and incubated at 37°C for 16 h. After the cells had been washed three times with DMEM base medium, they were incubated for 0, 30, 60, 90 or 120 with 100 µM resveratrol. The cells were washed twice with ice-cold PBS and then lysed by 62.5 mM Tris-HCl (pH 6.8) containing 2% SDS, 10% glycerol and 50 mM DTT (an SDS sample buffer) in the presence of inhibitor cocktails of proteases (Roche) and boiled for 10 min. The lysates were centrifuged at 14000 rpm for 10 min, and the resulting supernatants containing cytosolic and membrane proteins were collected. Proteins in the supernatant were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. After the membranes had been incubated at 4°C overnight with an anti-DC-SIGN mAb or an anti-β-actin mAb, they were incubated with an HRP-conjugated anti-mouse IgG. Immunoreactive proteins were detected by using ECL™ detection reagents (GE Healthcare, Piscataway, NJ).

**RESULTS**

**Inhibition of bacterial phagocytosis by resveratrol.** To examine whether resveratrol affects phagocytosis, human monocyte-like THP-1 cells and murine macrophage-like
RAW264.7 cells were used as phagocytes, and heat-killed gram-positive bacteria *S. aureus* and gram-negative bacteria *E. coli* were used as target bacteria after being conjugated with FITC. Phagocytosis assays were performed with various concentrations of resveratrol in serum-free medium in order to rule out the possibility of involvement of Fc- and complement-mediated opsonization of bacteria. FCM analysis showed that phagocytic activities of both THP-1 cells and RAW264.7 cells increased as the incubation time increased and that resveratrol significantly inhibited the activities in a dose-dependent manner at all incubation times (Fig. 1A and B). In addition, it was found that the activities increased as ratios of the number of bacterial cells to that of phagocytes increased and the activities at almost all ratios were suppressed by resveratrol (Fig. 1C and 1D). The activity level of the monocyte-like THP-1 cells was significantly lower than that of the macrophage-like RAW264.7 cells.

**Inhibition of the FSL-1-induced enhancement of bacterial phagocytosis by resveratrol.**

Recently, we have found that the TLR2 ligand FSL-1 enhances phagocytosis of bacteria by macrophages (13). Therefore, we next examined whether resveratrol affected the FSL-1-induced enhancement of phagocytosis. It was found that FSL-1 significantly enhanced phagocytosis of *E. coli* or *S. aureus* by RAW264.7 cells (Fig. 2A) and THP-1 cells (Fig. 2B) and that the enhancement was significantly inhibited by resveratrol in a dose-dependent manner (Fig. 2A, B).

In the following experiments, we tried to evaluate phagocytosis by another assay method using CLSM that enables the number of bacterial particles phagocytosed per cell to be counted (Fig. 3A). The analysis demonstrated that FSL-1 treatment caused a significant increase in the number of THP-1 cells phagocytosing multiple *E. coli* particles and that resveratrol treatment reduced the number of cells phagocytosing *E. coli* particles (Fig. 3B) as well as the percentage of the cells phagocytosing *E. coli* particles to total cell number.
analyzed (Fig. 3C).

Thus, resveratrol has the activity to inhibit the phagocytic activity of macrophages toward bacteria regardless of TLR stimulation.

**Inhibition of NF-κB activation by resveratrol.** To get an insight into the molecular mechanisms underlying the resveratrol-induced impairment of phagocytosis enhanced by TLR2 stimulation, we examined whether resveratrol suppressed the transcriptional activity of NF-κB, which is known to be activated downstream of the TLR2 signaling pathway. Neither the heat-killed bacteria nor FSL-1 could stimulate NF-κB in wild-type HEK293 cells in the absence or presence of resveratrol (data not shown). In contrast, the NF-κB activity in HEK293 cells stably expressing TLR2 (293/TLR2) was activated by FSL-1 in a dose-dependent manner and the FSL-1-induced enhancement was inhibited by resveratrol (Fig. 4A). The heat-killed bacteria also had activity to stimulate NF-κB in 293/TLR2 cells and the activity was also suppressed by resveratrol in a dose-dependent manner (Fig. 4B). Upon activation, the p65 element of NF-κB is translocated from the cytoplasm to the nuclei. Therefore, the intracellular localization of p65 in 293/TLR2 cells in response to these bacterial stimulation was examined. p65 of NF-κB was translocated into nuclei in 293/TLR2 cells, but the translocation was inhibited by resveratrol (Fig. 4C). These results also suggest that *E. coli* and *S. aureus* possess TLR2 ligands on the cell surfaces.

