Brucella Rough Mutant Induce Macrophage Death via Activating IRE1α Pathway of Endoplasmic Reticulum Stress by Enhanced T4SS Secretion

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Brucella is a Gram-negative facultative intracellular pathogen that causes the worldwide zoonosis, known as brucellosis. Brucella virulence relies mostly on its ability to invade and replicate within phagocytic cells. The type IV secretion system (T4SS) and lipopolysaccharide are two major Brucella virulence factors. Brucella rough mutants reportedly induce the death of infected macrophages, which is T4SS dependent. However, the underlying molecular mechanism remains unclear. In this study, the T4SS secretion capacities of Brucella rough mutant and its smooth wild-type strain were comparatively investigated, by constructing the firefly luciferase fused T4SS effector, BPE123 and VceC. In addition, quantitative real-time PCR and western blotting were used to analyze the T4SS expression. The results showed that T4SS expression and secretion were enhanced significantly in the Brucella rough mutant. We also found that the activity of the T4SS virB operon promoter was notably increased in the Brucella rough mutant, which depends on quorum sensing-related regulators of VjbR upregulation. Cell infection and cell death assays revealed that deletion of vjbR in the Brucella rough mutant absolutely abolished cytotoxicity within macrophages by downregulating T4SS expression. This suggests that up-regulation of T4SS promoted by VjbR in rough mutant ΔrfbE contribute to macrophage death. In addition, we found that the Brucella rough mutant induce macrophage death via activating IRE1α pathway of endoplasmic reticulum stress. Taken together, our study provide evidence that in comparison to the Brucella smooth wild-type strain, VjbR upregulation in the Brucella rough mutant increases transcription of the virB operon, resulting in overexpression of the T4SS gene, accompanied by the over-secretion of effector proteins, thereby causing the death of infected macrophages via activating IRE1α pathway of endoplasmic reticulum stress, suggesting novel insights into the molecular mechanisms associated with Brucella rough mutant-induced macrophage cytotoxicity.

Keywords: Brucella, Type IV secretion system, lipopolysaccharide, VjbR, endoplasmic reticulum stress
INTRODUCTION

Brucella is a Gram-negative facultative intracellular bacterial species that causes zoonotic brucellosis, characterized by reproductive disease in domestic animals and chronic debilitating disease in humans (Boschirol et al., 2001; Franco et al., 2007; Whatmore, 2009). Brucellosis in animals is endemic in most areas of the world, and it can become a serious public health problem that results in significant morbidity and economic losses (Boschirol et al., 2001; Atluri et al., 2011).

Brucella virulence relies mainly on its ability to invade and replicate within professional and non-professional phagocytes, among which macrophages are major target cells in infected mammals (Gorvel and Moreno, 2002; Celli, 2006). To date, many virulence factors have been identified, such as lipopolysaccharide (LPS), the type IV secretion system (T4SS), a two-component regulatory system (BvrS/BvrR), and cyclic β-1,2-glucan (CBG) (Byndloss and Tsolis, 2016). Two major Brucella virulence factors are LPS and T4SS. Brucella LPS is composed of lipid A, a core oligosaccharide, and the O-antigen. It is characterized by low stimulatory activity and toxicity to cells, and mediates lower superoxide and lysozyme production in infected cells (Goldstein et al., 1992; Rasool et al., 1992). Furthermore, Brucella LPS is critical in the inhibition of programmed cell death (apoptosis) and enhances the bacterium’s ability to survive within macrophages (Fernandez-Prada et al., 2003). Rough mutants of Brucella abortus that lack the O-antigen, induce infected macrophage death, and are taken up in greater numbers by macrophages than the smooth wild-type strains (Pei and Ficht, 2003; Bronner et al., 2013; Tian et al., 2014). In previous reports, the rough mutant VTRS1 of B. suis induced proinflammatory, caspase-2- and nuclear factor kappa B (NF-κB)-mediated macrophage cell death (Chen et al., 2011). Bronner and colleagues subsequently reported that the rough mutant of B. abortus RB51 induces a hybrid cell death, mediated by caspase-2 activation, with features of apoptosis and pyroptosis (Bronner et al., 2013). In further study, Bronner found that endoplasmic reticulum (ER) stress induced by RB51 activates the inflammasome via NLRP3- and caspase-2-driven mitochondrial damage (Chen et al., 2011; Bronner et al., 2015). However, the molecular mechanism underlying Brucella rough mutant modulation of ER stress to induce macrophage death remains unclear.

Cytotoxicity in macrophages that have been infected by Brucella rough mutants is reportedly T4SS dependent (Pei et al., 2008). Overexpression of T4SS in the Brucella smooth strain enhances its ability to induce macrophage death (Zhong et al., 2009). The T4SS encoded by the virB operon in Brucella comprises multiprotein complexes that translocate specific protein substrates across the bacterial cell envelope to the host cell, and guides trafficking of the Brucella containing vacuole to the ER-associated compartment within macrophages (Zechner et al., 2012; Byndloss et al., 2016). The Brucella virB operon is induced by lysosomal acidification and nutritional deprivation within macrophages, which is tightly controlled by several regulation-associated genes, such as the LuxR family VjbR regulator and integration host factor IHF (Porte et al., 1999; Boschirol et al., 2002; Sieira, 2013). The T4SS plays a significant role in Brucella trafficking, and is essential for Brucella to trigger a mild inflammatory response (Rolan et al., 2009). The effector protein VceC is translocated by T4SS to the ER, where it binds the ER chaperone Bip (binding immunoglobulin protein) and induces inositol-requiring enzyme 1α (IRE1α)-dependent ER stress during B. abortus infection, and this in turn affects the recruitment of the NOD-like receptors, NOD1 and NOD2, to induce NF-κB activation and expression of proinflammatory genes (de Jong et al., 2013; Keestra-Gounder et al., 2016). However, the manner in which the Brucella rough mutant regulates T4SS function has not been definitively determined.

