**Lactobacillus rhamnosus** GR-1 Ameliorates *Escherichia coli*-Induced Activation of NLRP3 and NLRC4 Inflammasomes With Differential Requirement for ASC

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**INTRODUCTION**

*Escherichia coli* is a frequent cause of bovine mastitis and a leading cause of clinical mastitis in bovine (Shaheen et al., 2015). The NLR family member pyrin domain-containing protein 3 (NLRP3) inflammasome is considered a suitable target for new alternatives to antibiotics to treat bovine mastitis (Thacker et al., 2012). Our previous study showed that probiotic *Lactobacillus rhamnosus* GR-1 ameliorates *E. coli*-induced inflammatory damage via...
attenuation of apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC)-independent NLRP3 inflammasome activation in primary bovine mammary epithelial cells (PBMCs) (Wu et al., 2016). Therefore, L. rhamnosus GR-1 represents a potentially promising therapeutic agent targeting inflammasome activity in E. coli-associated bovine mastitis.

Binding of lipopolysaccharide (LPS) from gram-negative bacteria to toll-like receptor (TLR) 4 increases cellular expression of NLRP3 protein through nuclear factor-κB (NF-κB) signaling, leading to rapidly NLRP3 activation (Afonina et al., 2017). Upon activation, NLRP3 nucleates the adaptor protein ASC through interaction with the pyrin domain (PYD). Pro-caspase-1 is subsequently autoproteolytically processed through CARD–CARD (caspase recruitment domain) interactions in the NLRP3/ASC complex scaffold and cleaves precursors of the proinflammatory interleukin (IL)-1 family into their biactive forms, IL-1β and IL-18. We found that L. rhamnosus GR-1 reduces E. coli-induced caspase-1 activation and production of IL-1β and IL-18. However, in contrast to increases in the expression of NLRP3 and caspase-1, expression of the adaptor protein ASC is decreased in PBMCs infected with E. coli, even in cells pretreated with L. rhamnosus GR-1 (Wu et al., 2016).

In contrast to the multiple stimuli that activate NLRP3, NLR4 is activated by flagellin and the rod protein EscI of the E. coli type III secretion system (T3SS) apparatus (Miao et al., 2010). NLR4 contains a CARD motif, through which it directly oligomerizes with caspase-1 independent of ASC; this complex activates caspase-1 without autoproteolysis, triggering pyroptosis, an inflammatory form of cell death (Broz et al., 2010b). However, ASC greatly enhances the efficiency of NLR4-mediated maturation of IL-1β and IL-18 by inducing caspase-1 autoproteolysis (Lamkanfi and Dixit, 2014). NLR4-dependent production of IL-1β is induced by pathogenic Salmonella or Pseudomonas but not commensal Lactobacillus plantarum, indicating that the NLR4 inflammasome specifically discriminates pathogens and probiotic bacteria (Franchi et al., 2012). However, the contributions of the NLR4 inflammasome to inflammatory responses that control E. coli infections are less clear in relation to L. rhamnosus GR-1.

NLRP3 and NLR4 inflammasomes play a crucial role in potentiating the host antimicrobial response (Guo et al., 2015). Studies using ASC-deficient cells from ASC−/− mice demonstrated the dual role of ASC in bridging NLRP3 and NLR4 inflammasomes and caspase-1 via PYD and CARD and regulating the result of inflammasome activation (Broz et al., 2010a; Gueya et al., 2014). ASC-dependent inflammasome activation results in the production of proinflammatory IL-1β family cytokines, whereas ASC-independent inflammasome activation induces cell pyroptosis. Given the significant potential of IL-1 family cytokines to cause detrimental inflammation and pyroptosis to control the spread of intracellular pathogens (Jorgensen et al., 2016; Lannitti et al., 2016), the role of ASC in regulating inflammasome activity during E. coli infection must be examined in detail to determine and how L. rhamnosus GR-1 regulates the immune response to prevent E. coli-associated bovine mastitis.

In the present study, we knocked out the ASC gene in bovine mammary epithelial (MAC-T) cells using the RNA-guided clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated nuclease 9 (Cas9) system. We hypothesized that during E. coli infection, the activity of NLRP3 and NLRC4 inflammasomes is differentially regulated by L. rhamnosus GR-1, inducing maturation of IL-1β and IL-18 or cell pyroptosis, depending on ASC. We provide evidence that L. rhamnosus GR-1 suppresses E. coli-induced ASC-dependent activation of NLRP3 and NLRC4 inflammasomes and thus decreases production of IL-1β and IL-18 during E. coli infection. In addition, L. rhamnosus GR-1 suppresses E. coli-induced cell pyroptosis, in part through attenuation of NLRC4 inflammasome and non-canonical caspase-4 activation, independent of ASC.

**MATERIALS AND METHODS**

**Biosecurity Statement**

All bacterial strains were treated in strict accordance with the *Regulations on Biological Safety Management of Pathogen Microbiology Laboratory* (000014349/2004-00195) from the State Council of the People's Republic of China. The *E. coli* CVC01450 was subjected to all necessary safety procedures to avoid pathogen transmission and infection.

