Original Article

Iranian Journal of Immunology
https://iji.sums.ac.ir

TLR9/NF-kB Pathway Regulates *Brucella* CpG DNA-mediated Cytokine Response in Human Peripheral Blood Mononuclear Cells

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ABSTRACT

Background: It was reported that targeting the Toll-like receptor 9 (TLR9) signaling pathway can be a promising therapeutic strategy for interventions in various inflammatory and infectious diseases. However, it was not known whether the human TLR9 is responsive to *Brucella* cytidine-phosphate-guanosine (CpG) DNA sequences and activates the host's innate immune system.

Objective: The present study aimed to identify the novel human TLR9 agonists from *Brucella* CpG oligodeoxynucleotide (ODN) candidates and verify their immune response regulatory mechanisms.

Methods: Molecular docking methods were used to discover potent agonists of the human TLR9. The potential molecules were further validated by Western blot and enzyme-linked immunosorbent assay (ELISA).

Results: The experiment results showed a strong interaction and good compatibility between the human TLR9 and *Brucella* ODN-1molecule. In addition, the induction of immune response by *Brucella* ODN-1 is a CpG-specific response. Moreover, the effects of *Brucella* ODN-1 on cytokine response are dependent on the TLR9-mediated NF-κB pathway.

Conclusion: These results indicated that the *Brucella* ODN-1 molecule can serve as a starting point to discover or designmore potent and specific TLR9 agonists that have the potential use in the treatment of infectious diseases.

Keywords: Toll-like receptor 9, *Brucella*; Cytidine-phosphate-guanosine (CpG) motif, Agonist

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Cite this article as:
Peng Y, Bai W, Wang Z, Yu H. TLR9/NF-kB Pathway Regulates *Brucella* CpG DNA-mediated Cytokine Response in Human Peripheral Blood Mononuclear Cells. *Iran J Immunol.* 2021; 18(4):268-278, doi: 10.22034/IJI.2021.84578.1665.

Received: 2019-12-17
Revised: 2021-11-22
Accepted: 2021-11-25

INTRODUCTION

Toll-like receptors (TLRs), a family of transmembrane proteins, are responsible for recognizing pathogen-associated molecular patterns (PAMPs) and activating immune effectors to form an essential bridge between the innate and adaptive immune systems (1, 2). They can initiate innate immune response through MyD88- and TRIF-dependent signaling pathways (3). Among TLRs, TLR9 is the sole family member for detecting bacterial DNA

based on the recognition of unmethylated cytidine-phosphate-guanosine (CpG) motifs (4). When TLR9 binds to specific unmethylated CpG motifs, it activates downstream signaling pathways including the NF-κB, which is involved in the immune and inflammatory responses. The activation of the NF-κB signaling pathway induces the increase of inflammatory cytokines, including interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) (5). It is well known that the TLR9-dependent immunostimulatory properties of bacterial DNA can be mimicked by synthetic CpG oligodeoxynucleotides (ODNs) (6). However, what kinds of natural bacterial DNA sequences are responsible for immunostimulation are still not entirely clear.

Brucella is a small gram-negative bacterium, which has adapted to survive and replicate inside the host immune cells, such as dendritic cells and macrophages (7). One pathway by which *Brucella* can modulate the host's immune response is through interference with TLR/NF-κB signaling (8). Previous studies have shown that *Brucella* DNA plays an important role in regulating the innate immune response via TLR9 (9). Some reports have also highlighted the molecular mechanism of immune cells activated by *Brucella* DNA. Gomes et al. found that *B. abortus*- derived CpG ODNs induced IL-12 and TNF-α production in macrophages in a TLR9-dependent manner (10). Our previous study also found that the mouse TLR9 recognized *Brucella* repetitive extragenic palindromic DNA sequences and modulated the innate immune response of the host (11). It is well known that the profile of CpG ODN-induced immune response in mice differs from that in humans (12). In contrast to mice, knowledge about *Brucella* CpG ODN-mediated effects on the human TLR9 is poor. Recently, some bacterial CpG DNA molecules were identified as natural ligands for TLR9 (13). The present study aims to identify the novel human TLR9 agonists from *Brucella* CpG ODN candidates. All these data could also be critical to understanding the host immune responses induced by *Brucella* infection.

MATERIALS AND METHODS

Bioinformatics Analysis

We used the cgplot and cgreport programs of EMBOSS to analyze CpG-rich sequences. The genomes analyzed were: *Brucella melitensis* (*B. melitensis*) NI chromosome 1 (RefSeq NC_003317.1), *B. melitensis* NI chromosome 2 (RefSeq NC_003318.1). We then searched for CpG motifs as reported previously (14). The similarity between sequences was computed using the Basic Local Alignment Search Tool (BLAST). The software MultAlin was used for sequence alignments. *Brucella* ODN-1 (CGT GCC GTC GCC AAG), ODN-2 (CCG TCG AAG ACG G), ODN-3 (CCG AAG GTT TCG G), and ODN-4 (CGC TCG CGC ATG ATG ATG GAG CG) were selected for a further test based on the evolutionary conservation, the length, the location and the surrounding bases of CpG motifs.