Brucella LPS is an important virulence factor, and the O-antigen is a crucial component that is synthesized in the cytoplasmic face of the bacterial inner membrane, and then exported to the periplasmic face of the inner membrane, based on an ATP-binding cassette (ABC) transporter system that is encoded by rfbE and rfbD genes (Godfroid et al., 2000; Tian et al., 2014). In a previous study, we found that the B. abortus rough mutant ΔrfbE, induced the death of infected macrophages (Tian et al., 2014), which is in line with the findings of other reports (Pei and Ficht, 2003; Chen et al., 2011). In Shigella, shortening of the LPS molecule by O-antigen glucosylation enhances the secretion of effector proteins and function of the type III secretion system (West et al., 2005). In view of these findings, we hypothesized that Brucella smooth LPS is crucial for T4SS function, which plays a vital role in intracellular survival of Brucella and its interaction with host cells. In this study, we demonstrated that VjbR upregulation in the Brucella rough mutant ΔrfbE enhances T4SS expression and secretion, both of which contribute to the death of infected macrophages via activation of the IRE1α pathway of ER stress.

MATERIALS AND METHODS

Strains, Plasmids, Macrophages, and Culture Conditions

All strains and plasmids used in this study are listed in Table 1. Brucella abortus S2308 and its derivatives were grown in tryptic soy broth (TSB) or on tryptic soy agar (TSA) (Difco, Franklin Lakes, NJ, USA) plates at 37°C with 5% CO2. Manassas, Virginia (VA, USA) was cultured at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, ThermoScientific, Grand Island, NY, USA).

Antibodies

The primary antibodies used in this study were: rabbit anti-firefly luciferase monoclonal antibody (Abcam, Cambridge, MA, USA); rabbit anti-Brucella VirB5 polyclonal antibody (prepared in our lab); rabbit anti-Brucella GAPDH polyclonal antibody (prepared in our lab); rabbit anti-IRE1 polyclonal antibody (phospho
S724, Abcam); and rabbit anti-β-actin monoclonal antibody (Cell Signaling Technology, Danvers, MA, USA). The secondary antibodies used for western blotting were: IRDye 800CW-conjugated donkey anti-Rabbit IgG polyclonal antibody (LI-COR Biosciences, Lincoln, NE, USA) and horseradish peroxidase-conjugated goat anti-rabbit IgG (Life Technologies, Eugene, OR, USA).

**Plasmid Construction**

All primers used in this study are listed in Table 2. Suicide plasmids were constructed, using an overlap PCR assay, as previously reported (Tian et al., 2014). Briefly, the upstream and downstream fragments of *virB123* (containing the *virB* promoter, *virB1*, *virB2*, and *virB3* genes) and *vjbR* were amplified by independent PCRs and extracted from agarose gels that were used as templates for a second round of PCR. The resultant product that contained joined flanking sequences was purified by gel extraction and cloned into a pSC plasmid, after being digested with XbaI, to generate the suicide plasmids pSCΔ*virB123* and pSCΔ*vjbR*.

Luciferase reporter plasmids of pBCSP31-BPE123-Luc, pBCSP31-Luc-VceC, pBCSP31-GST-Luc, and pVirB-Luc were previously reported.
### TABLE 2 | Primers used in the present study.

| Primers | Oligonucleotide sequences (5′-3′)<sup>a</sup> | Target genes<sup>b</sup> | Product size (bp) |
|----------|---------------------------------|------------------------|------------------|
| virB123-UF | GCTCTAGAGCCTATGCGGAGATCGCTTTC | Upstream fragment of virB123 | 1323 |
| virB123-UR | AAATCCGAGCCTAAGAATCGCTGCTGGTATT | Upstream fragment of virB123 | 1323 |
| virB123-DF | AATCGGACGATCGCTGCTGGTATT | Downstream fragment of virB123 | 1231 |
| virB123-DR | GCTCTAGAGCGTATCAGATGCGGCTTTC | Downstream fragment of virB123 | 1231 |
| vjbR-UF | GCTCTAGAGCCTGATCGCTGCTGGTATT | Upstream fragment of vjbR | 895 |
| vjbR-UR | GATATCGGACGATCGCTGCTGGTATT | Upstream fragment of vjbR | 895 |
| vjbR-DR | ATACGGGACGATCGCTGCTGGTATT | Downstream fragment of vjbR | 522 |
| mB-F | GCTCTAGAGCTATGCGGAGATCGCTTTC | Downstream fragment of mB | 522 |
| mB-R | GCTCTAGAGCTATGCGGAGATCGCTTTC | Terminator sequence of mB | 461 |
| P<sub>bcsp31</sub>-F | GCTCTAGAGCCTGATCGCTGCTGGTATT | Fragment of promoter bcsp31 | 112 |
| P<sub>bcsp31</sub>-R | GCTCTAGAGCCTGATCGCTGCTGGTATT | Fragment of promoter bcsp31 | 112 |
| Luc-CF | GCGATCCATCGATCGCTGCTGGTATT | Coding region of luciferase | 1653 |
| Luc-DR | GCTCTAGAGCCTGATCGCTGCTGGTATT | Coding region of luciferase | 1653 |
| Luc-NF | GCTCTAGAGCCTGATCGCTGCTGGTATT | Coding region of luciferase lacking termination codon (TAA) | 1650 |
| Luc-NR | GCTCTAGAGCCTGATCGCTGCTGGTATT | Coding region of luciferase lacking termination codon (TAA) | 1650 |
| BPE123-F | GCTCTAGAGCCTGATCGCTGCTGGTATT | Coding region of BPE123 | 471 |
| BPE123-R | GCTCTAGAGCCTGATCGCTGCTGGTATT | Coding region of BPE123 | 471 |
| vce-C-F | GCTCTAGAGCCTGATCGCTGCTGGTATT | Coding region of vceC | 363 |
| vce-C-R | GCTCTAGAGCCTGATCGCTGCTGGTATT | Coding region of vceC | 363 |
| GST-F | GCTCTAGAGCCTGATCGCTGCTGGTATT | Coding region of GST | 675 |
| GST-R | GCTCTAGAGCCTGATCGCTGCTGGTATT | Coding region of GST | 675 |
| mdrA-F | GCGATCCATCGATCGCTGCTGGTATT | Coding region of mda | 516 |
| mdrA-R | GCTCTAGAGCCTGATCGCTGCTGGTATT | Coding region of mda | 516 |
| blxR-F | GCTCTAGAGCCTGATCGCTGCTGGTATT | Coding region of blx | 708 |
| blxR-R | GCTCTAGAGCCTGATCGCTGCTGGTATT | Coding region of blx | 708 |
| P<sub>virB</sub>-F | GCTCTAGAGCCTGATCGCTGCTGGTATT | Fragment of promoter virB | 423 |
| P<sub>virB</sub>-R | GCTCTAGAGCCTGATCGCTGCTGGTATT | Fragment of promoter virB | 423 |
| RT-16S-F | GCTCTAGAGCCTGATCGCTGCTGGTATT | 16S rRNA | 87 |
| RT-16S-R | GCTCTAGAGCCTGATCGCTGCTGGTATT | 16S rRNA | 87 |
| RT-virB44-F | GCTCTAGAGCCTGATCGCTGCTGGTATT | virB44 | 156 |
| RT-virB44-R | GCTCTAGAGCCTGATCGCTGCTGGTATT | virB44 | 156 |
| RT-virB44-F | GCTCTAGAGCCTGATCGCTGCTGGTATT | virB44 | 156 |
| RT-virB44-R | GCTCTAGAGCCTGATCGCTGCTGGTATT | virB44 | 156 |
| RT-virB44-F | GCTCTAGAGCCTGATCGCTGCTGGTATT | virB44 | 156 |
| RT-virB44-R | GCTCTAGAGCCTGATCGCTGCTGGTATT | virB44 | 156 |
| RT-ihf-F | GCTCTAGAGCCTGATCGCTGCTGGTATT | ihf | 164 |
| RT-ihf-R | GCTCTAGAGCCTGATCGCTGCTGGTATT | ihf | 164 |
| RT-ihf-F | GCTCTAGAGCCTGATCGCTGCTGGTATT | ihf | 164 |
| RT-ihf-R | GCTCTAGAGCCTGATCGCTGCTGGTATT | ihf | 164 |
| RT-hutCF | GCTCTAGAGCCTGATCGCTGCTGGTATT | hutC | 155 |
| RT-ihf-F | GCTCTAGAGCCTGATCGCTGCTGGTATT | hutC | 155 |
| RT-ihf-R | GCTCTAGAGCCTGATCGCTGCTGGTATT | hutC | 155 |
| RT-blxR-F | GCTCTAGAGCCTGATCGCTGCTGGTATT | blxR | 248 |
| RT-blxR-R | GCTCTAGAGCCTGATCGCTGCTGGTATT | blxR | 248 |
| RT-blxR-F | GCTCTAGAGCCTGATCGCTGCTGGTATT | blxR | 248 |
| RT-blxR-R | GCTCTAGAGCCTGATCGCTGCTGGTATT | blxR | 248 |
| RT-mdra-F | GCTCTAGAGCCTGATCGCTGCTGGTATT | mdra | 174 |
| RT-mdra-R | GCTCTAGAGCCTGATCGCTGCTGGTATT | mdra | 174 |

