Legionella pneumophila infection-mediated regulation of RICTOR via miR-218 in U937 macrophage cells

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Abstract

Background: Inhalation of aerosolized *Legionella pneumophila*, a Gram-negative bacterium, can cause severe pneumonia. During infection, *L. pneumophila* replicates intracellularly in macrophages. The involvement of host microRNAs (miRNAs) in *L. pneumophila* infection is not fully understood.

Methods: The human macrophage-like cell line U937 was infected with *L. pneumophila*. The levels of miRNA and messenger RNA (mRNA) were measured using reverse transcriptase polymerase chain reaction. Release of lactate dehydrogenase was used to evaluate cytotoxicity. The expression of RICTOR and related proteins was examined by western blotting of cell lysates.

Results: *L. pneumophila* infection upregulated the expression of miR-218 and the host genes *SLIT2* and *SLIT3* in U937 cells. The expression of RICTOR, a component of the mechanistic target of rapamycin complex 2 (mTORC2), decreased during *L. pneumophila* infection. RICTOR protein expression was inhibited by the overexpression of miR-218, whereas knockdown of miR-218 restored the downregulation of *RICTOR* by *L. pneumophila*. *L. pneumophila* infection induced the expression of the proinflammatory cytokines IL-6 and TNF alpha, which was modulated by knockdown of miR-218 or *RICTOR*.

Conclusions: Our study revealed the involvement of miR-218 in regulating the inflammatory response of macrophages against *L. pneumophila* infection. These findings suggest potential novel roles for miR-218 and *RICTOR* as therapeutic targets of *L. pneumophila* infection.

Keywords: microRNA, *Legionella*, Rictor, macrophage

Introduction

Legionella pneumophila is a Gram-negative bacterium that lives in freshwater amoebae and is a pivotal cause of community- and hospital-acquired pneumonia [1]. *L. pneumophila* infection of alveolar macrophages in human lungs can cause Legionnaires' disease, an acute fibrinopurulent pneumonia [2]. Inside the host macrophages, *Legionella* induces non-endosomal replicative phagosomes. During the early phase of *L. pneumophila* infection, recognition of the pathogen-associated molecular patterns (PAMPs) by the innate immune system prevents invasion of *L. pneumophila* by alveolar macrophages, neutrophils, and natural killer (NK) cells [3]. The upregulation of proinflammatory cytokines such as tumor necrosis factor (TNF)-alpha and interleukin (IL)-1beta, for example, can modulate the innate host defense against *L. pneumophila* in infected alveolar macrophages [4].

MicroRNAs (miRNAs) control physiological and pathological cellular functions by repressing the expression of target genes post-transcriptionally [5]. The innate host immune response against pathogens is modulated by miRNAs [6, 7] including the NF-kB dependent miRNAs miR-146a and miR-155, which inhibit the inflammatory response [8]. Some bacterial infections control miRNA expression in macrophages. Infection with *Listeria monocytogenes*, a Gram-positive facultative intracellular pathogen, altered a set of miRNAs that included miR-146a and miR-125a in mouse macrophages in a MyD88-dependent manner [9]. Members of the miR-15 miRNA family are downregulated during *Salmonella* infection in a mechanism involving the recovery of cyclin D1 expression [10]. The cell envelope membrane of *Mycobacterium tuberculosis* decreased miR-125b expression, which partially inhibited TNF-alpha biosynthesis, in human macrophages [11]. These studies showed that bacteria can alter the expression of miRNAs in infected macrophages and uncovered important roles for these miRNAs in regulating the host immune response to bacterial infection.

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that regulates protein synthesis, lipid metabolism, and cellular growth [12]. The two major complexes, mTOR complexes 1 and 2 (mTORC1 and mTORC2), have mTOR as the catalytic subunit [13]. The first, mTORC1, comprises mTOR, mLST8 and rapamycin-sensitive adaptor protein of mTOR (Raptor), which activates protein synthesis and increases cell size [14]. The components of mTORC2 include mTOR, mSIN1, mLST8 and the rapamycin-insensitive subunit (Rictor), which promotes cell survival and cytoskeletal organization [15]. This complex directly phosphorylates protein kinase B (Akt) on serine 473 [16]. While the role of mTORC2 in inflammation in macrophages has been studied, the precise mechanism has not been determined [17].

Here, we discovered that miR-218 is upregulated by *L. pneumophila* in the human macrophage-like cell line U937 and elucidated the role of miR-218 during *Legionella* infection. We demonstrated that infection with *L. pneumophila* increased miR-218, which suppressed *RICTOR*, enhancing the production of inflammatory cytokines.

Materials and Methods

Reagents

L. pneumophila subsp. pneumophila (ATCC 33152) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). A Ginemez staining kit was obtained from Nikken-Bio-Medical-Laboratory (Kyoto, Japan). Rabbit antibodies against Rictor, phospho-Akt (serine 473), and total Akt were purchased from Cell Signaling Technologies (Beverly, MA, Canada). Mouse antibodies against beta-actin (ACTB) and peroxidase-conjugated antibody to rabbit IgG were purchased from Santa Cruz Biotechnology and MP Biomedicals (Santa Ana, CA, USA), respectively.

Amplification of L. pneumophila

L. pneumophila was plated on WYO-alpha agar for 2 days. A range of multiplicity of infection (MOI) of 0–100 was used for the experiments.

Cell culture

The human monocytic cell line U937 was obtained from ATCC and maintained at 37°C under 5% CO_2 in RPMI media containing 10% fetal bovine serum. Two days before infection with *L. pneumophila,* U937 was seeded onto 12-well plates and treated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) to induce differentiation into macrophage-like cells.

Gimenez staining

L. pneumophila-infected U937 cells were evaluated using a Gimenez-staining kit in accordance with the manufacturer's instructions. U937 cells were infected for 24 h, cell smears transferred to a glass slide, dried, fixed with methanol, stained with Carbol-Fuchsin working solution, and observed under oil immersion lens. Staining of *L. pneumophila* and

U937 cells appeared pink and blue/green, respectively.

Western blotting

U937 cell lysates were prepared using Laemmli sample buffer (Bio-Rad Laboratories Inc., Hercules, CA, USA) containing 50 mM DTT, 1 mM PMSF, 1 mM Na_3VO_4, and a protease inhibitors cocktail (Roche Diagnostics, Tokyo, Japan). The lysates were analyzed by western blotting as described previously [18] on nitrocellulose membranes using antibodies against RICTOR, total AKT, phosphor-AKT, and ACTB.