Molecular Modeling

All modeling and simulations were conducted using the Discovery Studio Version 4.5 (Accelrys Inc., USA). The structure model of the human TLR9 was obtained by homology modeling using the crystallographic structure of horse TLR9 as a template (PDB code: 3wpb) (https://swissmodel.expasy.org/repository/uniprot//Q9NR96). The two-dimensional chemical structures of CpG DNA molecules were built using Discovery Studio software. Docking calculations were carried out with program Hex version 6.12 (15). The shape plus electrostatic correlation algorithms were used in this study. The rest of the parameters were set at default values.

Cell Preparation

Human peripheral blood mononuclear cells (PBMCs) were isolated by using Ficoll density gradient centrifugation (Lymphoprep, MD PACIFIC Biotechnology COK. Tianjin, China). Cells were collected, washed three times with phosphate-buffered saline (PBS),

and suspended in RPMI-1640 medium (Gibco, Invitrogen Corporation, USA). PBMCs were then seeded at a density of 4.0×10^5 cells mL^{-1} in 24-well plates. This study was approved by the Ethics Committee of Baotou Medical College.

Cell Stimulation

Phosphorothioated ODNs were synthesized and purified (Sangon Biotech, Shanghai, China) and 5-methylcytidine was used in the methylated ODN synthesis. ODNs were then diluted in Opti-MEM medium supplemented with 10 μg/mL lipofectin (Thermo Fisher Scientific Inc. MA. USA). The final volume of the mixture was 0.3 mL. The resulting mixture was incubated for 5 min at room temperature. PBMCs in each well of 24-well plate were starved for 2 h in a pre-warmed serum-free medium and then incubated with ODNs (3 μM) in the above mixture in the absence or presence of 10 μM pyrrolidine dithiocarbamate (PDTC; Sigma-Aldrich). C class stimulatory CpG2395 (TCG TCG TTT TCG GCG CGC GCC G; Thermo Fisher Scientific Inc. MA. USA) was used as a positive control. CpG2395 non-CpG (TGC TGC TTT TGG GGG GCC CCC C) was used as a negative control. *Brucella* ODN-1 ^{methyl} CpG (^{m}CGT G^{m}C^{m}C GT^{m}C G^{m}C^{m}C AAG) and ODN-1 non-CpG (GCT GCC CTG CCC AAG) were also synthesized to determine whether cytokines production by ODNs is a CpG-specific response. Endotoxin levels in the synthetic ODNs used in this study were less than 0.1 EU/mg.

TLR9 siRNA Transfection

PBMCs in 24-well plates were transfected with siRNA targeting the human TLR9 by riboFECT™ CP transfection kit (Ribobio, Guangzhou, China) at a final concentration of 100 nM. The siR-Ribo™ negative sequence was used as negative siRNA control (16). The knockdown efficiency was verified 24 h after transfection.

ELISA Assay

The cells were treated with *Brucella* ODNs for 6, 12, or 24 h. Culture supernatants were collected and the production of IL-6 and TNF-α were measured using ELISA kits (R&D Systems, Minneapolis, MN, USA). All the experiments were carried out in duplicate and repeated at least three times.

Western Blot Analysis

PBMCs treated with *Brucella* ODNs were harvested at 20, 60, and 120 min post-stimulation. Total cell lysates were obtained using M-PER Mammalian Protein Extraction Reagent with a proteinase inhibitor cocktail (Thermo Fisher Scientific Inc. MA. USA). Concentrations of total protein were quantified using the Bicinchoninic Acid (BCA) reagent kit (Thermo Fisher Scientific Inc. MA. USA). Then the cell lysate was subjected to SDS-PAGE analysis (10% acrylamide gel, 120 v, 90 min) and followed by polyvinylidene difluoride (PVDF) membrane transfer (Millipore, USA). Immunodetection was carried out using a chemiluminescent substrate. GAPDH levels were measured as a control. The following antibodies obtained from Cell Signaling Technology (MA, USA) were used: rabbit anti-phospho-NF-κB p65 (1:1000 dilution), rabbit anti-NF-κB p65 (1:1000 dilution), rabbit anti-IKKα (1:1500 dilution), rabbit anti-phospho-IKKα (1:1500 dilution), and rabbit anti-GAPDH (1:1000 dilution) polyclonal antibodies. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (1:4000 dilution) was used as the secondary antibody.