<sup>a</sup>Underlining indicates restriction endonuclease recognition sequences.

<sup>b</sup>B. abortus locus tags listed are for genes in the B. abortus strain S2308.
the primers \( P_{\text{bcsp31}}-\text{F/R} \) or \( P_{\text{virB}}-\text{F/R} \), cloned into the pMCR plasmid and digested by KpnI and XhoI enzymes to generate the plasmids pBCSP31 and pVirB, respectively. The luciferase gene (\( \text{luc} \)) was amplified by PCR from the pNF\( \kappa \) plasmid (Beyotime, Jiangsu, China), using the primers Luc-F and Luc-R, and cloned into the XhoI- and PstI-digested pBCSP31 plasmids, to generate pBCSP31-Luc-N, and facilitate the generation of N-terminal in-frame fusions of the Luc protein. The \( \text{luc} \) gene was cloned into the pBCSP31 plasmid by BamHI and XbaI digestion to generate pBCSP31-Luc-C, in which the stop codon in the luc open reading frame (ORF) was removed, to allow C-terminal fusion of the Luc protein. Furthermore, the \( \text{luc} \) gene was cloned into the pVirB plasmid by XhoI and PstI digestion, to construct the pVirB-Luc plasmid and effect promoter activation of the \( \text{virB} \) operon. The ORF of BPE123 was amplified from S2308 by PCR using the primer BPE123-\text{F/R}, and cloned into the pBCSP31-Luc-N plasmid to generate pBCSP31-BPE123-Luc. At the C-terminal, 116 amino acids of the VceC protein that are necessary for secretion by T4SS was amplified by PCR using the primer vceC-\text{F/R}, and cloned into the pBCSP31-Luc-\text{C} plasmid to construct the pBCSP31-Luc-VceC plasmid. In addition, the glutathione transferase (GST) gene was amplified from the pGEX-4T-1 plasmid (Takara, Dalian, China) by PCR using the primer GST-\text{F/R} and cloned into the pBCSP31-Luc-N plasmid, to generate the negative control plasmid pBCSP31-GST-Luc.

Overexpression plasmids of pMdrA and pBlxR were constructed using conventional methods. The \( \text{mdrA} \) and \( \text{blxR} \) genes containing the promoter and terminator regions were amplified by independent PCRs using the primers \( \text{mdrA-} \text{F/R} \) and \( \text{blxR-} \text{F/R} \), respectively, and then cloned into the plasmid pBBR1-MCS, to generate the plasmids pMdrA and pBlxR, respectively.

All recombinant plasmids were propagated in \( \text{E. coli} \) DH5\( \alpha \) cells (Invitrogen Corp., Carlsbad, CA, USA) and then extracted to construct recombinant \( \text{Brucella} \) strains.

### Mutant Construction

The \( \Delta\text{rjbE}\Delta\text{virB} \) and \( \Delta\text{rjbE}\Delta\text{vjbR} \) mutants were constructed by allelic replacement, using a two-step strategy as previously reported (Kahl-McDonagh and Ficht, 2006; Tian et al., 2014). The suicide plasmids pSC\( \Delta\text{virB123} \) and pSC\( \Delta\text{vjbR} \) (0.5–1.0 \( \mu \)g) were transferred to the \( \text{bpe} \) plasmid by electroporation. The first exchanged recombinants were selected by plating on TSA containing ampicillin. The second round of exchanged recombinants was selected by plating on TSA containing 5% sucrose. Analyses of PCRs were carried out to identify clones.

Luciferase reporter strains and overexpression strains were also constructed by electroporation. The recombinants were then selected by plating on TSA containing chloramphenicol. The PCR or western blotting analyses were carried out to identify recombinants. The recombinant strains constructed in this study are listed in Table 1.

