Studies on Molecular Mechanisms of Placental Infection by *Listeria monocytogenes*

（リステリアモノサイトゲネスによる胎盤感染の分子機構に関する研究）

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CONTENTS

PREFACE

Listeriosis, caused by *Listeria monocytogenes*, is an important zoonotic food-borne disease. *L. monocytogenes* infects humans and various livestock, including cattle, pigs, sheep, and goats via ingestion of contaminated foods (Hernandez-Milian and Payeras-Cifre, 2014). In humans, this pathogen has the ability to cross the intestinal, placental, and blood-brain barriers, leading to gastroenteritis, maternofetal infections, and meningoencephalitis, respectively (Portnoy et al., 1992; Cossart, 2011). Though human listeriosis occurrence is sporadic throughout the world, it can result in severe damage during an outbreak. Especially, newborns, elderly people, pregnant women, and immunocompromised patients are generally at higher risk to acquire listeriosis (Hernandez-Milian and Payeras-Cifre, 2014). Therefore listeriosis is an important zoonotic food-borne disease of significant public health concern.

L. monocytogenes is a facultative intracellular gram-positive bacteria. A key aspect of the virulence of *L. monocytogenes* is its ability to proliferate within professional and non-professional phagocytic host cells by escaping from phagosome (Portnoy et al., 1992; Cossart, 2011). This ability allows bacterial replication in cytoplasm and cell-to-cell spread.

In pregnant women *L. monocytogenes* occasionally causes spontaneous abortion and stillbirths. Although detailed mechanism of infectious abortion is still unclear, it is well known that bacterial infection to placenta is required to induce abortion. Additionally, recent studies reported that abortion induced by bacterial infection was associated with cell death of placental immune cells, the trophoblast giant (TG) cells in mice (Kim et al., 2005; Tachibana et al., 2008).

TG cells are mouse placental cells that establish direct contact with endometrial tissues during implantation (Muntener and Hsu, 1977). TG cells play a crucial role in implantation, in the remodeling of the embryonic cavity and preventing maternal blood flow to the implantation site (Hu and Cross, 2010). TG cells also exhibit phagocytic activity like macrophages toward microorganisms including abortion-inducible bacteria such as *Brucella abortus* (Welsh and Enders, 1987; Amarante-Paffaro et al., 2004; Albieri et al., 2005; Kim et al., 2005). Moreover, my previous study showed that TG cells had the ability to phagocytose *L. monocytogenes* and *L. monocytogenes* replication was observed in TG cells (Watanabe et al., 2010).

Taken together, these findings suggest TG cells seem to be a key factor in infectious abortion. However, detailed function of TG cells in the abortion caused by *L. monocytogenes* infection is still unclear. To reveal roles of TG cells in infectious abortion by *L. monocytogenes*, in this study I investigated the following subjects:

1. Search of receptors responsible for bacterial uptake in TG cells. (Chapter 1)
2. Analysis of intracellular signaling dynamics of TG cells in infectious abortion. (Chapter 2)

CHAPTER 1

Mannose receptor, C type 1 contributes to bacterial uptake by placental trophoblast giant cells.

INTRODUCTION

Trophoblast giant (TG) cells are placental cells that establish direct contact with endometrial tissues during implantation (Muntener and Hsu, 1977). TG cells also show macrophage-like activity against microorganisms (Amarante-Paffaro et al., 2004; Albieri et al., 2005). Additionally, other report showed that TG cells also utilize their phagocytic activity to ingest intracellular bacteria such as *Brucella abortus* and *Listeria monocytogenes* that cause abortion in pregnant animals (Watanabe et al., 2009). However, details of molecular receptors and mechanisms for uptake by TG cells have not been clarified. In this study, I examined the receptors important for uptake of bacteria using a TG cell line differentiated from a trophoblast stem (TS) cell line *in vitro*.

In mammals, the first line of the defense mechanism includes antigen recognition by pattern recognition receptors (PRRs) such as Toll-like receptor (TLR), scavenger receptor (SR), and mannose receptor (MR) (Areschoug and Gordon, 2009; Gazi and Martinez-Pomares, 2009; Kumar et al., 2009). In my previous study, I demonstrated that TLR2 and class B scavenger receptor type 1 (SR-B1) play important roles in the uptake of abortion-inducible bacteria such as *L. monocytogenes* and *B. abortus* by TG cells

(Watanabe et al., 2010). However, knockdown of TLR2 and SR-B1 failed to reduce completely the uptake of *L. monocytogenes* and *B. abortus* by TG cells. In addition, the uptake of abortion-uninducible bacteria such as *Escherichia coli* was independent of those receptors. These findings indicated that common receptors are involved in the uptake of both abortion-inducible and abortion-uninducible bacteria in TG cells.

In this study, I focused on the mannose receptor, C type 1 (MRC1). MRC1 is a PRR that recognizes mannose, fucose, and N-acetylglucosamine sugar residues on the surface of microorganisms (Largent et al., 1984). To clarify whether MRC1 is a common receptor for uptake of abortion-inducible and abortion-uninducible bacteria, I investigated the involvement of MRC1 in the uptake of abortion-inducible gram-positive bacterium (*L. monocytogenes*) and abortion-uninducible gram-positive and -negative bacteria (*Bacillus subtilis* and *E. coli*, respectively) by TG cells. Knockdown of MRC1 inhibited the uptake of all of these bacteria. Blocking of MRC1 by MRC1 ligands also reduced the uptake of those bacteria, suggesting that MRC1 plays a fundamental role in the uptake of various bacteria by TG cells.

MATERIALS AND METHODS

Bacterial strains

L. monocytogenes EGD, *E. coli* DH5α, *E. coli* JM109, and *B. subtilis* 168 were used in this study. Bacterial strains were maintained as frozen glycerol stocks. *L. monocytogenes* EGD was cultured in brain heart infusion (BHI) broth (Becton Dickinson, Franklin Lakes, NJ, USA) or on BHI broth containing 1.5% agar (Wako, Osaka, Japan). *E. coli* DH5α, *E. coli* JM109, and *B. subtilis* 168 were cultured in Luria–Bertani (LB) broth (MO BIO Laboratories, Inc., Carlsbad, CA, USA) or LB broth containing 1.5% agar.

Cell culture

A mouse TS cell line was a gift from Dr. Tanaka (Tanaka et al., 1998; Watanabe et al., 2008). TS cells were cultured in mixed medium (TS medium : mouse embryonic fibroblast-conditioned medium = 3 : 7) containing 25 ng/ml fibroblast growth factor 4 (TOYOBO, Osaka, Japan) and 1 μg/ml heparin (Sigma, St. Louis, MO, USA) as described previously (Watanabe et al., 2008). TS medium was prepared by adding 20% fetal bovine serum (FBS), 1 mM sodium pyruvate, 100 μM β-mercaptoethanol, and 2 mM L-glutamine to RPMI 1640 medium. To induce differentiation of TS cells to TG cells, TS cells were cultured in the TS

medium alone for 3 days at 37 °C, 5% CO_2. RAW 264.7 cells were cultured in RPMI 1640 containing 10% FBS. TG or RAW 264.7 cells were seeded (1–2 × 10^5 per well) in 48-well or 12-well (4–8 × 10^5 per well) tissue culture plates.

Immunoblotting

TG or RAW 264.7 cells were washed twice with phosphate-buffered saline (PBS) and lysed in lysis buffer (ice-cold PBS containing 1% Triton X-100, 1 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, 100 mM sodium fluoride, and 1× Halt Protease Inhibitor Cocktail Kit (Thermo Fisher Science, Rockford, IL, USA) at 4 °C for 30 min, and sonicated for 10 s, three times. The cell lysates were centrifuged (16,000 × *g*, 4 °C, 20 min) and supernatants were collected. Protein concentrations were determined using Bio-Rad Protein Assay (Bio-Rad, Richmond, CA, USA). After separating 300 ng of each protein by SDS-PAGE with 10% polyacrylamide gels, the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% nonfat dry milk in Tris-buffered saline (TBS) at room temperature for 2 h, membranes were incubated with anti-mouse MRC1 rat monoclonal antibody (1 : 200; R&D Systems Inc., Minneapolis, MN, USA) or anti-mouse β-actin antibody (Sigma) at 4 °C overnight. After washing with TBS containing 0.02% Tween 20, membranes were incubated

with horseradish peroxidase-conjugated secondary antibody (0.01 μg/ml) at room temperature for 1 h and immunoreactions were visualized using the enhanced chemiluminescence detection system (GE Healthcare Life Science, Little Chalfont, UK).

RNA isolation and reverse transcription (RT)-PCR

Total RNA of TG cells was isolated using the RNAeasy Plus Mini Kit (Qiagen, Hilden, Germany). Purified RNA samples were stored at −80 °C prior to use. The RNA was quantified by absorption at 260 nm using the SmartSpec3000 spectrophotometer (Bio-Rad). RT-PCR was carried out using the SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen, Carlsbad, CA, USA). The sequences of primers were as follows. MRC1: 5′ -GCAAATGGAGCCGTCTGTGC-3′ and 5′ -CTCGTGGATCTCCGTGACAC-3′ (Bhatia et al., 2011), β-actin: 5′ -TGGAATCCTGTGGCATCCATGAAAC-3′ and 5′ -TAAAACGCAGCTCAGTAACAGTCCG-3′ (Nomura et al., 2002).

Efficiency of bacterial internalization and replication

Bacterial internalization assays were performed in a similar manner to that in a previous study (Watanabe et al., 2008). Bacterial strains were deposited onto TG cells cultured in 48-well plates with TS medium by centrifugation (150 × *g*, 10 min, room temperature). To

measure bacterial internalization efficiency after 30 min of incubation at 37 °C, the cells were washed once with TS medium and then incubated in a medium containing gentamicin (30 μg/ml) for 30 min. The cells were then washed three times with PBS and lysed with cold distilled water. Colony-forming unit values were determined by serial dilution on BHI or LB plates. Recombinant interferon-γ (IFN-γ) (Cedarlane Laboratories, ON, Canada) was added 24 h before infection. Mannan from Saccharomyces cerevisiae (Sigma), D-mannose (Sigma), L-fucose (Sigma), or D-galactose (Sigma) were added 15 min before infection.

Small interfering RNA (siRNA) experiment

The siRNA duplexes used for silencing mouse MRC1 (target sequence: 5′ -CAGCATGTGTTTCAAACTGTA-3′) and AllStars Negative Control siRNA were purchased from Qiagen. TG cells were transiently transfected using Lipofectamine RNAiMAX (Invitrogen) with or without siRNAs at a final concentration of 36 nM.

Measurement of phagolysosome maturation

Bacterial internalization assays were performed in a similar manner to that in a previous study (Watanabe et al., 2008). *E. coli* JM109 was introduced with pAcGFP1 (*E. coli* GFP+). Plasmid pAcGFP1 was purchased from Clontech (Mountain View, CA, USA). *E. coli* GFP+

was deposited onto TG cells on coverslips by centrifugation (150 × *g*, 10 min, room temperature), incubated at 37 °C for 30 min, and then incubated in TS medium containing gentamicin (30 μg/ml) for 0.5, 1, 2, and 4 h. The samples were washed twice with PBS. For vital staining of lysosomes, cells were incubated with LysoTracker red (Molecular Probes, Eugene, OR, USA) at 37 °C for 30 min. Cells were washed with PBS and fixed with 4% paraformaldehyde. Fluorescent images were obtained by the FluoView FV100 confocal laser scanning microscope (Olympus, Tokyo, Japan).

Microarray

Total RNA was extracted from TS or TG cells using TRIzol reagent (Invitrogen). The RNA was quantified by absorbance at 260 nm and the purity was assessed by the 260/280 nm ration. GENECHIP (Mouse Genome 430 2.0) and kits for cDNA synthesis, biotin-labeled cRNA synthesis, fragmentation of cRNA, and hybridization were obtained from Affymetrix (Santa Clara, CA, USA). All experiments were performed according to the manufacturer's recommendations. The results were obtained from three GENECHIPs.

Statistical analyses

Statistical analyses were performed using Student's *t*-test. Statistically significant

differences compared with control are indicated by asterisks (*, $P < 0.01$, **, $P < 0.05$). Data are the averages of triplicate samples from three identical experiments and the error bars represent standard deviations.

RESULTS

MRC1 is involved in bacterial uptake by TG cells

In my previous study, I demonstrated that TLR2 and SR-B1 play a central role in the uptake of the abortion-inducible bacteria *L. monocytogenes* and *B. abortus* (Watanabe et al., 2010). However, knockdown of these receptors failed to block the uptake completely. In addition, the uptake of abortion-uninducible bacteria such as *E. coli* was independent of TLR2 and SR-B1. These results indicated that other receptors were involved in the uptake of those bacteria. Since the uptake of *E. coli* was observed at the same levels in TG cells and immature TS cells, PRRs expressed at the same levels in TG and TS cells were searched from the data of microarray analysis (Table 1). MRC1 was one of the PRRs expressed in TG and TS cells. To examine whether MRC1 is expressed in TG cells, the expression was measured by immunoblotting and RT-PCR. MRC1 was expressed in TG cells at levels comparable to those of a MRC1-positive cell, RAW264.7 (Fig. 1A and B). To examine the influence of MRC1 on the uptake of bacteria, the expression of MRC1 in TG cells was blocked with MRC1-specific siRNA (Fig. 1A and B). Uptake of the abortion-inducible bacterium *L. monocytogenes* by TG cells was reduced significantly by siRNA (Fig. 1C). On the other hand, no reduction was observed in *L. monocytogenes* internalization in TG cells

transfected with AllStars Negative Control siRNA. Uptake of abortion-uninducible bacteria such as *E. coli* and *B. subtilis* by TG cells was also decreased in a similar manner to that of *L. monocytogenes*. These results suggest that MRC1 is a receptor for the uptake not only of abortion-inducible bacteria in TG cells but of abortion-uninducible bacteria.