**Inhibition of TNF-α production by resveratrol.** Since the transcription factor NF-κB plays an important role in the expression of a large amount of inducible genes, including genes for inflammatory cytokines (14), an experiment was carried out to determine whether resveratrol inhibites TNF-α production by THP-1 cells and RAW264.7 cells. It is thought that the gram-negative bacterium *E. coli* is mainly recognized by TLR2 and TLR4, whereas the gram-positive bacterium *S. aureus* is mainly recognized by TLR2, but not by TLR4 (20). Therefore, RAW264.7 cells and THP-1 cells were stimulated by both bacteria, and the amount
of TNF-α produced was measured by ELISA. It was found that *S. aureus* and *E. coli* stimulated both types of cells to induce production of TNF-α in a time-dependent manner (Fig. 5A, B). *S. aureus* and *E. coli* stimulated THP-1 cells more strongly than RAW264.7 cells (Fig. 5A, B), and RAW264.7 cells were stimulated more strongly by *E. coli* than by *S. aureus* (Fig. 5A). The difference in the profile of TNF-α production by between THP-1 and RAW264.7 cells might be explained by the difference of expression levels of TLR2 and TLR4, although we can not rule out other potential reasons for the differences in TNF-α production between these cell types. That is, the expression levels of TLR2 and TLR4 in THP-1 cells might be higher than those in RAW264.7 cells and/or the expression level of TLR4 might be higher than that of TLR2 in RAW264.7 cells.

**Inhibition of phagocytic receptor-mediated bacterial phagocytosis by resveratrol.** It has recently been reported that TLR-mediated signaling pathways lead to upregulation of mRNAs of phagocytic receptors, including scavenger receptors (SRs) and C-type lectin receptors (CLRs) (7). Several lines of evidence have indicated that SRs and CLRs function as PRRs and mediate phagocytosis of microbes by phagocytes (11, 16, 21, 22). We have also found that FSL-1 stimulation is able to induce upregulation of the expression of mRNAs of MSR1, CD36, DC-SIGN and Dectin-1 in THP-1 cells (Fig. 6A and (13)). Therefore, we examined whether resveratrol inhibited the FSL-1-induced upregulation of the expression of their mRNAs and found that resveratrol inhibited the FSL-1-induced upregulation (Fig. 6A). Therefore, inhibition of the FSL-1-induced enhancement of phagocytosis by resveratrol may be explained by downregulation of the expression of mRNAs these phagocytic receptors.

In order to further confirm this, we established HEK293 cells stably expressing DC-SIGN and examined the phagocytosis activity of the cells toward *E. coli* and *S. aureus*. It was found that the expression of DC-SIGN in the transfectant was reduced by resveratrol (Fig. 6B) and that phagocytosis activity of 293/DC-SIGN cells clearly increased as the ratios of bacteria/cells
increased, and resveratrol significantly inhibited the phagocytosis activity in a dose-dependent manner (Fig. 6C).

On the basis of these results, it was concluded that resveratrol inhibited the bacterial phagocytosis by macrophages by downregulating the expression of phagocytic receptors, including SRs and CLRs.

**DISCUSSION**

This study demonstrated that both the transcriptional activity and translocation into nuclei of NF-κB functioning downstream of TLR2, which had been activated by bacteria as well as TLR2 agonist FSL-1, were inhibited by resveratrol (Figs. 4 and 5). The first evidence of resveratrol affecting NF-κB was obtained by Draczynska-Lusiak et al. (8). They demonstrated that oxidized low-density lipoprotein treatment activated NF-κB in PC12 cells and that resveratrol attenuated the activation (8). Thereafter, there were many reports of resveratrol suppressing NF-κB activation in a variety of cell lines, including U-937, Jurkat and Hela cells, induced by several agents, including 12-O-tetradecanoylphorbol-13-acetate, lipopolysaccharide, H₂O₂ and ceramide (4). Therefore, there seems to be no doubt that resveratrol inhibits NF-κB activation. However, Youn et al. reported that resveratrol suppressed NF-κB activation in RAW264.7 cells downstream of TLR3 and TLR4 signaling pathways, but not TLR2 or TLR9 signaling pathways (23). That is, they concluded that resveratrol inhibited NF-κB activation induced by TRIF, but not by MyD88. Our results obtained by this study are in contrast to their findings. Even in their study, weak, but not significant, inhibition of NF-κB activation by the TLR2 agonist MALP2 was observed (23). Therefore, we think that the discrepancy can be explained by the level of inhibitory activity of resveratrol against MyD88 and TRIF-mediated NF-κB activation.

