Expression of cytokine and apoptosis-related genes in murine macrophages and dendritic cells stimulated with Brucella melitensis recombinant proteins

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Abstract

Background

Brucellosis, a zoonosis of public health and economic importance globally. Vaccination is the most efficient way to prevent the infection. Many protein antigens have been selected as diagnostic antigens recently, whereas, the exact mechanisms at the early stage of immune response are remain unknown. In this study, four *Brucella melitensis* cellular proteins were cloned, and the expressed recombinant proteins were purified. The expression of several cytokine genes (*IFN-β*, *IFN-γ*, *IL-4*, *IL-10*, *IL-12p40*, and *TNF-α*) was analyzed in a murine macrophage cell line (RAW264.7) and murine dendritic cells line(DCs) after stimulation with the recombinant proteins. Two apoptosis-related genes, *Bax* and *Bcl-2*, were also included in the analysis to find out the adverse effects of the proteins on the cells.

Results

Each protein induced a different pattern of cytokine expression depending on the antigen dose and cell type. The expression of Th1-type cytokine genes (*IFN-γ*, *TNF-α*, and *IL-12p40*), Th2-type cytokine genes (*IL-10* and *IL-4*), and *IFN-β* were induced with all of the proteins in RAW264.7 and DCs. The expression of apoptosis-related genes (*Bax* and *Bcl-2*) was also induced with most of the proteins in RAW264.7 and DCs. Specifically, the expression of two apoptosis-related genes was not altered with OMP25 protein treatment in DCs; prpA, wadC, and RomA can induce apoptosis-related gene expression both in RAW264.7 cell and DCs.

Conclusions

These results suggest that the four recombinant antigens of *B. melitensis* can induce pro- or anti-inflammatory cytokine genes at the mRNA level and these changes should be confirmed at the protein level. OMP25 might be a promising antigen for developing a vaccine, but the immunogenicity needs to be further assessed in vivo.

Introduction

Brucellosis is a highly contagious zoonosis disease that is transmitted from livestock and wild animals to humans. This disease is mainly caused by *Brucella abortus* or *Brucella melitensis*, which are Gram-negative, facultative intracellular pathogens [1]. Ten bacterial species have been identified based on the differences in pathogenicity and host preference [1, 2]. Brucellosis in animals often causes spontaneous abortion, reduced milk production, and impaired fertility, leading to significant economic loss worldwide [3, 4]. Human brucellosis is not usually fatal, but it can be debilitating with undulating fever. During chronic infection, it leads to lifelong complications, including endocarditis, meningitis, sacroiliitis, and

arthritis [5]. Annually, more than 500,000 human cases are reported globally, and it has been an important public health concern [6].

The innate immune response forms the first-line defense against brucellosis. However, *Brucella* develops multiple strategies to subvert or evade the immune responses for its adaption to the host. Protection against *Brucella* infection requires both innate and adaptive immune systems, involving an inflammatory response that promotes Th1-type immunity and a cell-mediated immune response that is considered mainly responsible for clearing intracellular bacteria [7, 8].

Cytokines play a pivotal role in the response to infection because they can amplify and coordinate the proinflammatory signals they induce the effector molecules to secrete, thus regulating the innate immunity against infection [9]. For this reason, the cytokine-mediated inflammatory responses to *Brucella* infection have been explored in recent years.

PrpA belongs to the family of proline racemases and it has been identified as a potent IL-10 inducer required for the establishment of chronicity after *Brucella* infection[10]. OMP25 is considered a controversial protein. Many researchers suggested that the OMP25 is a key virulence involved in the intracellular survival of *Brucella* [11–13], whereas it also has been reported that the protein is an immunogenic antigen that can effectively induce a cell-mediated and humoral immune response [14–16]. wadC and RomA proteins are involved in synthesis of *Brucella* lipopolysaccharide (LPS) and these two proteins acts as a shield against immune response via downregulation of the inflammatory response triggered by *Brucella*[17, 18].

Understanding the immune mechanisms is crucial for developing a vaccine because many bacterial proteins have been selected as diagnostic antigens without considering the immune response in vivo [19–21]. The mechanisms underlying the apoptotic activities involved in *B. melitensis* infection have not been revealed in the expression of cytokines and apoptotic-related genes. Therefore, for understanding the mechanism of the immune responses to *B. melitensis*, a murine macrophage cell line (RAW264.7) and dendritic cell line (DCs) were stimulated with four recombinant *B. melitensis* proteins. Cytokine expression and the expression of apoptotic-related genes were analyzed for understanding the induction of immune responses.

Materials And Methods

Bacterial strains

BL21(DE3) (Invitrogen, USA) was used for the expression of the outer membrane protein 25 (OMP25), proline-racemase protein A (prpA), glycosyltransferase (wadC), and periplasmic protein (RomA). The bacterial strains were grown at 37°C in Luria-Bertani (LB) broth or agar (Difco Dickinson). *B. melitensis* M5-90 was cultured in *Brucella* broth or agar (BD, USA).

Production and purification of the recombinant proteins

Cloning, expression, and purification of recombinant proteins have been described previously [22]. Briefly, the target genes were amplified by PCR from genomic DNA of *B. melitensis* M5-90 (Table 1). The PCR products were subjected to sequencing for confirmation of the nucleotide sequences. The amplified DNA fragment was cloned into a pET32a + vector (Novagen, Madison, WI, USA). Thirty milliliters of the culture were inoculated into 1 L of LB broth and supplemented with 250 μg ampicillin. After culturing at 220 rpm, 37°C for 3 h, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Amresco, USA) was added to induce expression of recombinant proteins, and the culture was further incubated for 7 h under the conditions described previously. Cells were harvested by centrifugation at 4,400 g for 15 min. The pellets were suspended in 25 mL of binding buffer (20 mM Tris–HCl, 8 M urea, 500 mM NaCl, 20 mM imidazole, 1 mM β-mercaptoethanol, pH 8.0), and sonicated at 10,000 Hz in ice water (50% pulse, 25 s pulse/50 s steps, 20 cycles) and centrifuged at 4,400 g for 10 min to collect supernatants. The recombinant proteins were purified using a His SpinTrap (GE Healthcare, UK) according to the manufacturer's protocol. The concentration of purified recombinant proteins was measured through a BCA kit (Bio-Rad, USA). The purity and identity of the recombinant proteins were analyzed by SDS-PAGE and western blotting with an anti-His antibody (Abcam, UK). Endotoxin contamination in the purified proteins was eliminated by 0.1% Triton X-114 in washing buffer in the purification process and was further confirmed using an endotoxin assay kit (Toxin Sensor™ Chromogenic LAL endotoxin Assay kit, GenScript) [23–25].