MRC1 ligands inhibit bacterial uptake by TG cells

It was reported that microbial phagocytic activity via MRC1 is allayed by MRC1 ligands (Miller et al., 2008; Macedo-Ramos et al., 2011). To confirm the involvement of MRC1 in the uptake of bacteria by TG cells, TG cells were treated with MRC1 ligands and infected with various bacteria. The MRC1 ligands mannan (0, 3, 15, and 30 mg/ml), D-mannose (0, 50, 100, and 200 mM), and L-fucose (0, 50, 100, and 200 mM) were selected. Treatment of TG cells with each ligand reduced the uptake of *L. monocytogenes*, *E. coli*, and *B. subtilis* in a dose-dependent manner (Fig. 2). In contrast, treatment with D-galactose (0, 50, 100, and 200 mM), a sugar not recognized by MRC1, showed no significant effect on uptake of those bacteria. These findings suggest that TG cells recognize specific sugar chains on the bacterial surface and ingest them using MRC1.

IFN-γ promotes bacterial uptake by TG cells

It has been reported that the expression of cell surface receptors is regulated by IFN-γ (Faure et al., 2001). Indeed, I demonstrated that IFN-γ augments the uptake of *L. monocytogenes* by TG cells (Watanabe et al., 2010). Next, I evaluated whether IFN-γ would affect the uptake of *L. monocytogenes*, *E. coli*, and *B. subtilis* by TG cells. TG cells were treated with IFN-γ for 24 h (0, 200, 400, and 1,000 units/ml) and internalization efficiency was measured. IFN-γ treatment increased the efficiency of *L. monocytogenes* uptake by TG cells in a concentration-dependent manner (Fig. 3B). Unexpectedly, IFN-γ failed to increase the uptake of *E. coli* and *B. subtilis*. To estimate the influence of IFN-γ on cell surface receptors, the levels of mRNA in MRC1 were measured by RT-PCR. IFN-γ treatment decreased the transcription levels of MRC1 (Fig. 3A). These findings suggest that decreased MRC1 expression levels inhibit the augmentation of *E. coli* and *B. subtilis* uptake by TG cells.

Maturation of lysophagosomes

To examine whether the bacteria internalized through MRC1 are killed by the phagocytic activity of TG cells, phagolysosome maturation was measured using LysoTracker red. Since *L. monocytogenes* shows strong cytotoxicity in TG cells and it is difficult to measure the maturation of phagolysosome, the maturation was observed using *E.*

coli. In TG cells, 20–40% of internalized *E. coli* were colocalized with LysoTracker red at 0.5–4 h post-infection (Fig. 4A and B). In contrast, in MRC1-knockdown TG cells, colocalization was slightly but efficiently increased. The same effect was observed when TG cells were treated with IFN-γ, and colocalization of *E. coli* and LysoTracker red was slightly augmented (Fig. 4C). These results indicate that the uptake of *E. coli* by MRC1 delays the maturation of phagolysosomes.

DISCUSSION

During pregnancy, maternal immune function is strictly controlled and immune tolerance is induced (Warning et al., 2011). There is little information about the mechanisms by which the fetus is protected from infectious microorganisms under the condition of immunosuppression at the fetal–maternal interface. TG cells are present in the placental labyrinth zone and play important roles in acquiring nutrition and space for embryonic attachment and development in the endometrium, which are key to a successful pregnancy (Welsh and Enders, 1987; Bevilacqua and Abrahamsohn, 1988, 1994; Amarante-Paffaro et al., 2004; Hu and Cross, 2010). TG cells also exhibit phagocytic activity toward foreign antigens in a similar manner to macrophages (Welsh and Enders, 1987; Amarante-Paffaro et al., 2004; Albieri et al., 2005). However, the molecular mechanisms of recognition and uptake of bacteria in TG cells remain unclear.

I previously reported that TLR2 and SR-B1 were involved in the uptake of abortion-inducible intracellular bacteria such as *B. abortus* and *L. monocytogenes* by TG cells (Watanabe et al., 2010). On the other hand, TLR2 and SR-B1 were not involved in the uptake of the abortion-uninducible bacterium *E. coli*. The uptake of *E. coli* was observed in

TG cells and immature TS cells at the same levels (Watanabe et al., 2010). Knockdown of TLR2 and SR-B1 resulted in partial reduction of uptake by TG cells. These results suggest the existence of other receptors that recognize and ingest various bacteria in TG and TS cells. Uptake of bacteria is controlled by a variety of receptors such as the TLR family, the SR family, MR, DEC-205-associated C-type lectin-1 (DCL-1), and dendritic cell-specific ICAM grabbing non-integrin (DC-SIGN) (Hsu et al., 1996; Faure et al., 2001; Kerrigan and Brown, 2009). Among these receptors, DCL-1 and DC-SIGN were not expressed in TG cells (Table 1). In the present study I focused on MRC1, since MRC1 was expressed in both TG and TS cells (Table 1, Fig. 1A and B). MRC1 is expressed on lymphatic and hepatic epithelia, kidney mesangial cells and retinal pigment epithelium (Shepherd et al., 1991; Lew et al., 1994; Linehan et al., 1999). This expression is also observed in a subpopulation of murine dendritic cells (McKenzie et al., 2007). MRC1, a C-type lectin, is known as a PRR. MRC1 recognizes mannose, fucose, and N-acetylglucosamine sugar residues on the surface of these microbes and is involved in the uptake of *Mycobacterium tuberculosis*, *Klebsiella pneumoniae*, and *Streptococcus pneumoniae* (Ezekowitz et al., 1991; Marodi et al., 1991; Schlesinger, 1993; O'Riordan et al., 1995; Chakraborty et al., 2001; Zamze et al., 2002). I demonstrated in this study that the inhibition of MRC1 by siRNA or MRC1 ligands decreases the uptake of both of abortion-inducible and abortion-uninducible bacteria (Fig.

1C and 2), indicating that MRC1 is an important receptor for the recognition and uptake of various bacteria by TG cells.

IFN-γ is one of the key factors related to infectious abortion. Previous study showed that anti-IFN-γ antibody decreased the induction of abortion caused by *B. abortus* in mouse (Kim et al., 2005). In addition, I demonstrated that IFN-γ enhanced the uptake of *L. monocytogenes* (Fig. 3B) and *B. abortus* by TG cells (Watanabe et al., 2010). It was reported that IFN-γ controls cell surface receptors (Faure et al., 2001). However, TLR2 and SR-B1 expression in TG cells was not regulated by IFN-γ (Watanabe et al., 2010), suggesting that IFN-γ facilitates the internalization step of bacteria, but not the binding step. It was reported that IFN-γ promoted bacterial internalization through extracellular-regulated kinase (ERK) 1/2 pathway in gut epithelial cells (Smyth et al., 2012). Although the expression of MRC1 is decreased by IFN-γ (Fig. 3A), *L. monocytogenes* may bind to TG cells through TLR2 or SR-B1, followed by up-regulation of uptake by IFN-γ. In contrast, the uptake of *E. coli* and *B. subtilis* by TG cells was not augmented by IFN-γ (Fig. 3B). It was reported that IFN-γ decreased MRC1 expression in murine macrophage (Harris et al., 1992). Indeed, the transcription level of MRC1 in TG cells was also decreased by IFN-γ treatment (Fig. 3A). Although the expression of MRC1 was decreased by IFN-γ, the uptake of *E. coli* and *B.*

subtilis was not impaired. These results imply that IFN-γ augments the internalization step of these bacteria, but the augmentation is inhibited by the reduction in MRC1 expression, since these bacteria are thought to be bound to TG cells mainly through MRC1.

Knockdown of MRC1 or IFN-γ treatment increased phagosomal maturation (Fig. 4), indicating that internalization of bacteria by MRC1 delays the maturation of phagolysosomes. This finding is consistent with other reports that the uptake of *M. tuberculosis* and *Mycobacterium avium* by MR limits phagosome maturation in murine bone marrow-derived macrophages and human macrophages (Kang et al., 2005; Sweet et al., 2010). For abortion-inducible bacteria such as *L. monocytogenes* and *B. abortus*, escaping from phagolysosomes is key to their pathogenicity in the placenta (O'Callaghan et al., 1999; Foulongne et al., 2000; Boschiroli et al., 2001, 2002; Bakardjiev et al., 2005; Le Monnier et al., 2007). Internalization to TG cells by MRC1 followed by a delay in phagolysosomal maturation may result in the escape of these bacteria. Unfortunately, *L. monocytogenes* exhibits strong cytotoxicity and it was impossible to identify the relationship between phagolysosome maturation and MRC1 for *L. monocytogenes* infection. In addition, there is little information about signal transductions downstream of MR, since it lacks any known motifs on its cytoplasmic tail and therefore is presumed to interact with unknown signaling

proteins. Further study is needed to clarify the relationship between infectious abortion and MRC1.

Overall, my findings in this study suggest that MRC1 is a fundamental receptor that plays an important role in the recognition and uptake of various bacteria. Although there are increasing numbers of reports maintaining that bacterial infection causes abortion or sterility in animals and humans (Low and Donachie, 1997; Bourke et al., 1998; Boschiroli et al., 2001; Lecuit, 2007), there is little information on how the fetus is protected from infectious microorganisms. Therefore, my finding may help to reveal the general mechanism of the placental immune system.

Table 1. Microarray analysis of TS and TG cells.

Gene ID	Gene title	TS signal	TG signal	TG/TS ratio
Mm. 282242	Scavenger receptor class B, member 1 (SR-B1)	659.17	2375.23	2.76
Mm. 87596	Toll-like receptor 2 (TLR2)	27.75	50.30	1.81
Mm. 2019	mannose receptor, C type 1 (MRC1)	27.05	27.6	1.02
Mm. 52281	CD209e antigen (DC-SIGN)	1.25	1.25	1.00
Mm. 2272281	CD302 antigen (DCL-1)	17.05	8.65	0.50

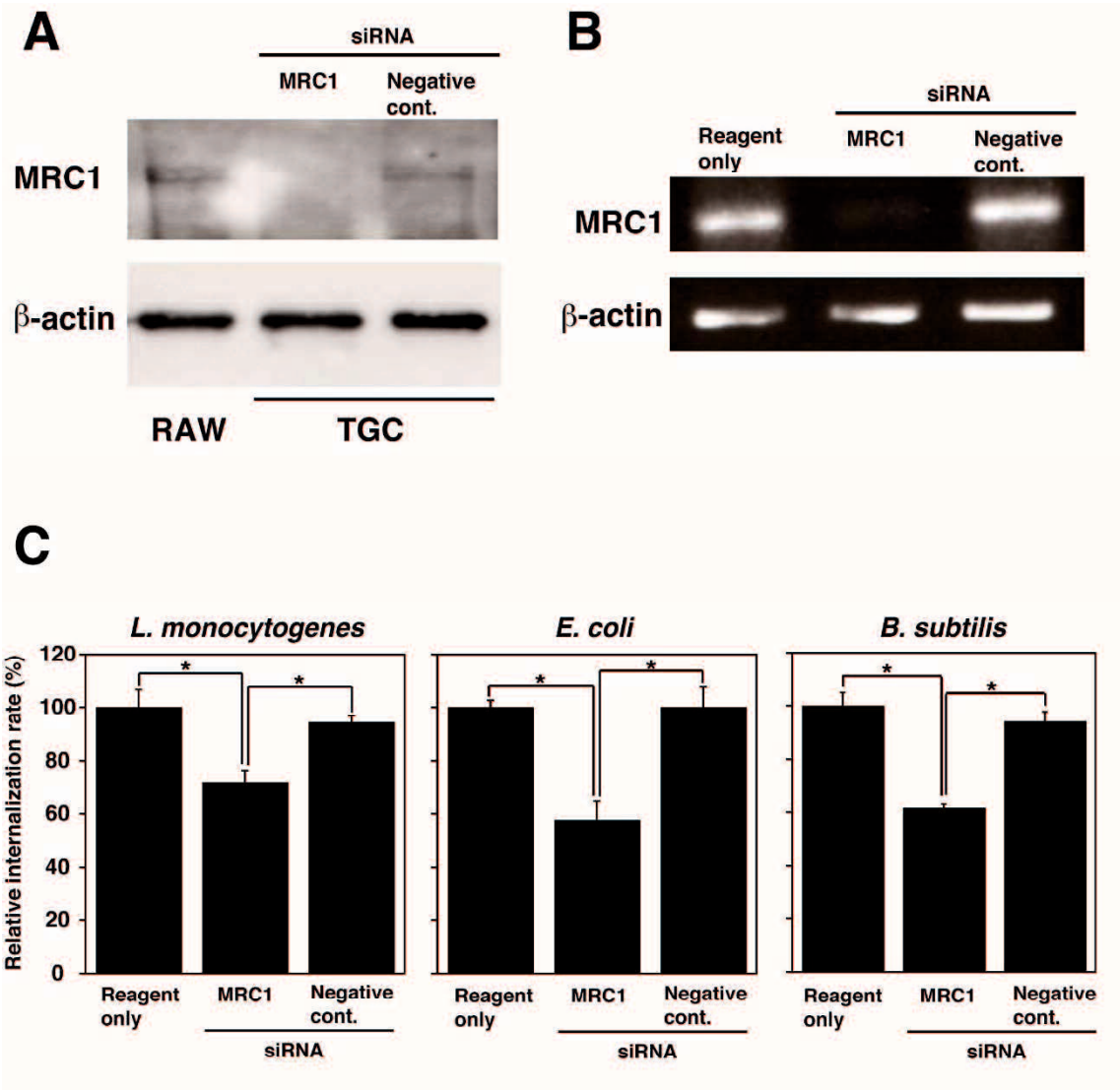

Fig. 1. MRC1 depletion inhibits bacterial internalization in TG cells.