Recently, we and others have reported that TLR-mediated signals leading to NF-κB
activation upregulate phagocytosis of bacteria by macrophages and dendritic cells (7, 9, 13, 15, 18). Therefore, we examined whether resveratrol affected bacterial phagocytosis by macrophages with or without TLR2 stimulation. This study demonstrated that phagocytic activities of both THP-1 and RAW264.7 cells toward bacteria were dose-dependently inhibited by resveratrol regardless of TLR-mediated signals (Figs. 1, 2 and 3). When macrophages were stimulated by the TLR2 agonist FSL-1, resveratrol inhibited bacterial phagocytosis by macrophages by downregulating the expression of phagocytic receptors including SRs and CLRs. This finding is supported by the finding of Leiro et al. (12) that phagocytosis of Kleyveromyces lactis by macrophages is inhibited by resveratrol, although they did not clarify the mechanism by which resveratrol inhibited bacterial phagocytosis of macrophages. However, resveratrol at low concentrations (1 to 10 µM) enhanced phagocytosis of Candida albicans in human macrophage-like cells (3). In the present study, resveratrol even at 10 µM significantly attenuated phagocytosis of E. coli or S. aureus by a human monocytic cell line, THP-1 cells. This discrepancy may be explained by the difference in cell surface components of target microbes, the eukaryotic microbe Candida albicans and the prokaryotic microbe bacteria. The C-type lectin Dectin-1 does not only function as a recognition receptor of Candida albicans but also as a phagocytic receptor (6, 10). Therefore, it is thought that the enhancement of phagocytosis of Candida albicans was mediated by Dectin-1. In addition, we have recently reported that HEK293 cells expressing Dectin-1 do not ingest E. coli and S. aureus (13). Thus, phagocytic receptors for yeast seem to be different from those for bacteria. This may explain the discrepancy described above, although the exact mechanism remains unknown.

Thus, the present study demonstrated that resveratrol does not only inhibit activation of NF-κB induced by TLR2-mediated signals but also inhibits phagocytosis of macrophages regardless of TLR stimulation. Judging from our results and the immunomodulatory effects
that have been reported (2, 4), there is no doubt as to that resveratrol potentially has anti-inflammatory properties against bacterial infection by repressing TLR-mediated recognition and/or subsequent phagocytosis.

ACKNOWLEDGEMENT

This work was supported by Grants-in-Aid for Science Research B 17390498 and 19390477 provided by the Japan Society for the Promotion of Science.

REFERENCES


FIGURE LEGENDS

Figure 1. Inhibitory effect of resveratrol on phagocytosis of FITC-conjugated *E. coli* and *S. aureus* by macrophages. FCM data are presented as MFI of each population. RAW264.7 cells (A) and THP-1 cells (B) were treated with the indicated dose of resveratrol for 1 h in a serum-free condition, and they were incubated for 0, 20, 40, 60 or 80 min with heat-killed FITC-*E. coli* at a cell:bacterium ratio of 1:100. RAW264.7 cells (C) or THP-1 cells (D) were treated with the indicated dose of resveratrol and were given FITC-*E. coli* and FITC-*S. aureus*. MFI of internalized heat-killed FITC-bacteria were plotted on the Y-axis versus dose of bacteria on the X-axis. The mean values and SD of triplicate experiments are shown. The statistically significant difference from vehicle was assessed by Student’s *t*-test; *p* < 0.05; **p** < 0.01. Representative data are from more than three independent experiments. Veh, vehicle.

Figure 2. Inhibitory effect of resveratrol on enhancement of bacterial phagocytosis by FSL-1 as revealed by FCM. FCM data are presented as MFI of each population. After pretreatment with the indicated dose of FSL-1 for 24 h, RAW264.7 cells (A) and THP-1 cells (B) were incubated with various concentrations of resveratrol and then given heat-killed FITC-*E. coli* or FITC-*S. aureus* at a cell:bacterium ratio of 1:10 and 1:100, respectively. The mean values and SD of triplicate experiments are shown. The statistically significant difference from vehicle was assessed by Student’s *t*-test; *p* < 0.05; **p** < 0.01. Representative data are from more than three independent experiments. Veh, vehicle.

Figure 3. Inhibitory effect of resveratrol on enhancement of bacterial phagocytosis by FSL-1 as revealed by CLSM. (A) THP-1 cells were incubated in the absence or presence of 100 nM FSL-1 for 24 h and treated with 100 µM resveratrol for 1 h. The cells were given
heat-killed FITC-\textit{E. coli} at a ratio of 1:100, stained with Alexa Fluor 488-conjugated concanavalin A to cell surface glycoproteins, and subjected to CLSM. The bar equals 25 µm. (B) The number of the same cells as those seen in A were counted to determine the number of \textit{E. coli} per individual cell. Data are presented as the number of THP-1 cells (out of 3,500) that phagocytosed 1, 2, 3, 4, or >5 \textit{E. coli} per cell. The pictures along the X-axis are representative of the cells counted. (C) Phagocytosis (%) in one field containing at least 100 cells is expressed as [the number of cells taking up bacteria]/[total number (>100) of cells in one field] x 100. Each value is the mean and SD of phagocytosis (%) obtained by 25 fields. The statistically significant difference was assessed by Student’s \textit{t}-test; **\textit{p} < 0.01.

