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Author(s)	Haque, Shamsul; Hasebe, Akira; Iyori, Mitsuhiro et al.
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1 **The TLR2 ligand FSL-1 is internalized via clathrin-dependent endocytic pathway**
2 **triggered by CD14 and CD36 but not by TLR2**

3

4 **Short Title: Internalization of the TLR2 ligand FSL-1**

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6 Haque M. Shamsul,^{1†} Akira Hasebe,^{1†} Mitsuhiro Iyori,^{1,2} Makoto Ohtani,^{1,3} Kazuto
7 Kiura,¹ Diya Zhang,^{1,4} Yasunori Totsuka,³ and Ken-ichiro Shibata^{1*}

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9 [†]Both authors contributed equally to this work.

10

11 Key Words: TLR2; clathrin-dependent endocytosis: CD14; CD36

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13 ¹ Laboratory of Oral Molecular Microbiology, Department of Oral Pathobiological
14 Science, Hokkaido University Graduate School of Dental Medicine, Japan.

15 ² Division of Research Fellow of the Japan Society for the Promotion of Science, Japan.

16 ³ Laboratory of Oral and Maxillofacial Surgery, Department of Oral Pathobiological
17 Science, Hokkaido University Graduate School of Dental Medicine, Japan.

18 ⁴ Department of Dentistry, Sir Run Run Shaw Hospital, affiliated with School of

1 Medicine, Zhejiang University, China.

2

3 *Correspondence: Ken-ichiro Shibata, Laboratory of Oral Molecular Microbiology,

4 Department of Oral Pathobiological Science, Hokkaido University Graduate School of

5 Dental Medicine, Nishi 7, Kita 13, Kita-ku, Sapporo 060-8586, Japan. E-mail:

6 shibaken@den.hokudai.ac.jp

7 Senior author: Ken-ichiro Shibata

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Abbreviations

1

2 TLR, Toll-like receptor; NF- κ B, nuclear factor- κ B; LP, lipoproteins; LPT, lipopeptide;

3 LTA, lipoteicoic acid; FITC-FSL-1, FITC-conjugated FSL-1; Alexa-Con A, Alexa Fluor

4 594-conjugated concanavalin A; Nys , nystatin; CPZ, chlorpromazine; MbCD,

5 methyl- β -cyclodextrin; HC, heavy chain; mAb, monoclonal antibody; PE,

6 phycoerythrin; TNF, tumor necrosis factor; PMs, peritoneal macrophages; HEK, human

7 embryonic kidney; WT, wild-type; FCM, flow cytometer ; MFI, mean fluorescence

8 intensity; CLSM, confocal laser scanning microscope; RNAi, RNA interference; siRNA,

9 small interfering RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

10

Summary

1
2 Little is known about how Toll-like receptor (TLR) ligands are processed after
3 recognition by TLRs. This study was therefore designed to investigate how the TLR2
4 ligand FSL-1 is processed in macrophages after recognition by TLR2. FSL-1 was
5 internalized into murine macrophage cell line, RAW264.7. Both chlorpromazine and
6 methyl- β -cyclodextrin, which inhibit clathrin-dependent endocytosis, reduced FSL-1
7 uptake by RAW264.7 cells in a dose-dependent manner, but nystatin which inhibits
8 caveolae- and lipid raft-dependent endocytosis, did not. FSL-1 was colocalized with
9 clathrin but not with TLR2 in the cytosol of RAW264.7 cells. These results suggest that
10 internalization of FSL-1 is clathrin dependent. In addition, FSL-1 was internalized by
11 peritoneal macrophages from TLR2-deficient mice. FSL-1 was internalized by human
12 embryonic kidney 293 cells transfected with CD14 or CD36 but not by the
13 non-transfected cells. Also, knockdown of CD14 or CD36 in the transfectants reduced
14 FSL-1 uptake.

15 In this study, we suggest that (i) FSL-1 is internalized into macrophages via a
16 clathrin-dependent endocytic pathway, (ii) the FSL-1 uptake by macrophages occurs
17 irrespective of the presence of TLR2, and (iii) CD14 and CD36 are responsible for the
18 internalization of FSL-1.

19

Introduction

1
2 Toll-like receptors (TLRs) are type-I transmembrane proteins with extracellular
3 leucine-rich repeat motifs and an intracellular Toll/interleukin-1 receptor domain, and
4 they play important roles in recognition of microbial invasion ¹. Numerous lines of
5 evidence have indicated that TLRs orchestrate not only the innate immune system but
6 also adaptive immune responses to microbial infections ². TLR signals are known to
7 induce activation of the nuclear factor- κ B (NF- κ B) in antigen presenting cells, which
8 results in the expression of various cytokine genes, induction of co-stimulatory
9 molecules, B7-1 (CD80) and B7-2 (CD86), and class II MHC molecules ³⁻⁵. Therefore,
10 TLRs are able to orchestrate the adaptive immune responses to microbial infections.

11 We have purified and characterized mycoplasmal diacylated lipoproteins (LP)
12 responsible for activation of macrophages and fibroblasts ^{6,7} and have synthesized a
13 diacylated lipopeptide (LPT) called FSL-1 [S-(2,3-bispalmitoyloxypropyl)
14 CGDPKHPKSF] on the basis of the N-terminal structure of a 44-kDa *Mycoplasma*
15 *salivarium* LP ⁷. We have also investigated various biological activities of FSL-1 ⁸⁻¹¹
16 and the mechanism by which it is recognized by TLRs ¹²⁻¹⁴. Recently, it was found that
17 FSL-1 can enhance phagocytosis of bacteria by macrophages through a TLR2-mediated
18 signaling pathway ¹⁰. In the course of these studies, we have become interested in how

1 the TLR2 ligand FSL-1 is processed by macrophages after recognition. Although
2 Triantafilou *et al.*¹⁵ recently reported that recognition of the TLR2 ligand lipoteicoic
3 acid (LTA) occurs at the cell surface and that LTA is internalized in a lipid
4 raft-dependent manner, details of internalization of TLR2 ligands after recognition
5 remain unknown.

6 This study therefore was designed to investigate how the TLR2 ligand FSL-1 is
7 processed in macrophages after recognition by TLR2.

8

Materials and methods

Chemicals and antibodies

FSL-1 was synthesized as described previously ⁷, and FITC-conjugated FSL-1 (FITC-FSL-1) was purchased from BioSynthesis (Lewisville, TX). Alexa Fluor 594-conjugated concanavalin A (Alexa-Con A), LysoTracker Red DND-99, and Alexa Fluor 594-conjugated anti-mouse IgG were purchased from Invitrogen-Molecular Probes (Eugene, OR); nystatin (Nys), chlorpromazine (CPZ) and methyl- β -cyclodextrin (MbCD) were obtained from Sigma-Aldrich (St. Louis, MO); anti-clathrin heavy chain (HC) monoclonal antibody (mAb) (clone X22) was obtained from Calbiochem-Novabiochem (La Jolla, CA); and anti-mouse/human TLR2 mAb (clone T2.5), and phycoerythrin (PE)-conjugated anti-mouse TLR2 mAb (clone 6C2) were obtained from eBioscience (San Diego, CA). Anti-human CD14 mAb (clone MY4) was obtained from Beckman Coulter (Fullerton, CA), and anti-human CD36 mAb (clone FA6-152) was obtained from Abcam (Cambridge, UK). ELISA kit used to determine the amount of tumor necrosis factor (TNF)- α in cell culture supernatants was obtained from BD Biosciences (San Diego, CA)

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

1

2 *Cell cultures and mouse peritoneal macrophages (PMs)*

3 Macrophage cell line, RAW264.7 cells (TIB-71; ATCC) were grown at 37°C and in 5%
4 CO₂ in RPMI 1640 medium (Sigma) supplemented with 10% (vol/vol) heat-inactivated
5 fetal bovine serum (FBS) (Gibco BRL, Rockville, MD), 100 units/ml penicillin (Sigma),
6 and 100 µg/ml streptomycin (Sigma) (complete medium). Human embryonic kidney
7 (HEK)293 cells (CRL-1573; ATCC) were grown in Dulbecco's modified Eagle's (DME)
8 complete medium (Sigma).