Statistical Analysis

All data were investigated for the normality of distribution. If data were normally distributed, student's unpaired t-tests were used to compare the two groups, and the one-way ANOVA tests followed by Tukey tests were performed to compare the three and more groups. If data were not normally distributed, then Mann-Whitney tests were used to compare variables between the two groups, and Kruskal-Wallis tests followed by Dunn tests were used to compare the

multiple groups. Values are presented as the mean±standard deviation (SD). Differences were deemed statistically significant at $P<0.05$.

RESULTS

Identification of the Bioactive Brucella ODNs

The complete genome sequence of *B. melitensis* NI was used to screen *Brucella* ODN sequences. We found 346,999 CpG ODNs in *B. melitensis* NI chromosome 1, and 193,039 CpG ODNs in *B. melitensis* NI chromosome 2. We then selected four *Brucella* ODN molecules sequence that had different sequences and numbers of the CpG motifs as well as various lengths of the ODNs for further investigation. The blast results showed that the selected four ODN molecules were present not only in *B. melitensis* but also in *B. abortus*, *B. suis*, and *B. ovis*, implicating that they were evolutionarily conserved and might play important roles in the immune recognition. To simulate the interactions between the human TLR9 and ODNs, the homology model of the human TLR9 was constructed using the crystal structure of horse TLR9 as a template. Figure 1A revealed that the interface was good compatibility between the protein and DNA molecule. Residues Trp47, Phe49, Lys51, Arg74, His76, His77, Ser104, Met106, Phe108, Pro109, Tyr132, Arg152, Tyr179, Tyr180, Lys181, Tyr208, Arg256, Arg262 and Asn263 in one protomer and Glu616, His641 and Thr642 in the other involved in the interaction between the human TLR9 and the *Brucella* ODN-1 (Figure 1B). Moreover, there were 4 intermolecular hydrogen bonds between *Brucella* ODN-1 and the human TLR9. These interactions lead to a large stabilization of *Brucella* ODN-1 in this region. The alignment of the energy-minimized docking model of *Brucella* ODN-1 with the crystal structure of agonistic DNA1668_12mer of TLR9 (PDB code: 3wpc) was performed (Figure 1C).

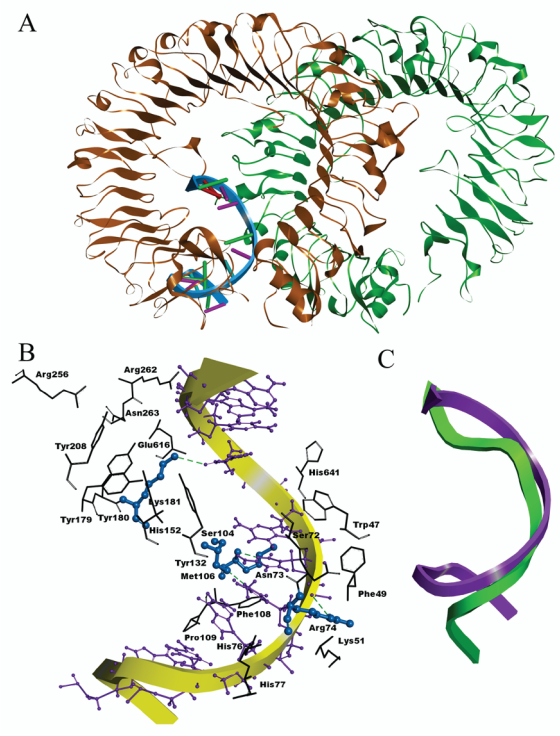

Figure 1. Proposed binding model of *Brucella* ODN-1 to the human TLR9 by the docking method. (A) The structure of the human TLR9 complexed with *Brucella* ODN-1. The structure of the human TLR9 is shown in a solid ribbon. *Brucella* ODN-1 is shown in the *blue* arrow. (B) Interactions between human TLR9 and *Brucella* ODN-1. The hydrogen bonds are labeled by green lines. Residues of the human TLR9 involved in the hydrogen bonds are highlighted in blue ball and stick. Other residues of the human TLR9 are highlighted in the black stick. (C) The energy-minimized docking model of *Brucella* ODN-1 (Purple) aligned to the crystal structure of agonistic DNA1668_ 12mer of TLR9 (Green) (PDB code: 3wpc).

A marked similarity of orientation (0.737) was observed. However, the pattern of other *Brucella* ODNs' interaction with the human TLR9 exhibited differences from that of the *Brucella* ODN-1 (data not shown). The interface between the human TLR9 and other *Brucella* ODNs was not geometrically complementary. In contrast to *Brucella* ODN-1, there are fewer hydrophobic interactions between the human TLR9 and other *Brucella* ODNs. Additionally, no intermolecular hydrogen bonds directly linking the human TLR9 to the other three *Brucella* ODNs were observed. The total energy of interactions

between the human TLR9 and Brucella ODNs were also presented in supplementary file Table S1. Our results implicated that the human TLR9 might interact with the *Brucella* ODN-1.

