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

Running title: Inhibition of bacterial phagocytosis by resveratrol

Mitsuhiro Iyori, Hideo Kataoka, Haque Mohammad Shamsul, Kazuto Kiura, Motoaki Yasuda, Takashi Nakata, Akira Hasebe, and Ken-ichiro Shibata*

Department of Oral Pathobiological Science, Hokkaido University Graduate School of Dental Medicine, Sapporo, Japan

* *Corresponding author*
Ken-ichiro Shibata
Laboratories of Oral Molecular Microbiology, Department of Oral Pathobiological Science, Hokkaido University Graduate School of Dental Medicine, Nishi 7, Kita 13, Kita-ku, Sapporo 060-8586, Japan.
Phone: +81 11 706 4240
FAX: +81 11 706 4901
shibaken@den.hokudai.ac.jp

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-trihydroxystilebene) 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). Althogh 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 acuired 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 phagogocytosis 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-trihydroxystilebene) 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-SIGN1 (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 ImmunoReserch 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% CO_2 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 METAFECTENE™ 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 resupended 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

116 treated at 37°C for 1 h with various concentrations (10, 50, 100 μM) of resveratrol. The cells
117 were then incubated for 1 h with 5 x 10^7 particles of FITC-conjugated *E. coli* or *S. aureus*.
118 After the cells had been washed three times with cold PBS, they were suspended in PBS
119 containing 0.2% (wt/vol) trypan blue to quench fluorescence caused by binding of bacteria to
120 the surface of the cells and 1% (wt/vol) paraformaldehyde to fix the cells. Flow cytometry
121 (FCM) was conducted using a FACS Caliber® machine (BD Biosciences, San Diego, CA)
122 and CellQuest software (BD Biosciences). Phagocytic activity was expressed as the mean
123 fluorescence intensity (MFI) obtained by CellQuest software.
124 For the phagocytosis assay by confocal laser scanning microscopy (CLSM), a 1.0-ml
125 suspension of THP-1 cells (1 x 10^6 /ml) was added to each well of a 24-well plate and
126 incubated at 37°C for 24 h with or without 100 nM FSL-1. After the cells had been washed
127 three times with RPMI 1640 base medium warmed at 37°C, they were treated for 1 h with 100
128 μM resveratrol or 0.1% (vol/vol) DMSO and then incubated for 1 h with 1 x 10^8 particles of
129 FITC-conjugated *E. coli* or *S. aureus*. The cells were then washed with PBS and reacted for
130 20 min with 50 μg/ml Alexa Fluor 594-conjugated concanavalin A (Molecular Probes) in PBS,
131 followed by fixation with PBS containing 3% (wt/vol) paraformaldehyde for 20 min. An
132 LSM510 invert Laser Scan Microscope (Carl Zeiss, Tokyo, Japan) using a 63 x objective
133 (Leica Microsystems, Tokyo, Japan) was used for image acquisition.
134 **Luciferase reporter gene assay.** HEK293 cells or HEK293 transfectant expressing TLR2
135 (293/TLR2 cells) were plated at 1 x 10^5 cells per well in a 24-well plate on the day before
136 transfection. The cells were transiently transfected by METAFECTENE™ Transfection
137 Reagent with 50 ng of an NF-κB reporter plasmid (pNF-κB-Luc, Stratagene, San Diego, CA)
138 and 5 ng of a construct directing expression of *Renilla* luciferase under the control of a
139 constitutively active thymidine kinase promoter (pRL-TK, Promega, Madison, WI) together
140 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 $MgCl_2$, 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 decribed 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 10^7 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

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

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

210 In the following experiments, we tried to evaluate phagocytosis by another assay method
211 using CLSM that enables the number of bacterial particles phagocytosed per cell to be
212 counted (Fig. 3A). The analysis demonstrated that FSL-1 treatment caused a significant
213 increase in the number of THP-1 cells phagocytosing multiple *E. coli* particles and that
214 resveratrol treatment reduced the number of cells phagocytosing *E. coli* particles (Fig. 3B) as
215 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

241 of TNF-α produced was measured by ELISA. It was found that *S. aureus* and *E. coli*
242 stimulated both types of cells to induce production of TNF-α in a time-dependent manner (Fig.
243 5A, B). *S. aureus* and *E. coli* stimulated THP-1 cells more strongly than RAW264.7 cells (Fig.
244 5A, B), and RAW264.7 cells were stimulated more strongly by *E. coli* than by *S. aureus* (Fig.
245 5A). The difference in the profile of TNF-α production by between THP-1 and RAW264.7
246 cells might be explained by the difference of expression levels of TLR2 and TLR4, although
247 we can not rule out other potential reasons for the differences in TNF-α production between
248 these cell types. That is, the expression levels of TLR2 and TLR4 in THP-1 cells might be
249 higher than those in RAW264.7 cells and/or the expression level of TLR4 might be higher
250 than that of TLR2 in RAW264.7 cells.