**Construction of CRISPR/Cas9 System Expression Vector**

Three guide RNAs (ASC-sgRNA1, ASC-sgRNA 2, and ASC-sgRNA 3) were designed to target the exon 1 regions of the bovine ASC gene (*Table 1*). A pair of oligos for each targeting site was annealed and ligated into the BbsI site of pCrispr-sg5, which was kindly provided by Professor Sen Wu (China Agricultural University, Beijing, China), to generate pCrispr-sg5-ASC-sgRNA1, pCrispr-sg5-ASC-sgRNA2, and pCrispr-sg5-ASC-sgRNA3 plasmids. All plasmids were confirmed by sequencing (Sangon Biotech, Shanghai, China).

**Cell Culture and Transfection**

MAC-T cells transferred with the SV40 T antigen (Huynh et al., 1991) was a gift from Dr. Ying Yu (China Agricultural University). MAC-T cells were cultured in Dulbecco's Modified Eagle medium/Ham's F-12 medium (1:1) supplemented with 10% heat-inactivated fetal calf serum, 100 U/mL of penicillin, and 1 g/mL of streptomycin (Invitrogen, Carlsbad, CA, United States) at 37°C in an atmosphere of 5% CO2 and 95% air at 95% relative humidity.

Plasmid DNA for cell transfection was prepared using an Omega Endo-free Plasmid Mini Kit II (Omega Bio-Tek Inc., Doraville, GA, United States). MAC-T cells (1 x 10^6) were electroporated with 1.5 µg of pCrispr-W9 plasmid, 1.5 µg of pCrispr-sg5-ASC-sgRNA plasmid, and 1 µg of PCAG-PBase plasmid using the T-020 program of an Amaza electroporator (Lonza, Allendale, NJ, United States), in which pCrispr-W9 encoded Cas9 nuclease and pCrispr-sg5-ASC-sgRNA encoded ASC-sgRNA. After electroporation, 300 cells...
TABLE 1 | Sequences of three guide RNAs designed to target the exon 1 region of the bovine ASC gene and primers for PCR amplification.

| Gene product | Primer | Accession number |
|--------------|--------|-----------------|
| ASC-sgRNA1   | F      | NM_174730.2     |
|              | R      |                 |
| ASC-sgRNA2   | F      | NM_174730.2     |
|              | R      |                 |
| ASC-sgRNA3   | F      | NM_174730.2     |
|              | R      |                 |
| ASC          | F      | NM_174730.2     |
|              | R      |                 |

were plated in a 10-cm dish using growth medium containing 350 μg/ml of selectable marker G418 (Sigma-Aldrich, St. Louis, MO, United States). After 10 days, individual clones were picked, and clonal cell populations were expanded. Before experiments, MAC-T cells were electroporated with pmaxGFP™ (Lonza) encoding green fluorescent protein to determine transfection efficiency using the T-020 and T-001 programs. MAC-T cells were chosen for CRISPR-Cas9 inactivation experiments due to their good transfection efficiency.

Sequencing and Protein Analysis of the Gene Target Site

Genomic DNA samples were extracted using a TIANamp Genomic DNA Kit (Tiangen, Beijing, China) according to the manufacturer’s instructions, and 50 ng of DNA template was used to amplify the 630-bp fragment encompassing the gene inactivation locus in 25 μl of PCR buffer (Takara, Shiga, Japan) using primer pairs listed in Table 1. The resulting PCR products were purified and subsequently sequenced to identify deletions. In addition, clonal cell population whole-cell extracts were analyzed by Western blotting.

Immunocytochemistry

The epithelial origin of MAC-T cells was tested by staining for cytokeratin 18. MAC-T cells (6 × 10^4 cells/well) were seeded into a 24-well culture plate with glass coverslips. After 24 h, cells were washed three times with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 15 min on ice. The cells were then permeabilized with 0.2% (v/v) Triton X-100 (Sigma-Aldrich) and blocked with 1% bovine serum albumin. Subsequently, cells were incubated with mouse anti-cytokeratin-18 primary monoclonal antibody at a dilution of 1:200 (Ab668; Abcam, Cambridge, United Kingdom) for 45 min at 4°C, following by incubation with secondary antibody, goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated IgG (F4143; Sigma-Aldrich). Cell nuclei were stained using 4’,6’-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Coverslips were imaged on an FV1000 confocal laser scanning biological microscope (Olympus, Tokyo, Japan).

Bacterial Strains and Growth Conditions

Lactobacillus rhamnosus GR-1 ATCC 55826 was purchased from the American Type Culture Collection (Manassas, VA, United States) and grown in De Man, Rogosa, and Sharpe (MRS) broth (Oxoid, Hampshire, United Kingdom) for 24 h at 37°C under microaerophilic conditions. After overnight incubation, L. rhamnosus GR-1 was subcultured at a dilution of 1:100 in fresh MRS broth for approximately 8 h until reaching mid-log phase [optical density (OD) at 600 nm (OD<sub>600</sub>) of 0.5] for all experiments.

Escherichia coli CVCC1450 (serotype O111:K58) was purchased from the China Institute of Veterinary Drug Center (Beijing, China) and grown in Luria–Bertani (LB) broth (Oxoid). After overnight incubation at 37°C with vigorous shaking, bacteria were diluted 1:100 in fresh LB and grown for approximately 3 h until reaching mid-log phase (OD<sub>600</sub> of 0.5).