Brucella ODN-1 Treatment Induced Cytokines Production

The cytokines concentration of PBMCs was determined with ELISA. Figure 2A showed that *Brucella* ODN-1 elevated the supernatant levels of IL-6 and TNF-α in cultured PBMCs at 24 h post-stimulation, compared to the negative control (P<0.05). However, the other three *Brucella* ODN molecules induced no or low levels of cytokines. Meanwhile, the kinetics of IL-6 and TNF-α concentrations following PBMCs stimulated with *Brucella* ODN-1 were determined. The cytokines levels were detectable as early as 12 h after *Brucella* ODN-1 stimulation (Figure 2B).

Brucella ODN-1-Induced Cytokines Production Associated with CpG Motif

To investigate whether the effects of *Brucella* ODN-1 on PBMCs cytokines response are CpG dependent, *Brucella* ODN-1 ^{methyl}CpG and *Brucella* ODN-1 non-CpG

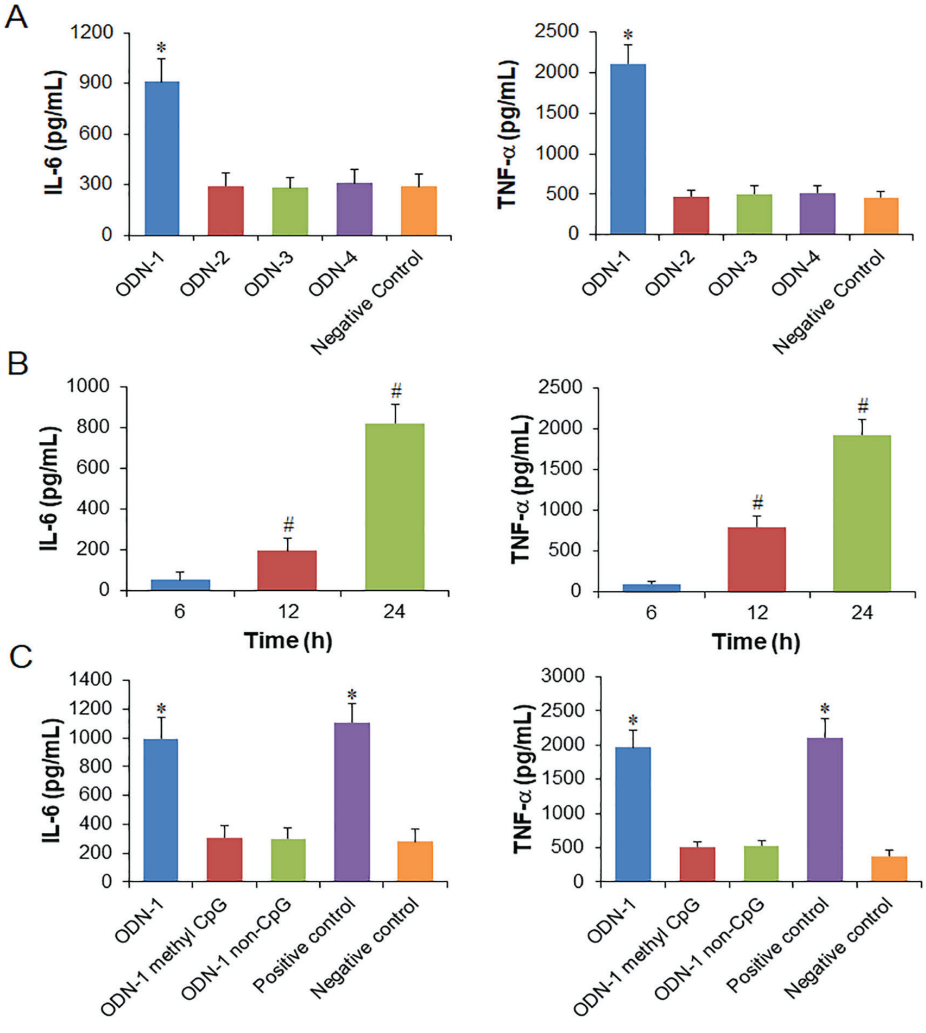

Figure 2. *Brucella* ODN-1 treatment induced cytokines production. (A) Effect of ODNs' stimulation on the levels of the IL-6 and the TNF-α in the supernatant of cultured PBMCs. After serum starvation for 2 h, PBMCs were treated with ODNs for 24 h. (B) Temporal kinetics of the IL-6 and the TNF-α levels in the supernatant of cultured PBMCs stimulated with *Brucella* ODN-1. After serum starvation for 2 h, PBMCs were treated with *Brucella* ODN-1 for 6, 12, and 24 h. (C) *Brucella* ODN-1-induced cytokines production associated with CpG motif. After serum starvation for 2 h, PBMCs were treated with *Brucella* ODN-1, ODN-1 ^{methyl}CpG, and ODN-1 non-CpG for 24 h, respectively. Values are expressed as the means±SD, *P<0.05, *vs* the negative control group, $^{\#}$P<0.05, *vs* 6 h group.

were evaluated. Supernatants were collected in 24 h post-stimulation with ODNs. In the *Brucella* ODN-1 ^{methyl}CpG group and the *Brucella* ODN-1 non-CpG group, there were no significant changes in levels of IL-6 and TNF-α as compared with the negative control group (P<0.05) (Figure 2C).