9 Sex-matched C57BL/6 mice [TLR2 (+/+) mice] were purchased from Japan Clea
10 (Tokyo, Japan). TLR2-deficient mice on the same background [TLR2 (-/-) mice] were
11 kindly provided by Dr. Shizuo Akira, Department of Host Defense, Research Institute
12 for Microbial Diseases, Osaka University (Osaka, Japan). All mice were maintained in
13 specific pathogen-free conditions at the animal facility of Hokkaido University, and all
14 experiments were approved by Hokkaido University Animal Care and Use Committee.
15 PMs were prepared from mice as described previously ¹⁰.

16

17 *HEK293 transfectants*

18 The cDNAs of human CD14, CD36 and TLR2 were obtained as described previously ¹⁰,

1^{13, 16}. Briefly, they were obtained by RT-PCR of total RNA isolated from a human
2 monocyte/macrophage cell line, THP-1 cells, and then they were cloned into a
3 pEF6/V5-His TOPO vector (Invitrogen, Carlsbad, CA) or pcDNA3.1-His-TOPO vector
4 (Invitrogen). Their transfection into wild-type HEK293 cells (HEK293WT) was
5 performed by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's
6 instructions. To obtain stable transfectants of CD14 (HEK293/CD14) or CD36
7 (HEK293/CD36), the cells were selected in the presence of blasticidin S (50 µg/ml)
8 (Invitrogen) with limiting dilution.

9 The cDNAs of TLR2 was cloned into a pEF6/V5-His TOPO vector (Invitrogen)
10 and transiently transfected into HEK293/CD14 (HEK293/CD14/TLR2) or
11 HEK293/CD36 (HEK293/CD36/TLR2) by using Metafectene (Biontex Laboratories
12 GmbH, Martinsried/Planegg, Germany).

13 The surface expression level of CD14, CD36 or TLR2 was confirmed by using a
14 flow cytometer (FCM), FACS Calibur (BD Biosciences). For FCM analysis, data for
15 30,000 cells falling within appropriate forward and side scatter gates were collected
16 from each sample. The results were analyzed by using CellQuest software (BD
17 Biosciences) or FlowJo software (Tree Star, Ashland, OR).

18

1 *FSL-1 uptake*

2 FSL-1 uptake by various types of cells was determined by modifying phagocytosis
3 assay described previously^{10, 11}. Briefly, a 2-ml cell suspension was incubated at 37 °C
4 for 2 h with FITC-FSL-1 (100 µg/ml) in base medium appropriate for each of the cells.
5 Then the cells were washed three times with cold PBS, suspended in PBS containing
6 0.2% (wt/vol) trypan blue to quench fluorescence caused by cell surface FITC-FSL-1,
7 and treated with 1% (wt/vol) paraformaldehyde. The uptake levels of FSL-1 by the cells
8 were analyzed by using FCM as described above and assessed by change in the mean
9 fluorescence intensity (MFI). For an assay using a confocal laser scanning microscope
10 (CLSM, LSM510 invert Laser Scan Microscope, Carl Zeiss, Tokyo, Japan), a 2-ml
11 suspension of the cells (1×10^5 /ml) was added to each well of a 6-well plate and
12 incubated at 37°C for 24 h. Then the cells were washed three times at 37 °C with
13 appropriate base medium and incubated with FITC-FSL-1. The cells were washed with
14 PBS and reacted for 20 min with 50 µg/ml Alexa-Con A in PBS and then treated with
15 PBS containing 3% (wt/vol) paraformaldehyde.

16

17 *Effects of chemicals on FSL-1 uptake*

18 To test the effects of Nys, CPZ and MbCD on FSL-1 uptake, RAW 264.7 cells were

1 treated for 30 min with various concentrations of the inhibitors. After the cells had been
2 washed with RPMI 1640 base medium, the uptake level of FSL-1 was determined as
3 described above.

4

5 *RNA interference (RNAi) and real-time PCR*

6 A mouse clathrin HC-specific small interfering RNA (siRNA)
7 (ACUAAGUAGCGAGAAAGGctt) and negative control siRNA were purchased from
8 Applied Biosystems (Foster City, CA). A 500- μ l suspension of RAW264.7 cells (5×10^5
9 cells/ml) in a 24-well plate was prepared with antibiotic-free RPMI complete medium.
10 The cells were incubated for 24 h and then transfected with the siRNA (20 pmol/well)
11 by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the
12 manufacturer's instruction. The medium was exchanged at 5 h and 24 h after
13 transfection, and the cells were examined for FSL-1 uptake at 48 h after transfection. To
14 confirm the effects of siRNAs, Real-Time TaqMan PCR was performed according to the
15 manufacturer's standard PCR protocol by using a StepOne Real-Time PCR system
16 (Applied Biosystems) with specific pre-made TaqMan probes for mouse clathrin HC
17 (CGTTAATTGACCAGGTTGTACAGAC, Applied Biosystems) and
18 glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

1 (GAACGGATTTGGCCGTATTGGGCGC, Applied Biosystems).

2 For downregulation of CD14 or CD36, their specific siRNA cocktails were
3 purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Eighty pmol of siRNA or
4 negative control siRNA were transfected into HEK293/CD14 or HEK293/CD36 by
5 using Metafectene (Biontex Laboratories GmbH, Martinsried/Planegg, Germany). The
6 effects of siRNA transfection on CD14 and CD36 expression level were confirmed by
7 FCM analysis.

8

Results

FSL-1 uptake by macrophages

First, we examined whether FITC-FSL-1 is able to activate macrophages, because there is a possibility that FITC conjugation affects the ability of FSL-1 to activate them¹³. It was found that both FITC-FSL-1 and FSL-1 induced TNF- α production by a murine macrophage cell line, RAW264.7 cells, in a dose-dependent manner (Fig. 1), suggesting that FITC-FSL-1 is also able to activate macrophages, possibly through TLR2. However, it remains unknown how FSL-1 is processed in macrophages after recognition by TLR2. To address this question, an experiment was carried out to determine whether FSL-1 is internalized by macrophages after recognition by TLR2. RAW264.7 cells were incubated with FITC-FSL-1 for 2 h at 4 °C (on ice) or at 37 °C, and then uptake of FSL-1 was determined. FSL-1 was found in the cell membrane but not in the cytosol of RAW264.7 cells at 4 °C (Fig. 2A and C). However, FSL-1 was found in both the cell membrane and the cytosol of the cells at 37 °C (Fig. 2B and D). These results suggest that FSL-1 is clearly internalized into the cells in a temperature-dependent manner.

Clathrin-dependent endocytosis of FSL-1

It has been demonstrated that modes of endocytosis of small soluble molecules can be

1 mainly divided into three pathways: clathrin-, caveolae-, and lipid raft-dependent
2 endocytic pathways ¹⁷. Therefore, experiments were carried out to determine the effects
3 of three chemicals, Nys, CPZ and MbCD, on internalization of FSL-1. Nys selectively
4 disrupts caveolae- and lipid raft-dependent endocytosis but has no effect on
5 clathrin-dependent endocytosis ¹⁸. CPZ disrupts clathrin-dependent endocytosis ¹⁹.
6 MbCD disrupts both lipid raft- and clathrin-dependent endocytosis ²⁰⁻²³. It has been
7 demonstrated that TLR2 is enriched in lipid rafts ²⁴ and that the TLR2 ligand LTA is
8 internalized into cells with TLR2 via lipid rafts ¹⁵. The present study demonstrated that
9 Nys had no effect on FSL-1 uptake by RAW 264.7 cells (Fig. 3A-C), suggesting that
10 FSL-1 is not internalized by caveolae- and lipid raft-dependent endocytosis. Both CPZ
11 and MbCD inhibited FSL-1 uptake by the cells in a dose-dependent manner (Fig. 3D-F
12 and G-I), suggesting that FSL-1 is internalized into macrophages by a
13 clathrin-dependent endocytosis.