Transfection of siRNA, miRNA mimics and miRNA inhibitors

Silencing RNAs (siRNAs) against Rictor, miR-218 mimic, miR-218 inhibitor and control oligos were purchased from Applied Biosystems (Foster City, CA, USA). A mixture of 50 nM siRNAs, 10 nM miRNA mimics, and 40 nM miRNA inhibitors was transfected into U937 cells using Lipofectamine RNAiMAX (Applied Biosystems) according to the manufacturer's instructions. The transfected cells were grown for 48 h in a CO_2 incubator and transduced with *L. pneumophila* at an MOI ranging from 0–100 for different hours.

RNA isolation and quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from U937 cells according to the manufacturer's protocol using a total RNA isolation kit (Ambion, Austin, TX, USA) and reverse-transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit and microRNA Reverse Transcription Kit (Applied Biosystems). Quantitative RT-PCR was performed using the FastStart Universal Probe Master (Roche Diagnostics, Indianapolis, IN, USA) and primers (*SLIT2* #Hs01061407_m1, *SLIT3* #Hs00935843_m1, *RICTOR* #Hs00380903_m1, *ACTB* #Hs01060665_g1, miR-218 #000521, and *U6* #001973). The data were normalized to the

expression levels of *ACTB* for mRNAs and *U6* for miRNAs.

Cytotoxicity assay

U937 cells were infected with *L. pneumophila* for 24 h and the culture media was harvested to measure lactate dehydrogenase (LDH) levels using the LDH Cytotoxicity Detection Kit (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. The values of LDH released from the lysis buffer of treated and non-treated cells were used as positive or negative controls, respectively. Cytotoxicity was calculated and shown as a percentage or relative ratio.

Statistical analysis

Data are presented as mean ± SD. Experiments were performed at least three times independently. Statistically significant differences were detected using unpaired *t*-tests and based on a significance cut-off of $p < 0.05$.

Results

L. pneumophila *infection increased miR-218 expression in U937 cells*

Because *L. pneumophila* is known to infect macrophages and replicate inside their cytoplasm, the human macrophage-like cell line U937 was used for this study. Gimenez staining of *L. pneumophila* in the cytoplasm of U937 cells infected with an MOI of 1 for 24 h is shown in Figure 1A. The cytotoxic effect of *L. pneumophila* on U937 cells was weak at MOIs of 1–10, but cells infected at an MOI of 100 were highly damaged (Fig. 1B). Subsequent infections were carried out at MOIs of < 10. Since there have been reports on the associations between miR-218 and miR-155 and the inflammatory response, we examined miR-218 and miR-155 expression after *L. pneumophila* infection in U937 cells. While the expression of miR-218 and miR-155 did not increase in the first 6 h after infection, both were upregulated in a dose-dependent manner at 24 h (Fig. 1C). Notably, the increase in expression of these miRNAs at MOIs of 0.5–5 did not affect the cell viability over 24 h (Fig. 1B).

L. pneumophila *increased expression of both SLIT2 and SLIT3 in U937 cells*

Mature miR-218 is created by two different primary miR-218s, which are encoded in the intron of each of the host genes *slit2* and *slit3* (Supplementary Fig. 1A). In this study, the expression of *SLIT2* increased after *L. pneumophila* infection for 6 h (Fig. 2A). Infection with *L. pneumophila* also upregulated *SLIT3* expression in a dose-dependent manner (Fig. 2B). This result suggested that miR-218 induced by *L. pneumophila* infection was derived from the primary miR-218 from the introns of both *SLIT2* and *SLIT3*.

miR-218 was not cytotoxic to U937 cells

To investigate the potential cytotoxic impact of miR-218 on U937 cells, we transfected the cells with precursor miR-218 and the scrambled control oligo. Overexpression

of miR-218 did not alter the cytotoxicity of U937 cells over 24 h (Figure 2C). To inhibit the increase in miR-218 after *L. pneumophila* infection, U937 cells were transfected with antisense miR-218 before being infected with *L. pneumophila*. The knockdown of miR-218 decreased endogenous miR-218 levels at MOIs of 0–10 (Supplementary Figure 1B). Antisense miR-218 did not induce the death of U937 cells with or without *L. pneumophila* infection (Figs. 2D and 2E), suggesting that the alteration in miR-218 was not cytotoxic to U937 cells.

***miR-218 induced by* L. pneumophila *suppressed RICTOR* expression**

~~In silico analysis and p~~Previous studies indicated that Rictor is one of the targets of miR-218 [19, 20]. In our study, expression of the RICTOR protein was inhibited by *L. pneumophila* infection in U937 cells at MOIs of 0.5 and 2.5 (Fig. 3A). Rictor phosphorylates the serine residue (S473) of Akt in U937 cells. Phosphorylation of Akt (S473) was suppressed by infection with *L. pneumophila* (Fig. 3A). However, while *RICTOR* mRNA expression was not significantly decreased in U937 cells that were infected at an MOI of < 10, it was significantly suppressed at a MOI of 10, which is quite higher (Fig. 3B). Overexpression of miR-218 inhibited the expression of Rictor (Fig. 3C). Infection at an MOI of 2.5 suppressed RICTOR expression; however, knockdown of miR-218 restored the decrease in RICTOR (Figs. 3D and 3E). Taken together, the results suggest that the regulation of RICTOR by *L. pneumophila* infection in U937 cells was mediated by miR-218.

Enhanced cytokine production by* L. pneumophila *infection was mediated by RICTOR

To examine the inflammatory impact of *L. pneumophila* infection on U937 cells, the levels of proinflammatory cytokines IL-6 and TNF-alpha were measured and shown to be upregulated by infection with *L. pneumophila* (Fig. 4A). This upregulation of both IL-6 and

TNF-alpha was enhanced by the knockdown of RICTOR (Fig. 4A), indicating that RICTOR could be a critical molecule in the control of IL-6 and TNF-alpha production during *L. pneumophila* infection. Moreover, the increase in expression of both IL-6 and TNF-alpha was blocked by the knockdown of miR-218 (Fig. 4B), suggesting that cytokine production by *L. pneumophila* infection was mediated through miR-218 and RICTOR (Fig. 4C).