### Cell Infection Assay

Monolayers of RAW264.7 cells were cultured in six- or 24-well plates and infected with \( \text{B. abortus} \) S2308 or its derivatives at a multiplicity of infection (MOI) of 100 or 1,000 colony forming units (CFU) per cell. To synchronize the infection, the infected plates were centrifuged at 400 \( \times \) g for 5 min, and cells were then incubated at 37\( ^\circ \)C with 5% CO\( _2 \) for 1 h. The monolayers were washed twice with phosphate buffered saline (PBS) (HyClone, GE Lifesciences, Logan, UT, USA) to remove extracellular nonadherent bacteria, and then incubated with DMEM containing gentamicin (100 \( \mu \)g/mL) for 1 h to kill extracellular bacteria. To maintain survival of the infected cells, the monolayers were incubated with DMEM containing gentamicin (20 \( \mu \)g/mL) and 2% FBS after being washed thrice with PBS.

### Cell Death Analysis

Macrophage death was detected, using two approaches. In the first approach, infected cells were stained with annexin V and propidium iodide (PI) at 3, 5, 8, and 12 h post infection (p.i.), using the annexin V-FITC/PI staining kit (Beyotime, Shanghai, China). In the second approach, the release of lactate dehydrogenase (LDH) in the supernatant of \( \text{Brucella} \)-infected RAW264.7 cells both with and without 4\( \mu \)g c (IRE1\( \alpha \) inhibitor, 100 \( \mu \)M, Selleck, Houston, TX, USA) treatment was determined at 3, 5, 8, and 12 h p.i., using a CytoTox 96 nonradioactive cytotoxicity assay (Promega, Fitchburg, WI, USA). Cell death was expressed as a percentage of maximum LDH release. The percentage was calculated as follows: (optical density at 490 nm [\( \text{OD}_{490} \)] of infected cells—\( \text{OD}_{490} \) of uninfected cells)/(\( \text{OD}_{490} \) of lysed uninfected cells—\( \text{OD}_{490} \) of uninfected cells) \times 100%.

### Determination of Luciferase Activity

For the determination of luciferase activity in the media culture, luciferase reporter strains of S2308(pVirB-Luc) and \( \Delta\text{rjbE}(\text{pVirB-Luc}) \) were cultured to exponential phase (\( \text{OD}_{600} = 1.0 \)), and then centrifuged at 8,000 \( \times \) g for 5 min to precipitate bacteria. The pellets of luciferase reporter strains were resuspended in 200 \( \mu \)L PBS and lysed by adding 200 \( \mu \)L B-PER\( \text{ Bacterial Protein Extraction Reagent} \) (Thermo Scientific). The lysate was centrifuged at 15,000 \( \times \) g for 5 min to separate soluble proteins, after which it was incubated for 15 min at room temperature. The luciferase activity (relative light units, RLUs) of lysate supernatants were measured using the Luc-creen\( \text{ Bepper gene assay system} \) (Abcam). Moreover, 100 \( \mu \)L of the luciferase reporter strains were serially diluted 10-fold with PBS and spread onto TSA plates to determine the bacterial CFU. All samples were analyzed in triplicate.

For the determination of luciferase activity in the cell culture, macrophage RAW264.7 cells were infected with luciferase reporter strains at a MOI of 1,000, as described previously. Infected cells were washed three times with PBS and lysed with 500 \( \mu \)L of 0.2% Triton X-100 in sterile water for 15 min at 3, 5, and 8 h p.i. The infected cell lysate (400 \( \mu \)L) was centrifuged at 12,000 \( \times \) g for 5 min. For cells infected with luciferase reporter strains of S2308(pVirB-Luc), \( \Delta\text{rjbE}(\text{pVirB-Luc}) \), \( \Delta\text{rjbE}\Delta\text{vjbR}(\text{BPE123-Luc}) \), \( \Delta\text{rjbE}(\text{BPE123-Luc}) \), \( \Delta\text{rjbE}\Delta\text{virB}(\text{BPE123-Luc}) \), S2308(Luc-VceC), \( \Delta\text{rjbE}(\text{Luc-VceC}) \), \( \Delta\text{rjbE}\Delta\text{virB}(\text{Luc-VceC}) \), S2308(GST-Luc), \( \Delta\text{rjbE}(\text{GST-Luc}) \), and \( \Delta\text{rjbE}\Delta\text{virB}(\text{GST-Luc}) \), the RLUs of lysate supernatants were measured by the Luc-creen\( \text{ Bepper gene assay system} \) (Abcam). For the cells
infected with luciferase reporter strains of S2308(pVirB-Luc) and ΔrfbE(pVirB-Luc), lysate pellets were resuspended in 200 µL PBS, after which 200 µL B-PER® Bacterial Protein Extraction Reagent (Thermo Scientific) was added to lyse the intracellular strains. The lysate of intracellular strains was centrifuged at 15,000 × g for 5 min, after which it was incubated for 15 min at room temperature. The RLUs of lysate supernatants were also measured using the Luc-Screen® reporter gene assay system (Abcam). Moreover, the remaining 100 µL of infected cell lysates were serially diluted 10-fold with PBS and spread onto TSA plates to determine the bacterial CFU. All samples were analyzed in triplicate.

RNA Extraction and Real-Time PCR
Total RNA was extracted from bacteria using the TRIzol® RNA Isolation Reagent (Invitrogen) according to the manufacturer's protocol. Genomic DNA contamination was removed through treatment with a Turbo DNA-free kit (Ambion). The RNA quantity and quality were evaluated using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc.). The RNA integrity was assessed by standard denaturing agarose gel electrophoresis, and RNA (1 µg) was reverse transcribed into cDNA, using a PrimeScript RT-PCR kit (TaKaRa) according to the manufacturer's instructions. A 20 µL RT-PCR mixture was made comprising 10 µL 2× GoTag qPCR master mix (Promega), 1 µL cDNA, 0.5 µL (each) forward and reverse primers (10 µM each), and 8 µL double-distilled water (ddH₂O). The mixture was incubated at 95°C for 2 min, and then subjected to 40 cycles at 95°C for 15 s, followed by 60°C for 1 min using a Mastercycler ep Realplex system (Eppendorf). All samples were analyzed in triplicate and relative transcription levels of each gene were determined by the 2−ΔΔCt method, using 16S RNA as an internal control for data normalization.