(A) TG cells were treated for 48 h with siRNA (targeting MRC1) or AllStars Negative Control siRNA. MRC1 expression in TG cells (TGC) and RAW 264.7 cells (RAW) was analyzed by immunoblotting. β-actin was used as an internal control. (B) TG cells were treated for 48 h with siRNA (targeting MRC1), transfection reagent, or AllStars Negative Control siRNA. Transcription efficiency of MRC1 and β-actin was

monitored by RT-PCR. (C) Bacterial internalization in MRC1-depleted TG cells was measured by bacterial internalization assay. All bacteria were ingested by TG cells treated with only reagent. Data represent the averages and standard deviations of triplicate samples from three identical experiments. Statistically significant differences of bacterial internalization in depleted TG cells and reagent or negative control are indicated by asterisks (*, $P < 0.01$).

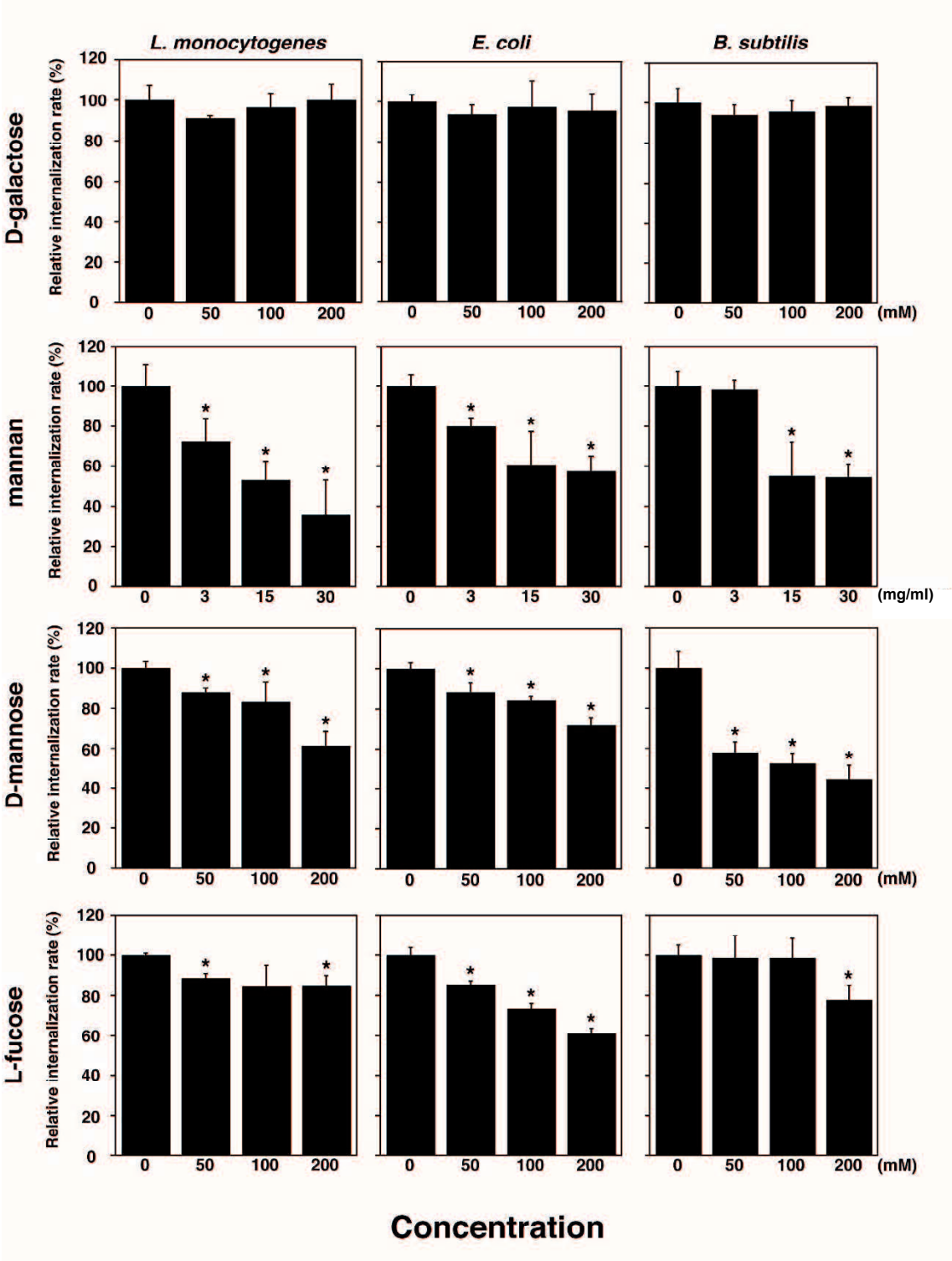

Fig. 2. MRC1 ligands block bacterial uptake efficiency of TG cells.

TG cells were treated with mannan, D-mannose or L-fucose at the indicated

concentrations for 15 min. Treated TG cells were infected with *L*. *monocytogenes*, *E*. *coli*, or *B*. *subtilis*. Bacterial internalization in TG cells treated with each ligand was measured by a bacterial internalization assay. All bacteria were ingested by TG cells without treatment. D-Galactose was used as control. Data represent the averages and standard deviations of triplicate samples from three identical experiments. Statistically significant differences between control (with no ligand treatment) and treated groups are indicated by asterisks (*, $P < 0.01$).

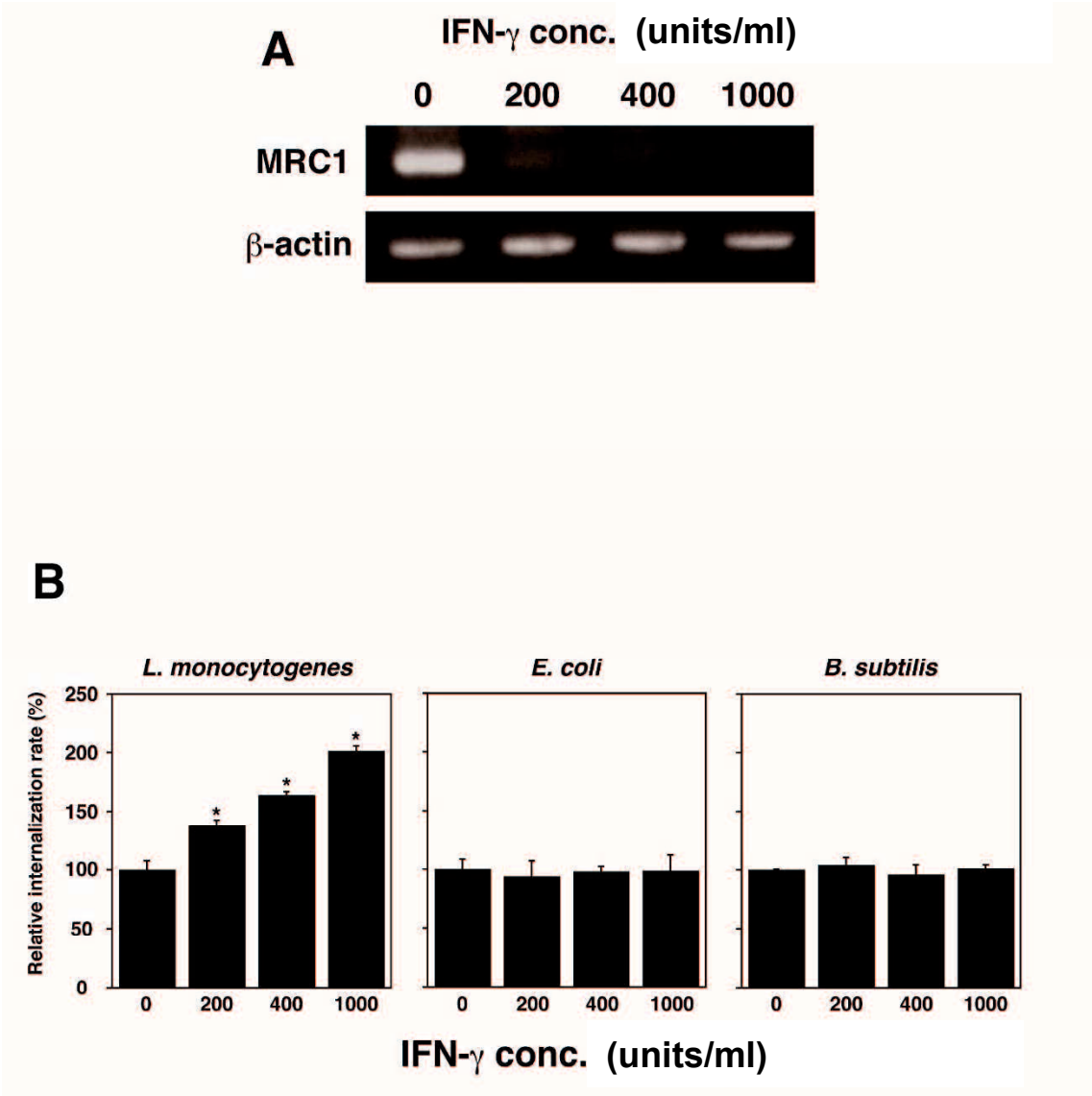

Fig. 3. IFN-γ regulates transcription efficiency of MRC1 in TG cells.

(A) TG cells were treated with IFN-γ at the indicated concentrations (0, 200, 400, and 1,000 units/ml) for 24 h. The transcription efficiency of MRC1 was monitored by RT-PCR. (B) TG cells treated with the indicated concentration of IFN-γ for 24 h were infected with *L. monocytogenes*, *E. coli*, or *B. subtilis*. Bacterial internalization in TG

cells treated with IFN-γ was measured by bacterial internalization assay. All bacteria were ingested by TG cells without IFN-γ treatment. Data represent the averages and standard deviations of triplicate samples from three identical experiments. Statistically significant differences between control (without IFN-γ treatment) and treated groups are indicated by asterisks (*, $P < 0.01$).

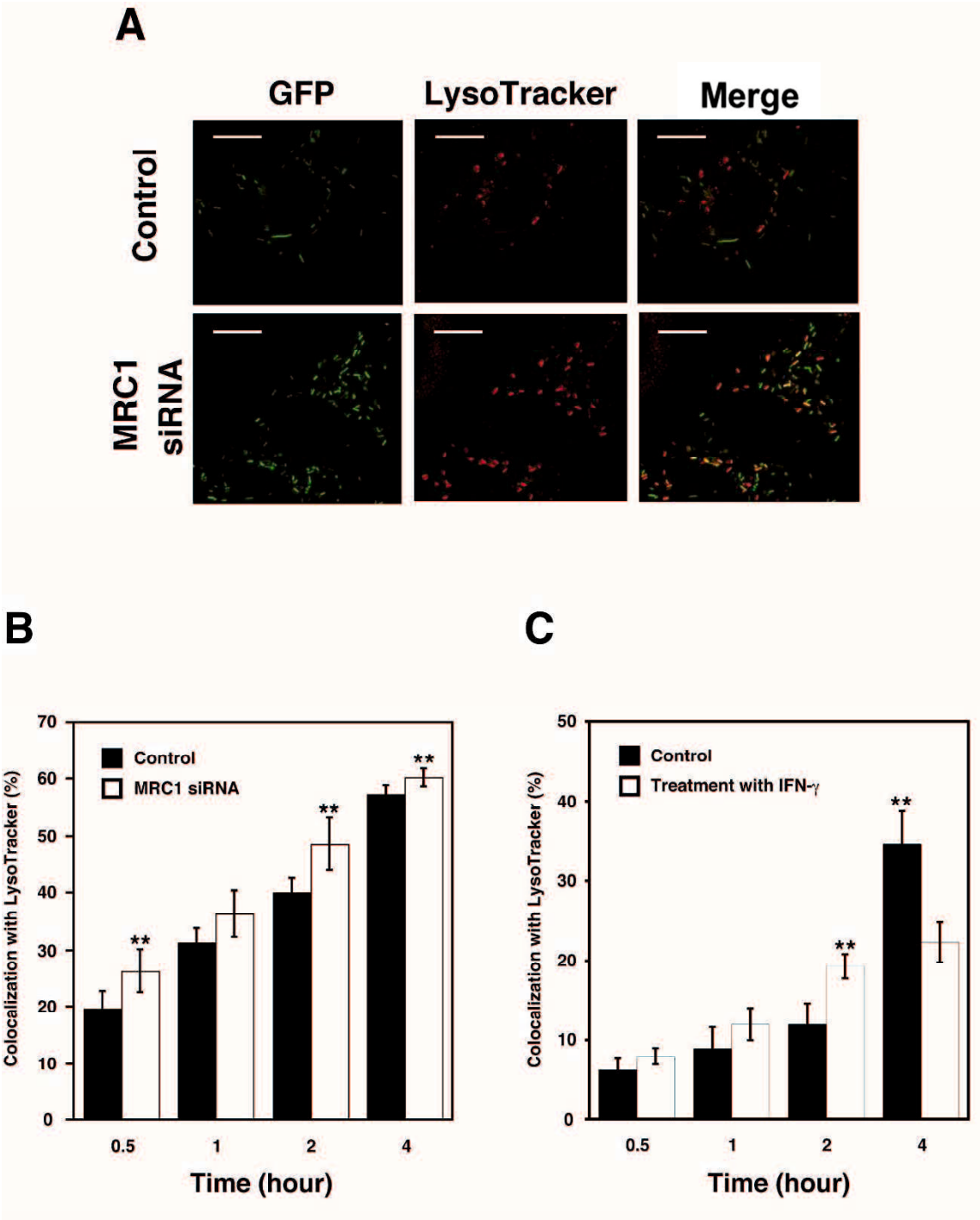

Fig. 4. MRC1 depletion upregulates maturation of phagolysosomes.