251 **Inhibition of phagocytic receptor-mediated bacterial phagocytosis by resveratrol.** It
252 has recently been reported that TLR-mediated signaling pathways lead to upregulation of
253 mRNAs of phagocytic receptors, including scavenger receptors (SRs) and C-type lectin
254 receptors (CLRs) (7). Several lines of evidence have indicated that SRs and CLRs function as
255 PRRs and mediate phagocytosis of microbes by phagocytes (11, 16, 21, 22). We have also
256 found that FSL-1 stimulation is able to induce upregulation of the expression of mRNAs of
257 MSR1, CD36, DC-SIGN and Dectin-1 in THP-1 cells (Fig. 6A and (13)). Therefore, we
258 examined whether resveratrol inhibited the FSL-1-induced upregulation of the expression
259 of their mRNAs and found that resveratrol inhibited the FSL-1-induced upregulation (Fig.
260 6A). Therefore, inhibition of the FSL-1-induced enhancement of phagocytosis by resveratrol
261 may be explained by downregulation of the expression of mRNAs these phagocytic receptors.
262 In order to further confirm this, we established HEK293 cells stably expressing DC-SIGN and
263 examined the phagocytosis activity of the cells toward *E. coli* and *S. aureus*. It was found that
264 the expression of DC-SIGN in the transfectant was reduced by resveratrol (Fig. 6B) and that
265 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, H_2O_2 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

316 that have been reported (2, 4), there is no doubt as to that resveratrol potentially has
317 anti-inflammatory properties against bacterial infection by repressing TLR-mediated
318 recognition and/or subsequent phagocytosis.
319
320 **ACKNOWLEDGEMENT**
321 This work was supported by Grants-in-Aid for Science Research B 17390498 and
322 19390477 provided by the Japan Society for the Promotion of Science.
323
324 **REFERENCES**
325 1. **Aderem, A., and D. M. Underhill.** 1999. Mechanisms of phagocytosis in macrophages.
326 Annu Rev Immunol **17:**593-623.
327 2. **Baur, J. A., and D. A. Sinclair.** 2006. Therapeutic potential of resveratrol: the in vivo
328 evidence. Nat Rev Drug Discov **5:**493-506.
329 3. **Bertelli, A. A., F. Ferrara, G. Diana, A. Fulgenzi, M. Corsi, W. Ponti, M. E. Ferrero,**
330 **and A. Bertelli.** 1999. Resveratrol, a natural stilbene in grapes and wine, enhances
331 intraphagocytosis in human promonocytes: a co-factor in antiinflammatory and
332 anticancer chemopreventive activity. Int J Tissue React **21:**93-104.
333 4. **Bhat, K. P., and J. M. Pezzuto.** 2002. Cancer chemopreventive activity of resveratrol.
334 Ann N Y Acad Sci **957:**210-29.
335 5. **Blander, J. M., and R. Medzhitov.** 2004. Regulation of phagosome maturation by
336 signals from toll-like receptors. Science **304:**1014-8.
337 6. **Brown, G. D.** 2005. Dectin-1: a signalling non-TLR pattern-recognition receptor. Nat
338 Rev Immunol.
339 7. **Doyle, S. E., R. M. O'Connell, G. A. Miranda, S. A. Vaidya, E. K. Chow, P. T. Liu, S.**
340 **Suzuki, N. Suzuki, R. L. Modlin, W. C. Yeh, T. F. Lane, and G. Cheng.** 2004. Toll-like

receptors induce a phagocytic gene program through p38. J Exp Med **199:**81-90.

8. **Draczynska-Lusiak, B., Y. M. Chen, and A. Y. Sun.** 1998. Oxidized lipoproteins activate NF-kappaB binding activity and apoptosis in PC12 cells. Neuroreport **9:**527-32.

9. **Fujita, M., T. Into, M. Yasuda, T. Okusawa, S. Hamahira, Y. Kuroki, A. Eto, T. Nisizawa, M. Morita, and K. Shibata.** 2003. Involvement of leucine residues at positions 107, 112, and 115 in a leucine-rich repeat motif of human Toll-like receptor 2 in the recognition of diacylated lipoproteins and lipopeptides and Staphylococcus aureus peptidoglycans. J Immunol **171:**3675-83.

10. **Gantner, B. N., R. M. Simmons, and D. M. Underhill.** 2005. Dectin-1 mediates macrophage recognition of Candida albicans yeast but not filaments. Embo J **24:**1277-86.

11. **Geijtenbeek, T. B., S. J. van Vliet, A. Engering, B. A. t Hart, and Y. van Kooyk.** 2004. Self- and nonself-recognition by C-type lectins on dendritic cells. Annu Rev Immunol **22:**33-54.

12. **Leiro, J., E. Alvarez, D. Garcia, and F. Orallo.** 2002. Resveratrol modulates rat macrophage functions. Int Immunopharmacol **2:**767-74.

13. **Mae, M., M. Iyori, H. Shamsul, H. Kataoka, K. Kiura, Y. Totsuka, and K. Shibata.** 2007. The diacylated lipopeptide FSL-1 enhances phagocytosis of bacteria by macrophages through Toll-like receptor 2-mediated signaling pathway. FEMS Immunol Med Microbiol**:**In press.

