



Evaluation of Reactivity of Monoclonal Antibodies Against Omp25 of *Brucella* spp.

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Brucellosis is a serious zoonosis occurring mainly in developing countries, and its diagnosis is largely dependent on serologic detection and bacterial culture. In this study, we developed the murine monoclonal antibodies (mAbs) against a conserved and major outer membrane protein 25 (Omp25) of *Brucella* species (*B. spp.*) for use in clinical diagnosis. The mAbs to Omp25 were produced by hybridoma technique, which were utilized for developing various immunoassays for detection of *Brucellae*, including Western blot (WB), enzyme-linked immunosorbent assay (ELISA), immunochemical staining (ICS), immunofluorescence staining (IFS), and flow cytometry assay (FCM). A number of five mAbs (2B10, 4A12, 4F10, 6C12, and 8F3) specific to Omp25 were selected, including 2 IgG1, 2 IgG2a, and 1 IgG2b. Among them, mAbs 6C12, 8F3, and 4A12 reacted highly with *B. melitensis* (M5-90), *B. abortus* (S19, 104M, and 2308), and *B. suis* strain (S2). No cross-reactivity with *Yersinia enterocolitica* O:9, *Salmonella* spp., and *Escherichia coli* was found. By mapping Omp25 epitopes, mAb 6C12 was found as reacting with a semi-conformational epitope, and mAbs 4A12 and 8F3 as recognizing a different linear epitope, respectively. The paired mAbs were tested for detecting *Brucella* species, suggesting that 8F3 was suitable for solid phase capture and 6C12 or 4A12 was suitable for conjugation with HRP for detection of *Brucella* Omp25 in ELISA. The FCM was established by mAb 6C12 for detecting intracellular *Brucellae*-infected peripheral blood mononuclear cells (PBMCs) from brucellosis patients. In conclusion, mAbs against Omp25 are precious reagents for detection of *Brucellae* in clinical samples with various immunoassays. mAb 6C12-based FCM could be potentially used for the monitoring of therapeutic efficacy for brucellosis in clinical practice.

Keywords: brucellosis, *Brucella*, Omp25, mAb, flow cytometry assay, diagnosis

INTRODUCTION

Brucellosis is a severe zoonotic disease distributed worldwide, especially in the developing world (Wang et al., 2015). Sheep, goats, cattle, and pigs infected by *Brucella* species are the main sources of human brucellosis (Ducrottoy et al., 2018). In the past 10 years, human brucellosis has increased rapidly in China. There were 37,947 new cases of human brucellosis nationwide (morbidity 2.7318/100,000) in 2018 as reported by the Chinese CDC (<http://www.nhc.gov.cn/jkj/s3578/201904/050427ff32704a5db64f4ae1f6d57c6c.shtml>). New cases of human brucellosis were reported in non-endemic areas such as the Guangdong province (Chen et al., 2014), which is located in the south of China and far away from the high prevalence areas such as Inner Mongolia, Heilongjiang, Xinjiang, and Shanxi in the north or west of China (Wang et al., 2015).

At present, diagnosis of brucellosis mainly depends on serological methods used to detect antibodies against *Brucellae* in infected animals or humans (Araj, 2010). Diagnosing brucellosis by means of *Brucellae* cultures themselves can take at least 10 days. Once a *Brucella* infection becomes chronic, patients are likely to carry the bacteria for their whole lives. Anti-*Brucellae* methods are the most important approach for treatment of brucellosis. However, currently there are no rapid, simple, and quantitative methods for the evaluation of therapeutic efficacy of brucellosis during hospitalization. *Brucella* outer membrane proteins (Omps) are excellent candidates for the serologic diagnosis of a *Brucella* infection and potential antigens for recombinant subunit vaccines against brucellosis (Ahmed et al., 2015; Yousefi et al., 2016). Omp25 is one major Omps of the *Brucella* species and is considered to be closely related to virulence of *Brucellae* (Salhi et al., 2003; Goel and Bhatnagar, 2012). *Brucella* species without Omp25 survive for a shorter period of time than wild-type strains in mice (Edmonds et al., 2001, 2002). As a structural protein, Omp25 is highly conserved in various types and subtypes of *Brucellae* and induces a strong immune response (Cloeckert et al., 2002; Goel et al., 2013; Ma et al., 2015). Therefore, it might be a useful diagnostic target for brucellosis. In a previous study, an antibody to Omp25 was used to identify rough *Brucella* isolates by means of a latex coagglutination test (Bowden et al., 1997). In this study, we aimed to generate novel monoclonal antibodies (mAbs) to Omp25 and to develop new immunoassays for diagnosis of brucellosis or evaluation of therapeutic efficacy of brucellosis in clinical practice.

MATERIALS AND METHODS

Bacterial Strains

Inactivated *Brucella* strains, including *B. melitensis* (M5-90), *B. abortus* (S19, 104M and 2308), and *B. suis* (S2), were provided from Shihezi University, Xinjiang, China. *Yersinia enterocolitica* O:9, *Salmonella* spp., and *Escherichia coli* (ATCC 23922) were provided from the Department of Microbiology, Southern Medical University (SMU), Guangzhou, China.

Recombinant *Brucella* Omps

Recombinant proteins of Omp31, Omp19, Omp16, and periplasmic protein 26 (BP26) were produced from *B. melitensis* strain in the laboratory (Qiu et al., 2012; He et al., 2016; Li et al., 2017). Omp25 gene (642 bp) from *B. melitensis* M5-90 genomic DNA was cloned into the pET30a expression vector (Zhang et al., 2014; Yousefi et al., 2016), and designated as pET30a-Omp25. Recombinant Omp25 (rOmp25) was expressed as an inclusion body in *Escherichia coli* (DH5 α) by induction with 1 mM IPTG. The rOmp 25 extract was denatured by 8 M urea and purified by Ni-NTA Agarose (GE Healthcare, Milwaukee, Wisconsin, USA), and then refolded by dialysis against 50 mM Tris-HCl buffer containing a declining gradient urea from 6, 4, 2, to 0 M (Qiu et al., 2012; Yousefi et al., 2016). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was utilized to analyze rOmp25 (Yousefi et al., 2016). The purified soluble rOmp25 was used for mouse immunization and development of serologic tests.