Table 1 Primer sequences for cloning *Brucella melitensis* genes

Gene	Primer sequence (5'-3')	Product size (bp)
Omp25-F	ATGCGCACTCTTAAGTCTCTCG	642
Omp25-R	TTAGAACTTGTAGCCGATGCCG	
prpA-F	TTATGCCACGCTGAACCCATGAGC	1002
prpA-R	TGGCAAGACATTCCTTCTTCTGCG	
wadC-F	CCAACTTCAAGCGCCGCCTTTC	879
wadC-R	CGATGGCGTTCGTCAATGCGCG	
RomA-F	ATCAGAGATCGACGTCGAGAAT	256
RomA-R	ATGCCGGGTGCAATAAACCGGC	

RAW264.7 and DCs culturing and stimulation

The murine macrophage RAW264.7 cell line (ATCC, TIB-71) and DCs were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco Life Technologies, Rockville, MD, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 100 U/mL penicillin, and 100 μg /mL streptomycin at 37°C with 5% CO_2. The RAW264.7 and DCs were respectively adjusted to 1×10^6 cells/mL in a 6-well plate and then stimulated with 30 or 50 μg/mL of the four different recombinant proteins for 24 h. Concanavalin A (ConA, 1 μg/mL) was used as a positive control.

Total RNA extraction from RAW264.7 and DCs

Total RNA was extracted from RAW264.7 and DCs using an RNeasy mini kit (CWBIO, Beijing, China), according to the manufacturer's protocols. Genomic DNA was eliminated by a TURBO DNA-free DNAse

(Ambio). Total RNA concentration was measured via Nanodrop 2000 (Thermo, USA). cDNA was synthesized from 2 μg of total RNA with a HiFiScript cDNA kit (CWBIO, Beijing, China), as per the manufacturer's instructions. The reaction products were used to analyze gene expression with real-time PCR.

Quantitative RT-PCR (qRT-PCR) analysis

Real-time RT-PCR was performed using ThermoFisher QuantStudio 3 RT PCR-well Q3 (Thermo Fisher, USA) with SYBR (CWBIO, Beijing, China). The reaction mixture contained 5 μL SYBR Green PCR Master Mix, 1 μL of the primers, 1 μL of the template cDNA, and 3 μL of RNase-free water. The mixture reaction conditions were as follows: initial denaturation at 95°C for 5 min followed by 50 cycles of 95°C for 30 s and annealing at 57°C or 60°C for 30 s. The expression levels of target genes were normalized against the expression of the *GAPDH* gene. The sequence primers for target genes are shown in Table 2.

Table 2 Murine primer sets for real-time PCR

Gene	Primer sequence (5'-3')	Annealing temperature (°C)	Product size (bp)
IL-10	F: GCTCTTACTGACTGGCATGAG	57	105
	R: CGCAGCTCTAGGAGCATGTG		
IL-12p40	F: TGGTTTGCCATCGTTTTGCTG	57	123
	R: ACAGGTGAGGTTCACTGTTTCT		
TNF-α	F: GACGTGGAACTGGCAGAAGAG	58	228
	R: TTGGTGGTTTGTGAGTGTGAG		
IFN-γ	F: ATGAACGCTACACACTGCATC	57	182
	R: CCATCCTTTTGCCAGTTCCTC		
IFN-β	F: CAGCTCCAAGAAAGGACGAAC	57	138
	R: GGCAGTGTAACTCTTCTGCAT		
IL-4	F: GGTCTCAACCCCCAGCTAGT	58	102
	R: GCCGATGATCTCTCTCAAGTGAT		
Bax	F: TGAAGACAGGGGCCTTTTTG	58	140
	R: AATTCGCCGGAGACACTCG		
Bcl-2	F: GTCGCTACCGTCGTGACTTC	58	284
	R: CAGACATGCACCTACCCAGC		
GAPDH	F: AGGTCGGTGTGAACGGATTTG	57	123
	R: TGTAGACCATGTAGTTGAGGTCA		

Statistical analysis

R version 3.6.1 was used to analyze the data. The student's *t-test* was used to compare the data between the two groups when the data conform to the normal distribution and homogeneity of the test of variance. If the data failed to obey the normal distribution and homogeneity of the variance test, the rank sum of two independent samples was tested using the Mann–Whitney *U* test. The Kruskal–Wallis rank-sum test was used to analyze the significance of the data between two groups. Statistical significance was defined as $p < 0.05$.

Results

The recombinant four different proteins of *B. melitensis* (OMP25, RomA, prpA, and wadC) were purified and analyzed by SDS-PAGE. The OMP25, RomA, prpA, and wadC proteins were approximately 25 kDa, 31 kDa, 30 kDa, and 27 kDa in size (Fig. 1A). Western blotting was used to confirm the specificity of the expressed proteins (Fig. 1B).