(A) TG cells were treated for 48 h with small interfering RNA (siRNA) (targeting

MRC1). After siRNA treatment, TG cells were infected with *E. coli* GFP+. For vital staining of lysosomes, cells were incubated for 30 min with LysoTracker red. Fluorescent images showing TG cells phagocytosing *E. coli* GFP+ after 2 h of bacterial infection were obtained using the FluoView FV100 confocal laser scanning microscope, employing a ×100 objective at a final magnification of ×1,000. Scale bars: 20 μm. (B) TG cells were treated for 48 h with siRNA (targeting MRC1). After siRNA treatment, TG cells were infected with *E. coli* GFP+. For vital staining of lysosomes, cells were incubated for 30 min with LysoTracker red. Colocalization of *E. coli* GFP+ with LysoTracker red (%) in one field containing more than 100 *E. coli* GFP+ in TG cells was calculated as (number of *E. coli* GFP+ colocalized with LysoTracker red)/(total number of internalized *E. coli* GFP+ in one field) × 100. Data represent the averages and standard deviations of triplicate fields from three identical experiments. Statistically significant differences between control and TG cells treated with siRNA (targeting MRC1) are indicated by asterisks (**, $P < 0.05$). (C) TG cells were treated for 24 h with 1,000 units/ml IFN-γ. After IFN-γ treatment, TG cells were infected with *E. coli* GFP+. Treated TG cells were incubated for 30 min with LysoTracker red. Colocalization with LysoTracker red (%) in one field containing more than 100 *E. coli* GFP+ in TG cells was calculated. Data represent the averages and standard deviations of triplicate fields from three identical experiments. Statistically significant differences between control

and TG cells treated with IFN-γ are indicated by asterisks (**, $P < 0.05$).

CHAPTER 2

Inactivation of the MAPK signaling pathway by *Listeria monocytogenes* infection promotes trophoblast giant cell death.

INTRODUCTION

Human listeriosis is a food-borne disease resulting from the ingestion of contaminated food, such as dairy products, vegetables, raw seafood, poultry, and processed meat (Hernandez-Milian and Payeras-Cifre, 2014). *Listeria monocytogenes* is the Gram-positive bacterium and causative agent of listeriosis. In humans, listeriosis causes gastroenteritis, maternofetal infections, and meningoencephalitis due to *L. monocytogenes* ability to cross the blood–brain, placental, and intestinal barriers. An important virulence feature of *L. monocytogenes* is its ability to escape from the killing mechanisms of phagocytic host cells, such as macrophages (Stavru et al., 2011a; Ribet and Cossart, 2015).

Pregnancy causes a widely suppression of the adaptive immune system, characterized by a decreased cell-mediated immunity and the suppressed response of cytotoxic T cells (Gluhovschi et al., 2015). Maternal rejection of the fetus is prevented by the immunosuppressed state; however, it has the unexpected effect of increasing maternal susceptibility to abortion-inducing pathogens (Dhama et al., 2015). *L. monocytogenes* is an intracellular pathogen and its immunity is principally mediated by cellular immune

responses (Parmer, 2004). In previous studies, the abortion was observed in pregnant mouse model infected with abortion-inducing bacteria, such as *L. monocytogenes* and *Brucella abortus* (Kim et al., 2005; Tachibana et al., 2008, 2011). In *Brucella* infection, compared to other organs, a large amount of bacterial colonization was found in the placenta, especially in the placental trophoblast giant (TG) cells. In contrast, an intracellular replication-defective mutant failed to induce abortion in a pregnant mouse model (Kim et al., 2005). In addition, infection of *B. abortus* induced a transient increase in interferon-γ (IFN-γ) levels in pregnant mice. Furthermore, this transient IFN-γ production leads to infectious abortion, and depletion of IFN-γ by neutralization inhibits infectious abortion (Kim et al., 2005). These reports of *B. abortus* infection imply that bacterial internalization and intracellular replication in TG cells are both key aspects in abortion and that TG cells are closely associated with the evasion of maternal immune rejection.

TG cells are essential for the establishment of pregnancy. TG cells are polyploid cells differentiated from trophoblast stem (TS) cells. TG cells have diverse functions that are crucial for implantation and subsequent placental function such as vasculature remodeling and uterine immune system (Hu and Cross, 2010). They form the fetal

component of the placenta (Parast et al., 2001). In particular, TG cells regulate maternal spiral artery remodeling and maternal blood flow into the placenta in mice (Hu and Cross, 2010). TG cells in the mouse placenta are analogous to extravillous cytotrophoblast (EVT) cells in the human placenta (Baczyk et al., 2004). *L. monocytogenes* and *Toxoplasma gondii* enter into EVT cells preferentially in primary human placental organ cultures (Robbins et al., 2010, 2012). Trophoblast cells show phagocytic feature. Trophoblast cells phagocytose stroma and uterine epithelial cells and invade maternal tissue during implantation (Welsh and Enders, 1987). Several of the mechanisms contributed to phagocytic ability of trophoblast cells have been published (Drake and Rodger, 1987), however, the detail process is still unclear. Another report showed that trophoblast cells have the ability of bacterial uptake and that IFN-γ treatment enhaces this activity (Amarante-Paffaro et al., 2004). Therefore, trophoblast cells may have phagocytic activity against pathogenic agents in a same way of macrophages. These studies suggest that trophoblast cells play roles in the placental defense system as well as in the development and maintenance of placenta.

Various cell types, such as dendritic cells (Guzmán et al., 1996), lymphocytes (Merrick et al., 1997), and hepatocytes (Rogers et al., 1996), are induced cell death *in*

vitro and *in vivo* by *L. monocytogenes* infection. The pore-forming toxin listeriolysin O (LLO) plays an important role in cell death induced by *L. monocytogenes*. LLO is the key factor responsible for degradation of the vacuole and escaping into the cytosol. In previous studies, collaborators and I found that reduction of heme oxygenase (HO)-1 expression by *L. monocytogenes* or *B. abortus* infection enhanced infectious abortions *in vivo* and cell death *in vitro* (Tachibana et al., 2008, 2011). HO-1 plays key roles in cytoprotection, antioxidation, and anti-inflammation. The majority of HO-1's physiological functions are associated with its enzymatic activity in heme catabolism (Hegazi et al., 2005; Nakahira et al., 2006). HO-1 deficiency causes an increased pro-inflammatory state and susceptibility to oxidative stress in humans (Yachie et al., 1999). HO-1 deficient mice acquire progressive chronic inflammatory disease (Poss and Tonegawa, 1997a) and express enhanced toxemia caused by lipopolysaccharide administration (Poss and Tonegawa, 1997b). Although the protective characteristics of HO-1 have been studied using various inflammatory models (Lee and Chau, 2002; Chora et al., 2007; Pamplona et al., 2007), the molecular mechanisms, timing, and mode of HO-1 function during disease remains largely unknown.

The mitogen-activated protein kinase (MAPK) signal transduction pathway is one of

the most important regulatory mechanisms in eukaryotic cells and a central signaling cascade that is essential for the host immune response (Krachler et al., 2011). In mammals, there are at least four subfamilies of MAPKs, including the extracellular signal-regulated kinases (ERKs), the c-Jun NH_2-terminal kinases (JNKs), the p38 isoforms (p38s), and ERK5 (Tanoue and Nishida, 2003; Turjanski et al., 2007). These kinases are organized as parallel cascades in which the activation of each component is regulated upstream and downstream by phosphorylation events (Chang and Karin, 2001). As in mouse TG cells, a large amount of phosphorylated MAPK family protein is detected in bovine TG cells. The localization of actin, its associated proteins, and phosphorylated MAPK family proteins, suggests their involvement in TG cell migration in bovine placentomes (Lang et al., 2004).

In this study, I investigated the dephosphorylation of MAPK family proteins during *L. monocytogenes* infection in TG cells. My results suggest that *L. monocytogenes* LLO contributes to the inhibition of MAPK signaling pathway activation and infectious abortion.

MATERIALS AND METHODS

Bacterial Strains

L. monocytogenes strains EGD, Δ*hly*, and Δ*hly*::*hly* (Hara et al., 2007) and *Escherichia coli* DH5α were used in this study. Two isogenic mutants of *L. monocytogenes*, Δ*hly* and Δ*hly*::*hly* were generated from parental strain using the method of homologous recombination and the expression level of LLO was shown in a previous study (Hara et al., 2007). *L. monocytogenes* strains, which were maintained in frozen glycerol stocks, were cultured overnight in brain heart infusion (BHI) broth (Becton Dickinson, Franklin Lakes, NJ, USA) at 37°C with shaking or on BHI broth containing 1.5% agar (Wako, Osaka, Japan) at 37°C under aerobic condition. *E. coli* DH5α was cultured in Luria–Bertani (LB) broth (MO BIO Laboratories, Inc., Carlsbad, CA, USA) or on LB broth containing 1.5% agar.

Cell Culture

A mouse TS cell line was gifted by Dr. Tanaka (Tanaka et al., 1998). Cell culture was done by using the same method described in chapter 1. TS cells were cultured in a mixed medium (TS medium: mouse embryonic fibroblast-conditioned medium = 3 : 7)

supplemented with 25 ng/ml fibroblast growth factor 4 (TOYOBO, Osaka, Japan) and 1 μg/ml heparin (Sigma, St. Louis, MO, USA), as described in chapter 1. TS medium was prepared by adding 20% (v/v) fetal bovine serum (FBS), 1 mM sodium pyruvate, 100 μM β-mercaptoethanol, and 2 mM L-glutamine to RPMI 1640 medium. TS cells were cultured in TS medium alone for 3 days at 37°C in an atmosphere containing 5% CO_2 for inducing cell differentiation of TS cells to TG cells. TG cells were seeded in 48-well (1–2 × 10^5 per well) or 12-well (4–8 × 10^5 per well) tissue culture plates. After differentiation into TG cells, the cells were incubated in RPMI 1640 medium for 24 h before use.

Immunoblotting

Immunoblotting was done by using the modified method described previously in chapter 1. TG cells were washed twice with phosphate-buffered saline (PBS) and lysed in lysis buffer, ice-cold PBS containing 1% (v/v) Triton X-100, 1 mM sodium orthovanadate (SOV), 2 mM phenylmethylsulfonyl fluoride, 100 mM sodium fluoride, and 1× Halt Protease Inhibitor Cocktail Kit (Thermo Fisher Science, Rockford, IL, USA) at 4°C for 30 min, and sonicated three times for 10 s each. The supernatants of cell lysates were collected by centrifugation (16,000 × *g*, 4°C, 20 min). Protein

concentrations were measured using by the Bio-Rad Protein Assay (Bio-Rad, Richmond, CA, USA). After separating 300 ng of each protein by SDS-PAGE using 12% polyacrylamide gels, the proteins were electrically transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After membranes were blocked for 2 h with 5% (w/v) non-fat dry milk in Tris-buffered saline (TBS) at room temperature, membranes were incubated with anti-mouse Phospho-c-Jun rabbit monoclonal antibody (1:1,000; Cell Signaling Technology, Danvers, MA, USA), anti-mouse c-Jun rabbit monoclonal antibody (1:1,000; Cell Signaling Technology), anti-mouse Phospho-p38 MAPK rabbit monoclonal antibody (1:1,000; Cell Signaling Technology), anti-mouse p38 MAPK rabbit monoclonal antibody (1:1,000; Cell Signaling Technology), anti-mouse Phospho-p44/42 MAPK rabbit monoclonal antibody (1:1,000; Cell Signaling Technology), anti-mouse p44/42 MAPK rabbit monoclonal antibody (1:1,000; Cell Signaling Technology), anti-mouse HO-1 rabbit polyclonal antibody (1:2,000; Stressgen, BC, Canada), anti-mouse TLR2 rat monoclonal antibody (1:200; R&D Systems Inc., Minneapolis, MN, USA), or anti-mouse β-actin antibody (1:5,000; Sigma) at 4°C overnight, as appropriate. After washing with TBS containing 0.02% (v/v) Tween 20, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (0.01 μg/ml) at room temperature for 1 h and

immunoreactions were visualized using the enhanced chemiluminescence detection system (GE Healthcare Life Science, Little Chalfont, UK). Multi gauge software (Fujifilm Life Science, Tokyo, Japan) was used for the quantification of the bands and all protein levels were normalized to the β-actin levels after densitometric scanning of the membranes by LAS-4000 mini Imaging System (Fujifilm Life Science).