Mouse Immunization and Monoclonal Antibody Production

BALB/c mice were obtained from the Animal Experimental Center of Southern Medical University (SMU), Guangzhou, China. BALB/c mice were immunized with purified rOmp25. The hybridoma cells, secreting mAbs to rOmp25, were generated and selected according to a previously reported method (Qiu et al., 2012; Patra et al., 2014; Li et al., 2017). To prepare the ascites fluid, BALB/c mice sensitized by the liquid paraffin were injected subcutaneously with 10⁶/ml hybridoma cells. The mouse ascites fluid was collected and purified by Recombinant Protein G NUPharose Fast Flow, rProtein G NUPharose FF (Nupotec, Hangzhou, China) (Divya et al., 2018).

All animal experimentations were approved by Southern Medical University (SMU) Animal Care and Use Committee (permit numbers: NFYY-2008-043 and NFYY-2010-076). All mouse surgery was performed under anesthesia, and all efforts were made to minimize suffering of animals.

mAb Isotyping

The isotype of mAbs was determined by IsoQuick Strips (a mouse mAb isotyping kit, Sigma-Aldrich, St Louis, Missouri, USA).

Blood Samples From Patients and Blood Donors

Plasma, or peripheral blood mononuclear cells (PBMCs), were isolated from healthy blood donors in Guangzhou and Harbin blood centers. Blood samples from brucellosis patients were collected at the General Hospital of Agricultural Reclamation Bureau, Harbin, Heilongjiang, China. All blood donor or patient samples were confirmed for negativity or positivity to *Brucella* infection by Standard Agglutination Test (SAT) and Polymerase Chain Reaction (PCR) (Mohammadi and Golchin, 2018). The PBMCs were prepared according to manufacturer's instructions (Ficoll Pague PLUS, GE Healthcare Life Sciences, USA).

Peptides

Peptides spanning 213 amino acids of Omp25 were synthesized as P1–P10 (Table S1) by a commercial company (Chinese Yuantai Company, Nanjing, China). A peptide of Omp31 (EP24: EYLTYDLGKRNLDVD) was used as a negative control. The purity of all peptides was more than 90% of total weight.

mAbs Titration and Affinity Measurement

The concentration of purified mAbs was determined by NanoDrop 2000 (Thermo Fisher Scientific, USA) as 1.14 mg/ml for 4F10, 2.1 mg/ml for 2B10, 7.4 mg/ml for 6C12, 3.8 mg/ml for 8F3, and 1.9 mg/ml for 4A12, respectively. The titer of mAbs was measured by indirect ELISA (Qiu et al., 2012; Divya et al., 2018). The Immuno MicroWell plate was coated with 5 µg/ml rOmp25 in 0.1 M carbonate buffer (CBS, pH 9.6) overnight at 4°C and blocked in 1% bovine serum albumin (BSA) for 1 h at 37°C. The purified mAbs as primary antibody were serially diluted from 1:100 to 1:1,968,300, and then added to microwell plate for 1 h at 37°C. ELISA plates were incubated for 1 h at 37°C with 1:10,000 diluted goat anti-mouse IgG and IgM HRP-conjugate antibodies (Rockland Immunochemicals, Inc, USA). The optical densities (OD) of reactions were detected with Epoch Microplate Spectrophotometer (BioTek Instruments, Inc. Winooski, USA). mAb titer was finally defined as the dilution fold at OD₄₅₀ value of 1 by indirect ELISA.

mAb's relative affinity was determined according to reactivity in different concentration of ammonium thiocyanate (NH₄SCN) solutions ranging between 0 and 4 M/L for 30 min at room temperature (Pullen et al., 1986; Macdonald et al., 1988). The appropriate concentration of primary mAbs was determined on the titration curve, which corresponded to the optical density observed near the top of the curve's linear portion (Macdonald et al., 1988). All experiments were carried out in triplicate and the mean of OD₄₅₀ values obtained from three independent experiments was used to calculate the relative affinity.

Lentivirus-Mediated Omp25 Expression in Cells

The infectious recombinant lentivirus (LV-HAGE-Omp25) mediated Omp25 expression in 293FT cell as described previously, mimicking *Brucella* Omp25 antigen in the infected mammalian cells (Zhang et al., 2012; Li et al., 2017).

Western Blot (WB)

The supernatant of sonicated proteins (SSPs) from *B. melitensis* (M5-90), *B. abortus* (S19, 104M, and 2308), and *B. suis* (S2) were considered as the native Omp25 lysates, which were prepared by breaking *Brucella* spp. stains with ultrasonication (Ultrasonic Apparatus XO-650, Xianou, Nanjing, China). The native membrane protein extracts (NMPs) was prepared by Membrane Protein Extraction Kit (Bestbio, China). The rOmp25 and native Omp25 were electrophoresed on SDS-PAGE and transferred to Polyvinylidene Fluoride membranes (PVDF membranes, Millipore, Billerica, Massachusetts, USA). The blotted membrane was incubated in 1:1,000 diluted mAb for 2 h at room temperature. The strip was washed in TBS containing 0.05% Tween[®] 20 (TBST) and incubated with 1:5,000 dilution

of goat anti-mouse IgG and IgM HRP-conjugate antibodies. The blot was visualized by adding immuno-chemiluminescence reagent (ECL, Millipore, Billerica, Massachusetts, USA). An HCV rNS3 mAb was used as unrelated negative control (Qiu et al., 2012). The SSPs from *E. coli* (ATCC23922) strain was used as negative control.