Discussion

Our study demonstrated that infection with *L. pneumophila* increased miR-218 expression in the human macrophage-like cell line U937. Downregulation of the key molecule of mTORC2, RICTOR, in *L. pneumophila*-infected U937 cells was expected as Rictor is a known target of miR-218. The upregulation of miR-218 during *L. pneumophila* infection enhanced the production of proinflammatory cytokines through suppression of *RICTOR* expression.

Bacterial pathogens including *Helicobacter pylori*, *Listeria monocytogenes*, *Salmonella enterica* serovar Typhi and *Mycobacterium tuberculosis* can modulate the expression of miRNAs in their host [21-23]. Macrophages play a crucial role in defending against a variety of such intracellular pathogens. The expression of miRNAs in macrophages is regulated by the inflammatory stimuli of bacterial infection [23]. In human and mouse macrophages infected with *M. tuberculosis*, for example, increased miR-142-3p expression inhibited actin filament assembly by suppressing N-Wasp [24]. In another study, the expression of miR-155 was upregulated by inflammatory stimulation in murine macrophages and increased miR-155–controlled inflammatory signaling [25]. Upregulation of miR-155 in murine macrophages was also observed during *H. pylori* infection [26]. Our findings showed that miR-155 was induced by *L. pneumophila* infection in U937 cells.

Several studies have shown alterations in the expression of multiple miRNAs during the infection of macrophages by *Legionella spp*. The expression of miR-125a-3p was upregulated by *L. pneumophila* infection in murine bone marrow-derived macrophages and the macrophage cell line RAW264.7 [27]. This increase in miR-125a-3p expression controls antigen stability and epitope generation by suppressing NTAN1. The same group also focused on the outer membrane vesicle secreted by Gram-negative bacteria. The outer membrane vesicles derived from *L. pneumophila*-infected, PMA-differentiated, macrophage-like THP-1

cells increased the expression of the anti-inflammatory miRNA known as miR-146a, which augments bacterial replication [28]. This is the first reported identification of another novel miRNA, miR-218, induced by *L. pneumophila* in U937 cells.

Acting as a tumor-suppressor miRNA, miR-218 inhibits cancer progression and metastasis [29]. The expression of miR-218 is downregulated in many types of tumors, including those of colorectal, gastric and lung cancers [30-32]. In general, miR-218 levels are lower in cancer specimens compared with their corresponding normal tissues [29]. There are two types of miR-218 precursors encoded by the host genes *slit2* and *slit3*, with patterns of expression that vary according to the type of cancer. In cases of lung squamous cell carcinoma, expression of both *slit2* and *slit3* was decreased to an equal degree; however, in some thyroid cancers reduced expression of *slit3* only was found [33, 34]. Our findings showed that both *SLIT2* and *SLIT3* can contribute to the upregulation of miR-218 in *L. pneumophila*-infected U937 cells. Further studies will be required to elucidate the dynamics of *slit2*, *slit3* and miR-218 regulation during the infection of macrophages with *L. pneumophila*.

In contrast to the established role of miR-218 in cancers, no previous reports have demonstrated a function of miR-218 in macrophages. Our study has elucidated miR-218–modulated cytokine production in the macrophage-like cell line U937. We chose to investigate the role of Rictor, a component of mTORC2, in macrophages because it has been shown to be a direct target of miR-218 in oral and prostate cancers [20, 35]. As shown in Figure 3C, overexpression of miR-218 downregulated RICTOR protein level in U937 cells, suggesting that Rictor may be one of the target genes of miR-218. In mammalian cells, miRNA generally binds onto the 3’ untranslated region (3’UTR) of target mRNAs to suppress protein synthesis. This inhibition of protein synthesis is regulated in two ways, mRNA destabilization or translational repression [36, 37]. In our experiments, U937 cells

infected with *L. pneumophila* increased miR-218 expression, which decreased the expression of RICTOR protein (Fig. 3A). However, *RICTOR* mRNA was not significantly affected by infection with *L. pneumophila* (Fig. 3B). These results suggested that the suppression of RICTOR protein by miR-218 might be regulated by translational repression of *RICTOR* mRNA.

The mTOR pathway plays pivotal roles in regulating cell growth and metabolism by sensing environmental alterations [38], and this network also controls the effector responses of innate immune cells [39]. In macrophages, mTORC1 shapes the balance of proinflammatory and anti-inflammatory responses. Treatment with rapamycin to block mTORC1 activity enhanced the production of proinflammatory cytokines by NF-kB [40]. Hallowell RW et al. demonstrated that deletion of mTORC2 signaling in macrophages promoted increased expression of TNF-alpha, IL-1beta and IL-6 [41]. *Rictor* was activated in macrophages derived from the fibrotic kidneys of mice, and a knockout of *Rictor* in macrophages reduced inflammatory cell accumulation, even though the production of inflammatory cytokines was upregulated in this model. In accordance with this recent finding, we found that knockdown of *RICTOR* enhanced the expression of TNF-alpha and IL-6 induced by *L. pneumophila* infection in U937 cells. Knockdown of miR-218, in turn, suppressed the increase in cytokine expression. Taken together, these results suggest that the miR-218–Rictor axis might be the key pathway in the modulation of infection-related inflammatory responses in macrophages.

In summary, we found that miR-218 was upregulated by *L. pneumophila* in the human macrophage-like cell line U937. The expression of *RICTOR*, a component of mTORC2, was shown to a target for suppression by miR-218, thereby enhancing the production of the proinflammatory cytokines TNF-alpha and IL-6. Our findings highlight the importance of miR-218 and *RICTOR* in innate immune reactions against *L. pneumophila* infection in

macrophages.

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Figure legends

Figure 1. *Legionella pneumophila* increases miR-218 expression in U937 cells. (**A**). *L. pneumophila*-infected U937 cells under light microscope (Gimenez stain x1,000). **(B).** Detection of lactate dehydrogenase (LDH) release to evaluate cytotoxicity. *L. pneumophila* at an MOI of 100, but not at MOIs of 1–10, increased LDH release in U937 cells. **(C).** The expression of miR-218 and miR-155 in U937 cells infected with *L. pneumophila* at different MOIs was measured using quantitative polymerase chain reaction. Data are presented as mean ± SD of three independent experiments. ***p** < 0.01.