Efficiency of Bacterial Internalization within Cultured Cells

Bacterial internalization assay was performed by using the modified method described in chapter 1. TG cells were infected with each bacterial strains at a multiplicity of infection (MOI) of either 10 or 5 by centrifugation at 150 × *g* for 10 min at room temperature. To measure bacterial internalization efficiency after 30 min of incubation at 37°C in atmosphere containing 5% CO_2, the cells were washed once with RPMI 1640 medium and then replaced with new media containing gentamicin (50 μg/ml) to kill any remaining extracellular bacteria. After 30 min of incubation at 37°C in atmosphere containing 5% CO_2, The cells were then washed three times with PBS and lysed with cold distilled water. The serial dilution of cell lysates was spread on BHI or LB plates for measuring colony-forming unit (CFU) values. Dimethyl sulfoxide (DMSO, Wako), SP600125 (Wako), SB203580 (Wako), or U0126 (Wako) were added 1

h before infection. SOV (Sigma) was added immediately before *L. monocytogenes* infection. These inhibitors were dissolved in 1% DMSO (v/v). Trypan blue dye exclusion staining was used for cell viability analysis.

Detection of Cytotoxicity

Cytotoxicity was detected using the Cytotoxicity Detection KitPLUS (LDH) according to the manufacturer's instructions (Roche Applied Science, Upper Bavaria, Germany). *L. monocytogenes* was deposited onto TG cells at a MOI of 10 or 5 by centrifugation at 150 × *g* for 10 min at room temperature. To measure cytotoxicity, the infected cells were incubated at 37°C in atmosphere containing 5% CO_2 for 30 min, after which the cells were incubated with RPMI 1640 medium containing gentamicin (50 μg/ml) for 30 min to remove extracellular bacteria. The cells were washed once with RPMI 1640 medium, and then incubated in RPMI 1640 medium at 37°C in atmosphere containing 5% CO_2 for 6 h. SOV (100 μM) was added immediately before *L. monocytogenes* infection.

Determination of Cell Death

Cell death was assessed using the JC-1 Mitochondrial Membrane Potential Assay Kit

(Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. Bacteria were deposited onto TG cells on coverslips by centrifugation (150 × *g*, 10 min, room temperature), incubated at 37°C in atmosphere containing 5% CO_2 for 30 min, and then incubated in RPMI 1640 medium containing gentamicin (50 μg/ml) for 30 min. The cells were washed once with RPMI 1640 medium, and then incubated in RPMI 1640 medium at 37°C in atmosphere containing 5% CO_2 for 6 h. For JC-1 staining, cells were incubated with JC-1 staining solution at 37°C for 15 min. The samples were washed twice with assay buffer. Fluorescent images were obtained using a FluoView FV100 confocal laser scanning microscope (Olympus, Tokyo, Japan). SOV (100 μM) was added immediately before *L. monocytogenes* infection. Mitochondrial membrane potential, ΔΨm, is used as an indicator of cell health. In this system, healthy cells show red fluorescence, while apoptotic or unhealthy cells show green fluorescence (Tachibana et al., 2008, 2011).

Small Interfering RNA (siRNA)

Small interfering RNA (siRNA) duplexes used for silencing mouse MRC1 (target sequence: 5′-CAGCATGTGTTTCAAACTGTA-3′), TLR2 (target sequence: 5′-TTGGATGTTAGTAACAACAAT-3′), and AllStars Negative Control siRNA were

purchased from Qiagen (Hilden, Germany). TG cells were transiently transfected with MRC1 siRNA using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) with or without siRNAs at a final concentration of 36 nM and transfected with TLR2 siRNA using X-tremeGENE siRNA Transfection Reagent (Roche Applied Science) with or without siRNAs at a final concentration of 10 nM.

RNA Isolation and qPCR Analysis of MRC1

RNA isolation was done by using the same method described previously (Tachibana et al., 2011). Total RNA for real-time PCR was prepared from TG cells by using the RNA Purification Kit (Qiagen), and the purified RNA samples were kept at –30°C until use. RNA was quantitated by absorption at 260 nm using a SmartSpec 3000 spectrophotometer (Bio-Rad). One microgram of total RNA was used to synthesize cDNA using a ReverTra Ace quantitative PCR (qPCR) reverse transcriptase (RT) kit (TOYOBO). Real-time PCR was performed using Thunderbird SYBR qPCR Mix (TOYOBO). The following primer sets were purchased from TAKARA BIO (Shiga, Japan): MRC1, forward 5′-AGCTTCATCTTCGGGCCTTTG-3′ and reverse 5′-GGTGACCACTCCTGCTGCTTTAG-3′; and β-actin, forward 5′-TGACAGGATGCAGAAGGAGA-3′ and reverse 5′-

GCTGGAAGGTGGACAGTGAG-3′ . All data were normalized to β-actin.

Murine Experiments

Eight to 12 week-old female ICR mice were mated individually with male ICR mice. The parent mice were obtained from Kyudo Co., Ltd. (Saga, Japan). All mice were maintained under specific pathogen-free conditions in sterile cages housed in a ventilated isolator. Fluorescent lights were cycled 12 h on/12 h off, and ambient temperature (23 ± 1°C) and relative humidity (40–60%) were regulated. A vaginal plug was observed at day 0.5 of gestation. The normal gestational time for these mice is 19 days.

Virulence in Pregnant Mice

Mouse virulence assay was done by using the modified method described previously (Tachibana et al., 2011). Groups of three pregnant mice were infected intravenously at 7.5 days of gestation with approximately 2×10^4 cells of *L. monocytogenes* strains in 0.1 ml PBS. To check the progression of the disease, infected mice were monitored by a veterinarian every 3–4 h during the day phase (7:00 am to 7:00 pm). At 18.5 days of gestation, their livers and spleens were removed, weighed and homogenized in PBS.

The tissue homogenates were serially diluted with PBS and plated on BHI agar plates to count the number of CFUs. Fetuses were classified as alive if there was a heartbeat and as dead if there was no heartbeat (Tachibana et al., 2011).

Ethics Statement

This study was performed in strict accordance with recommendations in the Guidelines for Proper Conduct of Animal Experiments stipulated by the Science Council of Japan. All experimental protocols involving the use of animals were approved by the Animal Research Committee of Yamaguchi University (Permission Number: 141). Animal studies were performed in compliance with the Yamaguchi University Animal Care and Use guidelines. The mice were sacrificed by overdose of isoflurane and all efforts were made to minimize suffering by using isoflurane anesthesia.

Statistical Analyses

Statistical analyses were performed using the ANOVA test with *post hoc* Tukey–Kramer test or Student's *t*-test. Statistically significant differences between groups are indicated by asterisks (*, $P < 0.05$). Values represent the means from three assays and

the error bars represent standard deviations (SD).

RESULTS

Dephosphorylation of MAPK Family Proteins by *L. monocytogenes* Infection of TG Cells

Because the MAPK pathway is involved in the infection of host cells by bacterial pathogens (Krachler et al., 2011), the phosphorylation of c-Jun, p38, and ERK1/2 in TG cells after *L. monocytogenes* infection was analyzed by immunoblotting. Phosphorylation of c-Jun, p38, and ERK1/2 was observed in uninfected control TG cells, whereas dephosphorylation of these proteins was observed in infected TG cells (Fig. 1A and B). These proteins were dephosphorylated at different times during bacterial infection. Dephosphorylation of c-Jun, p38, and ERK1/2 was observed 1–6 h, 0.5–6 h, and 0.5–6 h after infection, respectively (Fig. 1A and B).

Dephosphorylation of MAPK Family Proteins Induces Downregulation of HO-1

L. monocytogenes internalize into host cells and induce host cell death (Disson and Lecuit, 2013). In a previous study, I suggested that HO-1 inhibits cell death caused by *L. monocytogenes* infection (Tachibana et al., 2011). To investigate whether MAPK contributes to expression of HO-1 in TG cells, the effect of MAPK inhibitors on HO-1

expression in TG cells was analyzed by immunoblotting. HO-1 expression was observed in uninfected TG cells, whereas the HO-1 expression was decreased in infected TG cells (Fig. 2A). HO-1 expression was decreased by the treatment of uninfected TG cells with SP600125 (JNK inhibitor) and U0126 (ERK1/2 inhibitor), but not SB203580 (p38 inhibitor; Fig. 2A). DMSO may affect HO-1 expression slightly. It was difficult to analyze HO-1 expression after bacterial infection under the MAPK inhibitor treatment, because treatment of MAPK inhibitor inhibited bacterial internalization (see Fig. 3). These results suggest that the MAPK pathway contributes to HO-1 expression in TG cells.

I next investigated that the effect of SOV, an inhibitor of protein tyrosine phosphatases, on HO-1 expression in infected TG cells by immunoblotting. A decrease in HO-1 expression was observed in infected but untreated TG cells. The downregulation of HO-1 expression caused by bacterial infection was inhibited by SOV treatment (Fig. 2B). I also observed that dephosphorylation of MAPK family proteins was inhibited by SOV treatment (data not shown). In addition, I assessed the effect of SOV treatment on bacterial internalization into TG cells. The results showed that bacterial internalization into TG cells was significantly increased by SOV treatment

(Fig. 2C). SOV treatment did not affect cell viability that was confirmed by trypan blue dye exclusion staining.

To examine whether protein dephosphorylation induced by bacterial infection contributes to cell death, infection of *L. monocytogenes* to TG cells was done with or without SOV treatment and the rate of cytotoxicity and cell death was analyzed by measuring LDH release and the mitochondrial membrane potential. However, in this experimental system, cells with low mitochondrial membrane potential were detected as unhealthy cells (Fig. 2F). Since SOV treatment increased bacterial internalization in TG cells (Fig. 2C), I reduced the MOI to 5 from 10 when TG cells were treated with SOV to equalize the number of internalized bacteria. Therefore, MOI in SOV untreated and treated cells in Fig. 2B, D and E were 10 and 5, respectively. In this condition, the number of internalized bacterial was same as shown in Fig. 2C. Treatment with SOV inhibited cell death induced by *L. monocytogenes* infection in TG cells, which was not observed in infected, untreated TG cells (Fig. 2D and E).

Inhibition of MAPK Family Proteins Impairs Bacterial Internalization into TG Cells

Because HO-1 expression was decreased by treatment with JNK and ERK1/2 inhibitors, I next examined the effect of treatment with these inhibitors on bacterial internalization into TG cells. Internalization of *L. monocytogenes* into TG cells was decreased by treatment with SP600125 (JNK inhibitor), SB203580 (p38 inhibitor), and U0126 (ERK1/2 inhibitor) in a dose-dependent manner (Fig. 3A). Moreover, uptake of *E. coli* by TG cells was inhibited by treatment with these inhibitors (Fig. 3B). These results suggest that the MAPK pathway contributes to the bacterial internalization into TG cells. Inhibitor treatments did not affect cell viability that was confirmed by trypan blue dye exclusion staining.

Expression of MAPK Family Proteins in TG Cells upon MRC1 and TLR2 Knockdown

Previous studies have reported that Mannose receptor, C type 1 (MRC1) and Toll-like receptor 2 (TLR2) mediate bacterial uptake by TG cells (chapter 1; Watanabe et al., 2010). I hypothesized that activation or expression of MAPK family proteins may be mediated by MRC1 and TLR2. To examine whether MRC1 and TLR2 contribute to the activation or expression of MAPK family proteins, the phosphorylation and expression of c-Jun, p38, and ERK1/2 in TG cells were analyzed by immunoblotting after the

knockdown of these receptors. The amounts of endogenous MRC1 and TLR2 were reduced by transfecting TG cells with MRC1- or TLR2-specific siRNA duplexes. MRC1 expression was significantly reduced and TLR2 expression was no longer detectable 48 h after transfection of TG cells with MRC1- or TLR2-specific siRNA, however, transfection with control siRNA had no effect on expression (Fig. 4A and B). Because MRC1 is difficult to detect by immunoblotting, MRC1 expression was analyzed by reverse transcription quantitative PCR. Although MRC1 knockdown did not affect the phosphorylation or expression of c-Jun, p38, or ERK1/2 in TG cells (Fig. 4C, E and G), TLR2 knockdown reduced the expression of c-Jun, p38, and ERK1/2 (Fig. 4D, F and H). These results suggest that TLR2 plays critical roles in signal transduction through the MAPK pathway in TG cells.