Western Blotting
Sediments of the bacteria were collected, following centrifugation at 1,000 × g for 5 min and culture for various durations. The pellets were resuspended in Laemmli sample buffer and boiled for 10 min. The RAW264.7 cells were scraped into radioimmunoprecipitation assay buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP 40, 0.25% sodium deoxycholate, and 1 mM EDTA) that contained a protease inhibitor cocktail (Roche). The cell lysates were mixed with Laemmli sample buffer and boiled for 10 min. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto nitrocellulose membranes (Millipore) using a semidry transfer procedure. The membranes were blocked overnight at 4°C in Tris-buffered saline containing 5% skim milk or 5% Bovine Serum Albumin (BSA). Immunodetection of proteins in total cell lysates was performed with the respective primary antibody for 2 h at room temperature. After washing three times with Tris-buffered saline and Tween 20, the membrane was incubated with the respective secondary antibody for 1 h at room temperature. After washing three times with Tris-buffered saline and Tween 20, an Odyssey two-color infrared imaging system (LI-COR Biosciences) was used to develop the fluorescence for visualization. The gray intensity of the bands was quantified using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis
Statistical analysis was performed using the GraphPad Prism 6.0 software (GraphPad Software Inc., La Jolla, CA, USA). All p-values between identified samples were generated using unpaired two-tailed Student’s t-tests, or in the case of groups, two-way analysis of variance, followed by the Tukey’s test. All experiments were repeated at least three times and the results were presented as means ± SD from ≥3 replicates per condition.

RESULTS
Rough Mutant ΔrfbE Induced Macrophage Death Is T4SS Dependent
Brucella rough mutant induces macrophage death, a process that is T4SS dependent (Pei et al., 2008). Our previous studies have shown that rough mutant ΔrfbE also induces the death of macrophages (Tian et al., 2014). To identify the role of T4SS on ΔrfbE mutant-induced cytotoxicity for RAW264.7 macrophages, virB1, virB2, and virB3 genes were deleted from the ΔrfbE mutant, thereby generating a double-knockout strain (ΔrfbEΔvirB). Firstly, morphology of the RAW264.7 cells infected with S2308, ΔrfbE, ΔrfbE(pBBR-ΔrfbE), and ΔrfbEΔvirB were observed via light microscopy. As shown in Figure 1, ΔrfbE-infected cells exhibited obvious cell swelling and deformation at 8 and 12 h p.i.; however, the ΔrfbE(pBBR-ΔrfbE)- and ΔrfbEΔvirB-infected cells showed no cell lesions, which is consistent with our observations of the S2308-infected cells and mock cells. Furthermore, the death of Brucella-infected RAW264.7 macrophages was analyzed following annexin V-FITC and PI staining, which was used to detect translocation of phosphatidylserine from the inner cell membrane to the outer cell membrane during the early stages of apoptosis. The PI stains the DNA of necrotic cells and/or cells at the late stage of apoptosis (Tian et al., 2014). The results showed that macrophages infected with the ΔrfbE mutant exhibited some characteristics of necrosis and late apoptosis, accompanied by cellular membrane damage and PI staining of the nucleus at 5, 8, and 12 h p.i. Further disruption of the virB operon in the ΔrfbE mutant reduced its ability to induce cell death. Certainly, the S2308 and ΔrfbE(pBBR-ΔrfbE) with the smooth phenotype did not induce infected macrophage death in a similar manner to the mock cells (Figure 2A). These results are consistent with those of previous reports (Pei et al., 2008; Tian et al., 2014).

To evaluate cell death quantitatively, the release of LDH was determined for S2308-, ΔrfbE-, ΔrfbE(pBBR-ΔrfbE)-, and ΔrfbEΔvirB-infected cells. The levels of LDH released from the ΔrfbE-infected cells were significantly higher than those released from S2308-infected cells at 3, 5, 8, and 12 h p.i. (Figure 2B). However, the ΔrfbE(pBBR-ΔrfbE)- and ΔrfbEΔvirB-infected cells released similar levels of LDH as the S2308-infected cells at 3, 5, 8, and 12 h p.i. (Figure 2B). These results further confirmed that the rough mutant ΔrfbE induced macrophage death is T4SS dependent.
T4SS Secretion Is Enhanced in the *Brucella* Rough Mutant \(\Delta rfbE\)

The T4SS translocates effectors across the bacterial cell envelope to the host cell, and plays a central role in intracellular survival and replication of *Brucella* within the host (Ke et al., 2015). As macrophage death induced by the rough mutant \(\Delta rfbE\) is T4SS dependent, we hypothesized that the capacity of the rough mutant \(\Delta rfbE\), to secrete T4SS could be altered. For this purpose, we used the previously reported T4SS effector, BPE123 and VceC as target proteins, and GST as a negative control protein, to construct the luciferase reporter strains S2308(BPE123-Luc), \(\Delta rfbE\)(BPE123-Luc), \(\Delta rfbE\)\(\Delta virB\)(BPE123-Luc), S2308(Luc-VceC), \(\Delta rfbE\)(Luc-VceC), \(\Delta rfbE\)\(\Delta virB\)(Luc-VceC), S2308(GST-Luc), \(\Delta rfbE\)(GST-Luc), and \(\Delta rfbE\)\(\Delta virB\)(GST-Luc). The expression of luciferase fusion proteins was detected by western blotting analysis, indicating that luciferase was successfully expressed in the luciferase reporter strains, and the expression levels of luciferase fusion proteins in S2308, \(\Delta rfbE\), and \(\Delta rfbE\)\(\Delta virB\) were similar (Figure 3A).

Furthermore, the T4SS secretion capacity of S2308, \(\Delta rfbE\), and \(\Delta rfbE\)\(\Delta virB\) strains within host cells were determined. The RAW264.7 cells were infected with the luciferase reporter strains at a MOI of 1,000, and the secretion of BPE123 and VceC per 10^6 CFU of intracellular live *Brucella* were determined. Results showed that the rough mutant strain \(\Delta rfbE\) translocated significantly higher levels of BPE123 and VceC to the infected cells than its smooth wild-type strain S2308 at 3, 5, and 8 h p.i., indicating an increased T4SS secretion capacity of the rough mutant \(\Delta rfbE\) under the conditions of intracellular infection (Figures 3B,C). The increased T4SS secretion of the \(\Delta rfbE\) mutant was partially recovered by further deletion of *virB123* genes (Figures 3B,C), indicating that BPE123 and VceC oversecretion in the \(\Delta rfbE\) mutant was indeed dependent on T4SS function. Taken together, the T4SS secretion capacity of the rough mutant, \(\Delta rfbE\) was higher than that of the smooth wild-type strain, S2308.