14. **May, M. J., and S. Ghosh.** 1998. Signal transduction through NF-kappa B. Immunol Today **19:**80-8.

15. **Okusawa, T., M. Fujita, J. Nakamura, T. Into, M. Yasuda, A. Yoshimura, Y. Hara, A. Hasebe, D. T. Golenbock, M. Morita, Y. Kuroki, T. Ogawa, and K. Shibata.** 2004. Relationship between structures and biological activities of mycoplasmal diacylated lipopeptides and their recognition by toll-like receptors 2 and 6. Infect Immun

72:1657-65.

16. **Peiser, L., S. Mukhopadhyay, and S. Gordon.** 2002. Scavenger receptors in innate immunity. Curr Opin Immunol **14:**123-8.
17. **Shibata, K., A. Hasebe, T. Into, M. Yamada, and T. Watanabe.** 2000. The N-terminal lipopeptide of a 44-kDa membrane-bound lipoprotein of Mycoplasma salivarium is responsible for the expression of intercellular adhesion molecule-1 on the cell surface of normal human gingival fibroblasts. J Immunol **165:**6538-44.
18. **Suzuki, E., and K. Umezawa.** 2006. Inhibition of macrophage activation and phagocytosis by a novel NF-kappaB inhibitor, dehydroxymethylepoxyquinomicin. Biomed Pharmacother **60:**578-86.
19. **Takeda, K., and S. Akira.** 2005. Toll-like receptors in innate immunity. Int Immunol **17:**1-14.
20. **Takeuchi, O., K. Hoshino, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda, and S. Akira.** 1999. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity **11:**443-51.
21. **Taylor, P. R., L. Martinez-Pomares, M. Stacey, H. H. Lin, G. D. Brown, and S. Gordon.** 2005. Macrophage receptors and immune recognition. Annu Rev Immunol **23:**901-44.
22. **Underhill, D. M., and A. Ozinsky.** 2002. Phagocytosis of microbes: complexity in action. Annu Rev Immunol **20:**825-52.
23. **Youn, H. S., J. Y. Lee, K. A. Fitzgerald, H. A. Young, S. Akira, and D. H. Hwang.** 2005. Specific inhibition of MyD88-independent signaling pathways of TLR3 and TLR4 by resveratrol: molecular targets are TBK1 and RIP1 in TRIF complex. J Immunol **175:**3339-46.

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*. MFIs 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 enhacement 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 enhacement 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

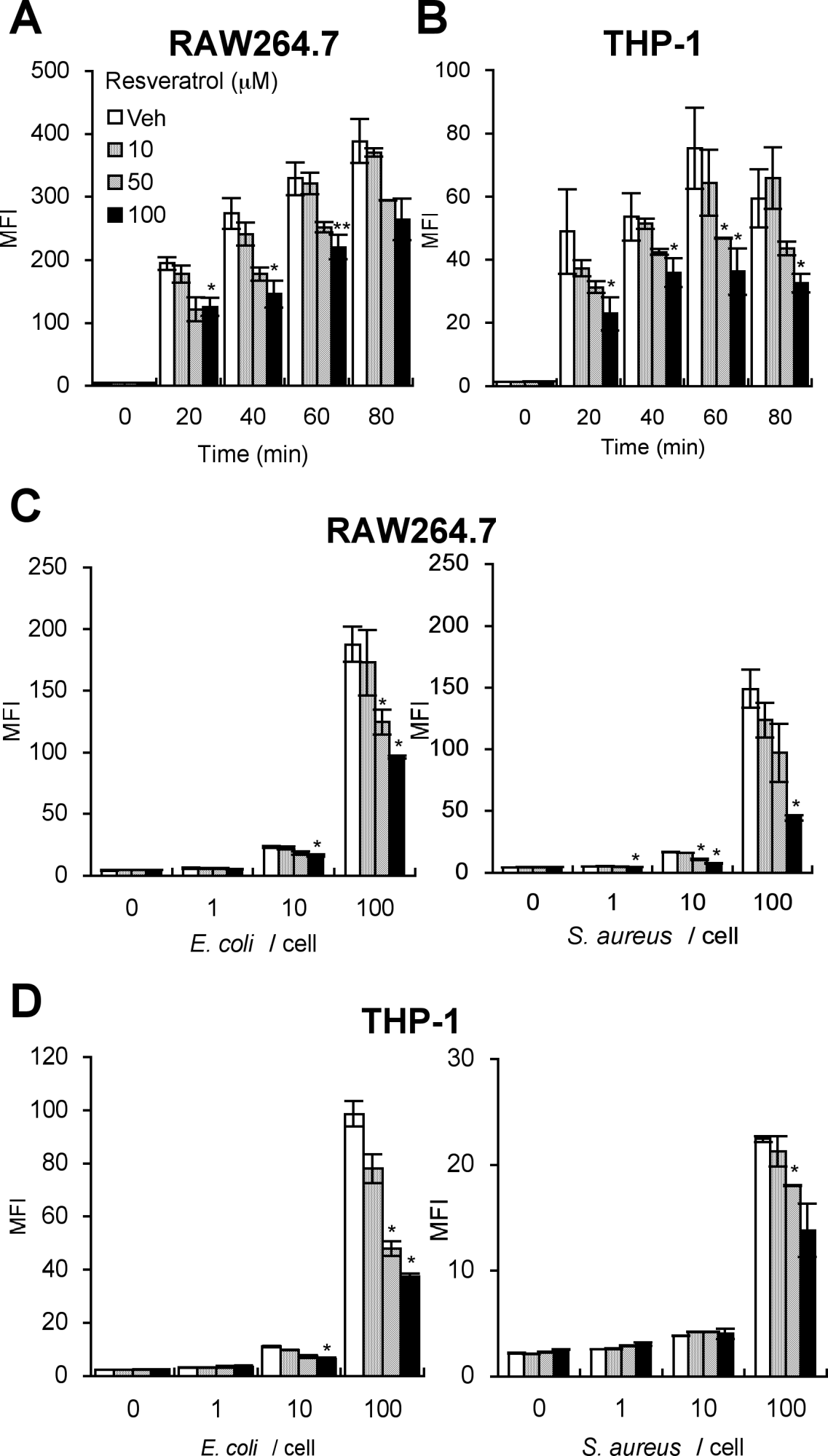
heat-killed FITC-*E. coli* at a ratio of 1:100, stained with Alexa Fluor 488-conjugated concanavalin A to cell surface glycoproteins, and subjected to CLSM. The bar equals 25 μm. (B) The number of the same cells as those seen in A were counted to determine the number of *E. coli* per individual cell. Data are presented as the number of THP-1 cells (out of 3,500) that phagocytosed 1, 2, 3, 4, or >5 *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 *t*-test; $^{**}p < 0.01$. Representative data are from at least two independent experiments. Veh, vehicle; Resv, resveratrol.