Infectious Abortion and Dephosphorylation of MAPK Family Proteins Depends on LLO

The thiol-activated, cholesterol-dependent, pore-forming toxin LLO is a major virulence factor of *L. monocytogenes* (Hernández-Flores and Vivanco-Cid, 2015). To investigate whether LLO contributes to the dephosphorylation of MAPK family proteins, TG cells were infected with wild-type *L. monocytogenes* EGD, an LLO

deletion mutant (Δ*hly*), and an LLO-complemented strain (Δ*hly*::*hly*). Subsequently, the phosphorylation of c-Jun, p38, and ERK1/2 in these cells was analyzed by immunoblotting. Phosphorylation of c-Jun, p38, and ERK1/2 was observed in uninfected TG cells and in TG cells infected with LLO deletion mutant. Dephosphorylation of these proteins was observed in TG cells infected with wild-type EGD and with LLO-complemented strain (Fig. 5A).

To investigate whether LLO contributes to the expression of HO-1 in TG cells, TG cells were infected with the three strains described above, and HO-1 expression in these cells was analyzed by immunoblotting. HO-1 expression was observed in uninfected TG cells and in TG cells infected with the LLO deletion mutant. HO-1 expression was decreased in TG cells infected with wild-type EGD and with the LLO-complemented strain infection (Fig. 5B).

Further, I investigated the role of LLO in infectious abortion induced by *L. monocytogenes*. In order to construct a mouse model of infectious abortion induced by *L. monocytogenes*, the numbers of fetuses aborted by infected mice were counted on day 18.5 of gestation. All fetuses were aborted when the mice were infected with wild-

type *L. monocytogenes* on day 7.5 of gestation. Almost fetuses were still alive when the mice were infected on days 10.5 and 11.5 of gestation. No abortion was observed when the mice were infected on day 14.5 of gestation. Therefore, I analyzed abortion caused by *L. monocytogenes* infection on day 7.5 of gestation. Infectious abortion was observed in pregnant mice infected with wild-type EGD and the LLO-complemented strain, whereas the LLO deletion mutant did not induce infectious abortion (Fig. 6A). Next, I examined bacterial colonization of the spleen and liver. Replicating bacteria were observed in both the spleen and liver of mice infected with wild-type EGD and the LLO-complemented strain, whereas replicating bacteria were undetectable in the spleen and liver of mice infected with the LLO deletion mutant (Fig. 6B and C). Infection with wild-type EGD and the LLO-complemented strain significantly increased the weight of the spleen (Fig. 6D), while bacterial infection had no significant effect on the weight of the liver (Fig. 6E).

DISCUSSION

MAPK activation induces the expression of multiple genes that regulate inflammatory responses. Intracellular pathogens manipulate MAPK pathways to increase their virulence (Krachler et al., 2011; Arthur and Ley, 2013). Several bacterial pathogens, including *Mycobacterium tuberculosis*, activate MAPK pathways and promote invasion of host cells (Schorey and Cooper, 2003). In contrast, *Yersinia* sp. and *Shigella* sp. inhibit phosphorylation of MAPK and negatively regulate proinflammatory responses (Orth et al., 1999; Reiterer et al., 2011). In this study, dephosphorylation of c-Jun, p38, and ERK1/2 was observed during *L. monocytogenes* infection of TG cells. When *L. monocytogenes* is internalized into endothelial cells, p38 is activated and IL-8 secretion is induced in a nucleotide-binding oligomerization domain (Nod) 1-dependent manner (Opitz et al., 2006). Although MAPK plays an important role in immune responses to *L. monocytogenes* infection, its role in the internalization of bacteria into TG cells was unclear. YopJ, a *Yersinia* sp. virulence factor, is injected directly into host cells through a needle-like complex called the type III secretion system (Espinosa and Alfano, 2004). YopJ inhibits MAPK pathways and the NF-κB pathway by preventing the activation of MAP kinase kinase (MKK) and IκB kinase b (IKKb) and promotes

host cell death (Orth et al., 1999). Since *L. monocytogenes* infection also induces cell death in TG cells (Tachibana et al., 2011), dephosphorylation of c-Jun, p38, and ERK1/2 is thought to contribute to TG cell death induced by bacterial infection.

MAPK pathways are also known to be associated with the production of HO-1 (Paine et al., 2010). HO-1 plays key roles in cytoprotection, antioxidation, and anti-inflammation (Hegazi et al., 2005; Nakahira et al., 2006). During spontaneous abortion, HO-1 is downregulated at the fetal-maternal interface in both human and mice (Yachie et al., 1999). Previous studies reported that HO-1 contributed to abortion caused by *L. monocytogenes* and *B. abortus* infection (Tachibana et al., 2008, 2011). *B. abortus* is a Gram-negative, intracellular, zoonotic bacterium. These bacteria cause downregulation of HO-1 in the placenta, resulting in abortion (Tachibana et al., 2008). In this study, I showed that inhibition of c-Jun and ERK1/2 pathway causes downregulation of HO-1. Inhibition of protein dephosphorylation by SOV treatment blocks the downregulation of HO-1 and TG cell death caused by bacterial infection. Furthermore, my results suggested that TG cells survived if downregulation of HO-1 was not induced, although a large number of bacteria were internalized into TG cells. Besides, not only down regulation of HO-1 but also bacterial infection is necessary for induction of TG cell

death (Tachibana et al., 2008, 2011). Therefore, it is speculated that the death of TG cells resulting from *L. monocytogenes* infection may be caused by the downregulation of HO-1 due to the dephosphorylation of MAPK family proteins. Therefore, dephosphorylation of MAPK family proteins by *L. monocytogenes* infection may be first step to infectious abortion, but detail mechanism of infectious abortion is still unclear.

Since SOV treatment enhanced internalization of *L. monocytogenes* into TG cells, phosphorylated proteins contributed to bacterial internalization. Phosphatidylinositol 3-kinase (PI3K)/Akt pathway is activated by binding to phosphorylated proteins (Wang et al., 2015), and my previous study showed that the activation of PI3K/Akt pathway contribute to *L. monocytogenes* infection in TG cells (Tachibana et al., 2015). In this study, I demonstrated that phosphorylation of MAPK family proteins also contributes to bacterial internalization. This data indicates that internalization of bacteria is controlled through various intracellular signaling pathway including MAPK and PI3K/Akt pathway. Although activation of the MAPK pathway is required to enhance bacterial internalization, *L. monocytogenes* induced dephosphorylation of MAPK family proteins in TG cells. The level of MAPK activity in TG cells is usually high, allowing TG cells

to internalize macromolecules, such as bacteria. Therefore, the ability of TG cells to internalize additional bacteria would decrease after *L. monocytogenes* infection. This phenomenon is believed to prevent rapid cell death by sequential bacterial infection. This method of infection control by *L. monocytogenes* in TG cells may be important for intracellular bacterial growth and the induction of infectious abortion.

In my previous studies, I showed that several receptors located on TG cells contribute to *L. monocytogenes* infection, such as MRC1 (chapter 1), TLR2, and class B scavenger receptor type 1 (SR-B1; Watanabe et al., 2010). Knockdown of these receptors inhibited the uptake of *L. monocytogenes* by TG cells. Although it is well known that signal transduction mediated by TLR2 activates the MAPK pathway (Re and Strominger, 2001), the role of MRC1 during the signal transduction remains unclear. The results of this study showed that TLR2 contributes to the expression of MAPK family proteins in TG cells, but that MRC1 was not involved. Since MAPK signaling pathway seemed to play important role in internalization of bacteria and cell death, TLR2 may be a key factor in controlling *L. monocytogenes* infection in TG cells.

LLO is known as a major virulence factor and contributes to bacterial escape from the

phagocytic vacuole in the host cell. Recent studies have revisited the role of LLO and are providing new functions of LLO (Hamon et al., 2012). Infection with *L. monocytogenes* causes fragmentation of the host mitochondrial network through the pore-forming action of its toxin LLO before bacterial entry (Stavru et al., 2011b). LLO has been described as highly lytic when applied to nucleated cells and can induce a wide range of cell death types. *L. monocytogenes* infection induces apoptosis in the cells of the spleen, lymph nodes, liver, and brain (Hernández-Flores and Vivanco-Cid, 2015). The in vitro results of LLO-mediated induction of apoptosis are in agreement with *in vivo* observations (Carrero et al., 2004). During *L. monocytogenes* infection of a macrophage cell line, the bacterium induces expansion of the endoplasmic reticulum (ER) and initiates a stress response to unfolded proteins (unfolded protein response or UPR). The induction of this ER stress response is dependent on the production of LLO. ER stress limits the number of intracellular bacterial, but sustained ER stress results in apoptotic cell death (Pillich et al., 2012). In this study, I showed that dephosphorylation of MAPK family proteins during *L. monocytogenes* infection in TG cells is dependent on LLO. A mutant lacking LLO did not induce infectious abortion, suggesting that the modulation of infection in the placenta should be advantageous toward *L. monocytogenes* infection.

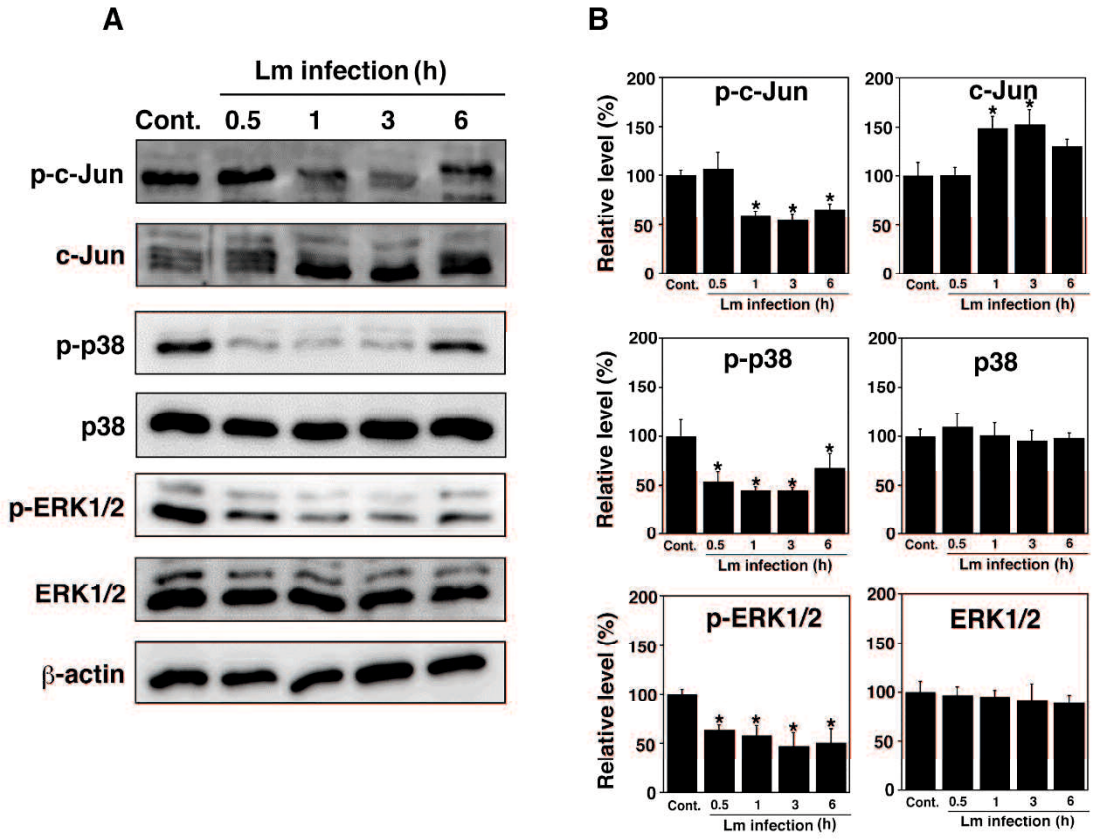

Fig. 1. Dephosphorylation of MAPK family proteins in trophoblast giant (TG) cells by *L. monocytogenes* infection.

(A) Expression of MAPK family proteins in TG cells infected with *L. monocytogenes*. TG cells were infected with or without (Cont.) *L. monocytogenes* for the indicated number of hours. Production of the indicated proteins was detected by immunoblotting. β-actin was used as an immunoblotting control. Phosphorylated c-Jun, p38, and ERK1/2 are designated p-c-Jun, p-p38, and p-ERK1/2, respectively. (B) Relative intensity levels of p-c-Jun, c-Jun, p-p38, p-38, p-ERK, and ERK. The intensity levels of all MAPK family proteins were measured using Multi gauge software and

normalized by the value of β-actin. Relative values to the intensity without infection (Cont.) were shown. All values represent the means and SD of three assays. *, $P < 0.05$ compared with Cont. by *post hoc* Tukey–Kramer test.