Brucella ODN-1-Induced Cytokines Production in a TLR-9-Dependent Manner

ODNs, which contain unmethylated CpG motif, have been shown to increase TLR9 expression. We then examined whether *Brucella* ODN-1 treatment will induce the expression of TLR9 in PBMCs. Figure 3A showed that *Brucella* ODN-1 treatment significantly increased the expression of TLR9, compared with the ODN-1 non-CpG negative control group (P<0.05). We further assessed whether the loss of TLR9 affected the release of cytokines from PBMCs. TLR9 siRNA was used to knock down the expression of TLR9 (Figure 3B). Meanwhile, TLR9 siRNA abolished *Brucella* ODN-1-induced release of cytokines (P<0.05) (Figure 3C), indicating that *Brucella* ODN-1- induced the production of IL-6 and TNF-α is mediated by TLR9.

Brucella ODN-1 Increased the Levels of Phosphorylated IKKα and NF-κB in PBMCs

Activation of transcription factor NF-κB is a prerequisite for cytokine secretion. We then checked whether NF-κB is involved in the signaling pathway triggered by *Brucella* ODN-1. The phosphorylation levels of IKKα and NF-κB in PBMCs transfected with the *Brucella* ODN-1 were carried out. Figure 4 showed that *Brucella* ODN-1 treatment significantly increased the levels of phospho-IKKα and phospho-NF-κB p65 at 60 min and 120 min post-transfection (P<0.05), respectively, compared with the *Brucella* ODN-1 non-CpG group.

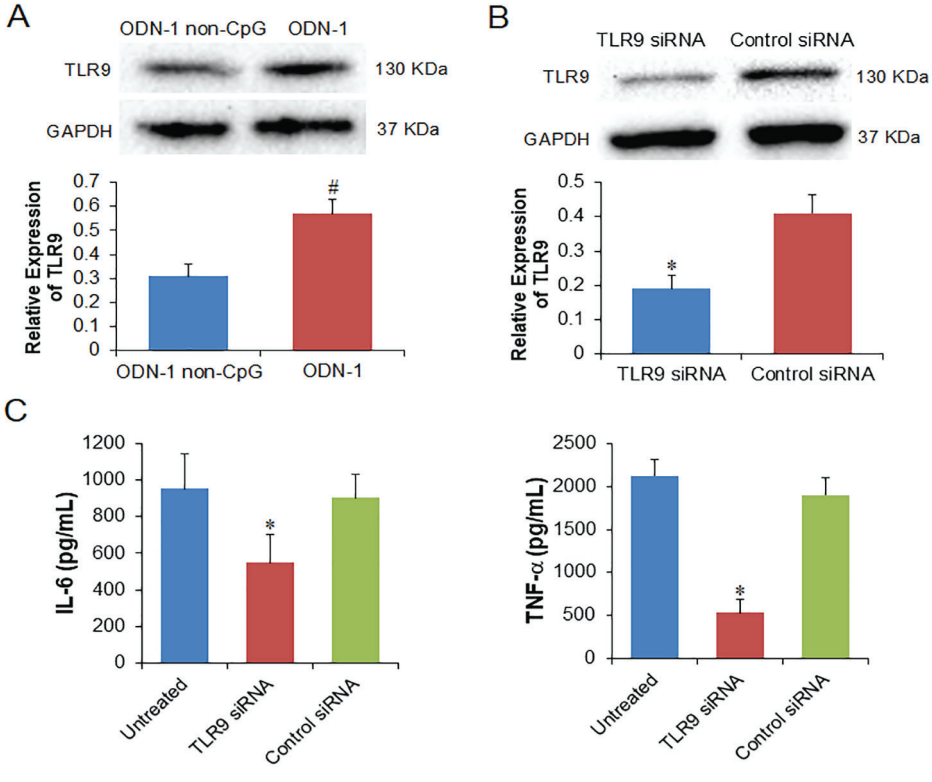

Figure 3. *Brucella* ODN-1 treatment increased cytokines release in a TLR9- dependent manner. (A) TLR9 expression significantly increased in PBMCs after the treatment with *Brucella* ODN-1 for 120 min. (B) The expression of the TLR9 was down-regulated by transfection of TLR9 siRNA for 24 h. (C) TLR9 siRNA treatment prevented *Brucella* ODN-1-induced release of the IL-6 and the TNF-α in the supernatant of cultured PBMCs. After transfection with the TLR9 siRNA or control siRNA for 24 h, the levels of cytokines were assessed using the ELISA kits. Values are expressed as the means±SD, *P<0.05, *vs* the control siRNA group, #P<0.05, *vs* the ODN-1 non-CpG group.