Enzyme-Linked Immunosorbent Assay (ELISA)

In indirect ELISA, rOmp25 or native Omp25 was used as a coating antigen. Purified mAbs were used as the primary antibody at optimum working concentration. The microwell plate was incubated with a 1:10,000 dilution of goat anti-mouse IgG and IgM HRP-conjugate antibodies as reported previously (Tiwari et al., 2013; Ahmed et al., 2015). In peptide-ELISA, peptides P1–P10 were used to identify antigenic epitopes recognized by each mAb (Qiu et al., 2012; Li et al., 2017). The double-antibody sandwich ELISA (DAS-ELISA) was used to detect *Brucella* Omp25 by cross-matching mAbs in pair with a capture mAb coated on the microwell plate and a detection mAb conjugated with HRP (Luo et al., 2012).

Immunofluorescent Staining (IFS)

293FT cells were transduced with recombinant lentivirus expressing Omp25 (LV-HAGE-Omp25) at multiplicities of infection (MOI = 5) for 8 h. The transduced 293FT cells were detected by immunofluorescent staining (IFS) with Omp25 mAbs as same as with Omp31 or BP26 described previously (Li et al., 2017). PBMCs were collected from two brucellosis patients (Guangzhou Center for Disease Prevention and Control) and were tested by IFS with mAb 6C12. PBMCs from a healthy blood donor were used as the negative control.

Immunochemical Staining (ICS)

Intact *Brucella* were detected by using ICS (Li et al., 2017). Briefly, after pretreatment with 3% H₂O₂ and washing with PBS three times, the *Brucella* smears on the glass-slide reacted with Omp25 mAbs and saturated with goat anti-mouse IgG and IgM HRP conjugate (Rockland Immunochemicals, Inc, USA). Bacteria were visualized with diaminobenzidine (DAB) substrate for color development.

Flow Cytometry Assay (FCM)

To explore the capacity of mAb to recognize intracellular *Brucellae* in PBMCs of patients with brucellosis, the purified mAb 6C12 was labeled with phycoerythrin (PE) by PE/R-Phycoerythrin Conjugation Kit according to the manufacturer's instructions (Abcam, Cambridge, United Kingdom). FITC-Anti-CD14 antibody (M5E2 clone; BD, Bioscience, USA) was used to recognize the lineage of mononuclear cells. PBMCs collected from 28 brucellosis patients and 55 non-*Brucella*-infected blood donors were stained for intracellular *Brucellae* by FACSCalibur flow cytometer with FITC-Anti-CD14 antibody (M5E2) and PE-6C12 specific to FCM Omp25 (Delaporte et al., 2008; Okumura et al., 2015). Briefly, PBMCs were treated with 5 µl FITC-M5E2 for 30 min at room temperature and cells were washed twice with sample buffer [PBS containing 1% bovine serum albumin (BSA);

Sigma-Aldrich, St Louis, Missouri, USA)]. After fixation and permeabilization with Intracellular Fixation & Permeabilization Buffer (eBioscience, California, USA), PBMCs were incubated with PE-6C12 for 45 min at 4°C and washed three times. Finally, 300 µl of resuspended PBMCs were tested by FCM. All steps were performed in the dark. The data were analyzed by FlowJo software version v10.0. The cutoff for non-Brucella-infected PBMCs from healthy blood donors was established as 1% by FCM.

Statistical Analysis

Computer software (SPSS, Version 20.0, SPSS, Inc., Chicago, IL) was used for statistical analysis. All experiments were repeated at least three times independently. The results were presented as the mean ± SD.

RESULTS

Production and Identification of mAbs to *Brucella* Omp25

Soluble rOmp25 was purified as an immunogen by Ni-NTA Agarose column. The purity of rOmp25 was about 95% of total

proteins (**Figure 1A**). Five mAbs (2B10, 6C12, 8F3, 4A12, and 4F10) were selected for reacting with rOmp25 and NMPs of *B. melitensis* by ELISA, respectively (**Figure 1B**). Western blot analysis showed all five mAbs were highly reactive with denatured rOmp25, while among them three mAbs (6C12, 8F3, and 4A12) strongly reacted with the denatured NMPs of *B. melitensis* and two (2B10 and 4F10) reacted weakly (**Figure 1C**). In addition, only mAb 6C12 reacted with intracellular *Brucella* Omp25 expressed in 293FT cells (**Figure 1C** and **Table 1**). No mAbs reacted with other recombinant proteins of *Brucella* (Omp31, BP26, Omp19, and Omp16).

Classification and Affinity Titration of *Brucella* Omp25 mAbs

mAbs 2B10, 6C12, 8F3, 4A12, and 4F10 were identified as 2 IgG1, 2 IgG2a and 1 IgG2b, respectively (**Table 1**). Antibody titers were determined by ELISA at value of 1 at OD₄₅₀. The titer of mAbs 6C12, 8F3, and 4A12 was measured up to 1:200,000, 2B10 up to 1:50,000, and 4F10 up to 1:1,000, respectively (**Figure 2A**). Correspondingly, the optimum working concentration of these five mAbs was 1.14 µg/ml (4F10), 0.042 µg/ml (2B10), 0.037 µg/ml (6C12), 0.019 µg/ml