The total RNA of RAW264.7 cells and DCs was extracted after stimulation with four recombinants proteins, followed by cDNA synthesis using the total RNA template. The mRNA expression of cytokines and apoptosis-related genes was confirmed using real-time PCR. Generally, the different patterns of cytokine expression were induced after stimulation with different doses of recombinant proteins. In RAW264.7 cells, the expression of apoptotic-related genes (*Bax* and *Bcl-2*) was not significantly affected by treatment with the OMP25 protein, except for the upregulation of the *Bax* gene after stimulation with 30 μg OMP25 ($p < 0.05$) (Fig. 2A and 2I). *IL-4* gene was significantly downregulated in a dose-dependent way after stimulation with 30 μg and 50 μg of OMP25 protein ($p < 0.05$, $p < 0.01$) (Fig. 2E and 2I). The IFN-beta (*IFN-β*) and *IL-12p40* genes were also downregulated after stimulation with 30 μg or 50 μg of OMP25, although not significantly (Fig. 2C, 2G, and 2I). The IFN-gamma (*IFN-γ*) gene was dose-dependently upregulated after stimulation with 30 μg or 50 μg of OMP25 protein ($p < 0.01$) (Fig. 2D and 2I). The *IL-10* and TNF-alpha (*TNF-α*) genes were efficiently induced in the cells treated with 30 μg and 50 μg of the OMP25 protein, but not in a dose-dependent way (Fig. 2F, 2H, and 2I). However, most cytokines and apoptotic-related genes were not greatly affected by stimulation with the OMP25 protein in DCs. Only the *IL-12p40* gene was induced significantly by treatment with 30 μg of OMP25 protein, and the *IL-10* gene was induced efficiently in a dose-dependent way. Additionally, the *TNF-α* gene expression was inhibited by stimulation with 50 μg of OMP25 protein in DCs ($p < 0.05$, $p < 0.01$) (Additional file 1F, 1G, 1H, and 1I).

Interestingly, the *Bax* gene was downregulated significantly in the RAW264.7 cells after treatment with 30 μg of prpA protein ($p < 0.01$), whereas it was greatly induced by stimulation of 50 μg of prpA ($p < 0.01$) (Fig. 3A and 3I). Although 50 μg of prpA protein was also able to induce higher *Bcl-2* gene expression, there was no statistical difference in contrast to the PBS group. *IL-10* gene expression was induced efficiently when the cells were stimulated with 30 μg and 50 μg of prpA protein ($p < 0.01$, $p < 0.001$) (Fig. 3F and 3I), and the *TNF-α* gene was also upregulated significantly by stimulation with 50 μg of prpA ($p < 0.05$) (Fig. 3H and 3I). However, the expression of *IFN-β*, *IL-4*, and *IL-12p40* genes were significantly inhibited dose-dependently after stimulation with prpA protein (Fig. 3C, 3E, 3G, and 3I). Intriguingly, the expression of the *IFN-β*, *IL-4*, and *IL-12p40* genes was significantly upregulated in the DCs treated with 50 μg of prpA protein ($p < 0.05$) (Additional file 2C, 2E, 2G, and 2I). In the DCs, only 50 μg of prpA protein-induced *Bax* and *Bcl-2* gene expression ($p < 0.05$) (Additional file 2A, 2B, and 2I). The expression of *IL-10*, *TNF-α*, and *IFN-γ* was not affected in the DCs by stimulation with the prpA protein.

After stimulation of the RAW264.7 cells with the wadC protein, the *Bax* gene was significantly induced by the treatment with the 30 ug and 50 μg of protein ($p < 0.05$) (Fig. 4A and 4I), whereas the *Bcl-2* and *IFN-γ*

gene expressions were not influenced by the wadC protein. The *IL-10* gene expression was markedly inhibited when the cells were stimulated with 30 μg and 50 μg of wadC protein ($p < 0.001$, $p < 0.0001$) (Fig. 4F and 4I). *IL-12p40* and *IL-4* gene expressions were also downregulated, but there was no statistical difference between the PBS and treatment groups. However, the *IFN-β* and *TNF-α* expressions were greatly upregulated after stimulation with the wadC protein ($p < 0.05$, $p < 0.05$) (Fig. 4C, 4H, and 4I). When the DCs were stimulated by the wadC protein, on the contrary, *Bax* and *Bcl-2* were both significantly downregulated ($p < 0.01$, $p < 0.01$) (Additional file 3A, 3B, and 3I). *IFN-β* and *TNF-α* were greatly inhibited in the DCs in contrast to the RAW264.7 cells (Additional file 3C, 3H, and 3I). However, the expression of *IFN-γ* and *IL-4* was induced by the treatment with 50 μg of wadC protein ($p < 0.05$) (Additional file 3D, 3E, and 3I). Interestingly, the expression of *IL-10* and *IL-12p40* was significantly downregulated in both the DCs and RAW264.7 cells. ($p < 0.05$, $p < 0.01$) (Additional file 3F, 3G, and 3I).

In the case of RomA protein stimulation, *Bax* gene expression was significantly downregulated in the RAW264.7 cells stimulated with 30 μg of protein ($p < 0.01$); however, *Bcl-2* gene expression was upregulated by the treatment with 50 μg of protein ($p < 0.001$) (Fig. 5A, 5B, and 5I). There were no meaningful changes in the gene expression of *IFN-β* and *IL-12p40* in RAW264.7 cells (Fig. 5C, 5G, and 5I). The *IFN-γ* gene expression was markedly induced in RAW264.7 cells stimulated with 30 μg and 50 μg of RomA ($p < 0.01$, $p < 0.01$) (Fig. 5D and 5I). The *IL-4* gene expression was not affected by the RomA protein stimulation, whereas the *IL-10* and *TNF-α* gene expressions in RAW264.7 cells were significantly downregulated by the treatment of the RomA protein ($p < 0.0001$, $p < 0.001$) (Fig. 5F, 5H, and 5I). In the DCs, both of the two apoptotic-related genes were upregulated by the stimulation of RomA protein ($p < 0.01$, $p < 0.01$) (Additional file 4A, 4B, and 4I). However, the expression of *IFN-β* and *IL-4* was efficiently induced after the treatment with RomA in the DCs, but not in the RAW264.7 cells (Additional 4C, 4E, and 4I). *TNF-α* gene expression was also significantly inhibited in the DCs treated with 50 μg of RomA protein ($p < 0.01$) (Additional file 4G and 4I). *IFN-γ*, *IL-10*, and *IL-12p40* gene expressions were not affected in the DCs after stimulation with RomA protein (Additional 4D, 4F, 4G, and 4I).