Adhesion Assay

Wild-type (WT) and ASC<sup>−/−</sup> MAC-T cells (3 × 10^5 cells/well) were seeded onto a six-well transwell collagen-coated polytetrafluoroethylene (PTFE) filter. Confluent cell monolayers were pretreated with L. rhamnosus GR-1 (3 × 10^7 CFU) for 3 h, and then were washed three times with PBS and exposed to E. coli (3 × 10^7 CFU). At 1.5, 3, and 6 h after E. coli challenge, the cell monolayers were washed four times with PBS to remove non-adherent bacteria and treated with 0.05% trypsin for 10 min at 37°C. Cells were harvested by centrifugation for 10 min at 4000 g and lysed using 100 μl of 0.2% Triton X-100 (Sigma-Aldrich) in sterile water. The populations of E. coli and L. rhamnosus GR-1 were determined on LB and MRS agar plates, respectively. The adhesion rate of E. coli was defined as the adhered E. coli population on the cells pretreated with L. rhamnosus GR-1 relative to the adhered E. coli population in the adhesion assay of E. coli infection alone.

Immunofluorescence

Confluent monolayers of WT and ASC<sup>−/−</sup> MAC-T cells (6 × 10^5 cells/well) grown on glass coverslips in a 24-well flat-bottom culture plate were treated under four different conditions, as follows: (i) medium alone (CONT); (ii) E. coli alone (6 × 10^6 CFU) at a multiplicity of infection (MOI) of 100:1 (ECOL); (iii) incubation with L. rhamnosus GR-1 (6 × 10^6 CFU) at a MOI of 100:1 for 3 h (LRGR); or (iv) pre-incubation with L. rhamnosus GR-1 (6 × 10^6 CFU) for 3 h prior to addition of E. coli (LRGR + ECOL). At 6 h after E. coli infection, the cells were washed, fixed with 4% paraformaldehyde for 15 min on ice, permeabilized with 0.2%
(v/v) Triton X-100 (Sigma-Aldrich), and blocked with 1% bovine serum albumin. Subsequently, the following primary monoclonal antibodies were used: mouse anti-cytokeratin-18 (Ab668, 1:200 dilution; Abcam), rabbit anti-ASC (10500-1-AP, 1:500 dilution; Proteintech Group, Chicago, IL, United States), and mouse anti-caspase-1 (22915-1-AP, 1:500 dilution; Proteintech Group). The cells were incubated with the primary antibody for 45 min at 4°C, followed by incubation with goat anti-rabbit Cy-3 (AP307F, 1:200 dilution; Sigma-Aldrich) or FITC-conjugated IgG (F-0382, 1:40 dilution; Sigma-Aldrich) as the secondary antibody. Cell nuclei were stained with DAPI. The coverslips and slides were visualized and photographed under an FV1000 confocal laser scanning biological microscope (Olympus).

Western Blotting
WT and ASC−/− MAC-T cells (6 × 10⁴ cells/well) were seeded onto a six-well transwell collagen-coated PTFE filter and treated with *E. coli* or *L. rhamnosus* GR-1 at a MOI of 100:1, as described above. Cells were also simultaneously treated with lactate at a concentration of 0.6 g/L (equivalent to 7 mM) and *E. coli* at a MOI of 100:1. At 1.5, 3, and 6 h after *E. coli* infection, cells were extracted using Radio-Immunoprecipitation Assay buffer (Sigma-Aldrich), as previously described (Wu et al., 2016). The primary antibodies were as follows: rabbit anti-NLRP3 (19771-1-AP, 1:200 dilution; Proteintech Group), rabbit anti-NLRC4 (12421, 1:1,000 dilution; Cell Signaling Technologies Inc., Danvers, MA, United States), mouse anti-caspase-1 (sc56036) (14F468, 1:500 dilution; Santa Cruz Biotechnology, Dallas, TX, United States), rabbit anti-cleaved caspase-4 (Glm81) (GTX86890, 1:250 dilution; GeneTex, Inc., San Antonio, TX, United States), and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 60004-1-Ig, 1:500 dilution; Proteintech Group). Horseradish peroxidase-conjugated AffiniPure goat anti-mouse IgG (SA00001-2, 1:5,000 dilution; Proteintech Group) or goat anti-rabbit IgG (SA00001-2, 1:5,000 dilution; Proteintech Group) were used as secondary antibodies. The detection of NLRP3 and caspase-1 proteins was performed in the same gel. After NLRP3 and caspase-1 proteins were visualized, blots were then stripped using Restore Western Blot Stripping Buffer (Solarbio, Beijing, China), and re-probed with the desired antibodies for GAPDH. The OD of each band was quantified by densitometric analysis using Quantity One software (Bio-Rad Laboratories, Richmond, CA, United States). Data were presented as the ratio of the NLRP3, NLRC4, caspase p10 intensity.

Lactate Dehydrogenase (LDH) Assay
The death of WT or ASC−/− MAC-T cells under the different conditions was assessed using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, United States) according to the manufacturer’s instructions. The assay measures the release of LDH into the supernatant, calculated as the percentage of total LDH content as determined from cell lysates (100%). LDH released by uninfected cells was used as a maximum-lysis control. The percentage of LDH released was calculated using the following equation: [(LDH infected–LDH uninjected)/(LDH total lysis–LDH uninjected)] × 100.

Enzyme-Linked Immunosorbert Assay (ELISA)
The concentrations of IL-1β and IL-18 in cell-free supernatants of WT or ASC−/− cells were determined at 1.5, 3, and 6 h after *E. coli* infection using commercially available ELISA kits specific for bovine IL-1β (DG90995Q) and bovine IL-18 (DG91524Q; Beijing Dongge Biotechnology Co., Beijing, China).