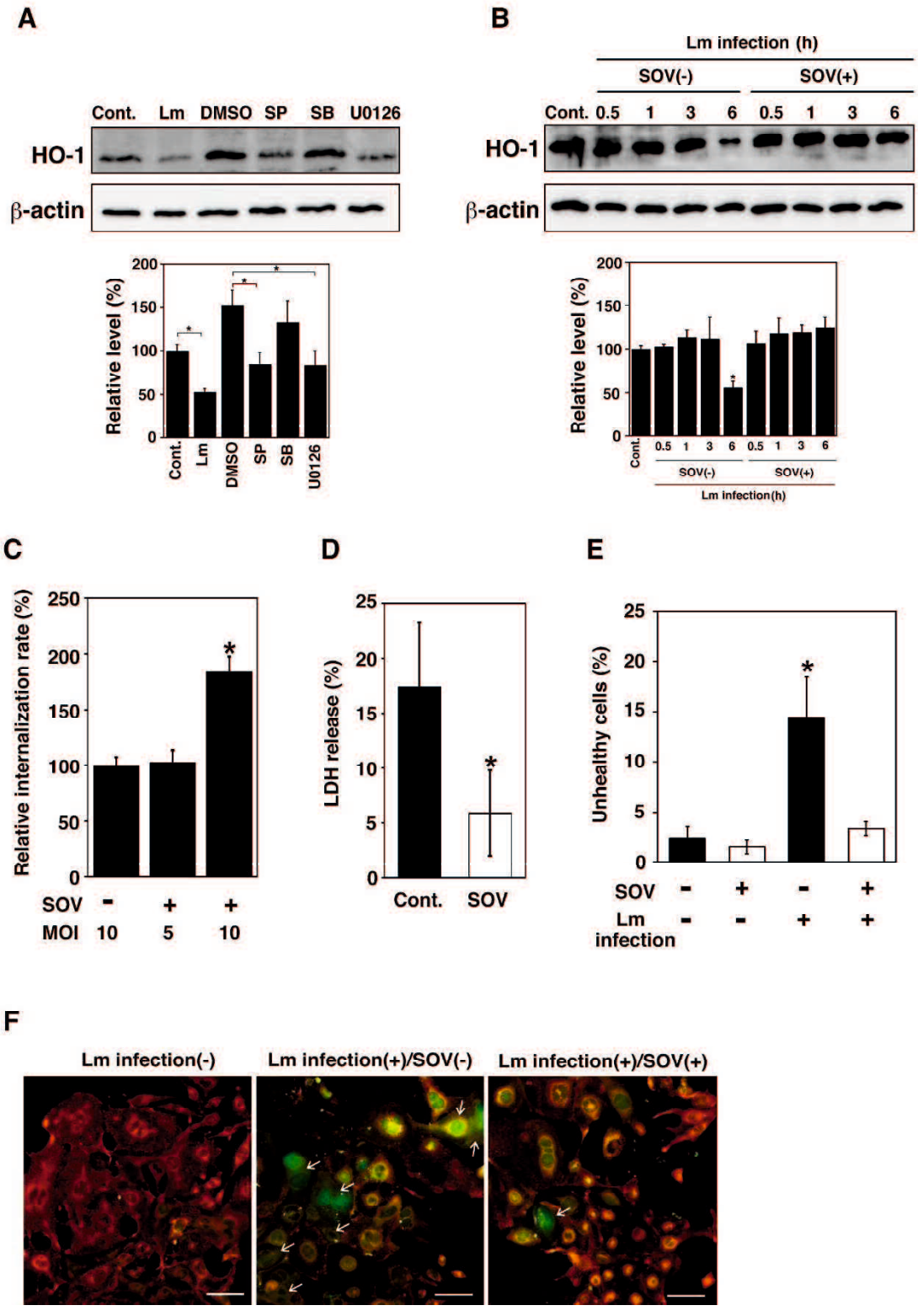

Fig. 2. Dephosphorylation of MAPK family proteins induces downregulation of HO-1 and TG cell death.

(A) Effect of MAPK inhibitor treatment on HO-1 expression. TG cells were infected with *L. monocytogenes* (Lm) for 6 h, treated with or without (Cont.) SP600125 (SP; JNK inhibitor, 25 μM), SB203580 (SB; p38 inhibitor, 25 μM), U0126 (ERK1/2 inhibitor, 25 μM), or DMSO 1 h before infection, respectively. Expression of HO-1 was detected by immunoblotting. β -actin was used as an immunoblotting control. (B) Effect of SOV treatment on HO-1 expression. TG cells were infected with or without (Cont.) *L. monocytogenes* in the presence (SOV+) or absence (SOV–) of SOV (100 μM) for the indicated number of hours. HO-1 expression was detected by immunoblotting. β-actin was used as an immunoblotting control. (A,B) The intensity levels of HO-1 expression were measured using Multi gauge software and normalized by the value of β-actin. Relative values to the intensity without infection (Cont.) were shown. All values represent the means and SD of three assays. *, $P < 0.05$ compared with Cont. by *post hoc* Tukey–Kramer test. (C) Relative bacterial internalization rate. TG cells were infected with *L. monocytogenes* in the presence (SOV+) or absence (SOV–) of SOV (100 μM), for 30 min at an multiplicity of infection (MOI) of 10 or 5. All values represent the means and SD of triplicate samples from three assays. *, $P < 0.05$ compared with bacterial internalization in TG cells lacking SOV by *post hoc* Tukey–Kramer test. (D) Detection of cytotoxicity by LDH release. TG cells were infected with

L. monocytogenes in the presence (SOV) or absence (Cont.) of SOV (100 μM) at an MOI of 10 (SOV–) or 5 (SOV+). The amount of LDH released was measured after 6 h of infection. All values represent the means and SD of triplicate samples from three assays. *, $P < 0.05$ compared with Cont. by Student's *t*-test. (E) Detection of cell death. TG cells were infected with *L. monocytogenes* in the presence (SOV+) or absence (SOV–) of SOV (100 μM) for 30 min at an MOI of 10 (SOV–) or 5 (SOV+). Cell death was determined using the JC-1 Mitochondrial Membrane Potential Assay Kit after 6 h of infection. The total number of live or dead cells was determined by examination of 100 TG cells per coverslip. All values represent the means and SD of three assays. *, $P < 0.05$ compared with control by *post hoc* Tukey–Kramer test. (F) Fluorescence microscopic images of cell death. Red or green fluorescence indicates healthy and unhealthy cells, respectively. Arrows indicate dead cells. Bar represents 100 μm.

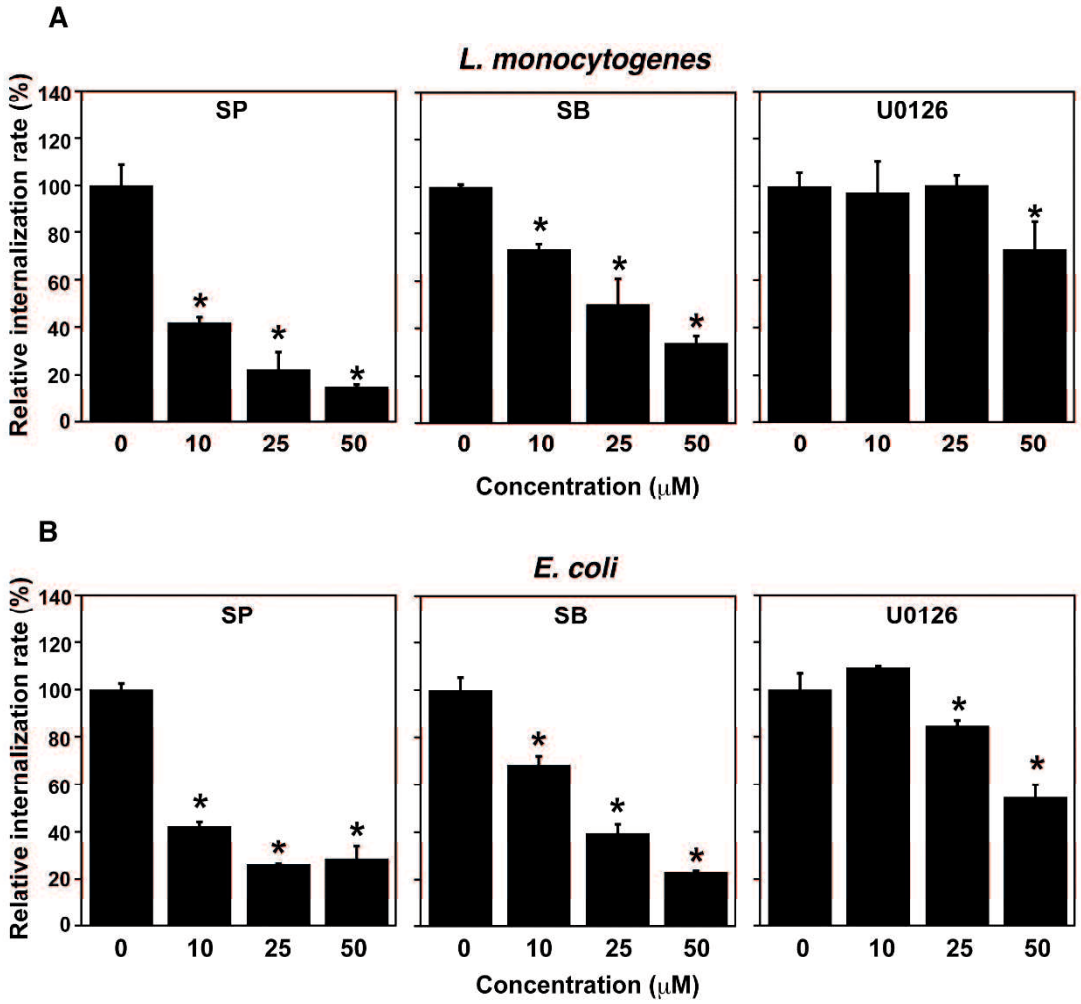

Fig. 3. Treatment with MAPK inhibitors inhibits *L. monocytogenes* infection in TG cells.

(A) Effect of MAPK inhibitor treatment on *L. monocytogenes* infection. TG cells were treated with SP600125 (SP; JNK inhibitor), SB203580 (SB; p38 inhibitor), or U0126 (ERK1/2 inhibitor) at the indicated concentration for 1 h and then infected with *L. monocytogenes* for 30 min at an MOI of 10. (B) Effect of MAPK inhibitor treatment on uptake of *E. coli*. TG cells were treated with MAPK inhibitors as described above

and then infected with *E. coli* for 30 min at an MOI of 10. (A,B) All values represent the means and SD of triplicate samples from three identical experiments. *, $P < 0.05$ compared with bacterial internalization in TG cells without treatment by *post hoc* Tukey–Kramer test.

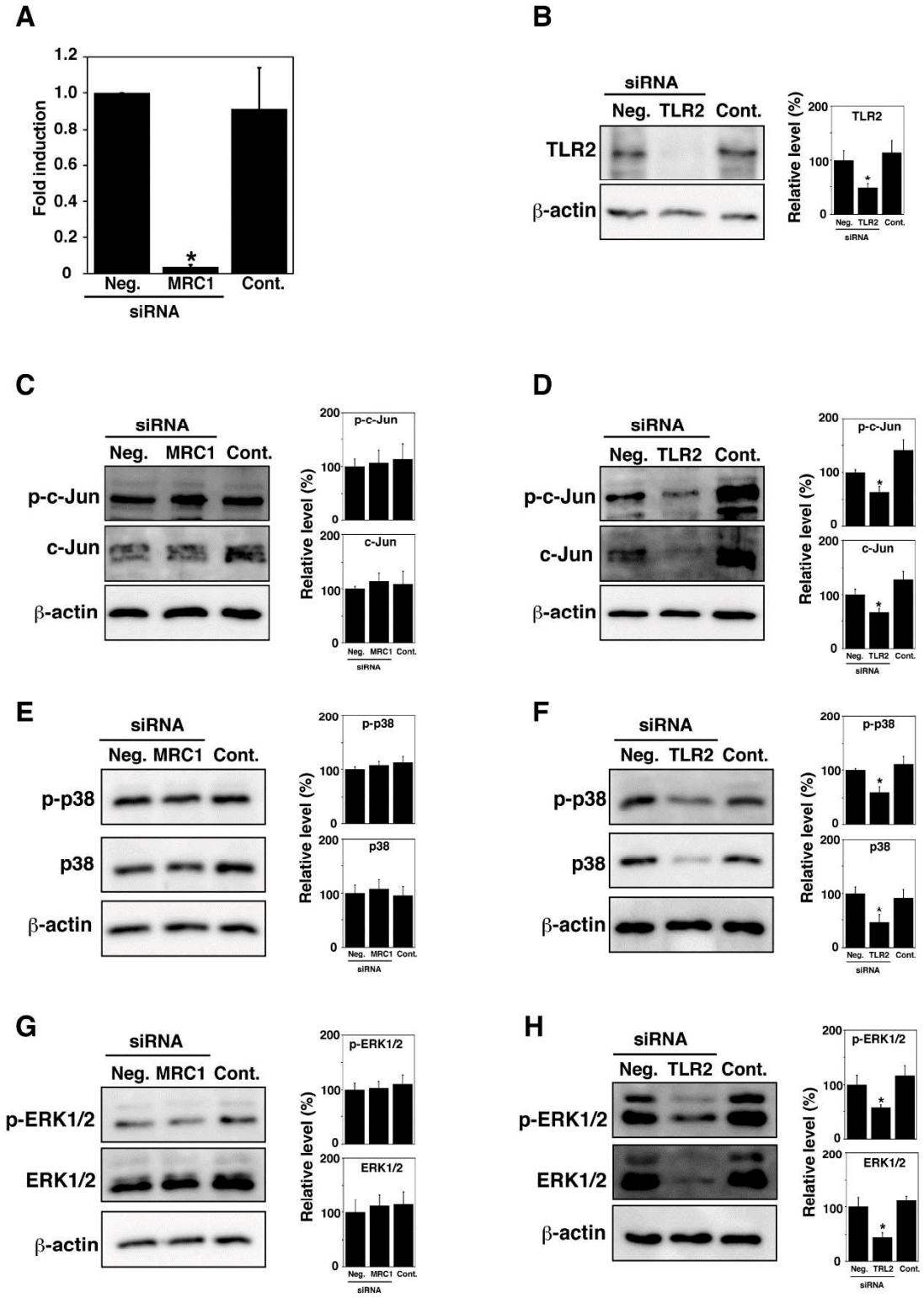

Fig. 4. Expression of MAPK family proteins in MRC1 and TLR2 knock-down TG cells.