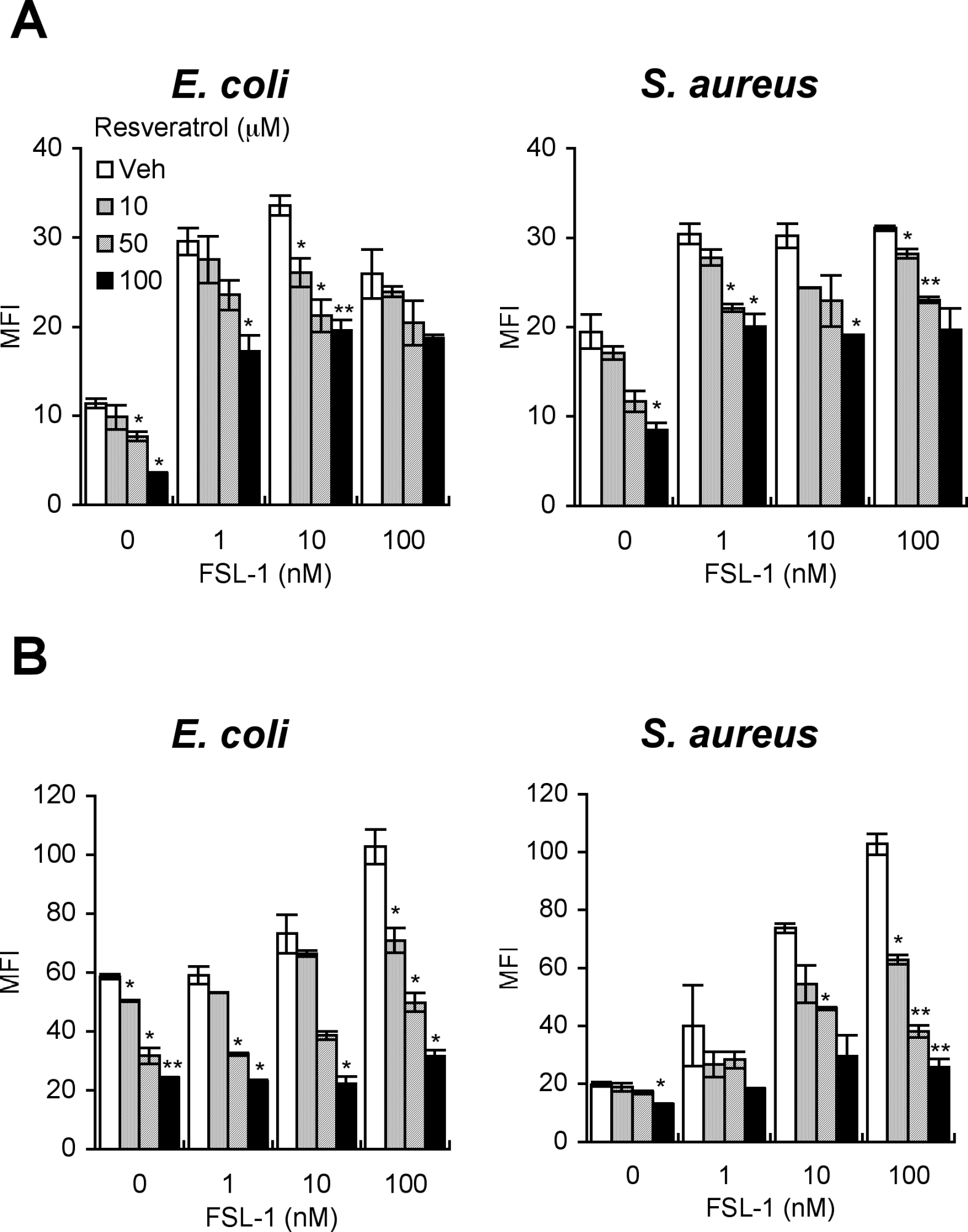
Figure 4. Effect of resveratrol on the recognition of FSL-1, *E. coli* or *S. aureus* by 293/TLR2 cells. 293/TLR2 cells were plated at 1 x 10^5 cells per well in 24 well plates on the day before transfection. The cells were transiently transfected with an NF-κB reporter plasmid and a construct directing expression of *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 *E. coli* (cell:bacterium = 1:1, 1:10, 1:100) or heat-killed *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 *t*-test; $^{*}p < 0.05$; $^{**}p < 0.01$. (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 *E. coli* at a ratio of 1:100 or heat-killed *S. aureus* at a ratio of 1:100 for 6 h. Nuclear translocation of the p65 element of NF-κB was