T4SS Overexpression in the *Brucella* Rough Mutant Contributes to Its Enhanced Secretion

To confirm that the enhanced T4SS secretion is associated with enhanced T4SS expression in the *Brucella* rough mutant, we evaluated the expression of the T4SS components, VirB4 and VirB5 in the S2308 and \(\Delta rfbE\) mutants at exponential phase in TSB, using qRT-PCR and western blotting. Results showed that *virB4* and *virB5* expression of the rough mutant \(\Delta rfbE\) was significantly upregulated at the exponential phase, compared to that of its smooth wild-type strain, S2308 (Figures 4A,B). Based on previous reports, it is evident that the *virB* operon of *Brucella* induced expression within host cells under the conditions of nutritional deprivation and an acidic environment (Boschirolli et al., 2002). To determine the T4SS expression under acidic conditions, the smooth wild-type strain S2308 and rough mutant \(\Delta rfbE\) were grown to exponential phase and exposed to TSB at pH 4.5 for 1 h. The qRT-PCR and western blotting analyses showed that *virB4* and *virB5* expression was induced in both strains at pH 4.5. Furthermore, much higher levels of *virB4* and *virB5* expression were induced in the rough mutant \(\Delta rfbE\), compared to those of the smooth wild-type strain S2308 (Figures 4A,B). To determine T4SS expression in nutritional deprivation, the S2308 and rough mutant \(\Delta rfbE\) were grown to log phase and exposed to RPMI 1640 for 3 h. The results showed that *virB4* and *virB5* expression was also induced in both strains, and the expression level of the \(\Delta rfbE\) mutant was significantly higher than that of the S2308 mutant (Figures 4A,B).

To further determine T4SS upregulation in the \(\Delta rfbE\) mutant, the promoter region of the *virB* operon was cloned and fused to the reporter *luc* gene, to generate luciferase reporter strains S2308(pVirB-Luc) and \(\Delta rfbE\)(pVirB-Luc). The promoter activity of the *virB* operon was assessed in both strains during the exponential phase in TSB, indicating that the \(\Delta rfbE\)(pVirB-Luc) strain displayed higher levels of luciferase activity than the S2308(pVirB-Luc) strain (Figure 4C). Furthermore, when exposed to TSB at pH 4.5 for 1 h, luciferase activity in both
FIGURE 2 | Macrophage death induced by Brucella rough mutant infection is T4SS dependent. RAW264.7 cells cultured in a 24-well plate were infected with S2308, \( \Delta rfbE \), \( \Delta rfbE(pBBR-rfbE) \), or \( \Delta rfbE \Delta virB \) strains at a MOI of 100, and cell death was determined at 3, 5, 8, and 12 hpi. (A) Annexin V-FITC/PI staining. The cells were stained with FITC-annexin (green) and PI (red), and observed using fluorescence microscopy at a magnification of \( \times 100 \). Uninfected RAW264.7 cells were used as negative controls (Mock). (B) LDH detection. The supernatants were collected and LDH release was detected using the CytoTox 96 nonradioactive cytotoxicity assay. The supernatants of uninfected RAW264.7 cells were used as negative controls (medium). ns, no significant difference, ***p < 0.0001.

Up-Regulation of T4SS Promoted by VjbR in Rough Mutant \( \Delta rfbE \) Contribute to Macrophage Death

We proved that the \( \textit{virB} \) operon was upregulated at the transcriptional level in the rough mutant, and further investigated whether T4SS overexpression in the rough mutant is associated with transcriptional regulators that directly bind to the \( \textit{virB} \) operon. Thus, we evaluated the transcriptional expression of \textit{Brucella} regulatory proteins that were found to be directly involved in transcriptional regulation of \( \textit{virB} \) expression, including VjbR, IHF, HutC, BlxR, BvrR, and MdrA (Sieira,
The qRT-PCR demonstrated that expression of vjbR was significantly upregulated, and that of the mdrA and blxR were evidently downregulated in the ΔrfbE mutant, compared to the S2308 strain (Figure 5A).

To determine whether macrophage death caused by infection with the ΔrfbE mutant is associated with VjbR, MdrA, and BlxR, we constructed ΔrfbEΔvjbR, ΔrfbE(pMdrA), and ΔrfbE(pBlxR) strains, respectively, to infect RAW264.7 macrophages. Under light microscopy, we observed no morphological changes in ΔrfbEΔvjbR-infected macrophages; however, obvious cell swelling and deformation were observed in ΔrfbE(pMdrA)- and ΔrfbE(pBlxR)-infected cells at 8 and 12 h p.i. (Figure 1). Furthermore, cell death was analyzed following annexin V-FITC and PI staining. The results showed that the ΔrfbEΔvjbR mutant was no longer cytotoxic to macrophages; however, the ΔrfbE(pMdrA) and ΔrfbE(pBlxR) strains induced macrophage death at 8 and 12 h p.i. (Figure 5B). In addition, the LDH release assay was performed to assess quantitatively the death of macrophages infected with the ΔrfbEΔvjbR, ΔrfbE(pMdrA), and ΔrfbE(pBlxR) strains. The results showed that the ΔrfbEΔvjbR mutant-infected macrophages reduced LDH release, compared to the ΔrfbE mutant infected cells, but similar to the smooth wild-type strain S2308 infected cells at 3, 5, 8, and 12 h p.i. Furthermore, the ΔrfbE(pBlxR)-infected cells also reduced LDH release at 5, 8, and 12 h p.i. compared to the ΔrfbE mutant; however, the LDH levels were much higher than those released from the S2308 infected cells (Figure 5C). The LDH release from ΔrfbE(pMdrA) infected cells showed no difference with those from the ΔrfbE mutant infected cells (Figure 5C). Taken together, these results indicated that VjbR upregulation was the key cause of ΔrfbE mutant-induced macrophage death. The BlxR downregulation played a partial role in macrophage death, but MdrA downregulation was not necessary for the ΔrfbE mutant to induce macrophage death.