14 Next experiment was therefore carried out to determine whether FSL-1 is
15 colocalized with clathrin in cells. RAW 264.7 cells were incubated for 2 h with
16 FITC-FSL-1, permeabilized with Cytofix/Cytoperm (BD Bioscience), and treated with
17 an anti-clathrin mAb. Both clathrin and FSL-1 were found in endosome-like
18 compartments of the cytosol, and some FSL-1-containing compartments were

1 colocalized with clathrin-coated compartments (Fig. 4A). These results also suggest that
2 clathrin is involved in the uptake of FSL-1. To further confirm this, effects of gene
3 silencing of clathrin mRNA on FSL-1 uptake were examined. The gene silencing
4 efficiency was confirmed by Real-Time TaqMan PCR using clathrin- or
5 GAPDH-specific TaqMan probes. PCR analysis revealed that the level of clathrin
6 mRNA was downregulated by approximately 35% (Fig. 4B). Then, effects of gene
7 silencing of these siRNAs on the level of FSL-1 uptake were determined. It was found
8 that the MFI of FSL-1 uptake without any siRNA was 1897, whereas MFIs transfected
9 with clathrin HC-specific siRNA and negative control RNA were 1036 and 1721 (Fig.
10 4C and D), respectively. Thus, downregulation of clathrin mRNA expression was
11 correlated with decrease in the level of FSL-1 uptake. These results strongly suggest
12 that FSL-1 is internalized into cells via a clathrin-dependent endocytic pathway.

13

14 **Maturation of FSL-1-containing endosomes**

15 Endosomes formed by endocytosis sequentially display specific markers dependent on
16 the maturation stage, early endosomes and late endosomes fused with lysosomes²⁵. To
17 investigate whether FSL-1-containing endosomes mature, LysoTracker Red was
18 employed since it is a dye specific for acidified compartments such as late endosomal

1 and lysosomal organelles²⁶. LysoTracker Red freely permeates cell membranes and
2 remains trapped in acidic compartments upon protonation²⁶. It was found that some
3 FSL-1-containing endosomes were colocalized with LysoTracker-containing ones (Fig.
4 5), suggesting that FSL-1-containing endosomes mature to acidified late endosomes.

5

6 **TLR2-independent uptake of FSL-1**

7 It has recently been demonstrated that triacylated LPT bind to TLR2 when they are
8 recognized by TLR2^{16,27,28}. On the basis of these findings, we thought that the complex
9 of TLR2 and FSL-1 is internalized into cells after recognition, because involvement of
10 receptors is indispensable for clathrin-dependent endocytosis²⁹⁻³¹. Therefore, at first, an
11 experiment was carried out to examine the intracellular localization of FSL-1 and TLR2.
12 Both FSL-1 and TLR2 were found to localize on the cell membrane as well as in the
13 cytosol, although no FSL-1 was found to colocalize with TLR2 in the intracellular
14 compartments (Fig. 6A). This result demonstrated that FSL-1 uptake by macrophages
15 occurs in a manner different from that of LTA, because LTA is internalized into a cell
16 and colocalized with TLR2¹⁵. Although no colocalization of FSL-1 with TLR2 cannot
17 rule out that TLR2 is involved in the FSL-1 uptake. Therefore, FSL-1 uptake by PMs
18 from TLR2 (+/+) and TLR2 (-/-) mice was examined in the next experiment. There was

1 no difference in the mode of FSL-1 uptake by these PMs (Fig. 6B-E). These results
2 suggest that FSL-1 uptake occurs irrespective of the presence of TLR2. In the next
3 experiment, we examined whether FSL-1 stimulation affects the expression level of
4 TLR2 on the surface of the cells, because TLR2 was detected in the cytosol after
5 incubation with FSL-1 (Fig. 6A). It was found that incubation with FSL-1 induced
6 downregulation of the surface expression level of TLR2 (Fig. 7A and B), suggesting
7 that FSL-1 stimulation is required for TLR2 internalization.

8

9 **CD14- and CD36-dependent uptake of FSL-1**

10 We speculated that receptor(s) that mediate(s) the uptake of FSL-1 are CD36 and CD14,
11 because they function as coreceptors for the recognition of a mycoplasmal diacylated
12 LPT, MALP-2³², and a triacylated LPT, Pam₃CSK₄^{16,33}, by TLR2. Therefore,
13 experiments were carried out to determine the roles of CD14 and CD36 in the uptake of
14 FSL-1 by using HEK293WT, HEK293/CD14, HEK293/CD36, HEK293/TLR2,
15 HEK293/CD14/TLR2 or HEK293/CD36/TLR2. They were incubated with FITC-FSL-1
16 for 2 h and then examined for the uptake of FSL-1 by CLSM and FCM (Fig. 8). It was
17 clearly demonstrated that FSL-1 internalization occur in both HEK293/CD14 (Fig. 8B)
18 and HEK293/CD36 (Fig. 8C) but not in both HEK293WT (Fig. 8A) and HEK293/TLR2

1 (Fig. 8D). In addition, cotransfection of TLR2 had no effect on the uptake of FSL-1 by
2 HEK293/CD14 (Fig. 8B and E) and HEK293/CD36 (Fig. 8C and F). These results
3 demonstrated that both CD14 and CD36 are responsible for the uptake of FSL-1.

4 To further confirm the involvement of CD14 and CD36 in FSL-1 uptake, the
5 experiments were carried out to investigate the effects of knockdown of CD14 and
6 CD36 on FSL-1 uptake. The gene silencing of CD14 and CD36 were attempted by
7 transfecting their specific siRNAs into HEK293/CD14 and HEK293/CD36, respectively.
8 FCM analysis revealed that the level of both CD14 and CD36 was significantly
9 downregulated by siRNA transfection (Fig. 9A and B). Then, effects of transfection of
10 these siRNA on the level of FSL-1 uptake were determined. It was found that the
11 internalization level was downregulated in both HEK293/CD14 by CD14 siRNA
12 transfection and HEK293/CD36 by CD36 siRNA transfection. Thus, downregulation of
13 CD14 and CD36 expression was correlated with decrease in the level of FSL-1 uptake
14 suggesting that CD14 and CD 36 are responsible for the uptake of FSL-1.