Quantification of Lactate Content
Cell culture supernatants were collected. Lactate content in the supernatants were quantified using the enzymatic kit K-DLATE (Megazyme, Bray, Ireland) that allows the measurement of both D-lactate and L-lactate.

Statistical Analysis
Statistical analysis was performed using the SAS statistical software package, version 9.1 (SAS Institute Inc., Cary, NC, United States). With regard to small sample sizes, normal distribution and homogeneity of variance were assumed using the UNIVARIATE (Shapiro–Wilk test) and HOVTEST procedures. Natural logarithm transformation was performed prior to analysis for IL-1β and IL-18 data to yield a normal distribution. Statistical significance of differences was tested using ANOVA procedures, following Tukey’s honestly significant difference post hoc test. Data of adhesion assay were compared by an unpaired two-tailed Student’s t-test. Data were visualized using GraphPad Prism5 software (GraphPad Software Inc., San Diego, CA, United States). Data from adhesion assay are presented as the mean ± standard deviation (SD) and data from Western blotting, LDH, ELISA and lactate quantification assays are presented as the mean ± standard error of the mean (SEM). Results are representative of three independent experiments, each performed in triplicate. P-values: *P < 0.05; **P < 0.01; ***P < 0.001.

RESULTS
CRISPR/Cas9 Mediates Knockout of ASC in MAC-T Cells
To demonstrate the role of ASC in *L. rhamnosus* GR-1 modulation of inflammasome activation during *E. coli* infection, we attempted knockout of the ASC gene in MAC-T cells using the CRISPR/Cas9 system. Upon immunocytochemistry analysis, MAC-T cells showed intense positive staining for epithelial cell-specific cytokeratin-18 in the cytoplasmic meshwork of cytokeratin fibrils (Supplementary Figure S1). Compared with program W-001, after electroporation with program T-020, MAC-T cells exhibited higher transfection efficiency (Figure 1A). Thus, the ASC gene knockout experiment was subsequently performed in MAC-T cells using program
T-020. Among the three designed sgRNA sequences, the specific 20-nucleotide sgRNA1 sequence targeted the exon 1 regions of the ASC gene and directed Cas9 nuclease to precisely introduce a DNA double-strand break in front of a protospacer adjacent motif (PAM). After cloning and sequencing of the DNA fragment, an 8-bp deletion was observed (Figure 1B). Western blot analysis did not show expression of ASC protein in sgRNA1 sequence-targeted MAC-T cells (Figure 1C). Furthermore, E. coli infection triggered assembly of ASC specks in WT cells, whereas pre-incubation with L. rhamnosus GR-1 attenuated E. coli-induced ASC speck assembly. No ASC specks were observed in ASC−/− cells, regardless of E. coli infection (Figure 1D). These results demonstrated that knockout of the ASC gene in MAC-T cells was successful.

Pre-incubation With L. rhamnosus GR-1 Reduces the Adhesion of E. coli to MAC-T Cell Monolayers

Escherichia coli or L. rhamnosus GR-1 exhibited similar adhesion capacity in WT and ASC−/− MAC-T cells. The number of adherent E. coli was about $1.29 \times 10^4 \pm 0.67 \times 10^2$ CFU (means ± standard deviation) at 1.5 h after E. coli infection,
and increased to $1.95 \times 10^4 \pm 1.17 \times 10^3$ CFU at 6 h. At 3 h after *E. coli* challenge, the number of adherent *E. coli* in ASC−/− cells was lower ($P = 0.007$) than in WT MAC-T cells (Figure 2A). *L. rhamnosus* GR-1 had a lower adhesion capacity and the number of adherent *L. rhamnosus* GR-1 was about $8.75 \times 10^3 \pm 1.56 \times 10^3$ CFU, regardless of infection time. Pre-incubation with *L. rhamnosus* GR-1 resulted in a reduction in the *E. coli* adhesion rate to 57% of that observed in MAC-T cells infected with *E. coli* alone (Figure 2B). No differences were observed in the number of *E. coli* recovered from the supernatant fraction among two groups (Figure 2C).

**Pre-incubation With *L. rhamnosus* GR-1 Attenuates *E. coli*-Induced NLRP3 Expression and Increases Lactate Content in the Supernatants**

Compared with untreated control WT cells, Western blot analysis showed an increase in NLRP3 protein expression at 1.5, 3, and 6 h after *E. coli* challenge in cells only infected with *E. coli*, but not in cells incubated with *L. rhamnosus* GR-1 alone ($P = 0.046$, $P < 0.001$, and $P < 0.001$, respectively; Figures 3A–C). In contrast, WT cells pre-incubated with *L. rhamnosus* GR-1 had a lower expression of NLRP3 protein than did WT cells only infected with *E. coli* at 3 and 6 h ($P < 0.001$ for both).

Compared with WT cells, ASC−/− MAC-T cells exhibited a similar differential response to *E. coli* challenge and *L. rhamnosus* GR-1 incubation. Compared with untreated control ASC−/− cells, at 1.5, 3, and 6 h after *E. coli* challenge, NLRP3 protein expression was elevated in ASC−/− cells only infected with *E. coli* ($P = 0.036$, $P = 0.005$, and $P < 0.001$, respectively; Figures 3A–C) but not in ASC−/− cells pre-incubated with *L. rhamnosus* GR-1.