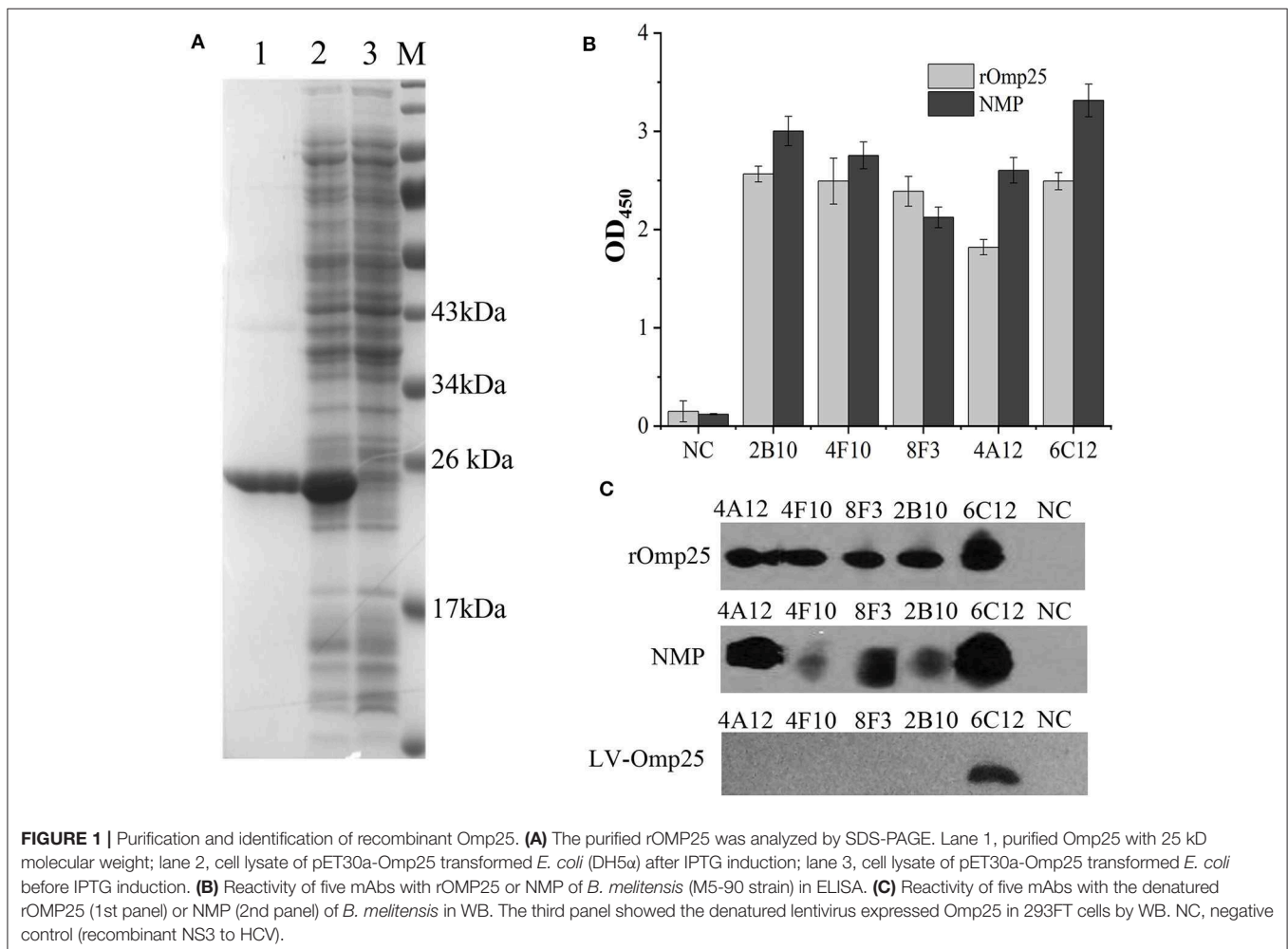
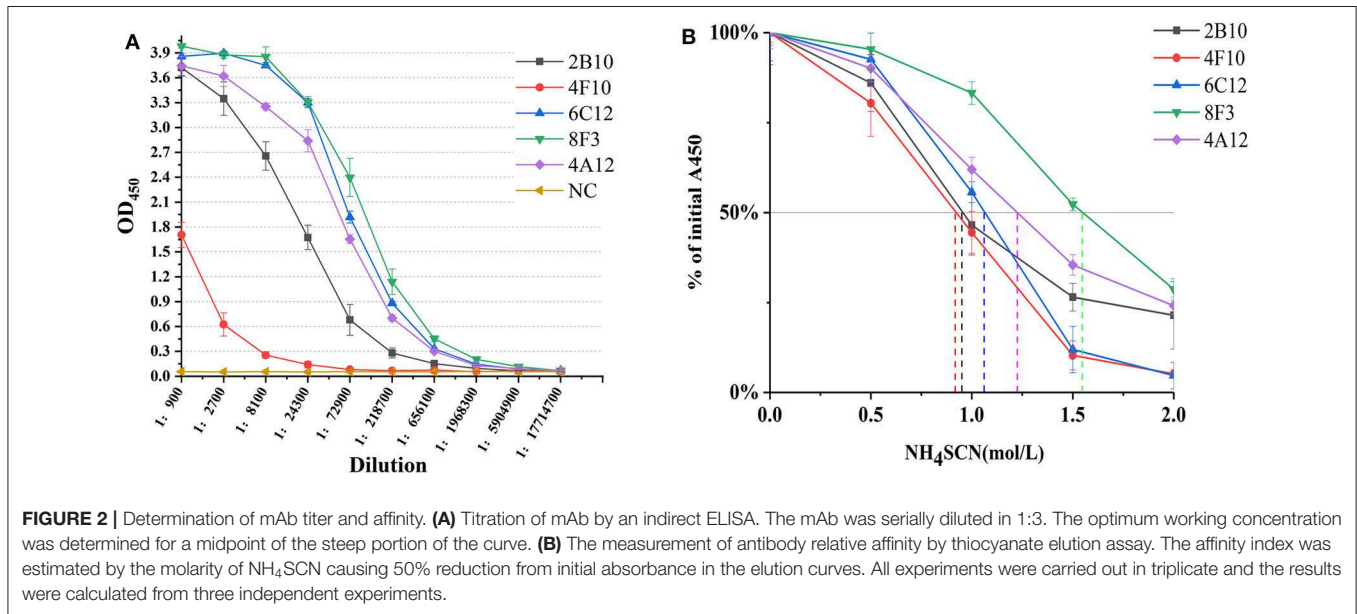


TABLE 1 | Characterization of mAbs reactive to *Brucella* Omp25.