15

Discussion

1
2 This study demonstrated that the diacylated LPT FSL-1 was incorporated into
3 mammalian cells through a clathrin-dependent endocytic pathway in which CD14 and
4 CD36 were involved. First we thought TLR2 is involved in the FSL-1 uptake, because
5 TLR2 is a receptor for FSL-1. However, TLR2 was not colocalized with FSL-1 in
6 cytosol of macrophages (Fig 6A) and FSL-1 was internalized into PMs from TLR2 (-/-)
7 mice (Fig. 6C and E). These results suggest the TLR2 is not involved in the FSL-1
8 uptake. This unique finding is supported by the recent findings of Triantafilou *et al.*¹⁵
9 on uptake of the other TLR2 ligand LTA. They found, by using HEK293 cells
10 transfected with both TLR2 and CD14, that TLR2 is recruited within lipid rafts
11 following LTA stimulation, that LTA is internalized in a lipid-raft dependent manner and
12 that TLR2 is colocalized with LTA in the Golgi apparatus¹⁵. However, they concluded
13 that LTA internalization is not dependent on TLR2, because LTA internalization occurs
14 even in HEK293 cells transfected with only CD14¹⁵. This is in a good agreement with
15 our finding that FSL-1 is internalized into PMs from TLR2 (-/-) mice (Fig. 6C and E).
16 However, their findings that LTA is internalized into a cell in a lipid raft-dependent
17 manner and is colocalized with TLR2 in the cytosol¹⁵ are in contrast to our findings that
18 FSL-1 is internalized in a clathrin-dependent manner (Fig. 3 and 4) and FSL-1 is not

1 colocalized with TLR2 in the cytosol (Fig. 6A). This discrepancy may be due to the
2 difference in cell types and the ligands used. Triantafilou *et al.* used nonphagocytic
3 HEK293 transfectants with LTA, whereas we used professional phagocytes, RAW264.7
4 cells. More recently, Triantafilou *et al.* ³⁴ have also reported that TLR2 is colocalized
5 with TLR6 and CD36 in the Golgi apparatus after stimulation with FSL-1 in HEK293
6 cells transfected with CD14, TLR2, TLR1, TLR6 and CD36, although they did not
7 investigate whether FSL-1 is colocalized with TLR2 in the cytosol ³⁴.

8 Taken together, these results suggest that TLR2 ligands are internalized into cells
9 irrespective of the presence of TLR2 after recognition by TLR2.

10 Therefore, we had a great interest to what kind of receptors other than TLR2 are
11 involved in the FSL-1 uptake. We speculated that CD14 or CD36 may mediate the
12 uptake, because they function as coreceptors of TLR2 to recognize LPT ^{32,33}. CD36 is
13 a glycosylated transmembrane protein that is expressed in various cell types and
14 tissues including monocytes/macrophages ³⁵. Especially for innate immune responses,
15 Hoebe *et al.* showed that CD36 is involved in recognition of TLR2/6 ligands ³². CD36
16 is also known as a class B scavenger receptor, and it has been reported that the
17 C-terminal cytoplasmic domain of CD36 is required for bacterial internalization ³⁶.
18 Therefore, it is reasonable that CD36 is responsible for FSL-1 uptake, although

1 Mairhofer *et al.* showed that most of CD36 are in the lipid-raft fraction ³⁷. CD14 is
2 found in a soluble form in serum or as a GPI-anchored protein on the cell membrane,
3 and is one of essential accessory proteins for LPS recognition ³⁸. It is also known that
4 CD14 functions as a coreceptor of TLR2 for the recognition of a triacylated LPT ^{16, 33}.
5 In addition, it has been reported that CD14 is constitutively presented in lipid rafts ³⁹
6 and it remains in lipid rafts after stimulation with LTA ¹⁵. In this study, we have shown
7 that both CD14 and CD36 were responsible for the uptake of FSL-1 (Fig. 8 and 9),
8 although it remains unknown how CD14 and CD36 in lipid rafts play roles in
9 clathrin-dependent endocytosis. Therefore, studies are in progress to elucidate the
10 detailed mechanism of FSL-1 uptake by CD14 and CD36.

11 Mycoplasmas are wall-less prokaryotes characterized by small genomes, and
12 known as the smallest self-replicating organisms ⁴⁰. LP, an integral component of
13 mycoplasmal cell membrane is a potent pathogenic factor in mycoplasmal infections
14 ⁴¹⁻⁴⁴. This study showed that the diacylated LPT FSL-1, the active entity of
15 mycoplasmal LPT, was internalized by a clathrin-dependent endocytosis. Some
16 pathogens, such as Influenza A viruses, adenoviruses and the bacterial pathogen
17 *Listeria monocytogenes* utilize clathrin-dependent endocytosis as an invasion
18 mechanism into target cells ^{45, 46}. Some mycoplasma species are also known to have

1 invasive properties to host cells ⁴⁰, but their invasion mechanism still remains unclear.
2 For example, *M. penetrans*, which is the most representative invasive mycoplasma, is
3 known to possess 65 kDa fibronectin binding protein, which is considered to play an
4 important role for its adhesion on a host cell ⁴⁷. Our finding that the lipopeptide FSL-1
5 derived from mycoplasmal membrane protein is internalized by a clathrin-dependent
6 endocytosis after recognition by TLR2 strongly suggests that membrane LP play a key
7 role in the invasion of mycoplasmas into host cells.

8 Studies to clarify roles of mycoplasmal LP in invasion into host cells are in
9 progress.

10

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1

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6 foundation (PK430031).

7

1

Disclosures

2 The authors have no financial conflict of interest.

3

Figure Legends

- 1
- 2 **Figure 1.** Induction of TNF- α production by RAW 264.7 cells stimulated with FSL-1 or
- 3 FITC-FSL-1. RAW 264.7 cells in 96-well plates were cultured and then stimulated at
- 4 37 °C for 15 h with various concentrations of FSL-1 or FITC-FSL-1 in RPMI base
- 5 medium. The culture supernatants were collected and examined for the amount of
- 6 TNF- α , which was determined by ELISA. Results are expressed as the means \pm SD of
- 7 three determinations.
- 8 **Figure 2.** Temperature dependency of FSL-1 internalization into RAW264.7 cells.
- 9 RAW 264.7 cells were incubated with FITC-FSL-1 at 4 °C (on ice) or 37 °C for 2 h.
- 10 The uptake level of FITC-FSL-1 was observed by using CLSM (A, 4 °C; and B, 37 °C)
- 11 and FCM (C, 4 °C; and D, 37 °C).
- 12 **Figure 3.** Effects of chemicals on FSL-1 uptake by RAW264.7 cells.
- 13 RAW264.7 cells were incubated for 30 min without (control) or with various
- 14 concentrations of Nys (A-C), CPZ (D-F), and MbCD (G-I) and then incubated for 2 h
- 15 with FITC-FSL-1. Results are shown as images obtained by CLSM (FITC-FSL-1,
- 16 green; Alexa-Con A, red) and as histograms (B, E, H) and MFI obtained from the
- 17 histograms by FCM.
- 18 **Figure 4.** Involvement of clathrin in FSL-1 uptake by RAW264.7 cells. RAW264.7 cells

1 were incubated for 2 h with FITC-FSL-1 (green) and then stained with anti-clathrin
2 mAb and second Ab (red). White arrows show colocalization of FSL-1-containing
3 compartments with clathrin-coated compartments (A). Downregulation of clathrin
4 mRNA by transfection of clathrin HC-specific siRNA was determined by using
5 Real-time TaqMan PCR (B). Effect of knockdown of clathrin mRNA on FSL-1 uptake
6 by RAW 264.7 cells was determined by FCM. Black line, cell only; gray area, cells with
7 FITC-FSL-1; red line, negative control siRNA-transfected cells with FITC-FSL-1; and
8 blue line, clathrin siRNA-transfected cells with FITC-FSL-1 (C) and MFI obtained from
9 the histogram (D, N, cells with FITC-FSL-1; SC, clathrin siRNA-transfected cells with
10 FITC-FSL-1; and NC, negative control siRNA-transfected cells with FITC-FSL-1) .

11 **Figure 5.** Maturation of FSL-1-containing endosome. The maturation was determined
12 by staining with LysoTracker in RAW 264.7 cells. White arrows show colocalization of
13 FSL-1-containing endosomes with LysoTracker-containing endosomes.