The lactate content (d-lactate plus l-lactate) in the supernatants was quantified. Compared with untreated control cells, the lactate content was increased in the supernatants from both WT and ASC−/− cells incubated with *L. rhamnosus* GR-1 alone or pre-incubated with *L. rhamnosus* GR-1, but not the cells only infected with *E. coli* at 1.5, 3, and 6 h after infection, regardless of ASC knockout ($P < 0.05$; Supplementary Figure S2A). Western blot analysis showed that compared with untreated control cells, at 6 h after *E. coli* challenge, NLRP3 protein expression was elevated in WT or ASC−/− cells infected with *E. coli*, but not in cells only treated with lactate alone ($P < 0.05$; Supplementary Figure S2B). Lactate addition did not attenuate *E. coli*-induced increase in NLRP3 protein expression in either WT or ASC−/− cells.
Effect of \textit{L. rhamnosus} GR-1 on NLRC4 Activation During \textit{E. coli} Infection

Compared with untreated control cells, expression of NLRC4 protein was elevated at 3 h after \textit{E. coli} infection in WT cells only infected with \textit{E. coli} (\( P = 0.027 \); Figure 4B) but not in WT cells incubated with \textit{L. rhamnosus} GR-1 alone or pre-incubated with \textit{L. rhamnosus} GR-1. Compared with WT cells only infected with \textit{E. coli}, expression of NLRC4 protein at 3 h was decreased in WT cells incubated with \textit{L. rhamnosus} GR-1 alone and pre-incubated with \textit{L. rhamnosus} GR-1 (\( P = 0.019 \) and \( P = 0.001 \), respectively). No differences were observed at 3 h in ASC\(^{-/-}\) cells, regardless of treatment.

Compared with untreated control cells, \textit{E. coli} challenge led to increased expression of NLRC4 protein at 6 h after \textit{E. coli} challenge in WT cells (\( P = 0.019 \)) and ASC\(^{-/-}\) cells (\( P < 0.001 \); Figure 4C). Expression of NLRC4 protein was lower in ASC\(^{-/-}\) cells incubated with \textit{L. rhamnosus} GR-1 alone and pre-incubated with \textit{L. rhamnosus} GR-1 (\( P = 0.001 \) for both) than in ASC\(^{-/-}\) cells only infected with \textit{E. coli}. No changes were observed at 1.5 h after \textit{E. coli} infection in WT cells or ASC\(^{-/-}\) cells, regardless of treatment (Figure 4A).

Pre-incubation With \textit{L. rhamnosus} GR-1 Attenuates \textit{E. coli}-Induced Caspase-1 Maturation

Immunofluorescence staining showed that compared with untreated control cells, \textit{E. coli} challenge triggered assembly of ASC specks in WT cells at 6 h after \textit{E. coli} challenge, and this was attenuated in WT cells pre-incubated with \textit{L. rhamnosus} GR-1 (Figure 5A). No ASC specks were observed in ASC\(^{-/-}\) cells, regardless of treatment. Compared with untreated control cells, bright foci indicative of increased caspase-1 expression was observed in WT cells only infected with \textit{E. coli} at 6 h after challenge; this increase was attenuated by incubation with \textit{L. rhamnosus} GR-1 (Figure 5B). Compared with WT cells, ASC deletion attenuated, but did not abolish, caspase-1 staining in response to \textit{E. coli} infection and pre-incubation with \textit{L. rhamnosus} GR-1. A punctate staining pattern for caspase-1 was observed in ASC\(^{-/-}\) cells only infected with \textit{E. coli}, and pre-incubation with \textit{L. rhamnosus} GR-1 inhibited the \textit{E. coli}-induced punctate caspase-1 staining pattern. Compared with untreated control cells, incubation with \textit{L. rhamnosus} GR-1 only did not result in
increase in caspase-1 activation either in WT or ASC\(^{-/-}\) cells (Figure 5B).

Compared with untreated control cells, increased maturation of procaspase-1 into its catalytic 10-kDa subunit was observed at 6 h after \(E.\ coli\) challenge in WT and ASC\(^{-/-}\) cells only infected with \(E.\ coli\) (\(P < 0.001\) for both; Figure 6C). However, maturation of caspase-1 declined in WT and ASC\(^{-/-}\) cells pre-incubated with \(L.\ rhamnosus\) GR-1 (\(P = 0.014\) and \(P = 0.006\), respectively) or incubated with \(L.\ rhamnosus\) GR-1 alone (\(P < 0.001\) for both), compared with WT cells infected with \(E.\ coli\) only. No differences were observed in caspase-1 maturation at 1.5 and 3 h after \(E.\ coli\) infection in WT cells or ASC\(^{-/-}\) cells, regardless of treatment (Figures 6A,B).

Pre-incubation With \(L.\ rhamnosus\) GR-1 Attenuates \(E.\ coli\)-Induced Caspase-4 Activation

At 3 h after \(E.\ coli\) infection, expression of cleaved caspase-4 (26 kDa) in WT and ASC\(^{-/-}\) cells pre-incubated with \(L.\ rhamnosus\) GR-1 was lower than in untreated control cells and cells only infected with \(E.\ coli\) (Figure 7B). Infection with \(E.\ coli\) resulted in increased expression of cleaved caspase-4 in WT and ASC\(^{-/-}\) cells (\(P = 0.001\) and \(P = 0.004\), respectively; Figure 7C) at 6 h. However, expression of caspase-4 decreased in WT and ASC\(^{-/-}\) cells pre-incubated with \(L.\ rhamnosus\) GR-1, compared with cells only infected with \(E.\ coli\) (\(P < 0.001\) and \(P = 0.001\), respectively). No differences were observed in the expression of cleaved caspase-4 at 1.5 h after \(E.\ coli\) infection in WT or ASC\(^{-/-}\) cells, regardless of treatment (Figure 7A).