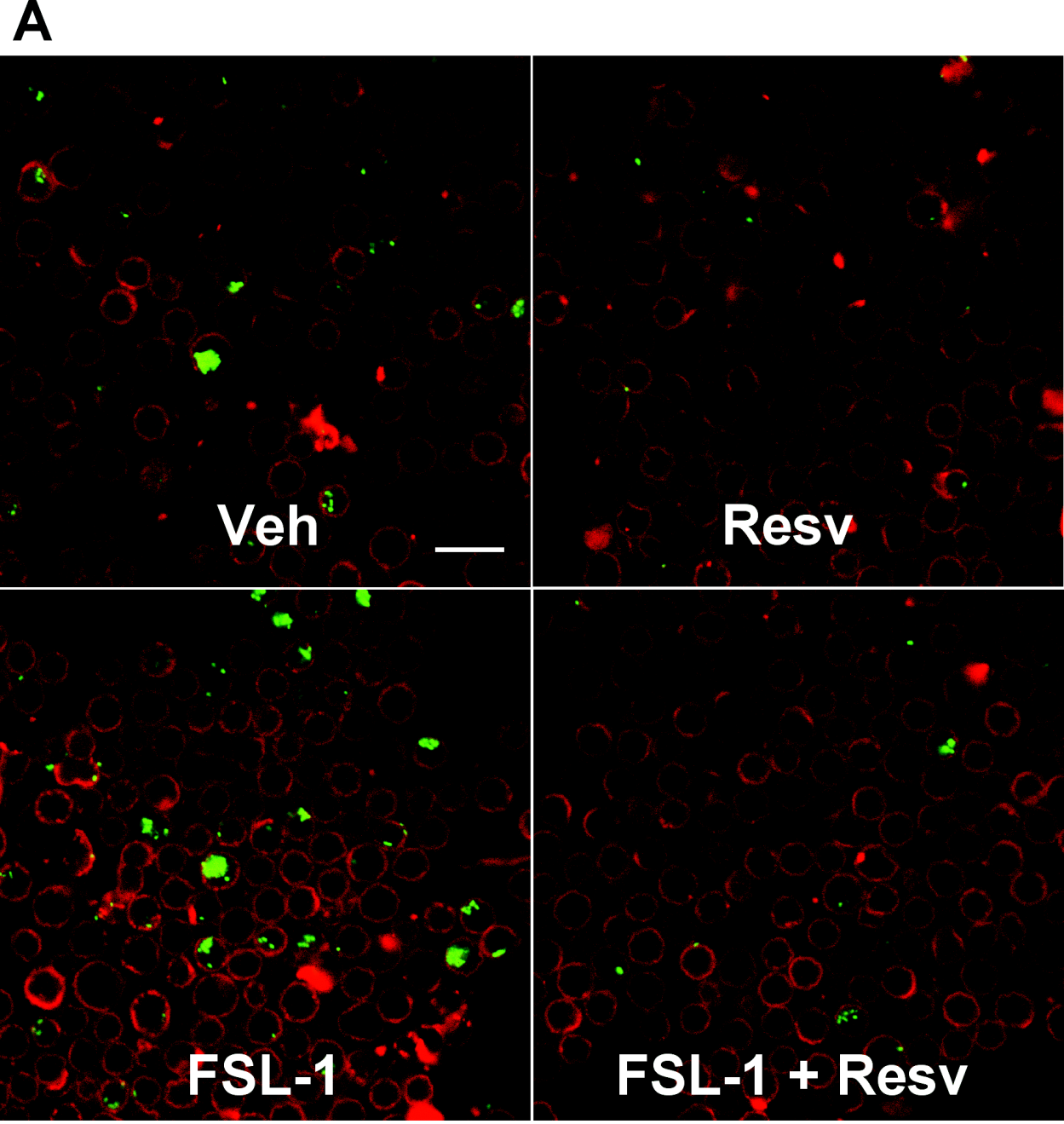
441 detected with a pAb against p65 of NF-κB (arrows). Representative data are from at least two
442 independent experiments. The bar equals 10 μm. Veh, vehicle.
443
444 **Figure 5. TNF-α production by RAW264.7 cells and THP-1 cells in response to**
445 **heat-killed *E. coli* or *S. aureus*.** RAW264.7 cells (A) and THP-1 cells (B) were in advance
446 treated for 1 h with various concentrations of resveratrol and incubated for 0, 2, 4 or 6 h in a
447 serum-free condition with heat-killed *E. coli* and *S. aureus* at a ratio of number of bacterial
448 particles to that of cells of 1:100. The amounts of TNF-α produced in the supernatants were
449 measured by ELISA. See text for details. The mean values and SD of triplicate experiments
450 are shown. The statistically significant difference from vehicle was assessed by Student's
451 *t*-test; $*p < 0.05$; $**p < 0.01$. Representative data are from more than three independent
452 experiments. Veh, vehicle.
453
454 **Figure 6. Inhibitory effect of resveratrol on impaired phagocytosis of bacteria mediated**
455 **by SRs and CLRs.** (A) THP-1 cells incubated for 24 h with 100 nM FSL-1 were treated with
456 100 μM resveratrol for 1 h in a serum-free condition. Expression of mRNAs of MSR1, CD36,
457 DC-SIGN, Dectin-1 and β-actin was confirmed by RT-PCR using total RNA isolated from the
458 cells. Representative data are from more than five independent experiments. (B)
459 293/DC-SIGN cells were incubated for 0, 30, 60, 90 or 120 min with 100 μM resveratrol. The
460 cells were then lysed in SDS sample buffer, and proteins were separated by electrophoresis on
461 10% SDS-poly-acrylamide gels and transferred to a nitrocellulose membrane. The membrane
462 was reacted with anti-DC-SIGN mAb or anti-β-actin mAb. (C) 293/DC-SIGN cells were
463 treated with the indicated dose of resveratrol for 1 h and given heat-killed FITC-*E. coli* or *S.*
464 *aureus* at various cell:bacteria ratios. The mean values and SD of triplicate experiments are
465 shown. The statistically significant difference from vehicle was assessed by Student's *t*-test;