Discussion

Brucellosis is a re-emerging zoonosis that causes huge world economic losses to animal production and human health. However, the real burden of brucellosis remains underestimated. Vaccination is the most efficient way to eradicate animal disease and prevent human brucellosis [26]. Recently, *Brucella* antigens have been screened and assessed for vaccine development, given their important role during infection [27]. In addition, it can also help to disclose the mechanism underlying the pathogenesis of Brucellosis.

Macrophages are considered to be a key element of the innate immune response against intracellular pathogens such as *Brucella*. Most pathogens are killed by macrophages at the early stage of infection; however, some of them still can reach replicative niches for survival [28]. Although several protein antigens have been identified and can stimulate a protective immune response in a murine model [29–32], the roles of some immunogenic proteins still remain unclear. Hence, three different antigens (prpA,

wadC, and RomA) with unknown potential in the induction of immune responses and OMP25 protein were selected for analysis of the effect on RAW264.7 and DCs in this study.

Inflammation is a well-known host response to microbial challenges during which various cytokines and chemokines are produced. Cytokines are crucial in innate immunity against infection because of their role as a soluble mediator of cell–cell interaction and amplify or coordinate proinflammatory signals to induce the secretion of effector molecules [33].

Apoptosis is an excellent host defense response against intracellular pathogens. However, *Brucella* has evolved multiple strategies that allow it to regulate the function of immune cells, such as the promotion or inhibition of apoptosis. BAX and BCL-2 proteins belong to the BCL-2 protein family that are respectively pro-apoptotic and anti-apoptotic [34]. In this study, prpA, wadC, and RomA proteins had a mild effect on *Bax* or *Bcl-2* gene expression in RAW264.7 cells, whereas these three proteins can obviously affect the *Bax* or *Bcl-2* gene expression in DCs. Hence, prpA, wadC, and RomA might affect apoptosis in DCs.

IFN-γ and TNF-α are two crucial cytokines for the host control of *Brucella* infection [35, 36]. IFN-γ can activate macrophages and promote antigen presentation to CD4 + T cells by enhancing MHC-II expression on their surfaces [37, 38]. It has been demonstrated that IFN-γ neutralized with monoclonal or *IFN-γ* gene knocked out mice was more susceptible and died in shorter days compared with the control group after infection with *B. abortus* 2308 [36, 39]. TNF is an important proinflammatory cytokine for the host to combat microbial infection [40]. It has been shown that TNF can recruit neutrophils to the infection site through induction of chemokines and adhesion molecules expression [33]. The macrophages from *TNF* receptor knock-out mice could not produce NO and IL-12, which resulted in a higher susceptibility to virulent *Brucella* [41]. In this study, most of the proteins efficiently induced *IFN-γ* and *TNF-α* gene expression in RAW264.7 cells, except RomA protein, which significantly inhibited *TNF-α* gene expression (Fig. 5H and 5I). However, most proteins remarkably downregulated *TNF-α* gene expression but did not affect *IFN-γ* gene expression in DCs. This result concurs with findings from a previous study showing that TNF-α production was negatively regulated in human DCs by stimulation with the *Brucella suis* and *B. suis* omp25 mutants triggered TNF-α production upon DCs invasion[11]. IL-12 is a cytokine that promotes the differentiation of Th1 cells and inhibits the cytokines required for Th2 cell development and proliferation [42]. Many studies reported that IL-12 is the predominant cytokine against *Brucella* infection [43–45]. Our results suggest that all of these recombinant proteins downregulate *IL-12p40* gene expression in RAW264.7 cells. Furthermore, the wadC protein could inhibit *IL-12p40* gene expression both in RAW264.7 and DCs, whereas OMP25 and prpA could induce *IL-12p40* gene expression in DCs.

IL-10 and IL-4 are Th2-type cytokines with a strong ability to suppress the Th1 immune response. Many studies revealed that IL-10 is crucial for intracellular pathogens' survival and chronic infection [46–49]. A major mechanism used by *Brucella* is to affect TLR signaling and then damp antigen-presenting cells (APCs) activation via induction of IL-10 [50]. IL-4 is an important cytokine that promotes M0 macrophage differentiation into M2 macrophages [51]. It has been reported that chronic brucellosis is associated with

IL-10–mediated M2 polarization and the levels of IL-4 in the peripheral blood of patients with chronic brucellosis are higher than in patients with acute brucellosis [52, 53]. Hence, IL-4 is also used by *Brucella* to regulate the function of immune cells for its survival. In our study, the OMP25 and prpA proteins are effective inducers of *IL-10* but significantly inhibit *IL-4* gene expression in RAW264.7 cells (Fig. 2E, 2F, 3E, and 3F). In addition, the prpA, wadC, and RomA proteins could upregulate *IL-4* gene expression in DCs (Additional file 2E, 3E, and 4E). Thus, the OMP25 protein may be associated with chronic infection in mice; the wadC and RomA proteins participate in *Brucella* chronic infection in humans, and the prpA protein is a key virulence factor in both mice and humans. However, the underlying mechanism needs to be further explored.

Traditionally, type I IFNs have been assigned a minor role in antibacterial host defenses [54]. However, it has been shown that the induction of type I IFN is detrimental to the host during *Listeria monocytogenes* infection and *Mycobacterium tuberculosis* [55, 56]. Leonardo A et al. [57] found that *Brucella* DNA can induce splenic apoptosis through induction of *Ifn-β* and *Ifn-αβR* KO mice are more resistant to infection. Hence, IFN-β can be utilized by *Brucella* to interfere with the immune response for intracellular survival. It has been shown that the wadC and RomA proteins are involved in the synthesis of the *Brucella* lipopolysaccharide (LPS) [17, 18]. In our study, the wadC and RomA proteins could markedly induce *IFN-β* gene expression in RAW264.7 cells (Fig. 4C and 5C), whereas the prpA and RomA proteins could upregulate *IFN-β* expression in DCs (Additional file 2C and 4C). This finding agrees with a report showing that *Brucella* LPS can induce *IFN-β* gene expression, and wadC and RomA may play a key role in LPS-mediated IFN-β production during *Brucella* infection [57].