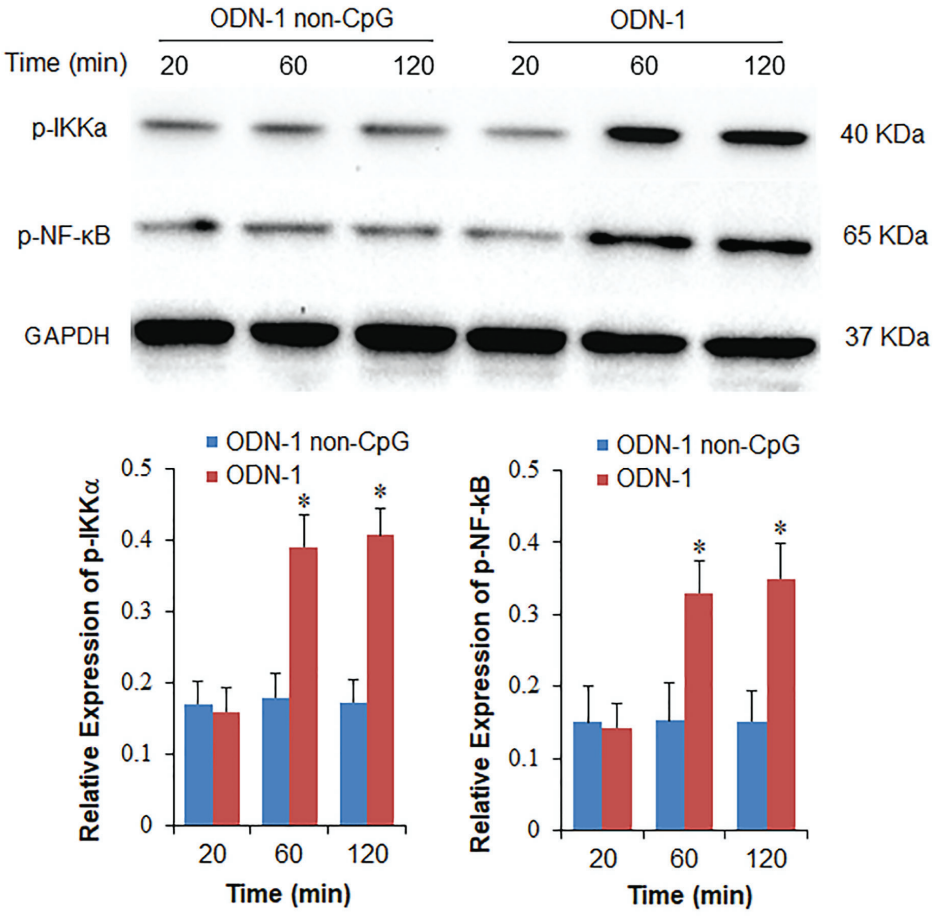

Figure 4. *Brucella* ODN-1 treatment increased the levels of phosphorylated IKKα and NF-κB in PBMCs. After serum starvation for 2 h, PBMCs were treated with *Brucella* ODN-1 or ODN-1 non-CpG for 20, 60, and 120 min, respectively. The levels of phosphorylated IKKα and NF-κB were evaluated using Western blotting. Values are expressed as the means±SD, *P<0.05, *vs* the *Brucella* ODN-1 non-CpG group.

Brucella ODN-1 Induced Cytokines Production Through TLR9/NF-κB Signaling Pathway

To determine the role of the TLR9/NF-κB signaling pathway in *Brucella* ODN-1-induced release of cytokines, we treated cells with TLR9 siRNA and *Brucella* ODN-1. Figure 5A showed that TLR9 siRNA treatment abolished *Brucella* ODN-1-induced expression of phospho-IKKα and phospho-NF-κB p65 (P<0.05), indicating that *Brucella* ODN-1-increased phosphorylation of IKKα and NF-κB is mediated by TLR9. Moreover, treatment of PBMCs with a specific NF-κB inhibitor, PDTC, abolished *Brucella* ODN-1-increased levels of IL-6 and TNF-α (P<0.05) (Figure 5B). These results suggested that *Brucella* ODN-1 could significantly upregulate the synthesis of IL-6 and TNF-α through the TLR9/NF-κB signaling pathway in PBMCs.