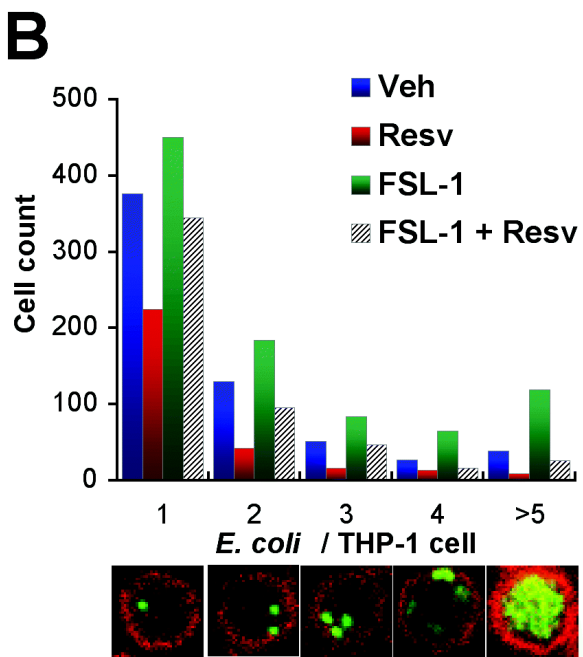
466 $*p < 0.05$; $**p < 0.01$. Representative data are from more than three independent experiments.

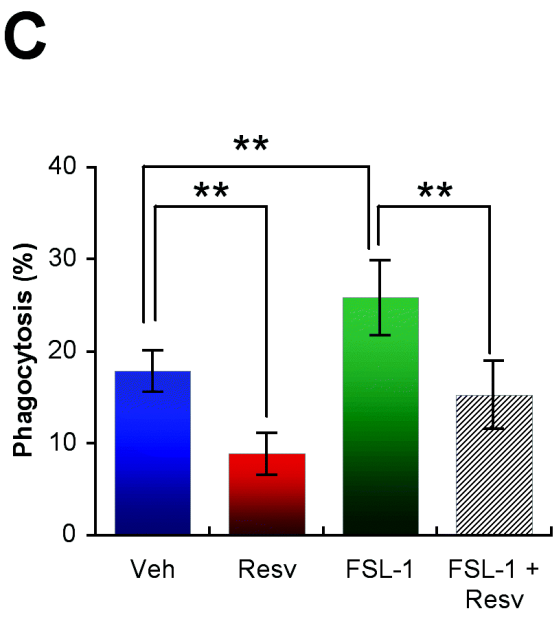
467 Veh, vehicle.