Figure 2. *Legionella pneumophila* upregulates *SLIT2* and *SLIT3* without affecting cellular viability in U937 cells. Expression of *SLIT2* **(A)** and *SLIT3* **(B)** was upregulated in *L. pneumophila*-infected U937 cells. Data are presented as mean ± SD of three independent experiments. ***p** < 0.01. **(C).** Overexpression of miR-218 did not affect cytotoxicity in U937 cells. **(D, E).** Knockdown of miR-218 did not affect cytotoxicity, with or without infection of *L. pneumophila.* Data are presented as mean ± SD of three independent experiments. ***p** < 0.01. NS: not significant.

Figure 3. RICTOR is regulated by miR-218 during *Legionella pneumophila* infection in U937 cells. (A). U937 cells were infected with *L. pneumophila* (MOI 0–2.5) for 2 days and the cell lysates were blotted. *L. pneumophila* infection decreased RICTOR expression and phosphorylation of AKT (ser473). **(B).** *L. pneumophila* infection at an MOI < 10 did not decrease mRNA expression of *RICTOR*. **(C).** Western blotting showed that overexpression of miR-218 inhibited RICTOR expression. **(D).** *L. pneumophila* (MOI 2.5) suppressed *RICTOR* expression, which was restored by knockdown of miR-218. **(E).** The quantification of (**D**). Data are presented as mean ± SD of three independent experiments. ***p** < 0.01 NS: not

significant.

Figure 4. IL-6 and TNF-alpha are regulated by *Legionella pneumophila* in U937 cells. (A, B). Measurement of mRNA expression of IL-6 and TNF-alpha in U937 cells transfected with silencing RNA of *RICTOR* and control, or antisense miR-218 and control, followed by infection with *L. pneumophila* (MOI 2.5) showed that: (**A**) silencing of Rictor enhanced *L. pneumophila*-induced IL-6 and TNF-alpha expression; and (**B**) knockdown of miR-218 diminished *L. pneumophila*-induced IL-6 and TNF-alpha expression. **(C)** Schematic representation showing increased miR-218 expression during infection with *L. pneumophila*. Upregulation of miR-218 inhibited *RICTOR* expression, leading to enhanced production of the proinflammatory cytokines IL-6 and TNF-alpha. Data are presented as mean ± SD of three independent experiments. ***p** < 0.01.

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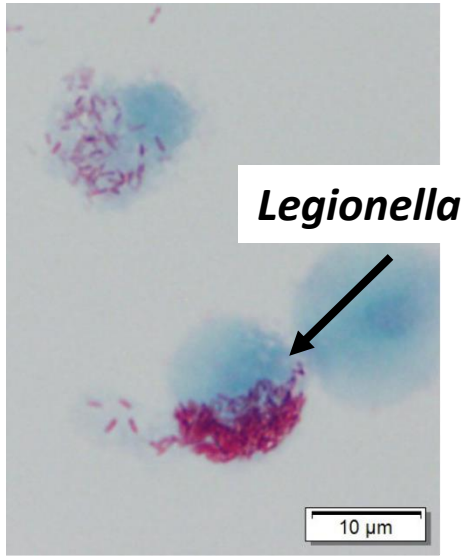
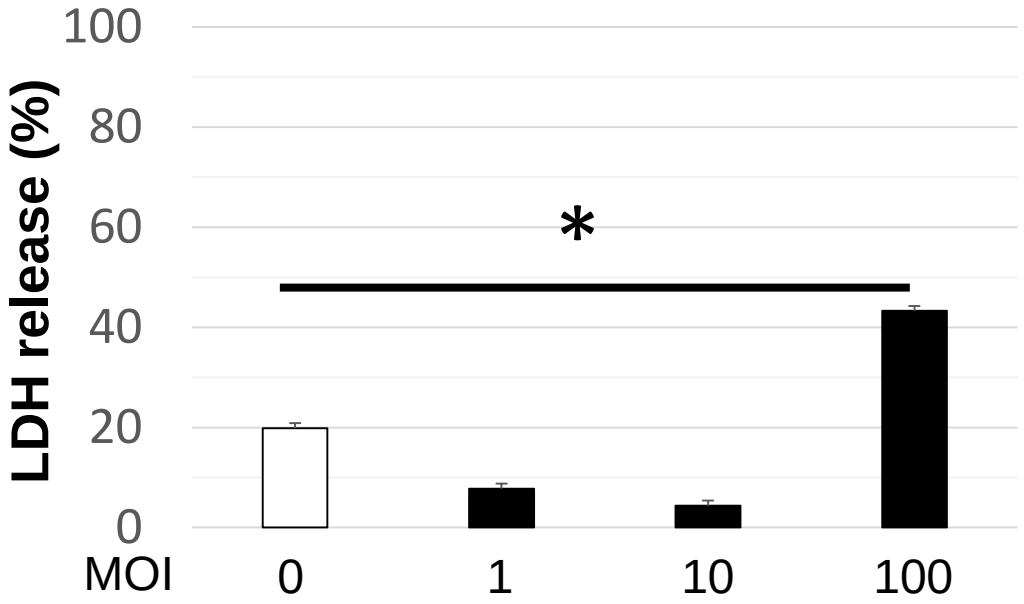
Figure 1