Intriguingly, in our results, OMP25 can significantly downregulate *IL-12* gene expression in RAW264.7 cells, but upregulate *IL-12p40* gene expression in DCs. The explanation for this result may be attributed to the different cellular properties [58, 59]. In addition, it has been reported that *Escherichia coli* O55:B5 LPS or p38MAPK inhibitor also has different effects on different cell types [60, 61].

These results suggest that the recombinant proteins can induce proper immune responses in the RAW264.7 and DCs. OMP25 might be a promising candidate antigen for developing a vaccine due to its effectiveness in the induction of *IFN-γ*, *TNF-α*, and *IL-10*, and inhibition of *IL-12p40*, *IFN-β*, and *IL-4* in RAW264.7 cells. Furthermore, the expression of *IL-12p40* was induced by the stimulation of OMP25 in DCs without adverse effects (such as apoptosis). However, further research is needed to validate whether OMP25 is involved in intracellular survival via the induction of IL-10 and the these cytokine changes should be confirmed at the protein level. The remaining three recombinant proteins (prpA, wadC, and RomA) can also induce an immune response in these two different cells. Most of the three proteins can significantly inhibit or not affect *IL-12*, *TNF-α*, and *IFN-γ* gene expression, while inducing *IFN-β*, *IL-10*, and *IL-4* in RAW264.7 or DCs. In addition, these three proteins can induce *Bax* or *Bcl-2* in both RAW264.7 and DCs. Hence, these three proteins might be involved in macrophages and DCs apoptosis. In conclusion, the four recombinant *B. melitensis* proteins examined in this study appear to induce effective pro- or anti-inflammatory cytokine genes at the mRNA level and these changes should be confirmed at the protein level. Specifically, the immunogenicity of OMP25 protein needs to be further assessed in vivo.

Abbreviations

Not applicable

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All of the data generated or analyzed in this study are available in the additional supporting files.

Competing interests

The author declare that they have no competing interest.

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Authors' contribution

H Z, Y-L W and Y-F W carried out the laboratory works, J-F Y, X-Y D, Z C-M and H-H L made contributions to the interpretation of data, N-N Y, Y-M W, Z-Q L and J-L S made contributions to the analysis of data, Z W, Y W, Y-Z W and C-F C made contributions to the design of the work, H Z,Y-Z W and C-F,C drafted and revised the manuscript. All authors have read and approved the final manuscript.

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Figures

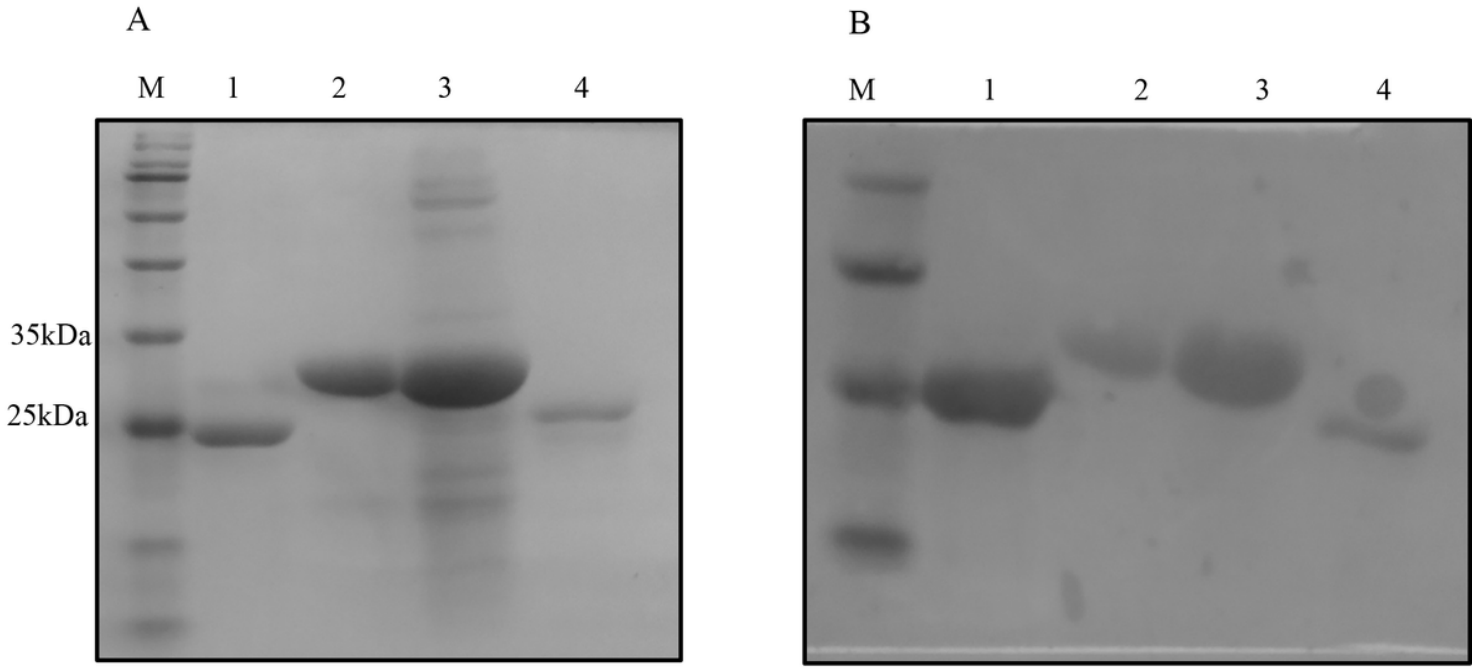

Figure 1

Analysis of the purified recombinant proteins. 15ug of each protein was loaded on SDS-PAGE (A) and western blotting (B) with an anti-His antibody. Lane M: molecular weight marker (CWBIO, Beijing, China), lane 1: outer membrane protein 25, lane 2: RomA protein, lane 3: prpA protein, lane 4: wadC protein. The cropping was performed on the original gel and blot.