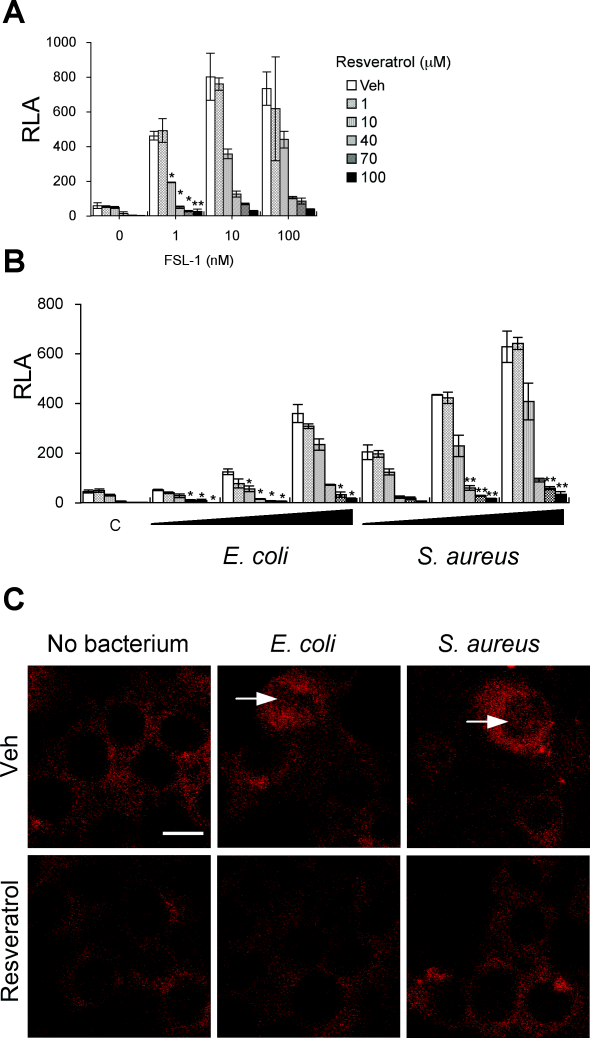

A
RAW264.7
Resveratrol (μM)
Veh
10
50
100
MFI
Time (min)
B
THP-1
MFI
Time (min)
C
RAW264.7
MFI
E. coli / cell
MFI
S. aureus / cell
D
THP-1
MFI
E. coli / cell
MFI
S. aureus / cell