Pre-incubation With \(L.\ rhamnosus\) GR-1 Attenuates \(E.\ coli\)-Induced Production of IL-1\(\beta\) and IL-18 and Cell Pyroptosis

Compared with untreated control cells, IL-1\(\beta\) production was increased at 3 and 6 h after \(E.\ coli\) infection in WT cells only infected with \(E.\ coli\) (\(P < 0.001\) for both; Figure 8A). IL-1\(\beta\) production was lower at 3 and 6 h both in WT cells incubated with \(L.\ rhamnosus\) GR-1 alone (\(P = 0.002\) and \(P = 0.004\), respectively) and cells pre-incubated with \(L.\ rhamnosus\) GR-1 (\(P = 0.013\) and \(P = 0.011\), respectively) than in WT cells only infected with \(E.\ coli\). Compared with WT cells, ASC deletion led to a decrease in production of IL-1\(\beta\) in ASC\(^{-/-}\) cells in response to different treatments. Challenge with \(E.\ coli\) resulted in elevated production of IL-1\(\beta\) at 1.5, 3, and 6 h after \(E.\ coli\) challenge.
infection in ASC−/− cells only infected with E. coli (P = 0.009, P = 0.021, and P = 0.001, respectively; Figure 8A) compared with untreated control cells; however, the E. coli-induced increase in IL-1β production was attenuated at 3 and 6 h by incubation with L. rhamnosus GR-1 alone (P = 0.014 and P = 0.007, respectively) and pre-incubation with L. rhamnosus GR-1 (P = 0.009 and P = 0.003, respectively).

IL-18 exhibited similar differential production as IL-1β. Compared with untreated control cells, production of IL-18 was increased at 3 and 6 h after E. coli challenge both in WT (P = 0.001 for both) and ASC−/− (P < 0.001 for both) cells only infected with E. coli (Figure 8A). However, incubation with L. rhamnosus GR-1 alone and pre-incubation with L. rhamnosus GR-1 attenuated the E. coli-induced elevation in the concentration of IL-18 at 3 and 6 h after E. coli challenge both in WT and ASC−/− cells. Compared with untreated control ASC−/− cells, the concentration of IL-18 was elevated at 3 and 6 h in ASC−/− cells pre-incubated with L. rhamnosus GR-1 (P = 0.008 and P = 0.002, respectively).

Cell pyroptosis was quantified by monitoring the release of LDH into the supernatants after E. coli challenge. Compared with untreated control cells, the percentage of pyroptotic cells at 3 and 6 h was increased in WT cells only infected with E. coli (P < 0.001 for both; Figure 8B) but not in WT cells incubated with L. rhamnosus GR-1 alone and pre-incubated with L. rhamnosus GR-1. Compared with WT cells, ASC deletion led to a similarly differential but attenuated cell pyroptosis. Compared with untreated control cells, the percentage of pyroptotic cells was increased at 3 and 6 h in ASC−/− cells only infected with E. coli (P = 0.020 and P < 0.001, respectively), whereas incubation with L. rhamnosus GR-1 alone and pre-incubation with L. rhamnosus GR-1 ameliorated the E. coli-induced increase in pyroptotic cell death at 6 h (P < 0.001 and P = 0.002, respectively). No changes were observed in WT and ASC−/− cells, regardless of treatment.

**DISCUSSION**

Bacterial adhesion to host epithelial cells is an essential step in the initiation of infection. Lactobacillus can reduce pathogen adhesion to epithelial cells and exert direct antimicrobial activity due to accumulation of antimicrobial substances (Gudina et al., 2015). We found that L. rhamnosus GR-1 did not directly kill E. coli, but did decrease the level of E. coli adhesion to 57% of that observed in MAC-T cells infected with
E. coli alone. We previously revealed that live and ultraviolet-irradiated L. rhamnosus GR-1 rather than culture supernatant of L. rhamnosus GR-1 and medium acidified with lactate lead to a decrease in the E. coli adhesion rate in bovine mammary epithelial cells (Wu et al., 2016). The reduced E. coli adhesion level mediated by L. rhamnosus GR-1 may be attributed to steric hindrance due to competition for attachment sites (Ardita et al., 2014; Tytgat et al., 2016).

NLRP3 is activated by a wide variety of stimuli, including pore-forming toxins, extracellular adenosine triphosphate, RNA-DNA hybrid molecules, and pathogens (Jo et al., 2016). We found that E. coli infection also increased the expression of NLRP3 protein from 1.5 to 6 h, but L. rhamnosus GR-1 pretreatment inhibited this increase. NLRP3 must be primed before activation. Escherichia coli LPS binds to TLR4 to induce expression of NLRP3 protein via NF-κB signaling. Bacterial mRNA from viable E. coli cells that have been phagocytosed enters the cytosolic compartment, resulting in assembly of the NLRP3 inflammasome (Sander et al., 2011). Intake of L. plantarum CECT 7315/7316 downregulates expression of Nlrp3 in the ileum of rats (Vilahur et al., 2015). However, the NLRP3 inflammasome is also activated by L. rhamnosus GG and LC705 originating from dairy sources (Miettinen et al., 2012).