To determine whether vjbR and blxR are essential for virB upregulated expression in the ΔrfbE mutant, ΔrfbEΔvjbR, and ΔrfbE(pBlxR) strains were evaluated for virB expression using qRT-PCR. Results demonstrated that deletion of vjbR in the ΔrfbE mutant restored virB transcription to a level similar to that of the smooth strain S2308 (Figure 5D). In comparison to the ΔrfbE mutant, virB4 was significantly downregulated when blxR was robustly over-expressed in the ΔrfbE(pBlxR) mutant (Figure 5E), suggesting that virB upregulated expression in the ΔrfbE mutant was associated with the regulatory proteins, VjbR and BlxR.

Taken together, T4SS overexpression induced by VjbR regulation in the Brucella rough mutant plays a key role in macrophage death.
Rough Mutant ΔrfbE Induces Macrophage Death via Activating IRE1α Pathway of ER Stress

The Brucella T4SS effector protein, VceC, is associated with triggering ER stress by activating the unfolded protein response (UPR) sensor, inositol-requiring enzyme 1α (IRE1α) (de Jong et al., 2013; Keestra-Gounder et al., 2016). To investigate the ER stress induced by Brucella rough mutant infection, activation of the UPR sensor, IRE1α was analyzed using western blotting. The results showed that compared to the Brucella smooth wild-type strain, the levels of P-IRE1α in the Brucella rough mutant were significantly increased at 3, 5, and 8 h p.i. (Figure 6A), indicating that the Brucella rough mutant induced stronger ER stress. To determine whether P-IRE1α is involved in macrophage death caused by the ΔrfbE mutant, the inhibitor of IRE1α, 4µ8c, was used to treat the macrophages before infection, which blocks the access of the substrate to the active site of IRE1α, and selectively inactivates both Xbp1 splicing and IRE1α-mediated mRNA degradation (Cross et al., 2012). We infected 4µ8c-treated macrophages with S2308 and the ΔrfbE mutant, and then evaluated cell death quantitatively, using the LDH release assay. The results demonstrated that in comparison to macrophages that had not been subjected to 4µ8c treatment, LDH levels were diminished in 4µ8c-treated macrophages infected with the ΔrfbE mutant at 3, 5, and 8 h p.i. (Figure 6B), indicating that IRE1α inhibition reduced macrophage death caused by the ΔrfbE mutant. The 4µ8c treatment did not affect LDH release from the macrophages infected with S2308 at 3, 5, and 8 h p.i. (Figure 6B).

DISCUSSION

The cytotoxicity induced by Brucella rough mutants within macrophages was originally described more than 50 years ago (Freeman et al., 1961; Freeman and Rumack, 1964). The T4SS is essential for cytotoxic death of macrophages induced by Brucella infection (Pei et al., 2008). Brucella T4SS is tightly regulated by various regulatory proteins under specific conditions, such as acidification and nutritional deprivation. Deletion or overexpression of virB is detrimental to intracellular survival of Brucella (Zhong et al., 2009). In addition, shortening
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FIGURE 5 | Brucella VjbR regulates T4SS expression in the rough mutant to induce macrophage death. (A) The qPCR analysis. Compared to the smooth wild-type strain S2308, upregulation of vjbR and downregulation of mdrA and blxR were evident in the rough mutant ΔrfbE. ns, no significant difference, **p < 0.001 and ***p < 0.0001. (B) Annexin V-FITC/PI staining. RAW264.7 cells cultured in a 24-well plate were infected with S2308, ΔrfbE, ΔrfbEΔvjbR, ΔrfbEΔvjbRΔvirB, ΔrfbEΔvjbRΔvirBΔmDrA, or ΔrfbEΔvjbRΔvjbRΔvirB at a multiplicity of infection (MOI) of 100. The cells were stained at 8 and 12 hpi with FITC-annexin (green) and PI (red) and observed by fluorescence microscopy at a magnification of ×200. Uninfected RAW264.7 cells were used as negative controls (Mock). (C) Determination of LDH release. RAW264.7 cells were infected with S2308, ΔrfbE, ΔrfbEΔvjbR, ΔrfbEΔvjbRΔvirB, ΔrfbEΔvjbRΔvjbRΔmDrA, and ΔrfbEΔvjbRΔvjbRΔmDrA at an MOI of 100. The supernatants were collected at 8 and 12 hpi, and LDH release was detected using the CytoTox 96 nonradioactive cytoxicity assay. The supernatants of uninfected RAW264.7 cells were used as negative controls (medium). ns, no significant difference, *p < 0.05 and ***p < 0.0001. (D) VirB expression in the ΔrfbEΔvjbR was recovered to the similar level of the smooth wild-type strain S2308, as determined by qRT-PCR. **p < 0.001 and ***p < 0.0001. (E) VirB expression in the ΔrfbEΔvjbR was partly recovered, compared to the ΔrfbE mutant, as determined by qRT-PCR. *p < 0.05 and ***p < 0.0001.

of the LPS molecule enhances the type III secretion system in *Shigella* (West et al., 2005). In this study, we confirmed that the capacity for T4SS secretion and the effectors being translocated to macrophages were highly increased in the ΔrfbE mutant.

The *virB* mRNA level has been shown to be very low when *Brucella* is grown in a rich medium at neutral pH, and *virB* transcription is upregulated when cultured in acidic conditions or minimal medium (Boschirioli et al., 2002). However, the use of *lacZ* reporter gene fusions has shown that the *virB* operon of *B. abortus* S2308 is expressed during the stationary phase, without the requirement for acidic induction conditions (Sieira et al., 2000). According to the analysis of mRNA levels, our results demonstrated that in comparison to the smooth wild-type strain S2308, T4SS expression of the rough mutant ΔrfbE was significantly upregulated at the exponential phase under conditions of both a rich medium at neutral pH and
nutrient-deprived or acidic conditions. In the rich medium at neutral pH, the parental *B. abortus* and *B. melitensis* strains constitutively produced *virB5* and *virB8* (Rouot et al., 2003). However, in this study, the smooth wild-type strain, S2308, produced a low level of the *virB5* protein in a rich medium at neutral pH; whereas *virB5* expression levels of the rough mutant, Δ*rfbE*, were significantly increased. After exposure to acidic conditions, *Brucella* easily produces detectable levels of *virB8* (Rouot et al., 2003). Under nutrient-deprived or acidic conditions, we confirmed that the *virB5* protein is easily detected, and that expression of *virB5* in the rough mutant Δ*rfbE* was higher than that in the S2308 strain. Thus, enhanced expression of T4SS in the Δ*rfbE* mutant might account for its increased capacity for T4SS secretion.