mAb	Isotype	Titers	Relative affinity	Omp25 WB	NMP WB	LV WB	Omp25 ELISA	NMP ELISA	LV IFS	<i>B. m</i> ICS	Epitope (aa)/peptide
2B10	IgG2a(K)	1:2 × 10 ⁴	0.5–1.0	++	–	–	2.6	3.0	+	–	L(36–156)/P7
4A12	IgG1(K)	1:1 × 10 ⁵	1.0–1.5	++	++	–	1.8	2.6	+	+	L(24–42)/P2
4F10	IgG2b(K)	1:1 × 10 ³	0.5–1.0	++	–	–	2.4	2.7	+	–	L(24–42)/P2
6C12	IgG2a(K)	1:1 × 10 ⁵	1.0–1.5	++	++	+	2.5	3.3	++	+	SC(24–213)
8F3	IgG1(K)	1:1 × 10 ⁵	1.5–2.0	++	+	–	2.4	2.0	+	+	L(68–86)/P4

NMP, native outer membrane proteins extracts of *B. melitensis* (*B. m*); LV, Lentivirus LV-HAGE-Omp25 transduced 293T cells; The numbers indicate the OD₄₅₀ value in ELISA; The reactivity levels are indicated by ++ (strongly reactive), + (reactive), or – (non-reactive) by WB, IFS, and ICS; L, linear epitope; SC, semi-conformational epitope.



(8F3), and 0.01 μg/ml (4A12), respectively. An appropriate dilution of 1:2,700 for 2B10, 1:900 for 4F10, 1:8,100 for 6C12 and 8F3, and 1:2,700 for 4A12 was used as initial concentration of mAb to determine its relative affinity. The relative affinity of mAb was estimated by thiocyanate elution assay, calculated at 50% reduction from initial antibody reactivity reached by increasing molarity of NH₄SCN in the elution curves (**Figure 2B**). Relative affinity of mAbs was around 1.0 (**Table 1**), among them the affinity of 2B10 and 4F10 ranged from 1.0 to 1.5, 4A12 and 6C12 from 1.0 to 1.5, and 8F3 from 1.5 to 2.0, respectively.

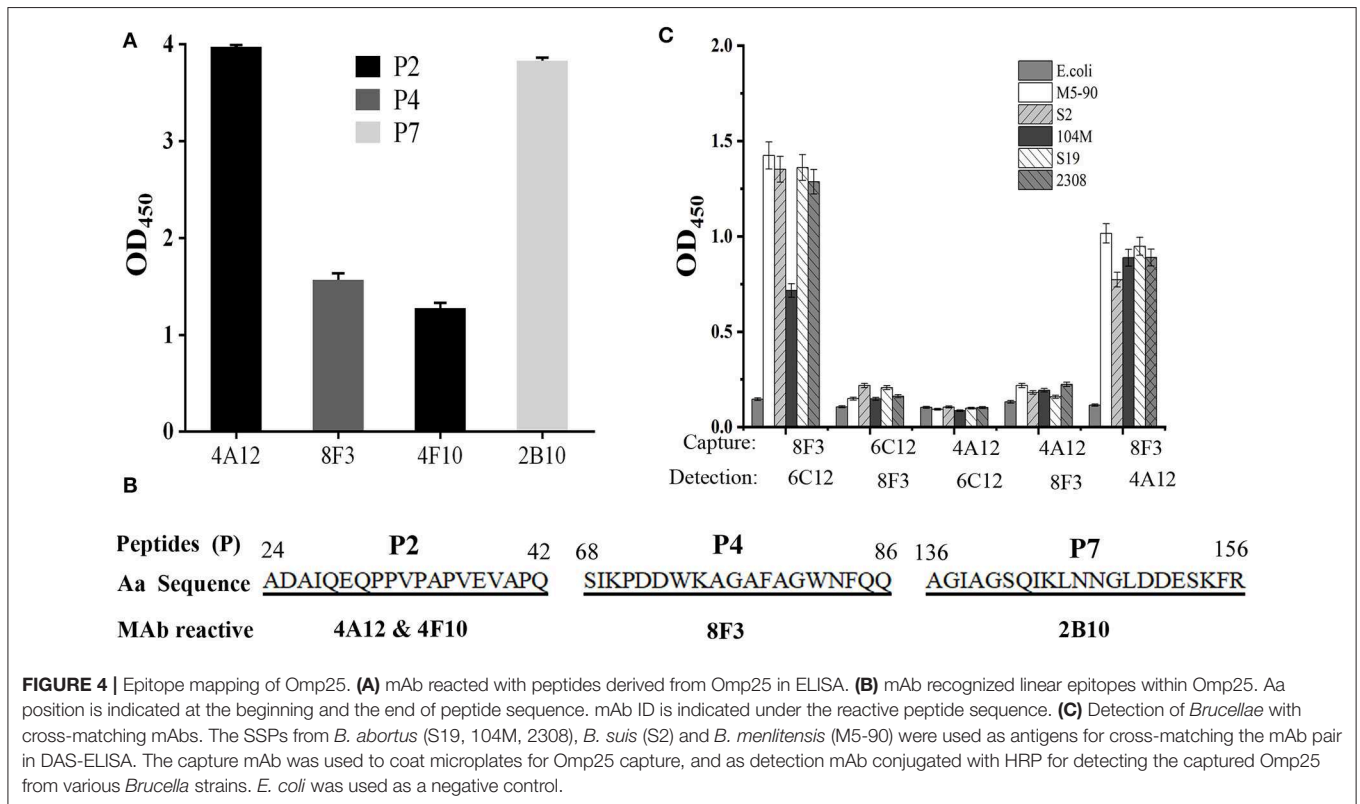
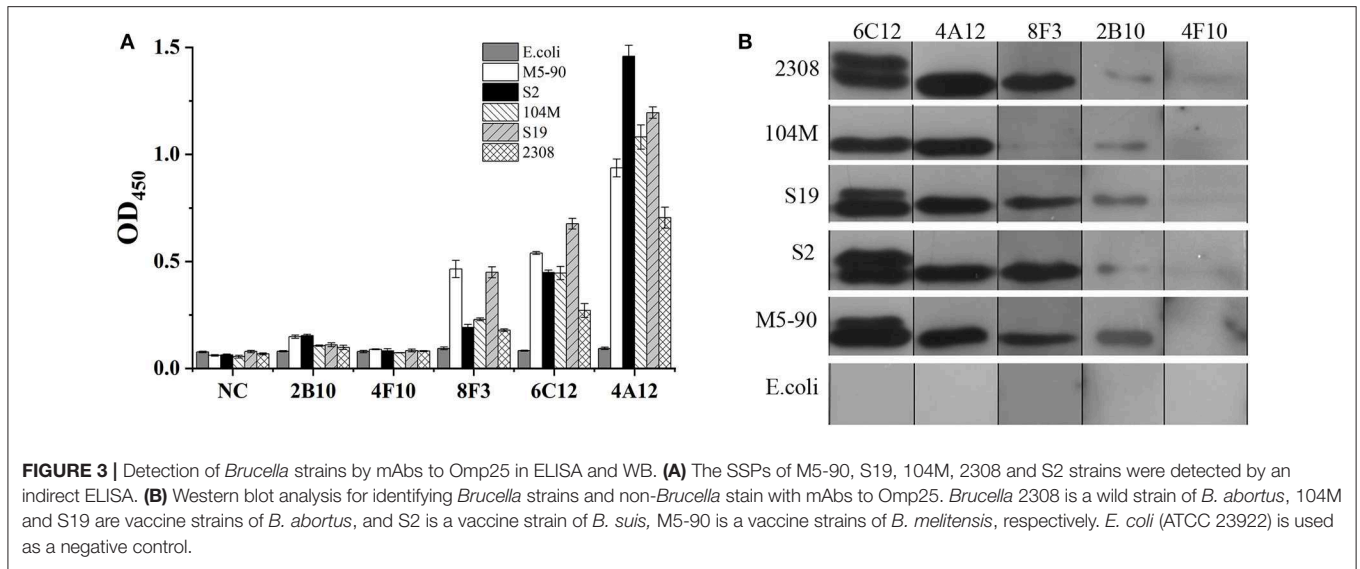
Reactivity of mAbs With *Brucella* Species