In a mouse immune hepatitis model, lactate treatment was shown to attenuate hepatic and pancreatic injury by negatively regulating TLR4-mediated activation of the NLRP3 inflammasome and production of IL-1β through arrestin β2 and G-protein-coupled receptor 81 (Hoque et al., 2014). In the present study, there was a higher lactate content in the supernatants of cells incubated with L. rhamnosus GR-1. However, additional lactate treatment did not attenuate the E. coli-induced increase in expression of NLRP3. This indicates that the elevated lactate content in the supernatant is a secondary effect of L. rhamnosus GR-1 treatment and cannot account for attenuation of E. coli-induced activation of NLRP3. Previously, we have shown that L. rhamnosus GR-1 attenuates E. coli-induced TLR4 expression in bovine mammary epithelial cells (Wu et al., 2016). This may contribute to attenuating the priming step and subsequent activation of NLRP3 through TLR4-mediated NF-κB signaling (Bauernfeind et al., 2009). The mitogen-activated protein kinase (MAPK) subfamilies, c-Jun N-terminal kinase (JNK) and extracellular regulated protein kinase (ERK) are essential for NLRP3 inflammasome activation and addition of JNK1/2 inhibitor SP600125 or upstream MAPK/ERK kinase inhibitor PD98059 of ERK inhibits the LPS-induced increase in NLRP3 protein expression (Liao et al., 2013). Culture medium of L. rhamnosus GR-1 inhibits LPS-induced JNK
activation in macrophages or monocytic THP-1 cells (Kim et al., 2006). Histamine derived from Lactobacillus reuteri 6475 inhibits activation of ERK in THP-1 cells (Thomas et al., 2012). It is possible that L. rhamnosus GR-1 attenuates E. coli-induced NLRP3 activation through reducing the adhesion of E. coli to MAC-T cells and subsequently negatively regulating the functional synergy between NF-kB and JNK/ERK MAPK pathways mediated by some uncertain soluble factors. Further studies are required to determine active components derived from L. rhamnosus GR-1 and elucidate possible mechanisms underlying the antagonistic effects of L. rhamnosus GR-1 on NLRP3 activation during E. coli infection.

NLRP3 contains only a PYD, which engages the PYD of ASC, leaving the CARD of ASC to interact with the CARD-containing region of pro-caspase-1. Caspase-1 is thought to be activated by a proximity-induced dimerization and autoproteolytic process in the NLRP3/ASC complex platform (Shi, 2004). Active caspase-1 cleaves pro-IL-1β and pro-IL-18 into mature IL-1β and IL-18, which are essential for coordination of immune responses to pathogen infection through allograft neutrophil sequestration, mononuclear phagocyte recruitment, and T-cell activation (Samuel Weigt et al., 2017). In the present study, L. rhamnosus GR-1 attenuated E. coli-induced caspase-1 activation in response to E. coli infection. Caspase-1 is activated via NLRP3-independent mechanisms, but it is essential for NLRP3-dependent and ASC-dependent caspase-1 processing and IL-1β maturation in response to E. coli infection (Kayagaki et al., 2011). In addition, binding of LPS to human caspase-4 or murine caspase-11 via the CARD directly induces cell pyroptosis, independently of NLRP3 and ASC (Shi et al., 2014). A recent study revealed that outer-membrane-vesicle-mediated cytoplasmic delivery of extracellular E. coli LPS activates murine caspase-11 to induce pyroptosis and IL-1β maturation (Vanaja et al., 2016). Bovine caspase-4 is a homolog of human caspase-4 and mouse caspase-11 and plays a role in the processing of IL-1β and IL-18 precursors (Koenig et al., 2001; Martinon and Tschopp, 2004). In the present study, caspase-4 was activated by E. coli at 6 h; however, L. rhamnosus GR-1 pretreatment attenuated this activation.

![Figure 8](image)
During Escherichia coli Infection

**FIGURE 9** | *Lactobacillus rhamnosus* GR-1 attenuated activation of ASC-dependent and ASC-independent inflammasomes during *E. coli* infection. *L. rhamnosus* GR-1 ameliorates *E. coli*-induced inflammatory damage via attenuation of both ASC-dependent and ASC-independent inflammasome activation in MAC-T cells. *L. rhamnosus* GR-1 inhibits activation of ASC-dependent NLRP3 and NLRC4 inflammasomes and production of the downstream proinflammatory cytokines IL-1β and IL-18 during *E. coli* infection. *L. rhamnosus* GR-1 suppresses *E. coli*-induced cell pyroptosis, in part through attenuation of NLRC4 inflammasome activation, independently of ASC. In addition, *L. rhamnosus* GR-1 inhibits non-canonical caspase-4 activation, which subsequently synergizes with the NLRP3 inflammasome to attenuate caspase-1 activation and potentially inhibit caspase-1-independent cell pyroptosis and IL-1β and IL-18 production. Full lines represent the results of the present study, and dashed lines represent the conclusions drawn in other studies.