14 **Figure 6.** Involvement of TLR2 in FSL-1 uptake. Colocalization of FITC-FSL-1
15 (green) and TLR2 (red) in RAW 264.7 cells was observed by CLSM (A). FSL-1 uptake
16 by PMs from TLR2 (+/+) mice and TLR2 (-/-) mice was determined by FCM (B, C) and
17 CLSM (D, E). Small histograms in B and C show the surface expression level of TLR2
18 in each PMs.

1 **Figure 7.** The time course of cell surface TLR2 expression after FSL-1 stimulation.
2 RAW264.7 cells were stimulated with FSL-1 (10 nM), and cell surface expression level
3 of TLR2 was determined by using FCM. The results were shown as both histograms (A,
4 black line, cell only; gray line, cells with isotype control Ab; faint green area, TLR2
5 expression on unstimulated cells; blue line, TLR2 expression on 1-h stimulated cells;
6 red line, TLR2 expression on 2-h stimulated cells) and MFI obtained from the
7 histogram (B).

8 **Figure 8.** Involvement of CD14 or CD36 in FSL-1 uptake. HEK293WT (A),
9 HEK293/CD14 (B), HEK293/CD36 (C), HEK293/TLR2 (D), HEK293/CD14/TLR2 (E)
10 or HEK293/CD36/TLR2 (F) were incubated with FITC-FSL-1 for 2 h. Results are
11 shown as images obtained by CLSM (FITC-FSL-1, green; Alexa-Con A, red) and as
12 histograms obtained by FCM (faint gray area, cell only; black line, cell + FITC-FSL-1).

13 **Figure 9.** Effects of knockdown of CD14 in HEK293/CD14 and knockdown of CD36
14 in HEK293/CD36 on the uptake of FSL-1. HEK293/CD14 and HEK293/CD36 cells
15 were transiently transfected with si RNA against CD14 and CD36 respectively. The cell
16 surface expression levels of CD14 (A) and CD36 (B) were confirmed by using FCM.
17 FSL-1 uptake in the cells transfected with siRNA against CD14 (C) and CD36 (D) were
18 shown as both relative MFI and the histograms (E, F).

19

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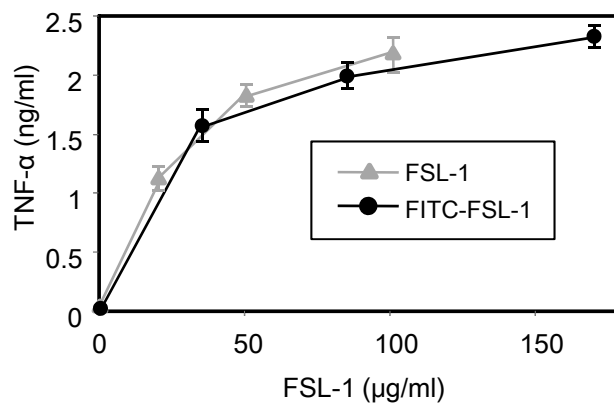


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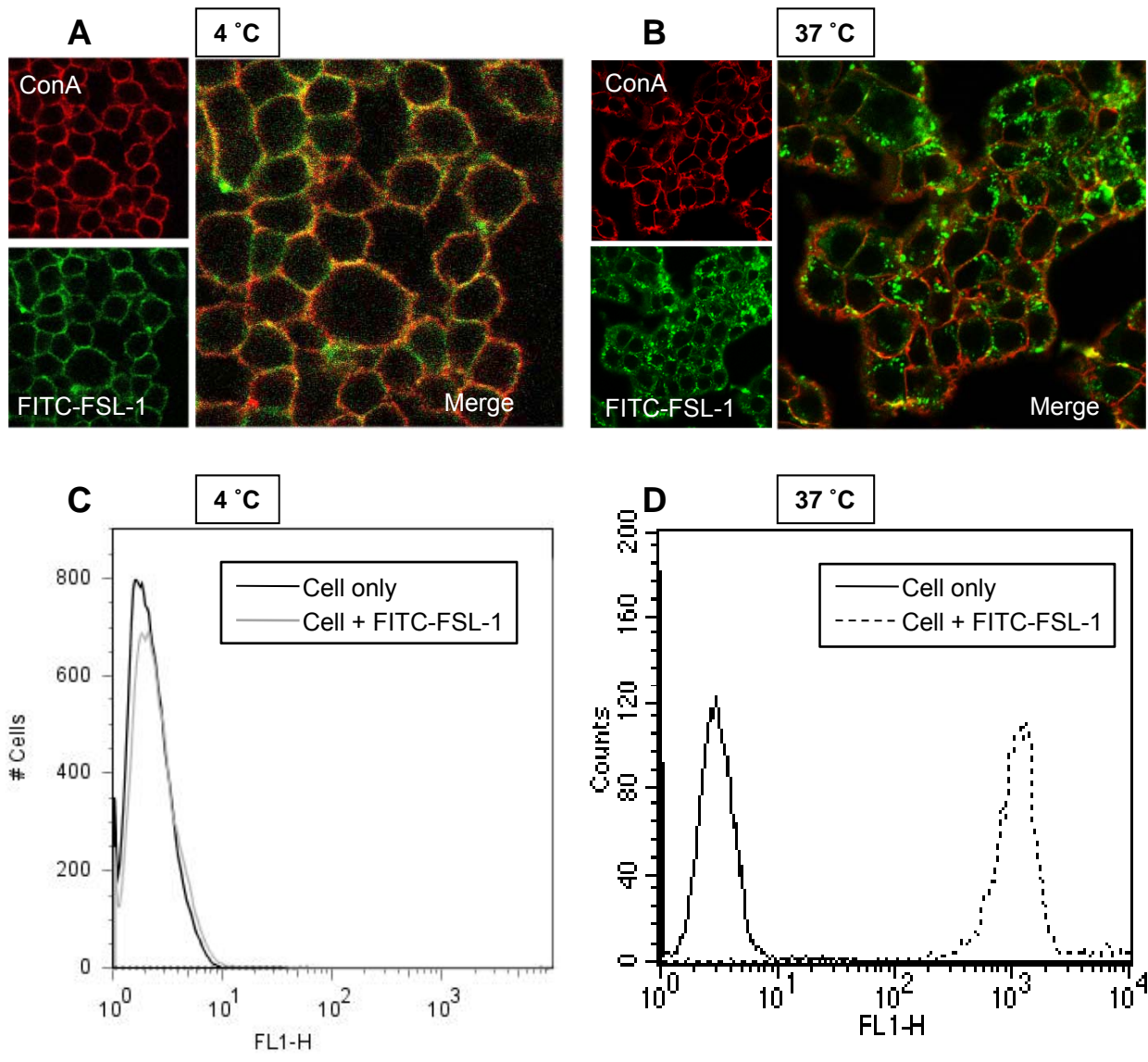