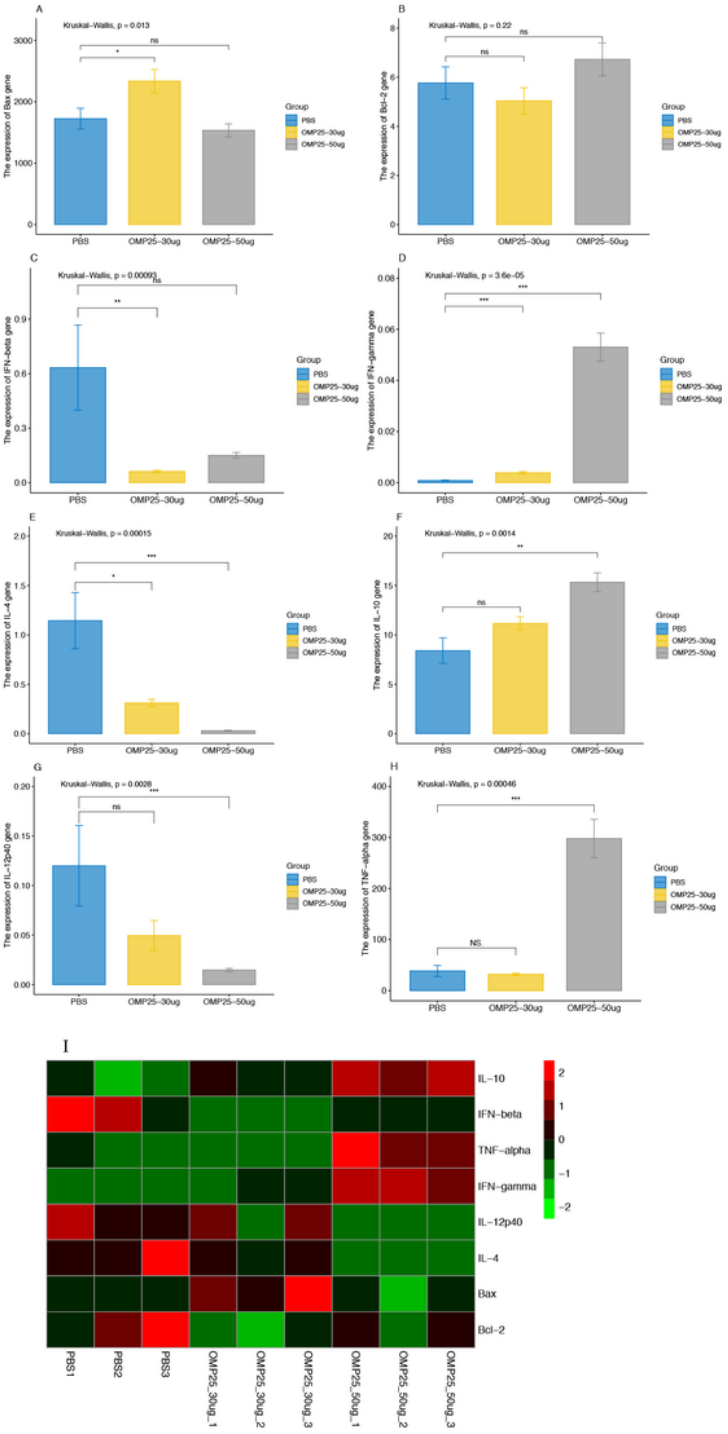

Figure 2

Expression of several cytokine and apoptosis-related genes after treatment with OMP25 protein in RAW264.7 cells. RAW264.7 cells were stimulated with 30 μg and 50 μg of OMP25 protein of *B. melitensis* at 6 h. Gene expression was analyzed by real-time quantitative RT-PCR and normalized by the expression of *GAPDH*. A: The expression of *Bax*, B: the expression of *Bcl-2*, C: the expression of IFN-beta (*IFN-β*), D: the expression of IFN-gamma (*IFN-γ*), E: the expression of *IL-4*, F: the expression of *IL-10*, G: the

expression of *IL-12*, H: the expression of TNF-alpha (*TNF-α*), I: The heatmap shows the expression levels of cytokine and apoptosis-related genes between PBS and OMP25 protein stimulated cells. These results are expressed as the means ± standard deviations from at least three independent experiments.