The ability of mAbs to Omp25 from different strains of *Brucella*, the SSPs extracted from *B. melitensis* (M5-90), *B. abortus* (S19, 104M, and 2308), and *B. suis* (S2) were tested by ELISA and WB. mAbs 4A12, 6C12, and 8F3 reacted with all three of *Brucella* species by ELISA (**Figure 3A**). The reactivity of 4A12 and 6C12 reached a relatively higher level than 8F3, but 2B10 and 4F10 reacted at low level or did not react. Western blot analysis showed these mAbs had a

reaction pattern similar to ELISA (**Figure 3B**). Specifically, mAb 6C12 presented two reactive bands with *Brucella* strains 2308 (*B. abortus*), S19 (*B. abortus*), S2 (*B. suis*), and M5-90 (*B. melitensis*), corresponding to Omp25 (the lower) and Omp25d (the upper), respectively. The other four mAbs reacted only with Omp25 (**Figure 3B**). No mAbs reacted with lysates of *Yersinia enterocolitica* O:9, *Salmonella* spp., and *Escherichia coli*.

mAb Recognition for Omp25 Epitopes

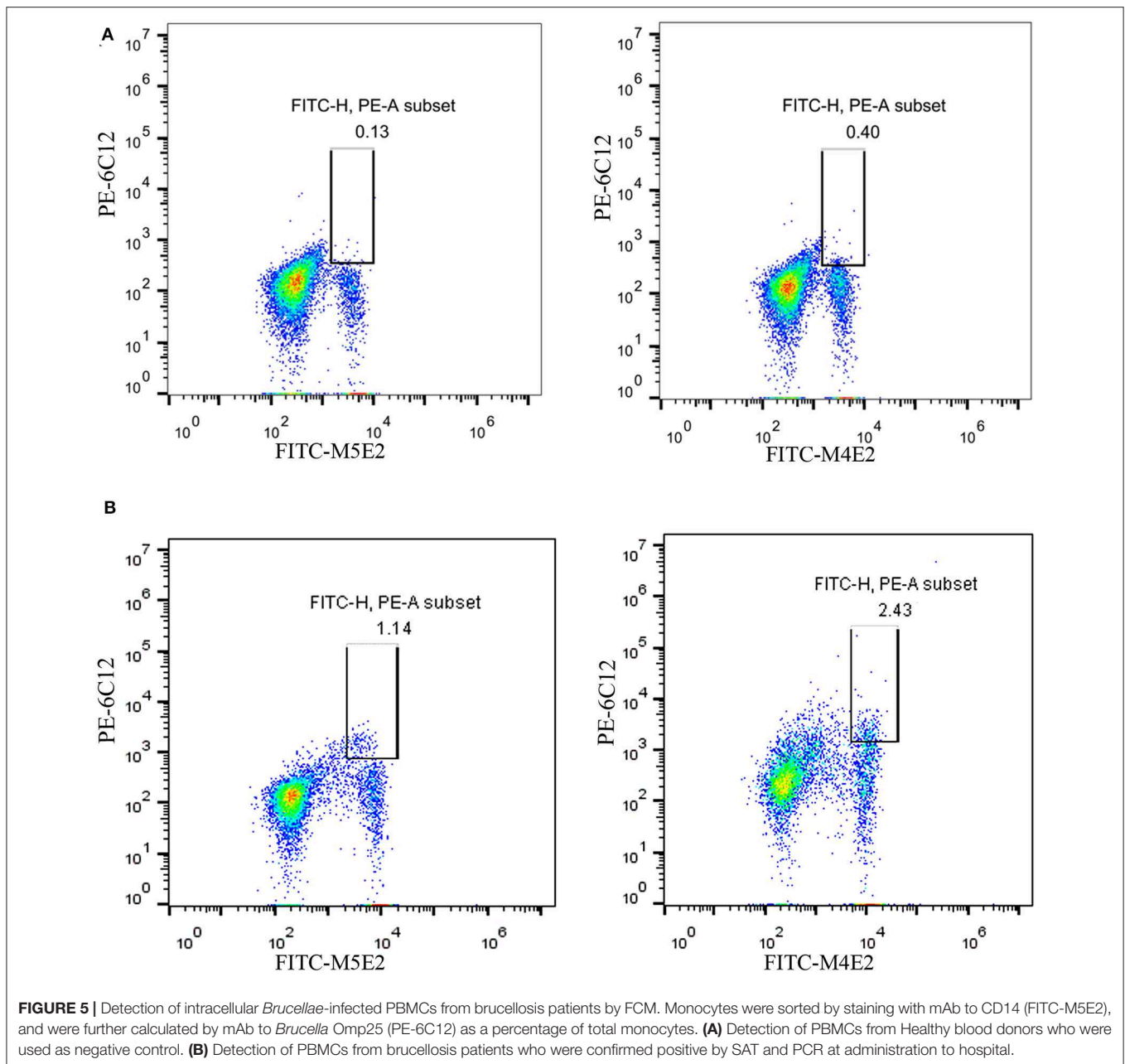
In order to identify the Omp25 antigenic epitopes, all mAbs were tested in ELISA with 10 peptides derived from the 213 amino acid (aa) sequence of *B. melitensis* Omp25. Four mAbs (4A12, 8F3, 4F10, and 2B10) reacted with three different peptides (**Figure 4A**) identified as recognition of linear epitopes. mAb 6C12 did not react with any peptides but with denatured native Omp25 (**Figure 3**), corresponding to the recognition of a semi-conformational epitope (**Table 1**). The amino acid sequences of the three linear epitopes of Omp25 are presented in **Figure 4B** (19–20 mers peptides P2, P4, and P7).