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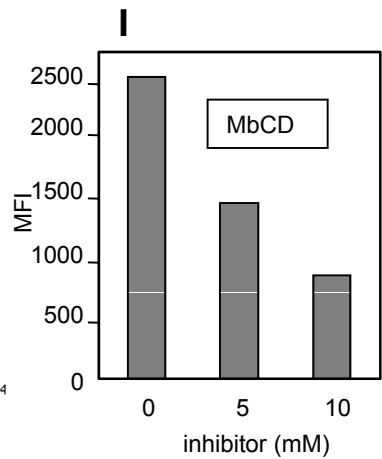
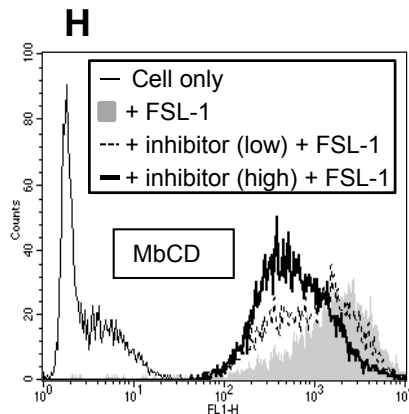
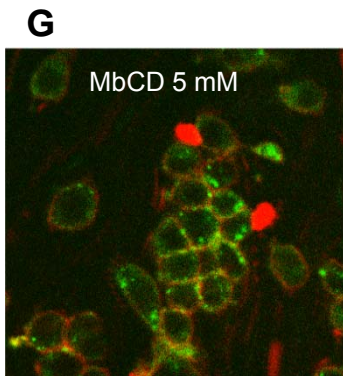
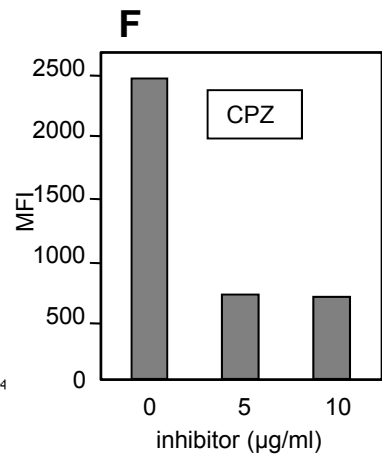
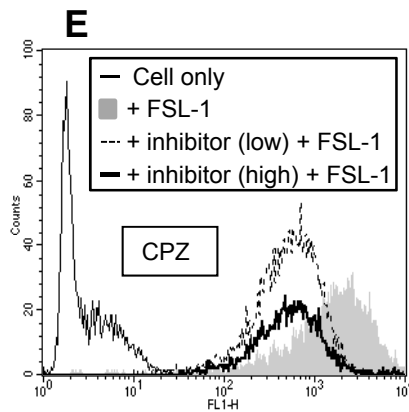
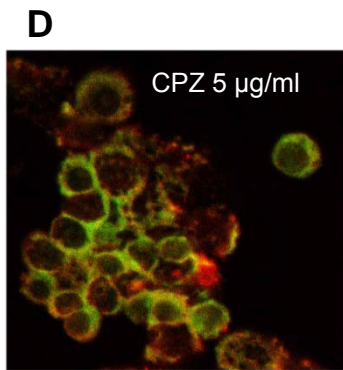
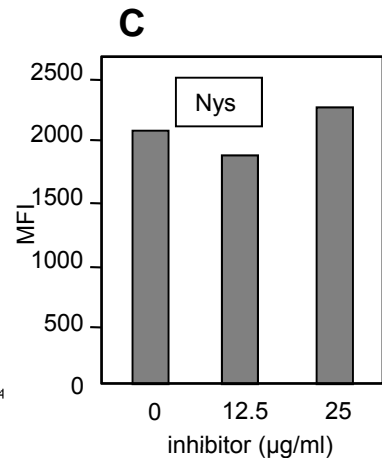
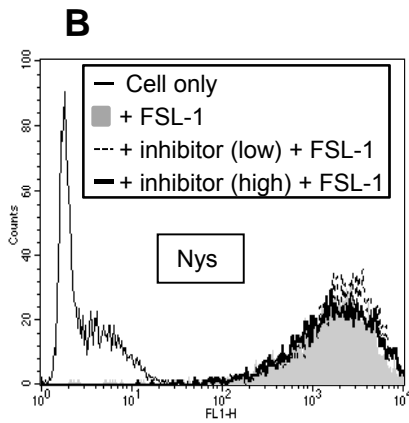
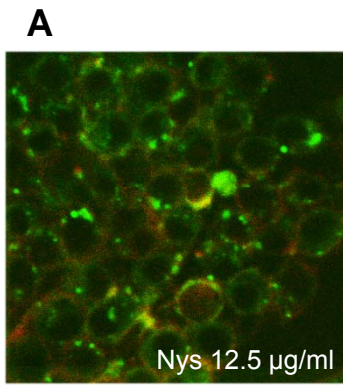
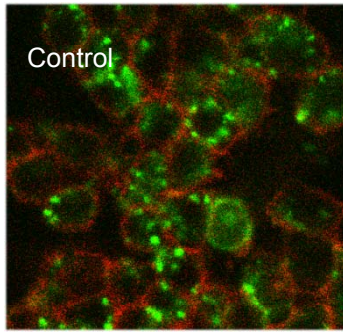


Fig. 3

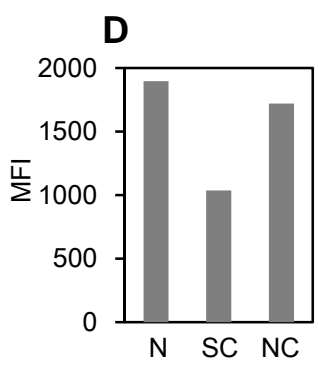
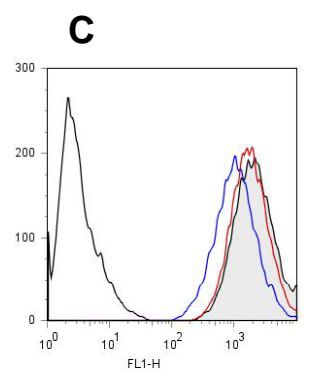
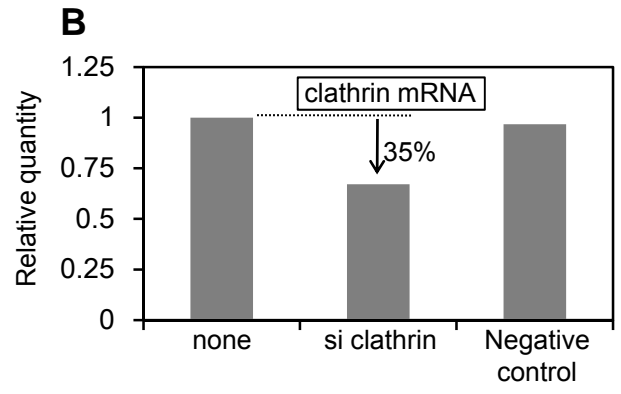
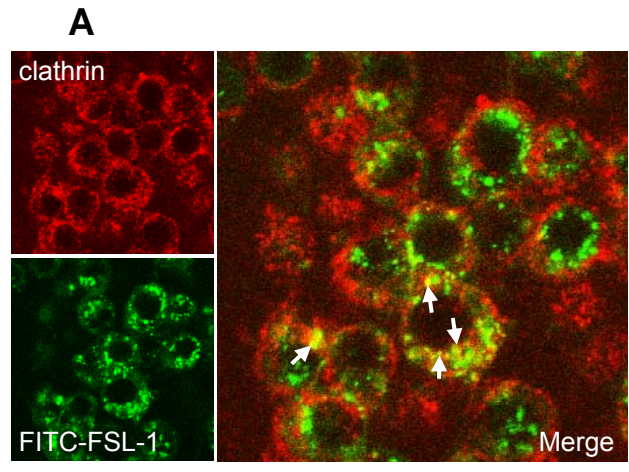


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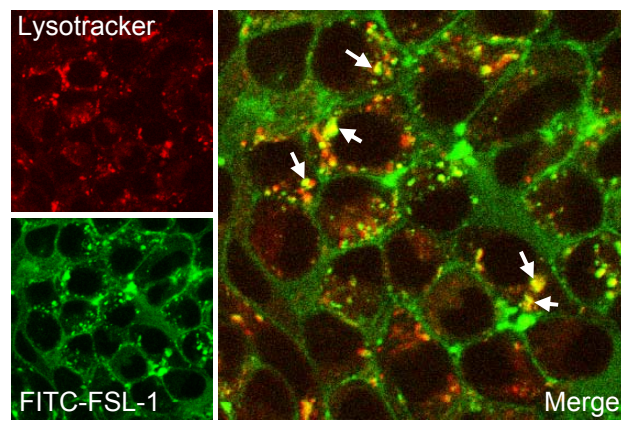