Intracellular induction of *virB* expression has been observed to be transient, and the translocation and activity of VirB-secreted effectors within the host cell might be determined by the timing of expression of the *virB* operon (Sieira, 2013). In this work, we analyzed the activity of the *virB* promoter in the smooth wild-type strain S2308, and its rough mutant Δ*rfbE*, using the luciferase reporter assay in a rich medium and in an intracellular environment. In compared to the wild-type strain S2308, the *virB* promoter activity of the rough mutant Δ*rfbE* was significantly increased in the rich medium and in acidic conditions, both of which enhanced T4SS expression and secretion in the rough mutant Δ*rfbE*. Once *Brucella* is internalized in macrophages, the transcriptional activity of the *virB* promoter reaches a maximum level at 5 h.p.i., and the promoter is then turned off, when *Brucella* reaches its replicative niche (Sieira et al., 2004). Our results demonstrated that the activity of the *virB* promoter was increased in the intracellular environment of both the smooth wild-type strain S2308, and the rough mutant Δ*rfbE*, at an early stage of infection. Furthermore, T4SS expression and secretion of the Δ*rfbE* mutant was notably upregulated in comparison to that of the S2308 strain within macrophages. However, the *virB* promoter activity in wild-type strain S2308 did not stop at 8 h.p.i. in this study, which may be due to different setting up of the time point in the cell infection assays.

On further study, we investigated the expression of *vjbR*, *blxR*, and *mdrA* genes that have been proven to regulate T4SS expression directly in the smooth *Brucella* strain. We found that *vjbR* expression was upregulated, and the expression of both *blxR* and *mdrA* were downregulated in the Δ*rfbE* mutant. The VjbR protein belongs to the LuxR family, a group of transcriptional regulators involved in the cell-to-cell communication process referred to as quorum sensing (QS), This process allows bacteria...
to sense changes in population density and coordinate adaptive responses, and acts as the main regulator of expression of the virB operon (Miller and Bassler, 2001; Uzureau et al., 2010; Weeks et al., 2010). A vjbR mutant of B. melitensis exhibits downregulated expression of both the virB operon and flagellar genes, either during vegetative growth or during intracellular infection, and is strongly attenuated in a mouse model of infection (Delrue et al., 2005). In addition, VjbR regulates exopolysaccharide synthesis or export, as well as the production of several outer membrane proteins, some of which are involved in virulence (Uzureau et al., 2007). In the present study, we found that deletion of vjbR in the ΔrfbE mutant significantly reduced its cytotoxicity in macrophages. The BlxR protein is the second QS-related regulator of Brucella that contains both the DNA- and AHL-binding domains characteristic of the LuxR-type proteins (Rambow-Larsen et al., 2008; Sieira, 2013). Deletion of blxR affects virulence and intracellular survival of Brucella, but to a lesser extent than deletion of vjbR (Rambow-Larsen et al., 2008). A previous report suggests that BlxR negatively modulates activity of the virB promoter in B. abortus (Caswell et al., 2012). Our results confirmed that BlxR negatively modulates the activity of the virB promoter in B. abortus, and overexpression of blxR in the ΔrfbE mutant reduces to some extent, the cytotoxicity within macrophages. However, overexpression of mdrA in the ΔrfbE mutant did not reduce cytotoxicity within macrophages. The significance of mdrA downregulation in the ΔrfbE mutant requires further study. Thus, it is evident that a QS-related transcriptional regulator plays important roles in Brucella rough mutant-induced macrophage death. The QS-related transcriptional regulators might function in sensing environmental changes, such as cell density, acidification, and nutritional deprivation. One possible explanation is that loss of LPS in Brucella makes it sensitive to environmental stress that dysregulates the QS-related transcriptional regulators and upregulates T4SS to secrete a greater number of effectors. This probably accounts for the cytotoxicity in macrophages infected by Brucella rough mutants.

During Brucella interaction with host cells, the Brucella T4SS effector protein VceC, is involved in the induction of inflammatory responses by binding chaperone BiP, to trigger ER stress (de Jong et al., 2013). The ER stress induces the UPR in macrophages, and activates IRE1α, which in turn, recruits the NOD-like receptors NOD1 and NOD2, to induce activation of NF-κB and expression of pro-inflammatory genes (Keestra-Gounder et al., 2016). Brucella abortus inhibits cell death of infected macrophages (Fernandez-Prada et al., 2003; He et al., 2006), and chronically persists under conditions of a mild inflammatory response that leads to granuloma formation (Silva et al., 2011). However, the ER stress sensor IRE1α, induced by the rough mutant RB51, induces ROS-dependent NLRP3 translocation to mitochondria, and NLRP3 stimulates the caspase-2-Bid mitochondrial damage pathway, thereby leading to the release of mitochondrial danger signals that activate the inflammasome (Bronner et al., 2015). In this study, we found that the rough mutant ΔrfbE, secreted more effector proteins and induced stronger IRE1α pathways of ER stress, in comparison to the smooth wild-type strain S2308. These actions might excessively activate the IRE1α pathway and further activate the inflammasome via NLRP3- and caspase-2- driven mitochondrial damage, and result in cell death of macrophages. The crucial components associated with activation of the IRE1α pathway of ER stress to promote macrophage death in rough mutants remain to be identified.

Taken together, this study provided evidence that VjbR upregulation in the Brucella rough mutant ΔrfbE increases transcription of the virB operon, resulting in T4SS overexpression, accompanied by over-secretion of T4SS effector proteins. This in turn, strongly activates the IRE1α pathway of ER stress to cause the death of infected macrophages. This study provides novel insights into molecular mechanisms of Brucella rough mutant ΔrfbE-induced macrophage cytotoxicity.

AUTHOR CONTRIBUTIONS

SY, MT, and CD conceived and designed the experiments; PL and MT mainly performed the experiments and analyzed the data; YB, HH, JL, YY, and SW helped to perform some experiments; PL wrote the paper, SY revised the manuscript and coordinated the research. All authors have read and approved the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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