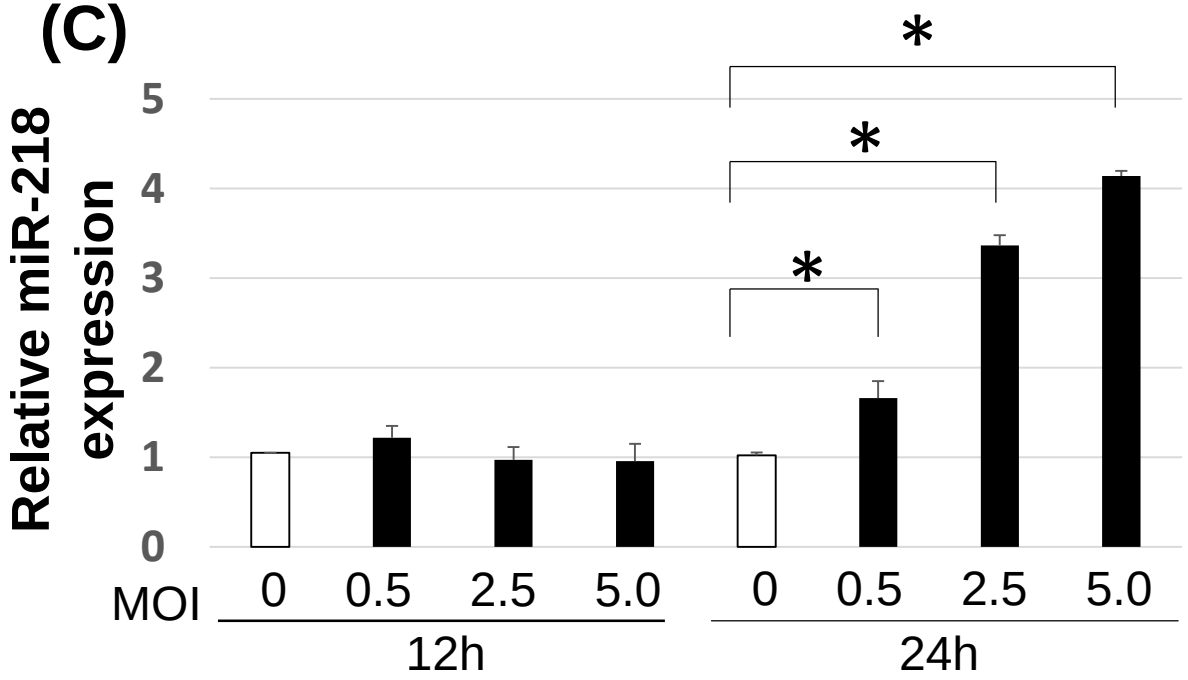
(A)


Legionella pneumophila
10 μm

(B)


LDH release (%)
100
80
60
40
20
0
*
MOI
0
1
10
100

(C)


Relative miR-218 expression
5
4
3
2
1
0
*
*
*
MOI
0
0.5
2.5
5.0
0
0.5
2.5
5.0
12h
24h

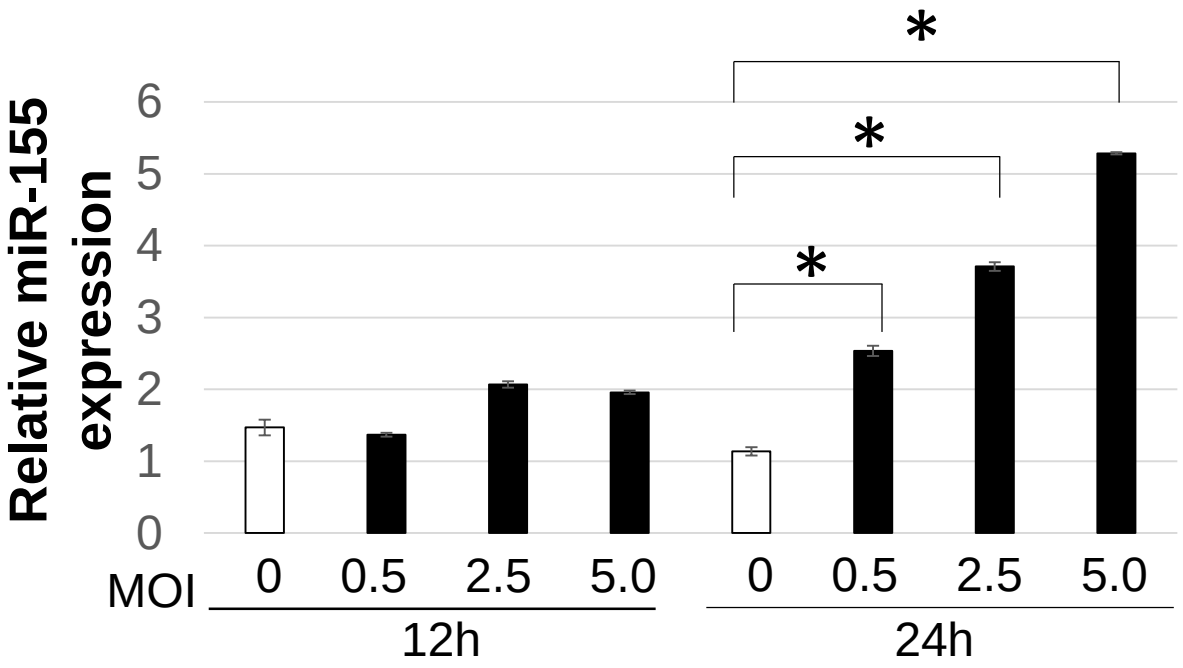

Relative miR-155 expression
6
5
4
3
2
1
0
*
*
*
MOI
0
0.5
2.5
5.0
0
0.5
2.5
5.0
12h
24h