Fig. 5

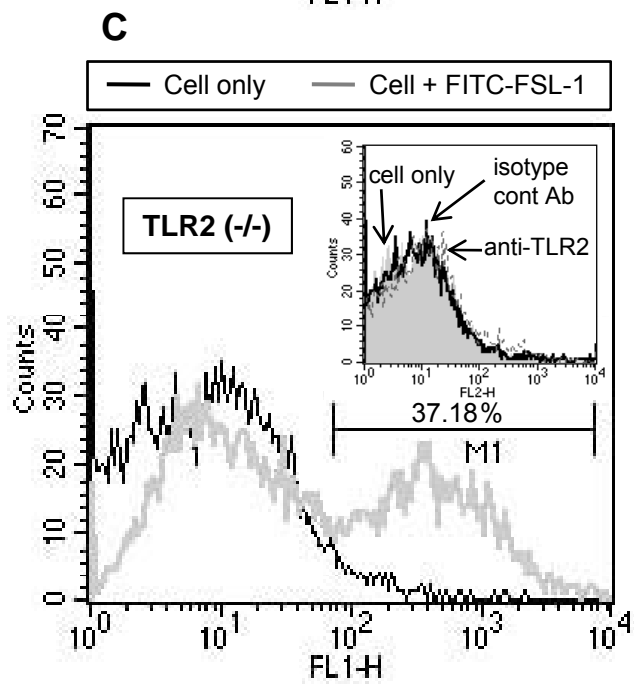
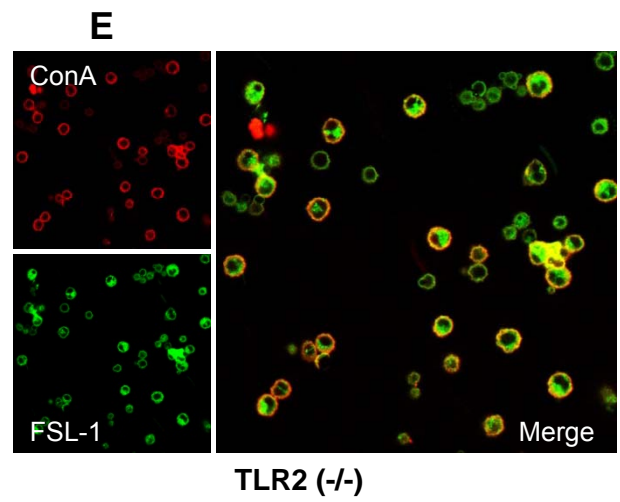
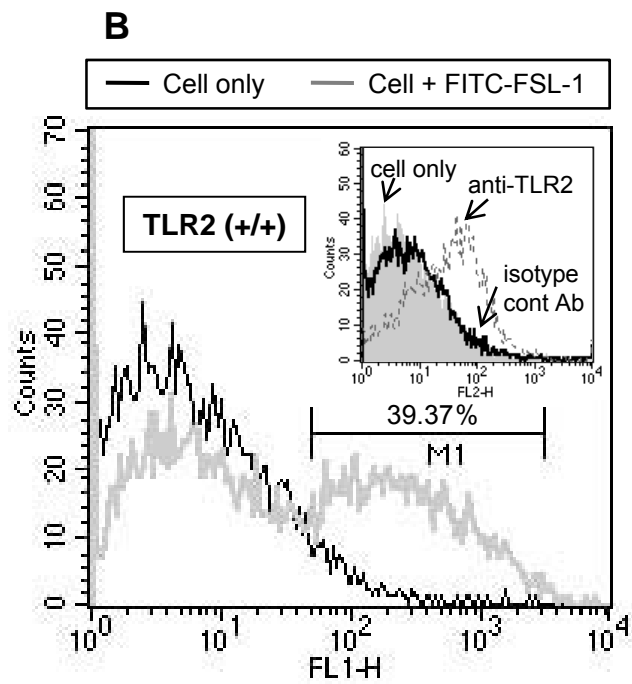
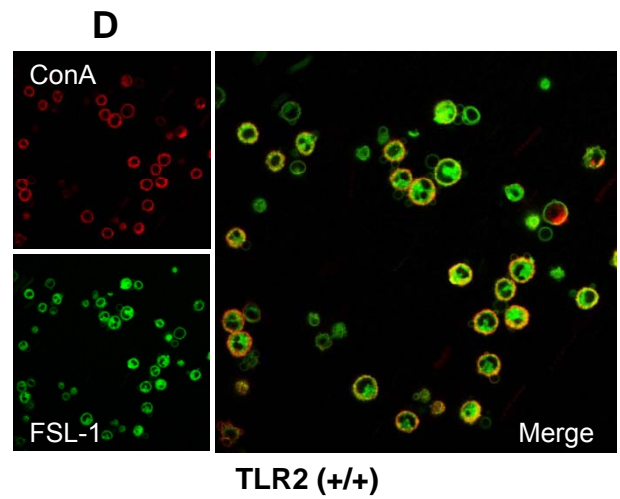
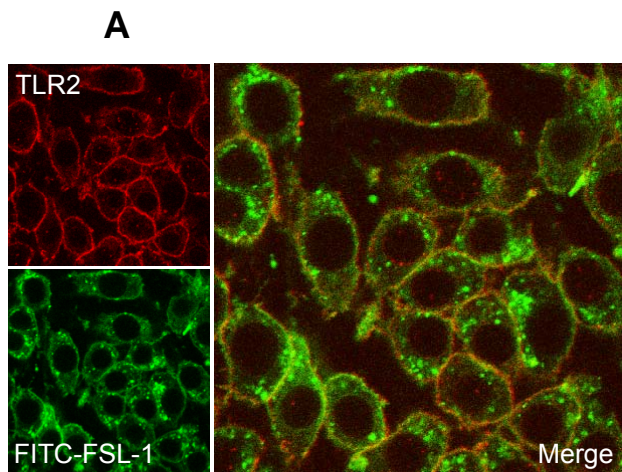


Fig. 6

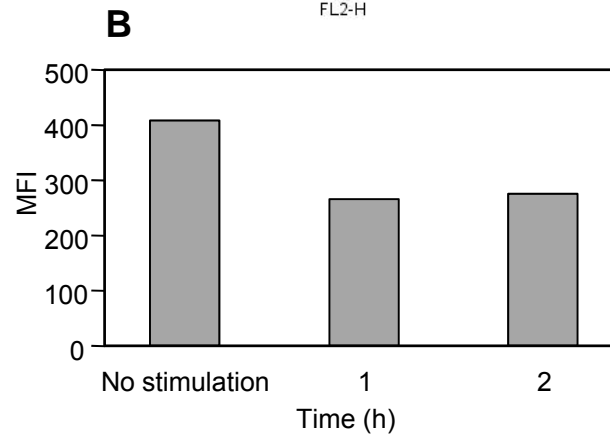
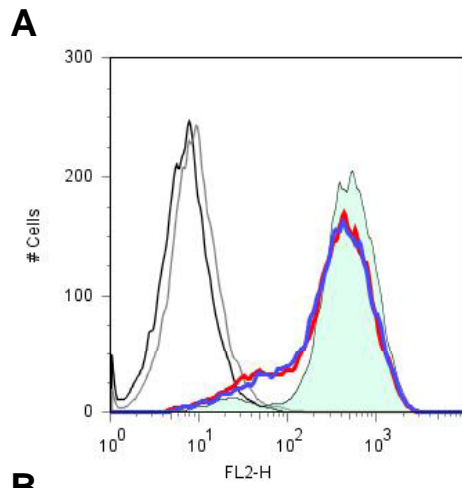