Detection of *Brucellae* With Cross-Matching mAbs in ELISA

Five mAbs were tested for reactivity with Omp25 by cross-matching pairs with capture and detection antibodies by DAS-ELISA. Five pairs of mAbs (4A12/6C12-HRP, 4A12/8F3-HRP, 6C12/8F3-HRP, 8F3/4A12-HRP, and 8F3/6C12-HRP)

reacted with rOmp25 (Table S2). These five pairs were tested for reactivity with the SSPs of 2308, 104M, S19, S2, and M5-90 strains from *B. melitensis*, *B. abortus*, or *B. suis*. Two mAb pairs, 8F3/6C12-HRP and 8F3/4A12-HRP, were found the be more reactive for detection of various *Brucella* species (Figure 4C).



Detection of *Brucellae* by Immunostaining With Omp25 mAbs

To determine the ability of Omp25 mAbs to detect intracellular *Brucellae* or intact *Brucella* strains by microscopy, infected cells or intact bacteria were stained by IFS or ICS, respectively. mAbs 6C12, 2B10, and 4F10 showed a strong fluorescent reactivity with the lentivirus-Omp25 transduced 293FT cells by IFS (**Figure S1A**). In addition, by IFS with mAb 6C12 from two patients with brucellosis, visible fluorescent intracellular *Brucellae* infected PBMCs were observed, but not seen with controls from healthy blood donors (**Figure S1B**).

By ICS, the cultured *Brucella* could be seen by microscopy after staining with HRP-conjugated 6C12, 8F3, or 4A12 on a glass slide (**Figure S2**). The overall results are presented in **Table 1**.

Clinical Detection of *Brucellae*-Infected PBMCs From Brucellosis Patients by FCM

On the basis of the above analysis, mAb 6C12 was considered the most functional antibody for *Brucella* detection. Hence, mAb 6C12 was selected for labeling with PE to detect intracellular *Brucellae*-infected PBMCs by FCM (**Figure 5**). The mononuclear cells were sorted from freshly isolated PBMCs by FITC-Anti-CD14 antibody (M5E2), and the ratio of *Brucella*-infected

monocytes was calculated by staining with PE-6C12. Stained monocytes from healthy blood donor controls over brucellosis patients were stratified as the cutoff or threshold between two levels of intracellular *Brucellae*: <1.0%, indicating a lower level or negative intracellular *Brucellae*-infected cells (**Figure 5A**), and $\geq 1.0\%$, indicating a higher level of intracellular *Brucellae*-infected cells (**Figure 5B**). A total of 28 brucellosis patients and 20 blood donors were tested by FCM with both mAbs M5E2 and 6C12, showing that 46.4% (13/28) of brucellosis patients but none of blood donors carried intra-PBMCs *Brucellae* ($\geq 1.0\%$) (**Table 2**). Only one patient (No. 8) had 1.13% of stained monocytes with unclear boundary by FCM. These results suggested that FCM with mAb 6C12 might be a practical assay to determine the frequency of intracellular *Brucellae*-infected PBMCs in individual brucellosis patients with clinical diagnosis.

DISCUSSION

Several early studies focused on the immunogenicity and protective activity of *Brucella* Omp25 (Bowden et al., 1998). In this study, three non-overlapping linear epitopes (P2, P4, and P7) were recognized by mAbs 4A12 and 4F10, 8F3, and 2B10, respectively (**Figure 4B**). Previously mAb A59/05F01/C09 was reported to recognize a linear epitope of Omp25 (aa 24-43) (Salhi et al., 2003), which overlaps with the epitope P2 (aa24-42) recognized by mAbs 4A12 and 4F10 in this study. P1 peptide (aa1-30) has seven amino acids (24-ADAIQEQ-30) overlapping with P2 peptide (**Table S1**) but does not react with these mAbs, suggesting that mAbs 4A12 and 4F10 likely recognize the linear epitope PPVPAPVEVAPQ (aa 31-42) within Omp25. Another mAb A76/02C12/C11 to Omp25 was reported to strongly bind to rough *Brucella* isolates except *B. ovis* tested with a latex coagglutination assay (Bowden et al., 1997), but the epitope was not clearly defined. In addition to the three different linear epitopes (P2, P4, and P7) revealed by mAbs 4A12 and 4F10, 8F3, and 2B10, a semi-conformational epitope within the denatured native *Brucella* Omp25 was identified by mAb 6C12 recognition. Interestingly, mAb 6C12 recognized two bands of 25 kD and 26 kD proteins from *Brucella* strains in WB (**Figure 3B**). The larger band (26 kD) was designated as Omp25d as previously described (Salhi et al., 2003), while the other four mAbs reacted only with the linear epitopes of 25 kD protein (Omp25). This might explain that 6C12 had better binding capacity in FCM than other mAbs for detecting *Brucellae* of PBMCs in comparison with mAb 5H3 to Omp31 or 5A5 to BP26 (Yang et al., 2019). These Omp25 mAbs had no cross-reactivity with *Yersinia enterocolitica* O:9, *Salmonella* spp., and *Escherichia coli* reported previously (Muñoz et al., 2015). However, previous studies found the high serological cross-reactivity between *Brucella* and other *Alphaproteobacteria* such as *Ochrobactrum* and *Sinorhizobium* (Clockaert et al., 1999; Velasco et al., 2000; Delpino et al., 2004). By aligning *Brucella* Omp25 aa sequence with *Ochrobactrum anthropi* and *Rhizobiales* 63-22 strains, the high homologous sequences are observed including the linear epitopes (P2, P4, and P7) within *Brucella* Omp25 recognized by mAbs 4A12/4F10, 8F3, and 2B10