Figure 2

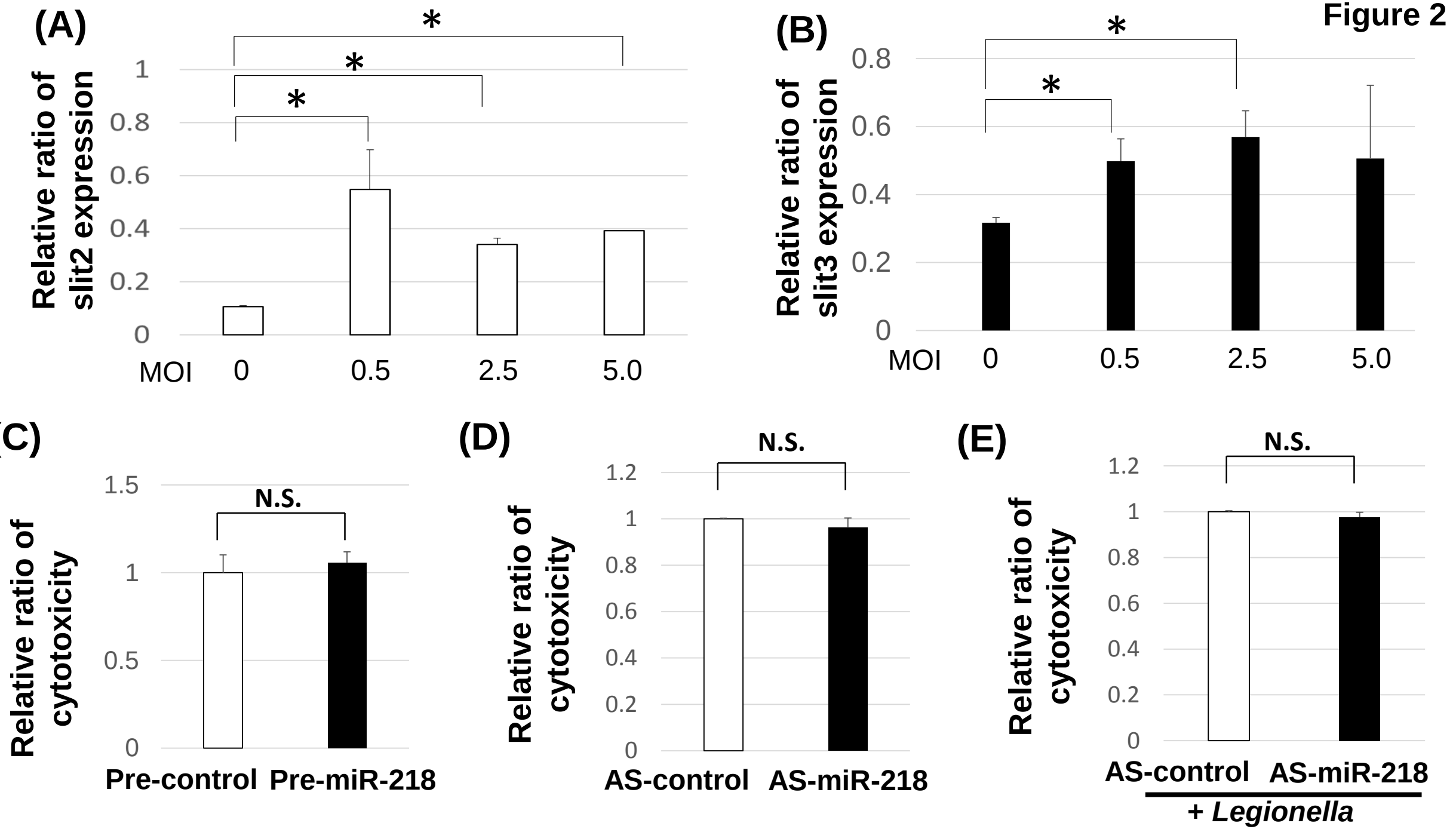
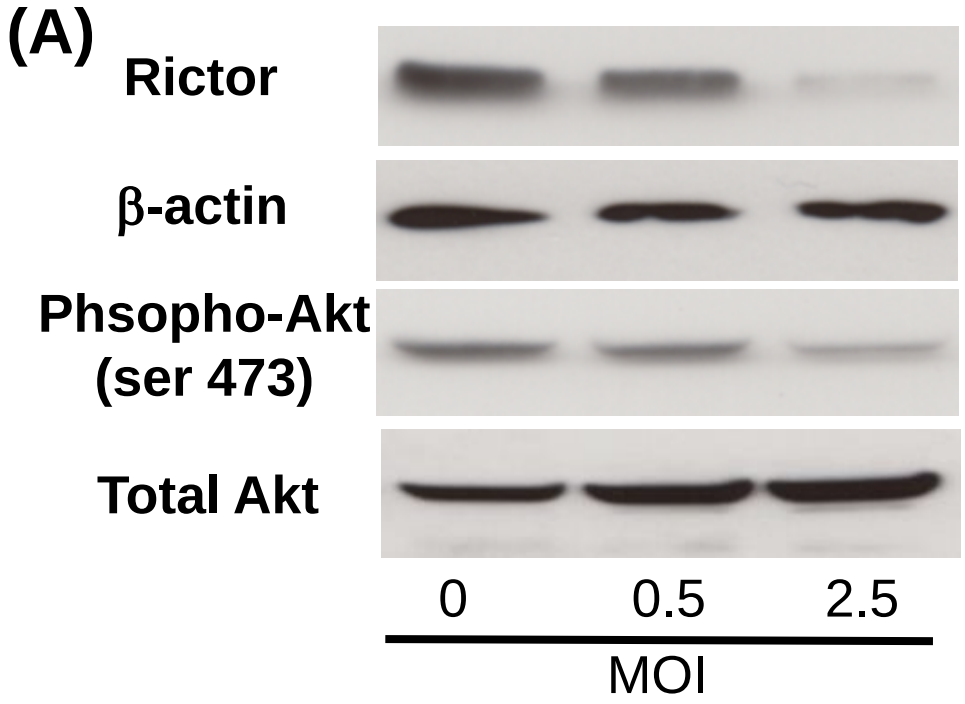