Fig. 7

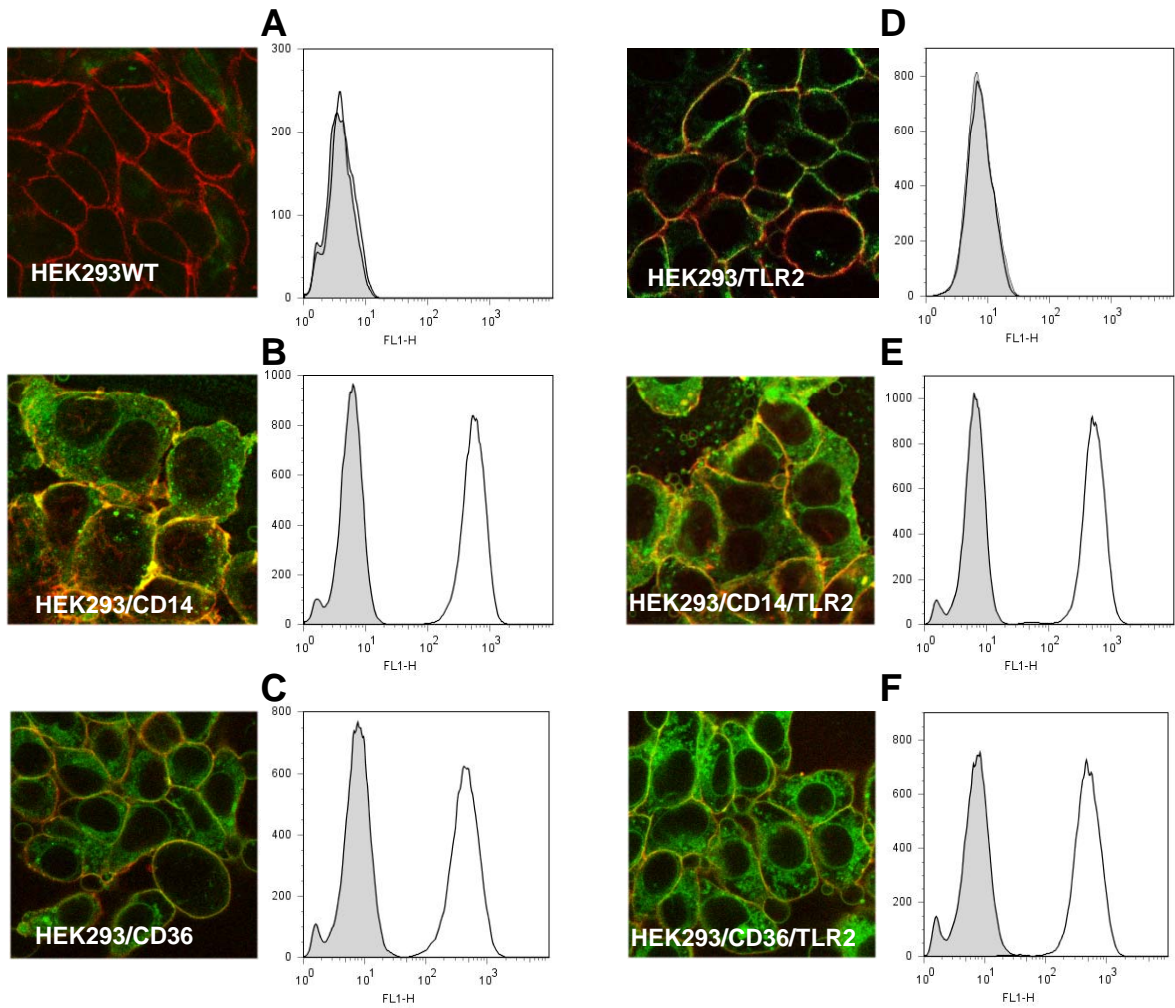


Fig. 8

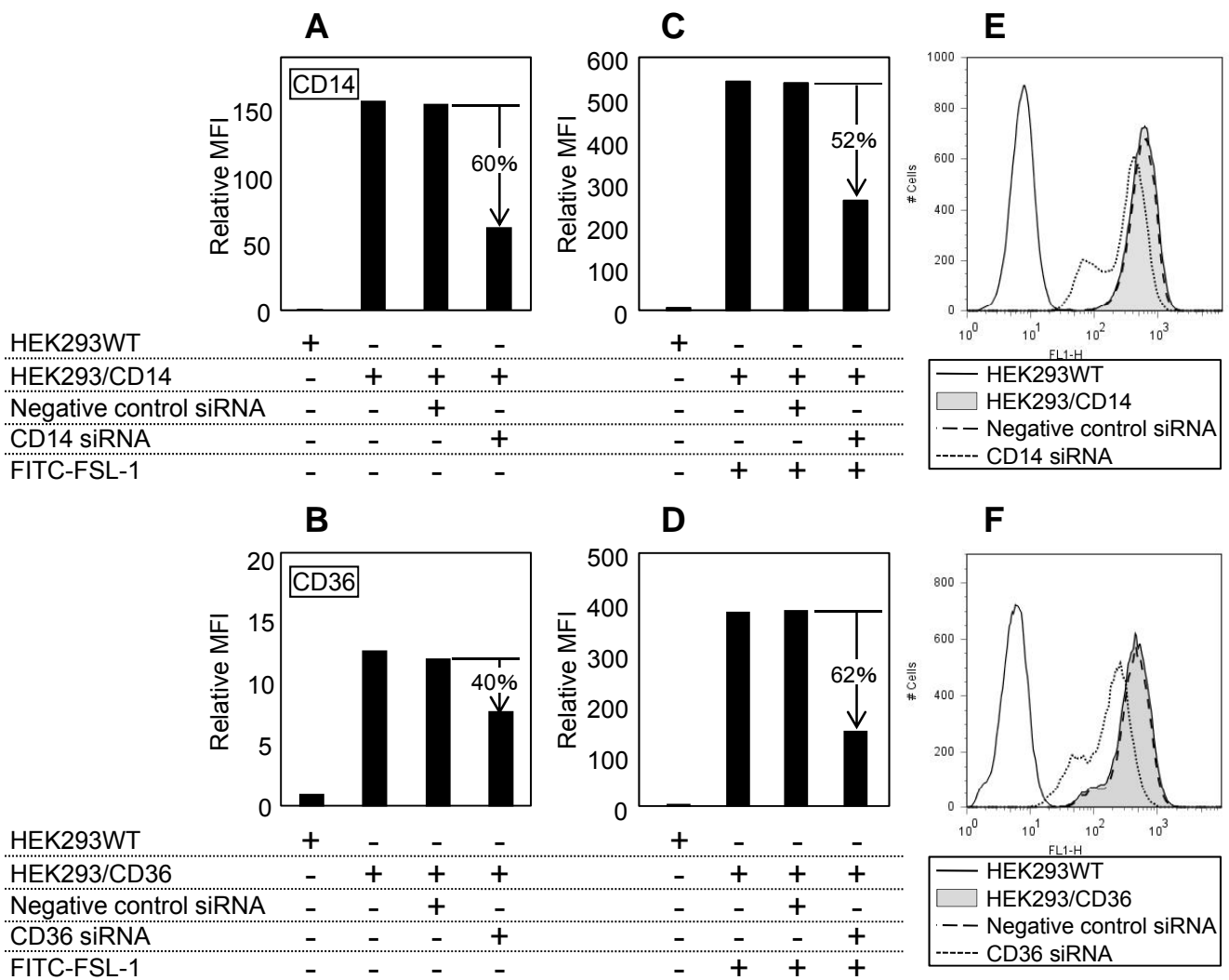


Fig. 9