(A,B) Expression of MRC1 and TLR2. TG cells were treated for 48 h with or without (Cont.) siRNA-targeting MRC1 (A) or TLR2 (B). AllStars Negative Control siRNA (Neg.) was used for a negative control. Production of the indicated proteins was monitored by qPCR (A) or immunoblotting (B). β-actin was used as a control. (C,D) Detection of c-Jun and phosphorylated c-Jun (p-c-Jun) in MRC1 (C) or TLR2 (D) knock-down TG cells by immunoblotting. (E,F) Detection of p38 and phosphorylated p38 (p-p38) in MRC1 (E) or TLR2 (F) knock-down TG cells by immunoblotting. (G,H) Detection of ERK1/2 and phosphorylated ERK1/2 (p-ERK1/2) in MRC1 (G) or TLR2 (H) knock-downed TG cells by immunoblotting. (B–H) The intensity levels of each protein expression were measured using Multi gauge software and normalized by the value of β-actin. Relative values to the intensity of Neg. were shown. (A–H) All values represent the means and SD of three assays. *, $P < 0.05$ compared with Neg. by *post hoc* Tukey–Kramer test.

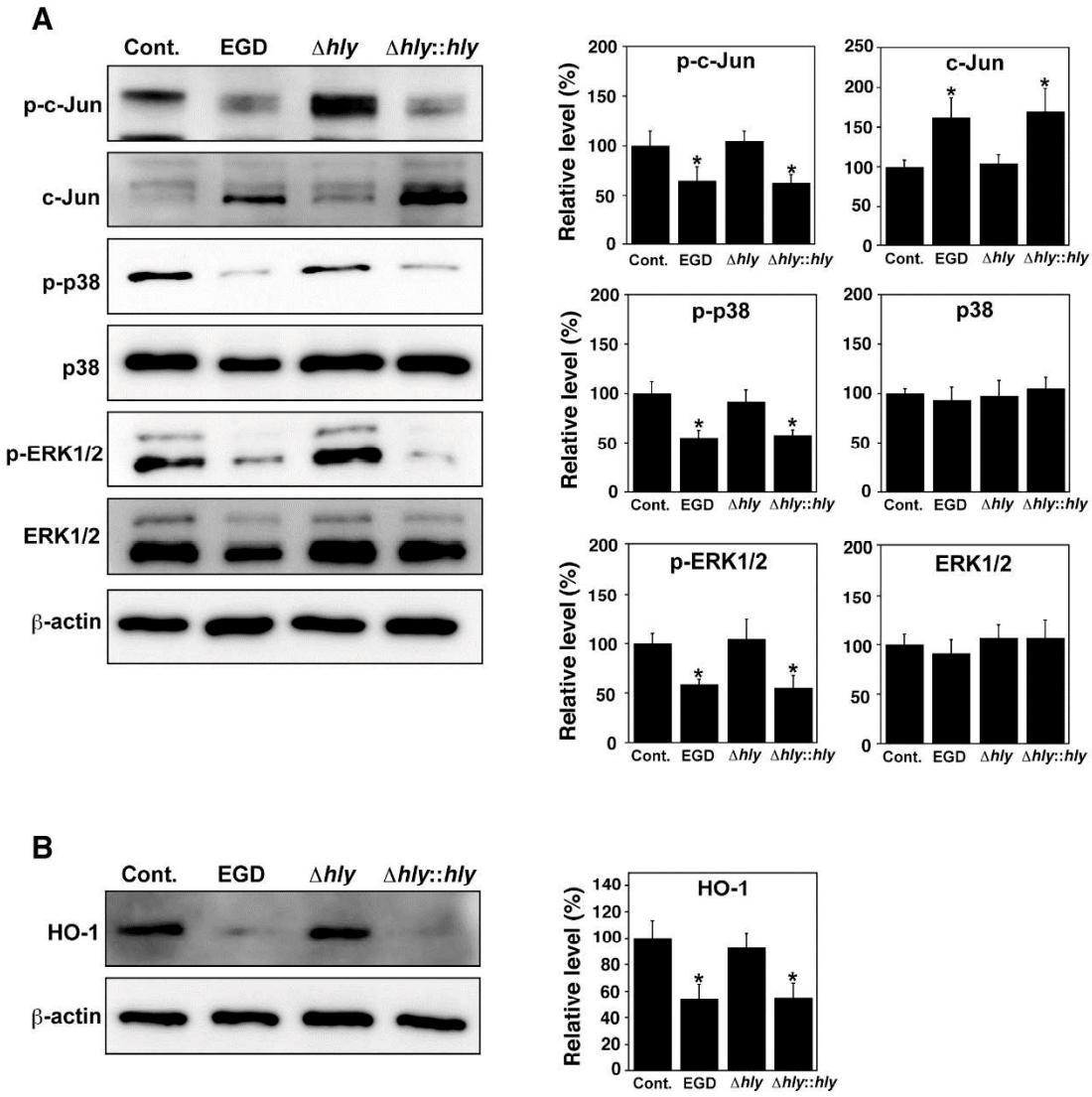

Fig. 5. LLO contributes to dephosphorylation of MAPK family proteins and downregulation of HO-1.

(A) Expression of MAPK family proteins. TG cells were infected with or without (Cont.) *L. monocytogenes* wild-type strain EGD, LLO deletion mutant (Δ*hly*), or LLO-complemented strain (Δ*hly*::*hly*) for 3 h. Expression of the indicated proteins was detected by immunoblotting. β-actin was used as an immunoblotting control.

Phosphorylated c-Jun, p38, and ERK1/2 are indicated as p-c-Jun, p-p38, and p-ERK1/2, respectively. (B) Expression of HO-1. TG cells were infected with the *L. monocytogenes* strains described above for 6 h. Expression of HO-1 was detected by immunoblotting. β-actin was used as an immunoblotting control. (A,B) The intensity levels of each protein expression were measured using Multi gauge software and normalized by the value of β-actin. Relative values to the intensity without infection (Cont.) were shown. All values represent the means and SD of three assays. *, $P < 0.05$ compared with Cont. by *post hoc* Tukey–Kramer test.

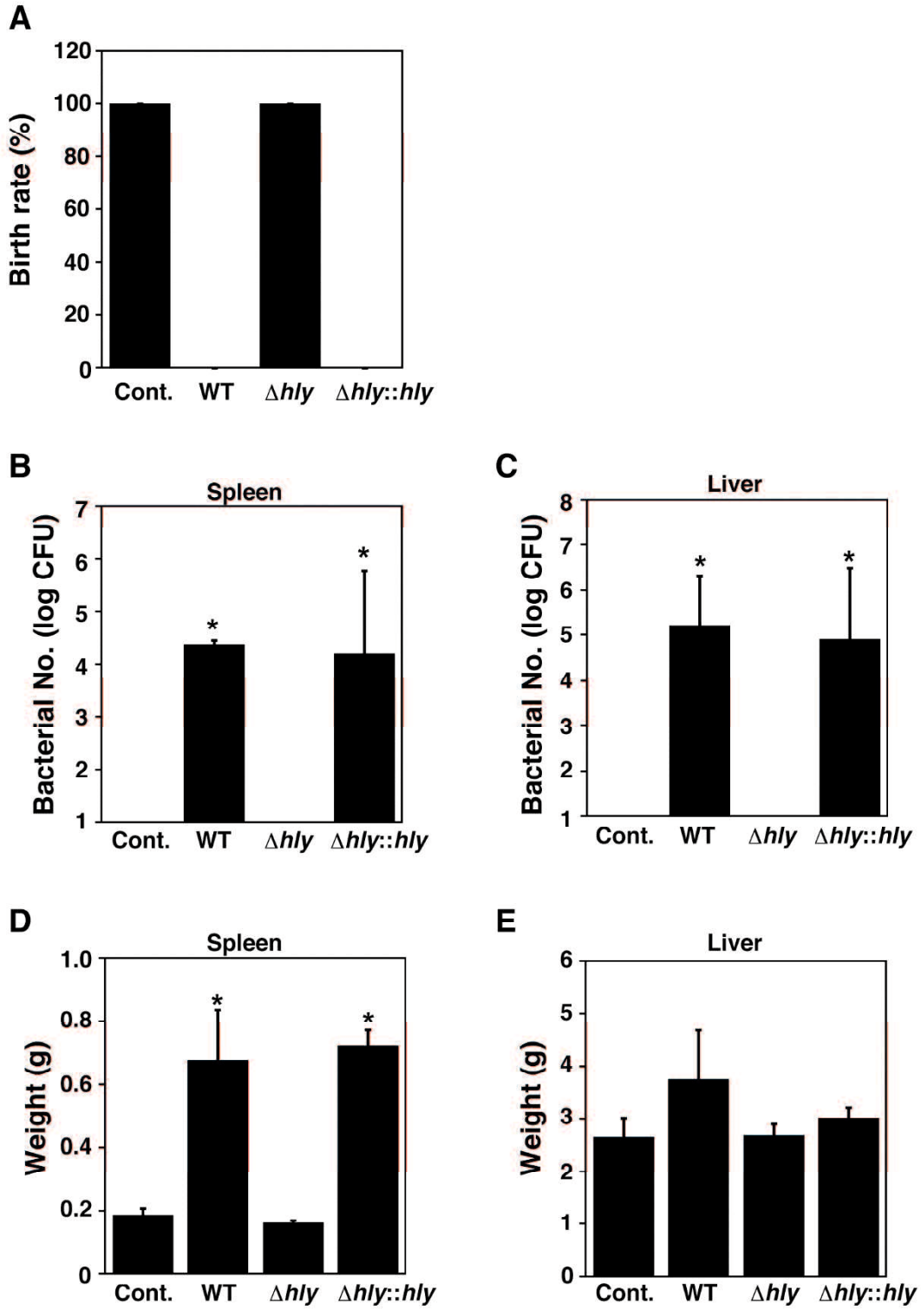

Fig. 6. LLO contributes to infectious abortion.

(A) Birth rate. Groups of three pregnant animals were infected intravenously at 7.5

days of gestation with approximately 2×10^4 cells of *L. monocytogenes* wild-type strain EGD, LLO deletion mutant (Δ*hly*), and LLO-complemented strain (Δ*hly*::*hly*). (B,C) Number of bacteria in the spleen (B) and liver (C) of mice infected with the *L. monocytogenes* strains described above. At 18.5 days of gestation, their spleens and livers were removed and homogenized. The serially diluted tissue homogenates were plated on BHI agar plates and the number of CFU was determined. (D,E) Weights of spleen (D) and liver (E) from mice infected with the *L. monocytogenes* strains described above. (B–E) All values represent the means and SD (n = 9). *, $P < 0.05$ compared with Cont. by *post hoc* Tukey–Kramer test.

CONCLUSION

Infectious abortion is a major public health concern, but the molecular mechanisms of infectious abortion are still unclear. In this study, I focused on TG cell, an immune cell in placenta, and investigated the receptors responsible for the uptake of bacteria and intracellular signaling dynamics.

In chapter 1, I demonstrated that MRC1 was an important receptor of TG cells to ingest various bacteria. Internalization of bacteria through MRC1 delayed the maturation of phagolysosomes compared to bacterial internalization through other cell surface receptors such as TLR2. This phenomenon seemed to allow intracellular bacteria such as *L. monocytogenes* to escape from phagolysosomes. Escape of *L. monocytogenes* from phagolysosomes and replication in cytoplasm are essential step to induce infectious abortion. However, intracellular dynamic of TG cells infected with *L. monocytogenes* was still unclear.

In chapter 2, I investigated the intracellular signaling dynamics in TG cells and demonstrated that escaped *L. monocytogenes* from phagolysosome induced

dephosphorylation of MAPK family proteins. This dephosphorylation resulted in the down regulation of HO-1, a cytoprotective protein, leading to cell death. Since TG cells are essential for establishment of placenta, TG cell death seemed critical step of infectious abortion.

Altogether, in this study I demonstrated that MRC1 is an important receptor for *L. monocytogenes* to infect to TG cells, and dephosphorylation of MAPK family proteins is a key event to induce infectious abortion. Infectious abortion is not only a public concern but also an animal hygiene problem. These days, the therapeutic approaches other than antibiotics are strongly desired worldwide. Since MRC1 and MAPK family proteins are key factors of infectious abortion, these factors would be suitable candidates for therapeutic target.

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