Interestingly, there was a lower number of adherent *E. coli* in *ASC−/−* MAC-T cells at 3 h after *E. coli* challenge compared with WT MAC-T cells. It must be noted that ASC deficiency reduced, but did not abolish, caspase-1 processing, IL-1β and IL-18 maturation, and cell pyroptosis during *E. coli* infection. Decreased *E. coli* adhesion may delay the expression of NLRC4 receptor and the downstream activation of caspase-1, maturation of proinflammatory cytokines and cell pyroptosis in *ASC−/−* MAC-T cells. Caspase-1 activation by *E. coli* requires NLRP3 and ASC, but caspase-11 processing and cell pyroptosis do not (Kayagaki et al., 2011). A previous study reported weaker oligomerization of both ASC and caspase-1 in macrophages infected with *E. coli* compared with the canonical NLRP3 inflammasome activator nigericin, but with comparable production of IL-1β (Rathinam et al., 2012). Caspase-11 interacts with caspase-1 in infected cells, forming a heterodimeric complex (Kayagaki et al., 2011). These data suggest that caspase-4/11 amplify caspase-1 activation independently of ASC by enabling caspase-1 autoprocessing through heterodimerization. Indeed, in the present study, caspase-4 activation was enhanced in *ASC−/−* cells compared with WT cells, which could have compensated for the loss of caspase-1 activation due to ASC-dependent NLRP3 inflammasome activation. Our findings indicate that *E. coli* infection activates caspase-4, subsequently resulting in cell pyroptosis and maturation of IL-1β and IL-18 via an NLRP3 inflammasome-dependent and ASC-independent pathway. *Lactobacillus rhamnosus* GR-1 suppresses ASC-dependent NLRP3 inflammasome activation and ASC-independent caspase-1 processing by inhibiting caspase-4 activation, thereby attenuating cell pyroptosis and cytokine production and thus preventing establishment of *E. coli* infection.

In contrast to NLRP3 activation in response to diverse stimuli, upon *E. coli* infection, NLRC4 responds to bacterial rod protein of the T3SS apparatus and flagellin (Zhao et al., 2011). We found that *L. rhamnosus* GR-1 inhibited *E. coli*-induced NLRC4 expression, as was also observed in *ASC−/−* cells. NLRC4 contains a CARD motif through which it directly interacts with caspase-1 to induce pyroptosis, independently of ASC. This NLRC4-dependent/ASC-independent cell death pathway proceeds in the absence of caspase-1 autoproteolysis. Interestingly, we observed weaker staining for caspase-1 in *ASC−/−* cells. Caspase-1 autoproteolysis is often used as an indicator of caspase-1 activation. However, it was also reported that uncleaved caspase-1 is enzymatically active in *ASC−/−* cells and can induce pyroptosis. In contrast to the formation of a single large ASC/caspase-1 focus for efficient IL-lβ and IL-18 processing, pro-caspase-1 could be recruited to NLRC4, with which it forms a smaller complex that induces pyroptosis (Broz et al., 2010b). Although NLRC4 contains a CARD, ASC amplifies NLRC4 inflammasome activity because ASC is essential for NLRC4-induced caspase-1 autoprocessing and maturation of IL-lβ and IL-18 (Brubaker et al., 2015). Our data suggest that *L. rhamnosus* GR-1 inhibits *E. coli*-induced cell pyroptosis via suppression of ASC-independent NLRC4 inflammasome.
 activation. During *E. coli* infection in the present study, *L. rhamnosus* GR-1 decreased the secretion of IL-1β and IL-18, in part due to suppression of ASC-dependent NLRC4 inflammasome activation.

This MAC-T cell model of *E. coli* and *L. rhamnosus* GR-1 co-incubation presents an *in vitro* framework for assessing bovine mammary immune response to pathogen infection, and evaluating the efficiency of *Lactobacillus*-based intervention in preventing bovine mastitis. The results require further confirmation in other cell lines and *in vivo* studies. However, several concerns need to be addressed before the clinical application of *Lactobacillus* in bovine mastitis. Oral ingestion of probiotics promotes mucosal immune response to pathogen infection in the gut. The mechanism underlying how the immunomodulatory effect extends to the mammary glands remains unclear. The means of probiotic supplementation (e.g., mixed into the feed, oral capsules or intra-mammary infection) and dose effect also needs to be studied in more details. The molecular mechanism underlying regulation of inflammasome activity by *Lactobacillus* requires further investigation. Our findings identify NLRP3 and NLRC4 inflammasomes as potential targets for bovine mastitis therapy and could strengthen the development for other inflammasome-targeted therapies in *E. coli*-associated mastitis.

**CONCLUSION**

In conclusion, our findings suggest that *L. rhamnosus* GR-1 ameliorates *E. coli*-induced inflammatory damage by attenuating both ASC-dependent and ASC-independent inflammasome activation in MAC-T cells (Figure 9). *L. rhamnosus* GR-1 inhibits activation of ASC-dependent NLRP3 and NLRC4 inflammasome activation and production of the downstream proinflammatory cytokines IL-1β and IL-18 during *E. coli* infection. In addition, *L. rhamnosus* GR-1 suppresses *E. coli*-induced cell pyroptosis, in part through attenuation of NLRC4 inflammasome activation, independently of ASC. Furthermore, *L. rhamnosus* GR-1 inhibits non-canonical caspase-4 activation, which subsequently synergizes with NLRP3/ASC-dependent caspase-1 activation to potentially inhibit ASC-independent caspase-1 activation, thus suppressing cell pyroptosis and IL-1β and IL-18 production.

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**AUTHOR CONTRIBUTIONS**

QW, Y-HZ, and J-FW conceived and designed the experiments, analyzed the data, and wrote the manuscript. QW, JX, XL, CD, and M-JW performed the experiments.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01661/full#supplementary-material