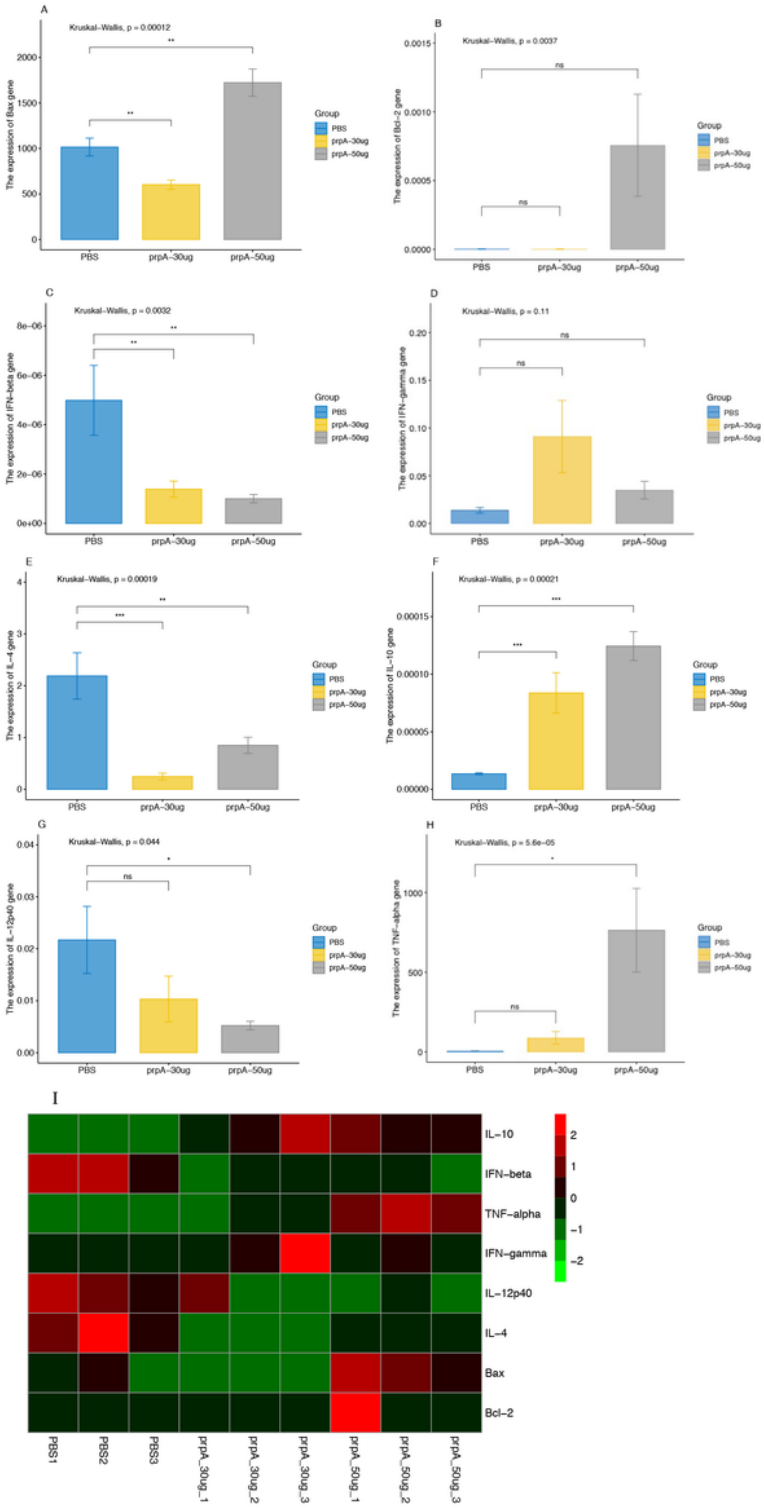

Figure 3

Expression of several cytokine and apoptosis-related genes after treatment with prpA protein in RAW264.7 cells. RAW264.7 cells were stimulated with 30 μg and 50 μg of prpA protein of *B. melitenisis* at 6 h. Gene expression was analyzed by real-time quantitative RT-PCR and normalized by the expression of *GAPDH*. A: the expression of *Bax* gene, B: the expression of *Bcl-2*, C: the expression of IFN-beta (*IFN-β*), D: the expression of IFN-gamma (*IFN-γ*), E: the expression of *IL-4*, F: the expression of *IL-10*, G: the expression of *IL-12*, H: the expression of TNF-alpha (*TNF-α*), I: The heatmap shows the expression levels of cytokine and apoptosis-related genes between PBS and prpA protein stimulated cells. These results are expressed as the means ± standard deviations from at least three independent experiments.