DISCUSSION

Brucella infections cause NF-κB-mediated pro-inflammatory cytokine release that is essential for host protective immunity (8). This inflammatory response has previously been shown to be partially associated with TLR9 (10). Identifying the *Brucella* TLR9 ligands is important for the understanding of brucellosis pathogenesis and for the design of new therapeutic approaches aimed at modulating the inflammatory responses to *Brucella*. Recent evidence suggested that *Brucella* CpG ODNs stimulated inflammatory responses by the mouse TLR9-dependent mechanisms (11). CpG ODNs have species-

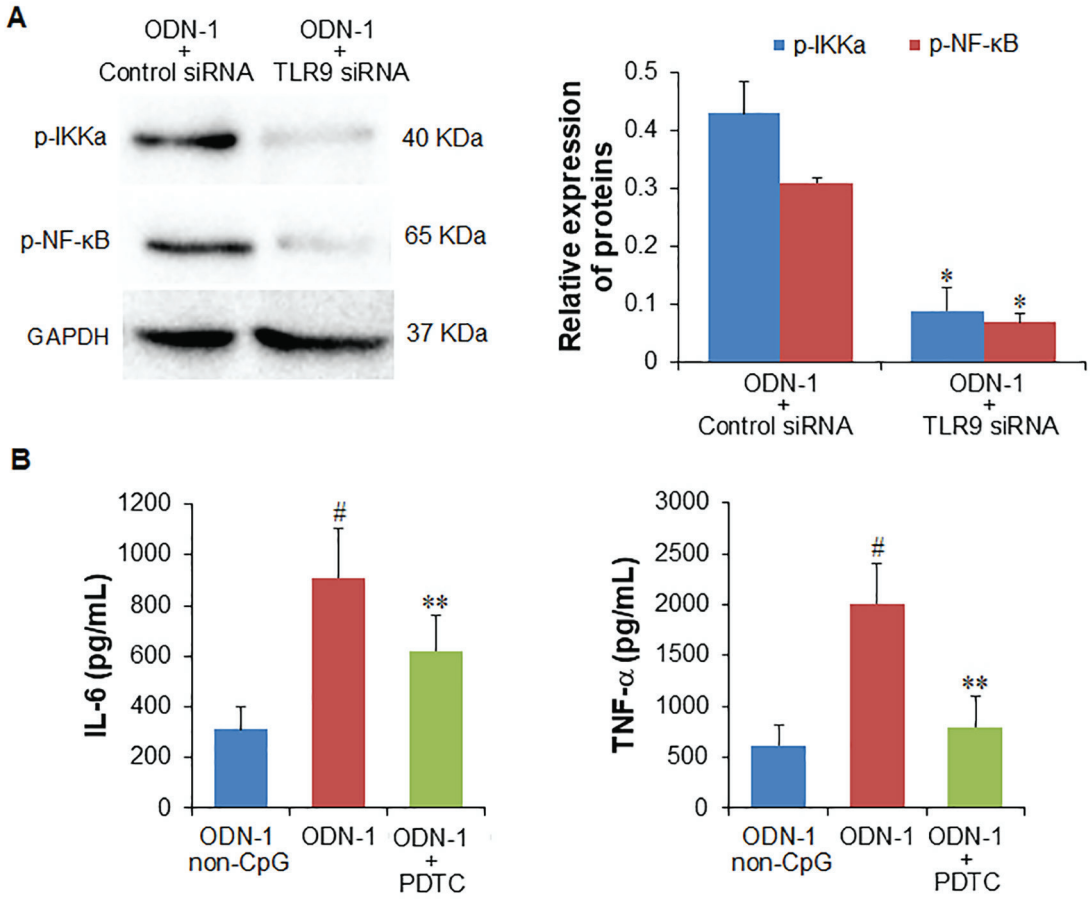

Figure 5. TLR9/NF-κB pathway involves *Brucella* ODN-1-mediated cytokines release. (A) TLR9 siRNA treatment abolished *Brucella* ODN-1-induced expression of phospho-IKKα and phospho-NF-κB p65 in PBMCs. After transfection with TLR9 siRNA or control siRNA for 24 h, PBMCs were treated with *Brucella* ODN-1 for 120 min, the levels of phosphorylated IKKα and NF-κB were evaluated using Western blotting. (B) NF-kB inhibition abolished *Brucella* ODN-1-induced cytokines production in the supernatant of cultured PBMCs. After serum starvation for 2 h, PBMCs were stimulated by *Brucella* ODN-1 or ODN-1 non-CpG with or without the NF-κB inhibitor PDTC for 24 h. The levels of cytokines were assessed using ELISA kits. Values are expressed as the means±SD, *P<0.05, *vs* the control siRNA group, #P<0.05, *vs* the ODN-1 non-CpG group, **P<0.05, *vs* the ODN-1 group.

specific immunostimulatory activity, which is determined by the nucleotide sequence of CpG ODNs (17). Up to now, the specific interaction of *Brucella* CpG ODNs with the human TLR9 is poorly understood, and how this interaction results in activation of downstream signaling have not been characterized sufficiently. This prompted us to identify novel human TLR9 agonists by molecular modeling methods and to elucidate the molecular mechanism of action of an agonist by *in vitro* biological analyses.