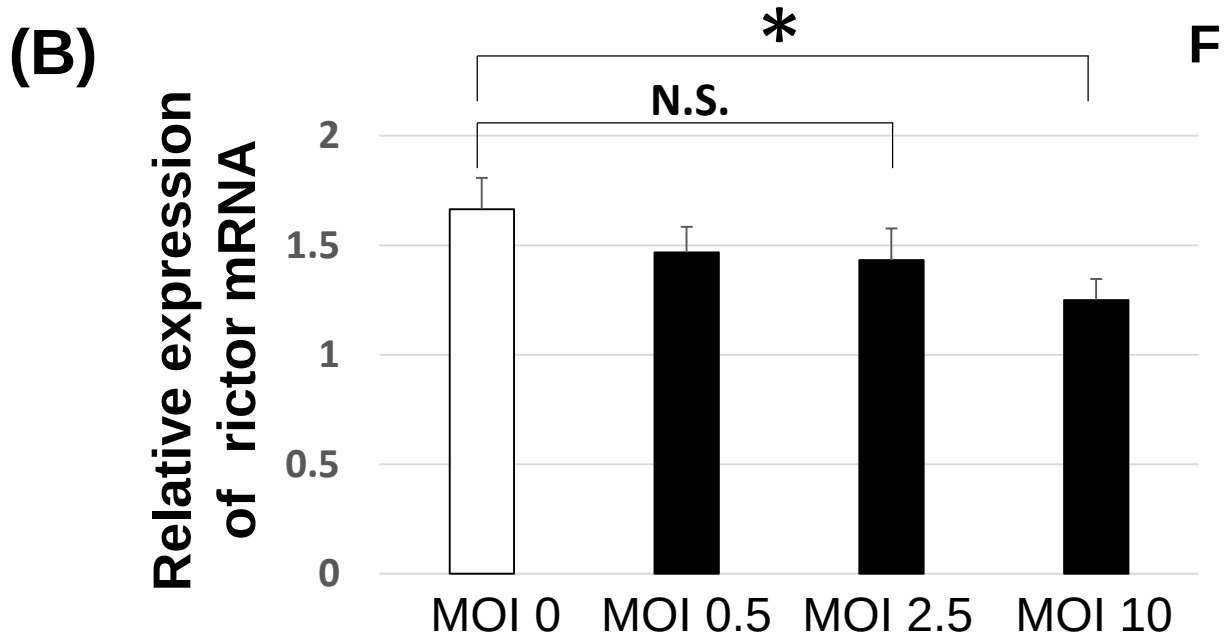
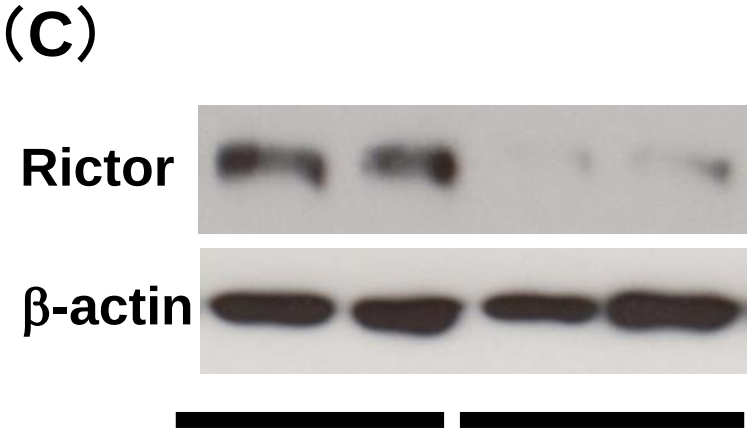
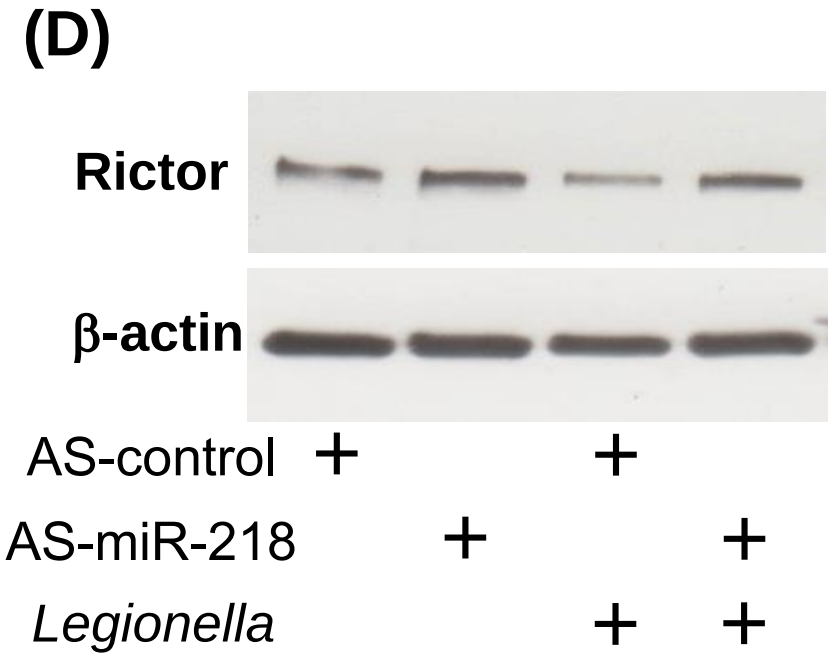