Representative data are from at least two independent experiments. Veh, vehicle; Resv, resveratrol.

\textbf{Figure 4. Effect of resveratrol on the recognition of FSL-1, \textit{E. coli} or \textit{S. aureus} by 293/TLR2 cells.} 293/TLR2 cells were plated at 1 x 10^5 cells per well in 24 well plates on the day before transfection. The cells were transiently transfected with an NF-κB reporter plasmid and a construct directing expression of \textit{Renilla} luciferase under the control of a constitutively active thymidine kinase promoter. After 24-h incubation, 293/TLR2 cells were pretreated for 1 h with various concentrations of resveratrol and stimulated for 6 h with FSL-1 (1, 10, 100 nM) (A), heat-killed \textit{E. coli} (cell:bacterium = 1:1, 1:10, 1:100) or heat-killed \textit{S. aureus} (cell:bacterium = 1:1, 1:10, 1:100) (B) in a serum-free condition. The mean values and SD of triplicate experiments are shown. The statistically significant difference from vehicle was assessed by Student’s \textit{t}-test; *\textit{p} < 0.05; **\textit{p} < 0.01. (C) Representative CLSM images of p65 element of NF-κB in 293/TLR2 cells were shown. The cells were treated with or without 100 µM resveratrol for 1 h and stimulated with heat-killed \textit{E. coli} at a ratio of 1:100 or heat-killed \textit{S. aureus} at a ratio of 1:100 for 6 h. Nuclear translocation of the p65 element of NF-κB was
detected with a pAb against p65 of NF-κB (arrows). Representative data are from at least two independent experiments. The bar equals 10 µm. Veh, vehicle.

**Figure 5.** TNF-α production by RAW264.7 cells and THP-1 cells in response to heat-killed *E. coli* or *S. aureus*. RAW264.7 cells (A) and THP-1 cells (B) were in advance treated for 1 h with various concentrations of resveratrol and incubated for 0, 2, 4 or 6 h in a serum-free condition with heat-killed *E. coli* and *S. aureus* at a ratio of number of bacterial particles to that of cells of 1:100. The amounts of TNF-α produced in the supernatants were measured by ELISA. See text for details. The mean values and SD of triplicate experiments are shown. The statistically significant difference from vehicle was assessed by Student’s *t*-test; *p* < 0.05; **p** < 0.01. Representative data are from more than three independent experiments. Veh, vehicle.

**Figure 6.** Inhibitory effect of resveratrol on impaired phagocytosis of bacteria mediated by SRs and CLR. (A) THP-1 cells incubated for 24 h with 100 nM FSL-1 were treated with 100 µM resveratrol for 1 h in a serum-free condition. Expression of mRNAs of MSR1, CD36, DC-SIGN, Dectin-1 and β-actin was confirmed by RT-PCR using total RNA isolated from the cells. Representative data are from more than five independent experiments. (B) 293/DC-SIGN cells were incubated for 0, 30, 60, 90 or 120 min with 100 µM resveratrol. The cells were then lysed in SDS sample buffer, and proteins were separated by electrophoresis on 10% SDS-poly-acrylamide gels and transferred to a nitrocellulose membrane. The membrane was reacted with anti-DC-SIGN mAb or anti-β-actin mAb. (C) 293/DC-SIGN cells were treated with the indicated dose of resveratrol for 1 h and given heat-killed FITC-*E. coli* or *S. aureus* at various cell:bacteria ratios. The mean values and SD of triplicate experiments are shown. The statistically significant difference from vehicle was assessed by Student’s *t*-test;
*p < 0.05; **p < 0.01. Representative data are from more than three independent experiments.

Veh, vehicle.
**Figure A**

**E. coli**

- Resveratrol (μM)
  - Veh
  - 10
  - 50
  - 100

- MFI vs. FSL-1 (nM)
  - 0
  - 1
  - 10
  - 100

**S. aureus**

- MFI vs. FSL-1 (nM)
  - 0
  - 1
  - 10
  - 100

**Figure B**

**E. coli**

- MFI vs. FSL-1 (nM)
  - 0
  - 1
  - 10
  - 100

**S. aureus**

- MFI vs. FSL-1 (nM)
  - 0
  - 1
  - 10
  - 100
A

Resveratrol (μM)

- Veh
- 1
- 10
- 40
- 70
- 100

RLA

0 100 200 300 400 500 600 700 800

FSL-1 (nM)

0 1 10 100

B

RLA

0 200 400 600 800

C

No bacterium

E. coli

S. aureus

Veh

Resveratrol

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