The in-silico analysis showed that the *Brucella* genome contains numerous CpG motifs. Based on our *in silico* analysis, we chose four CpG ODN sequences with various nucleotide contexts of the CpG motifs. These four CpG ODNs were strongly conserved in genomes of *B. abortus*, *B. melitensis*, *B. suis*, and *B. ovis*. The molecular docking approach was then applied to predict the human TLR9-ODN complex. It has been reported the crystal structure of TLR9 is bound to agonistic CpG-DNA, which showed that agonistic CpG-DNA was recognized by both protomers in the dimer, in particular by the amino-terminal fragment (LRRNT-LRR10) from one protomer and the carboxy-terminal fragment (LRR20-LRR22) from the other (18). Our results demonstrated that the interaction pattern between *Brucella* ODN-

1 and the human TLR9 was similar to that of the previous report(18). Also, there are 4 intermolecular hydrogen bonds in the human TLR9-*Brucella* ODN-1 complex. These observations are consistent with the previous report that hydrogen-bond interactions are principally responsible for specific recognition between proteins and DNA (19). Thus, we suppose that *Brucella* ODN-1 might demonstrate a potential high binding affinity for the human TLR9 as an agonist.

To further validate our molecular docking results described above, *Brucella* ODN-1 was tested for its ability to induce cytokines production in PBMCs. In the present study, the levels of the IL-6 and TNF-α were detected following stimulation of PBMCs with *Brucella* ODN-1. In contrast, the other three *Brucella* ODNs did not induce significant cytokines response. The results were in agreement with the above molecular docking analysis. Moreover, the dynamic changes of inflammatory cytokines treated by *Brucella* ODN-1 were observed. Many studies usually choose 24 h after stimulation as the time point (20). Our preliminary experiments found that cytokines levels changed in 12 h, so we choose the 6, 12, and 24 h after stimulation as the time point to explore the role of *Brucella* ODN-1 in this experiment.

It has been reported that the immunostimulatory effects of bacterial DNA heavily rely on the presence of unmethylated CpG dinucleotides (13). We then turned the CpG motif of *Brucella* ODN-1 into either a non-CpG sequence or methylated CpG motif. We found that ODNs with methylated or deleted CpG motifs had no stimulatory effect. The results confirmed that the induction of cytokines production by *Brucella* ODN-1 is indeed CpG dependent.

To date, all the evidence indicates that TLR9 is required for cellular responses to unmethylated CpG motifs (21). We, therefore, determined whether *Brucella* ODN-1 induced high levels of cytokines in a TLR9-dependent manner. TLR9-siRNA was then transfected into PBMCs to down-regulate TLR9 expression. We found that the levels of the IL-6 and TNF-α induced by *Brucella* ODN-1 significantly decreased. In addition, ODN-1 treatment increased the expression of TLR9. These experiments proved that the immunostimulatory effects of *Brucella* ODN-1 were mediated by the TLR9 receptor, which is in agreement with our molecular docking results. These observations were generally consistent with the idea that bacterial DNA could be novel targets to regulate TLR9-mediated innate immune responses (22).

The ability of bacterially-derived molecular patterns to promote innate immune responses through the TLR9-mediated NF-κB signaling pathway has been characterized extensively (23). We then checked whether the NF-κB is involved in the signaling pathway triggered by *Brucella* ODN-1. Our results demonstrated that the phosphorylation levels of the NF-κB p65 and the IKKα significantly increased in PBMCs stimulated with *Brucella* ODN-1. Inhibition of TLR9 contributed to a reduction of ODN-1-induced phosphorylation of IKKα and the NF-κB. Moreover, inhibition of the NF-κB blocked the *Brucella* ODN-1-induced increase in cytokines levels. Therefore, our data suggested that the activation of the NF-κB signal might in part contribute to the TLR9-mediated immunological responses induced by *Brucella* ODN-1. The results were in agreement with previous findings that DNA from microflora could be exerting stimulatory effects mediated by the TLR9/NF-κB pathway (20).

CONCLUSION

To the best of our knowledge, this is the first study to identify the human TLR9 agonist from the *Brucella* DNA. Our data also clearly illustrate that the obtained active molecule *Brucella* ODN-1 induced the release of cytokines in a CpG-dependent manner, which is involved in the immunostimulatory effects mediated by the TLR9/NF-κB pathway.

ACKNOWLEDGMENT

This study was supported by the National Natural Science Foundation of China (no. 81360242).

Conflicts of Interest: The authors declare that there is no conflict of interest.

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Supplementary File

Table S1. Total energy of interactions between human TLR9 and *Brucella* ODNs.

Name	Binding energy (KJ/mol)
Brucella ODN-1	-427.1
Brucella ODN-2	-109.3
Brucella ODN-3	-121.0
Brucella ODN-4	-152.7