(A)
Relative ratio of slit2 expression
1
0.8
0.6
0.4
0.2
0
MOI 0 0.5 2.5 5.0
*
*
*
(B)
Relative ratio of slit3 expression
0.8
0.6
0.4
0.2
0
MOI 0 0.5 2.5 5.0
*
*
(C)
Relative ratio of cytotoxicity
1.5
1
0.5
0
N.S.
Pre-control Pre-miR-218
(D)
Relative ratio of cytotoxicity
1.2
1
0.8
0.6
0.4
0.2
0
N.S.
AS-control AS-miR-218
(E)
Relative ratio of cytotoxicity
1.2
1
0.8
0.6
0.4
0.2
0
N.S.
AS-control AS-miR-218
+ Legionella

Figure 3


(A)
Rictor
β-actin
Phsopho-Akt
(ser 473)
Total Akt
0
0.5
2.5
MOI


(B)
*
N.S.
Relative expression of rictor mRNA
2
1.5
1
0.5
0
MOI 0
MOI 0.5
MOI 2.5
MOI 10


(C)
Rictor
β-actin
Pre-control
Pre-miR-218


(D)
Rictor
β-actin
AS-control + +
AS-miR-218 + +
Legionella + +

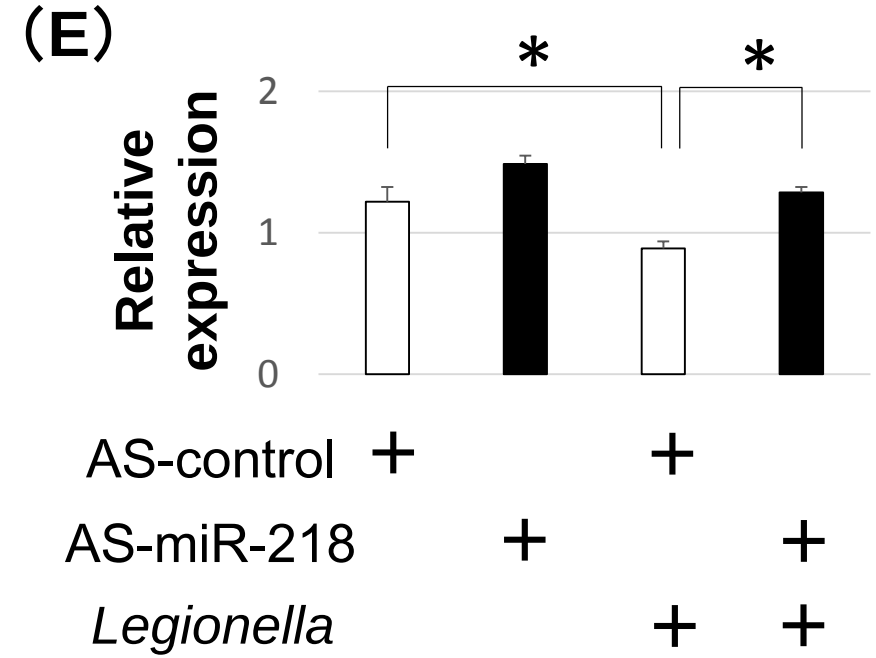
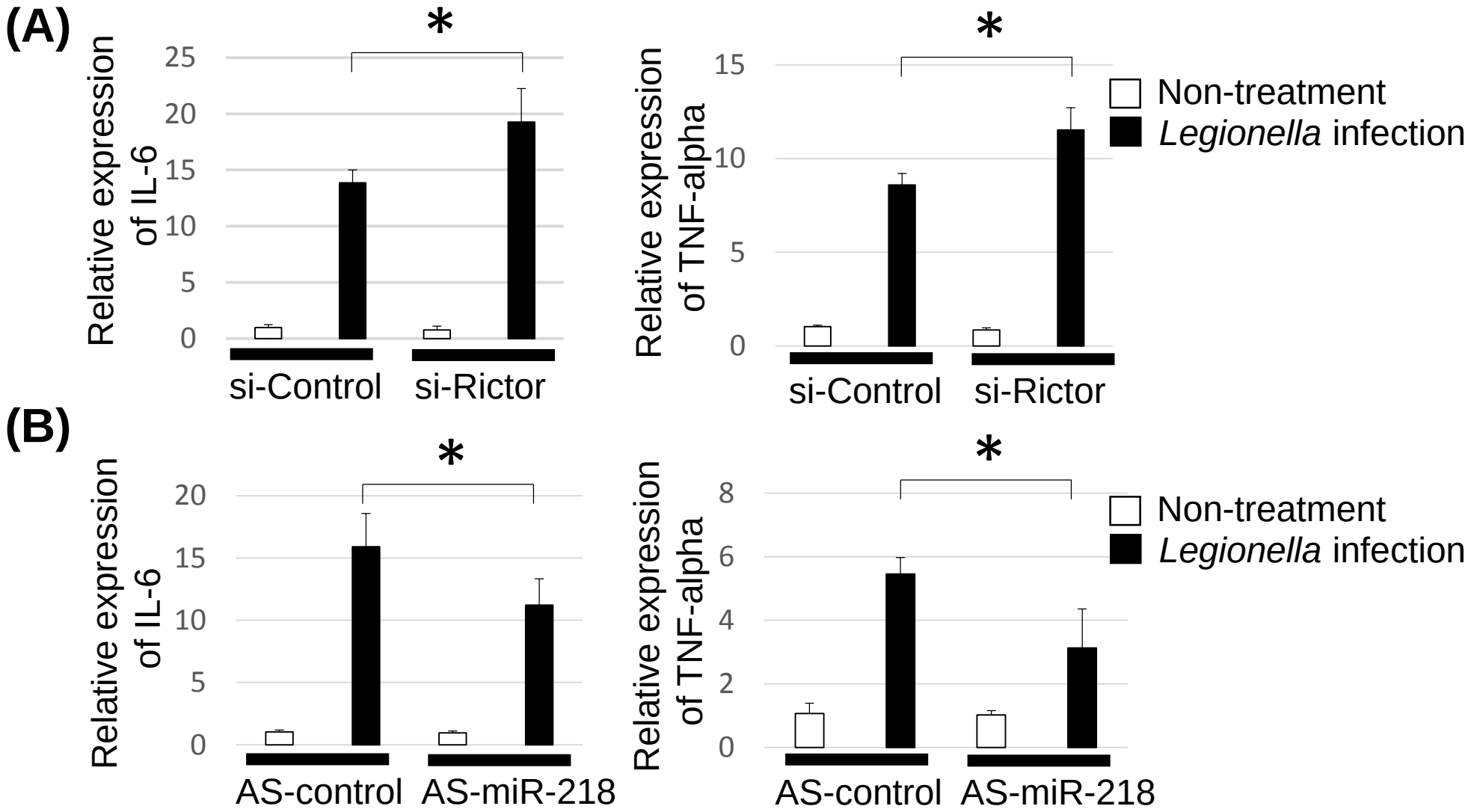
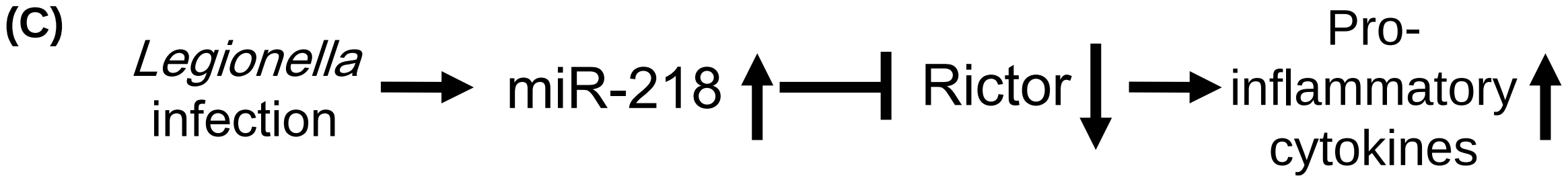

(E)
*
*
Relative expression
2
1
0
AS-control + +
AS-miR-218 + +
Legionella + +

Figure 4


(A)
Relative expression of IL-6
25
20
15
10
5
0
*
si-Control
si-Rictor
Relative expression of TNF-alpha
15
10
5
0
*
si-Control
si-Rictor
Non-treatment
Legionella infection
(B)
Relative expression of IL-6
20
15
10
5
0
*
AS-control
AS-miR-218
Relative expression of TNF-alpha
8
6
4
2
0
*
AS-control
AS-miR-218
Non-treatment
Legionella infection


(C)
Legionella infection
miR-218
Rictor
Pro-inflammatory cytokines

Supplementary Figure 1