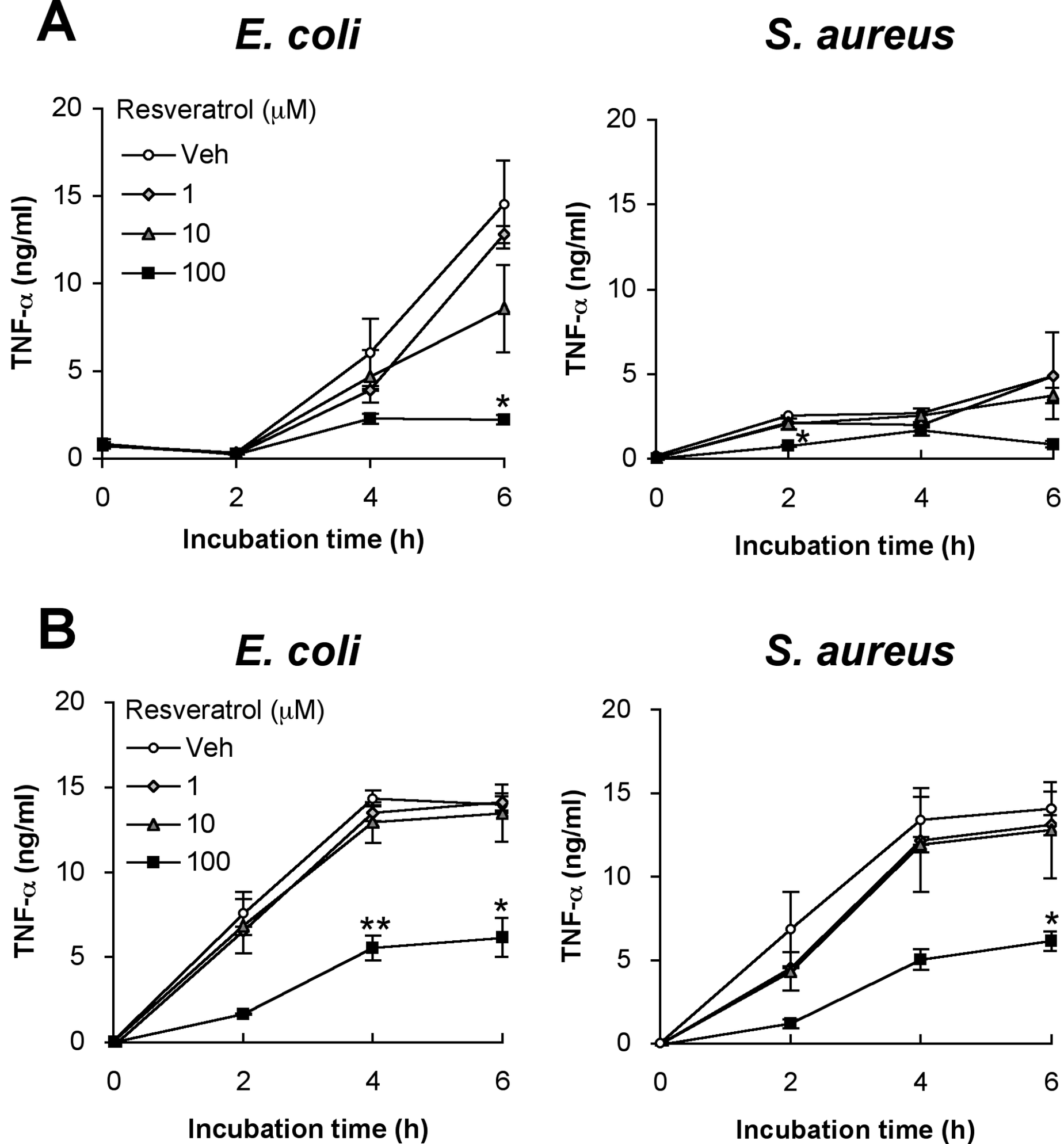

A
E. coli
S. aureus
Resveratrol (μM)
Veh
10
50
100
MFI
FSL-1 (nM)
0
1
10
100
B
E. coli
S. aureus
MFI
FSL-1 (nM)

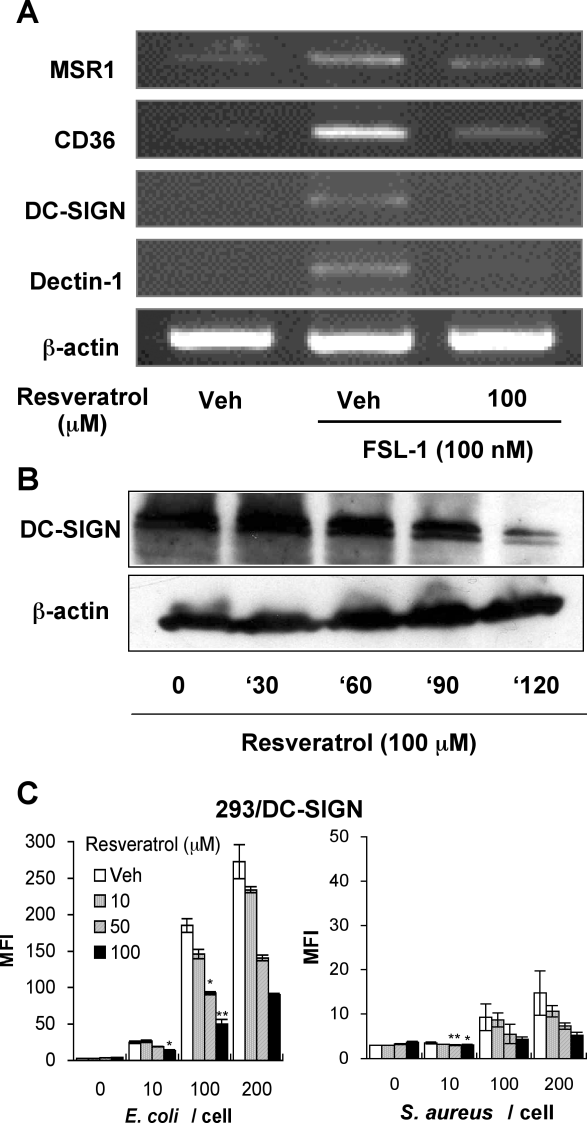

A
Veh
Resv
FSL-1
FSL-1 + Resv


B
Veh
Resv
FSL-1
FSL-1 + Resv
Cell count
500
400
300
200
100
0
1
2
3
4
>5
E. coli / THP-1 cell


C
**
**
**
Phagocytosis (%)
40
30
20
10
0
Veh
Resv
FSL-1
FSL-1 + Resv


A
RLA
1000
800
600
400
200
0
0
1
10
100
FSL-1 (nM)
Resveratrol (μM)
Veh
1
10
40
70
100
B
RLA
800
600
400
200
0
C
E. coli
S. aureus
C
No bacterium
E. coli
S. aureus
Veh
Resveratrol


A
E. coli
S. aureus
Resveratrol (μM)
Veh
1
10
100
TNF-α (ng/ml)
Incubation time (h)
0
5
10
15
20
0
2
4
6
*
B
E. coli
S. aureus
Resveratrol (μM)
Veh
1
10
100
TNF-α (ng/ml)
Incubation time (h)
**
*


A
MSR1
CD36
DC-SIGN
Dectin-1
β-actin
Resveratrol (μM)
Veh
Veh
100
FSL-1 (100 nM)
B
DC-SIGN
β-actin
0
'30
'60
'90
'120
Resveratrol (100 μM)
C
293/DC-SIGN
Resveratrol (μM)
Veh
10
50
100
MFI
E. coli / cell
S. aureus / cell