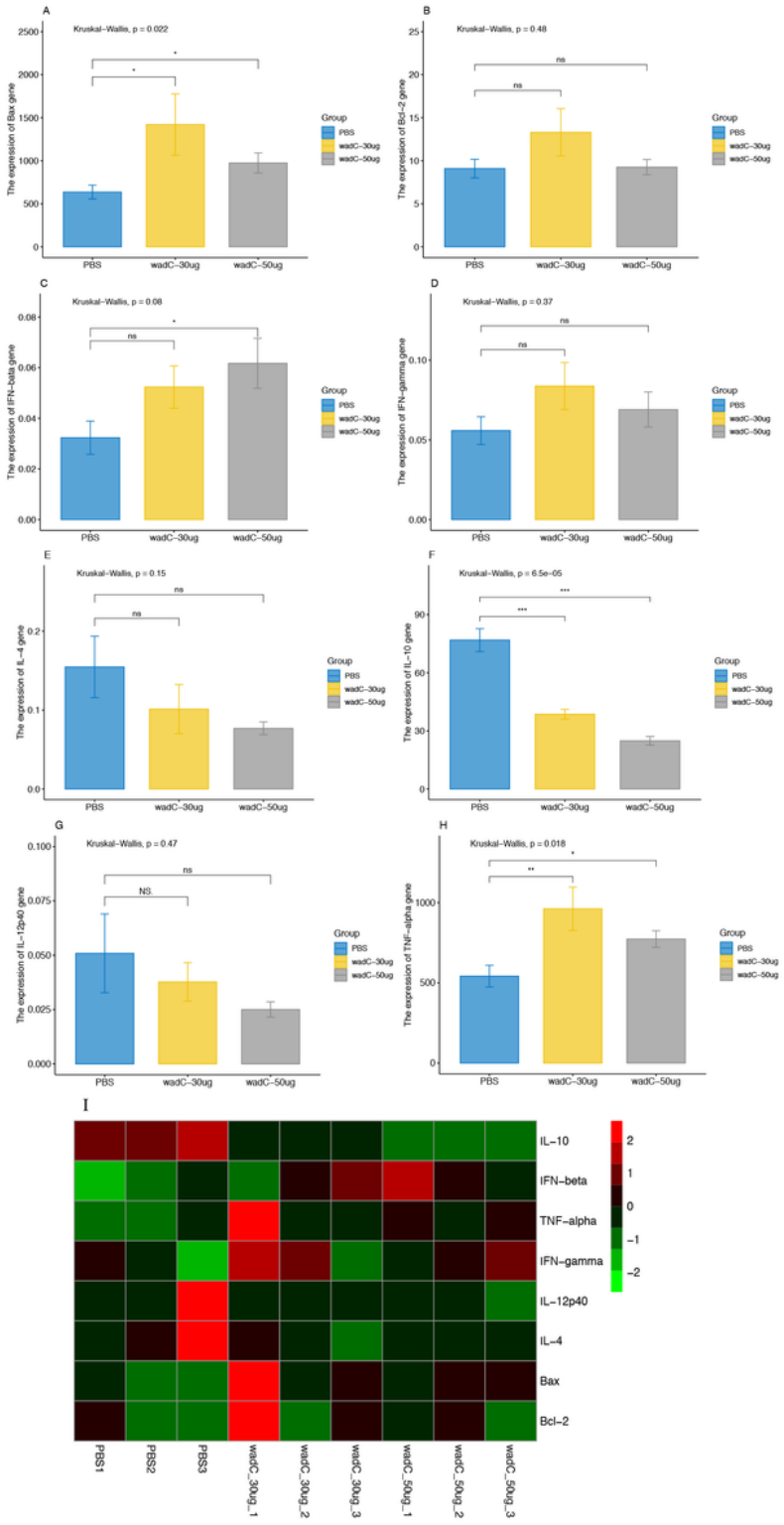

Figure 4

Expression of several cytokine and apoptosis-related genes after treatment with wadC protein in RAW264.7 cells. RAW264.7 cells were stimulated with 30 μg and 50 μg of wadC protein of *B. melitenisis* at 6 h. Gene expression was analyzed by real-time quantitative RT-PCR and normalized by the expression of *GAPDH*. A: the expression of *Bax*, B: the expression of *Bcl-2*, C: the expression of IFN-beta (*IFN-β*), D: the expression of IFN-gamma (*IFN-γ*), E: the expression of *IL-4*, F: the expression of *IL-10*, G: the

expression of *IL-12*, H: the expression of TNF-alpha (*TNF-α*), I: The heatmap shows the expression levels of cytokine and apoptosis-related genes between PBS and wadC protein stimulated cells. These results are expressed as the means ± standard deviations from at least three independent experiments.

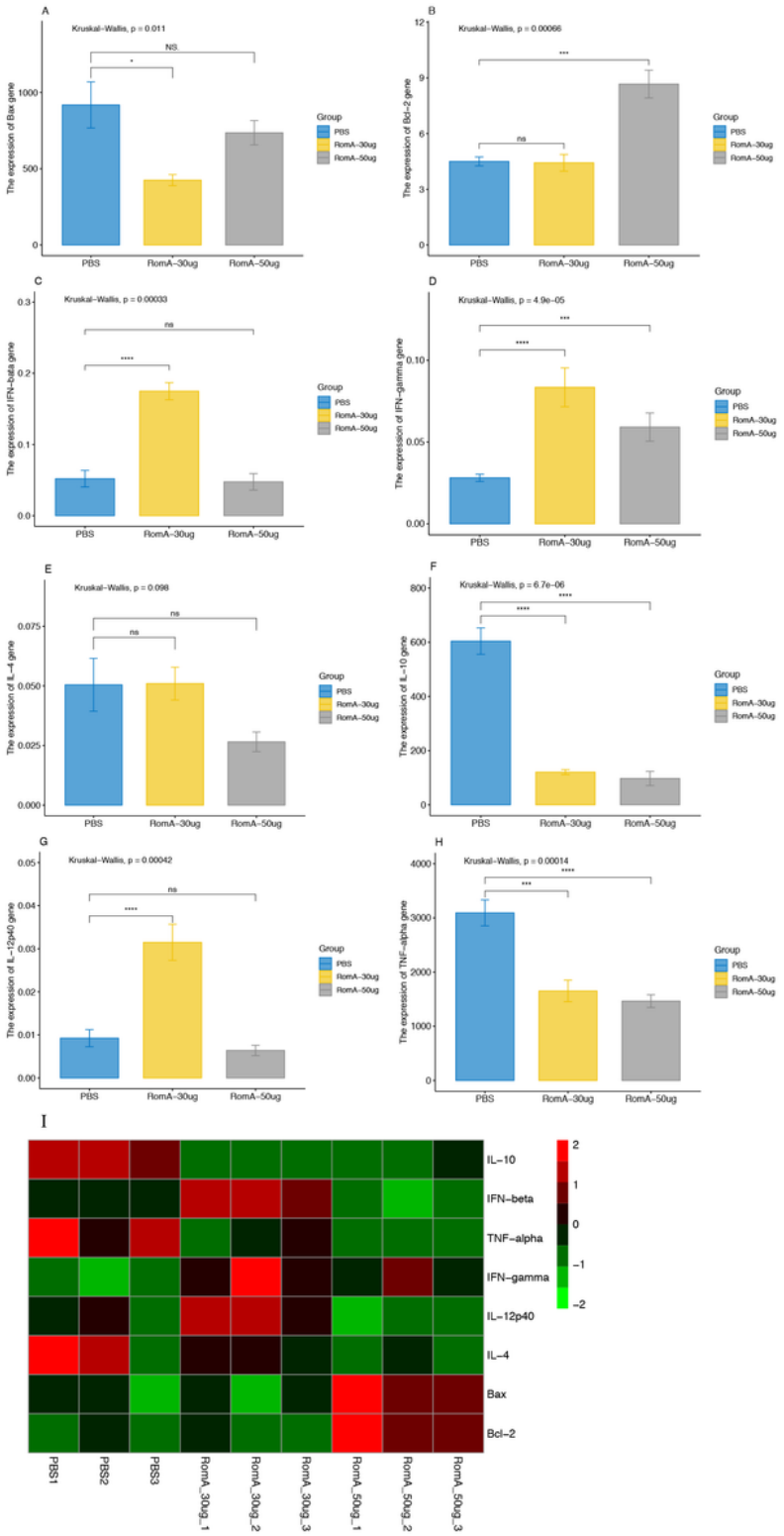

Figure 5

Expression of several cytokine and apoptosis-related genes after treatment with RomA protein in RAW264.7 cells. RAW264.7 cells were stimulated with 30 μg and 50 μg of RomA protein of *B. melitenisis* at 6 h. Gene expression was analyzed by real-time quantitative RT-PCR and normalized by the expression of *GAPDH*. A: the expression of *Bax*, B: the expression of *Bcl-2*, C: the expression of IFN-beta (*IFN-β*), D: the expression of IFN-gamma (*IFN-γ*), E: the expression of *IL-4*, F: the expression of *IL-10*, G: the expression of *IL-12*, H: the expression of TNF-alpha (*TNF-α*), I: The heatmap shows the expression levels of cytokine and apoptosis-related genes between PBS and RomA protein stimulated cells. These results are expressed as the means ± standard deviations from at least three independent experiments.

Supplementary Files

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