TABLE 2 | Characterization of blood samples and detection results of *Brucellae*-infected PBMCs by FCM with mAb PE-6C12.

Number	Sample source	Status	FCM (%)	PCR	SAT
1	Brucellosis patient	Acute	0.99	+	1:200
2	Brucellosis patient	Chronic	0.39	+	1:100
3	Brucellosis patient	Acute	1.59	+	1:100
4	Brucellosis patient	Chronic	0.57	+	1:100
5	Brucellosis patient	Chronic	0.97	+	1:400
6	Brucellosis patient	Chronic	0.61	+	1:100
7	Brucellosis patient	Acute	1.26	+	1:800
8	Brucellosis patient	Acute	1.13 (unclear boundary)	+	1:400
9	Brucellosis patient	Acute	2.04	+	1:50
10	Brucellosis patient	Acute	0.73	+	1:100
11	Brucellosis patient	Acute	0.79	+	1:100
12	Brucellosis patient	Chronic	2.41	+	1:50
13	Brucellosis patient	Acute	1.19	+	1:800
14	Brucellosis patient	Acute	0.69	+	1:400
15	Brucellosis patient	Chronic	0.75	+	1:100
16	Brucellosis patient	Chronic	0.8	+	1:200
17	Brucellosis patient	Acute	0.79	+	1:100
18	Brucellosis patient	Chronic	1.38	+	1:100
19	Brucellosis patient	Chronic	0.65	+	1:100
20	Brucellosis patient	Chronic	1.37	+	1:50
21	Brucellosis patient	Acute	1.18	+	1:100
22	Brucellosis patient	Acute	1.15	+	1:400
23	Brucellosis patient	Acute	1.97	+	1:200
24	Brucellosis patient	Chronic	2.43	+	1:50
25	Brucellosis patient	Chronic	3.12	+	1:100
26	Brucellosis patient	Chronic	4.14	+	1:50
27	Brucellosis patient	Acute	0.43	+	1:100
28	Brucellosis patient	Chronic	0.46	+	1:50
29	Blood donor		0.49	-	-
30	Blood donor		0.31	-	-
31	Blood donor		0.18	-	-
32	Blood donor		0.41	-	-
33	Blood donor		0.1	-	-
34	Blood donor		0.4	-	-
35	Blood donor		0.15	-	-
36	Blood donor		0.22	-	-
37	Blood donor		0.28	-	-
38	Blood donor		0.25	-	-
39	Blood donor		0.3	-	-
40	Blood donor		0.58	-	-
41	Blood donor		0.23	-	-
42	Blood donor		0.37	-	-
43	Blood donor		0.47	-	-
44	Blood donor		0.29	-	-
45	Blood donor		0.35	-	-
46	Blood donor		0.52	-	-
47	Blood donor		0.46	-	-
48	Blood donor		0.24	-	-

FCM, flow cytometry; SAT, standard agglutination test.

(Figure S3), which indicate the cross-reactivity of these mAbs may exist in other *Alphaproteobacteria*.

B. melitensis, *B. abortus*, and *B. suis* are three major pathogenic strains for human brucellosis transmitted from infected sheep and goats, cattle, or pigs (Chen et al., 2014; Ducrotoy et al., 2018). Among five mAbs, 8F3, 4A12, and 6C12 had higher relative affinity (>1) reacted with Omp25 of five strains (M5-90, S19, 104M, 2308, and S2) from three *Brucella* in various immunoassays. mAb 8F3 presented a strong capacity as a capture antibody (Figure 4C), while 6C12 and 4A12 appeared to be the best detection antibody when conjugated with HRP or fluorescein (Figure 4C, Figures S1, S2). The best-matched antibody pairs were 8F3/6C12-HRP and 8F3/4A12-HRP for detection of *Brucellae* in DAS-ELISA, while mAb 6C12 was the most suitable for detection of intracellular *Brucellae* in PBMCs from patients by FCM (Figure 5).

Currently, there is no an optimal immunoassay for detecting intracellular *Brucellae* in terms of counting the infected monocytes for evaluation of anti-*Brucella* efficacy in clinical treatment of brucellosis. Conventional method of blood culture requires a biosafety level 3 laboratory (BSL-3) and takes 1–2 weeks (Pappas et al., 2006). In this study, we sought to develop an Omp25 mAb-based FCM assay to detect intracellular *Brucellae*-infected PBMCs from brucellosis patients, which might be helpful for evaluating therapeutic efficacy by determining the percentage of *Brucella*-infected monocytes before or after treatment of individual patients with brucellosis. By analyzing 28 patients with brucellosis, who were found positive upon admission to hospital by SAT and PCR, eight out of 28 (28.6%) and five out of 28 (17.8%) brucellosis patients had more than 1 or 2% intracellular *Brucellae* infected PBMCs, respectively. Overall, 46.4% (13 out of 28) patients carried a higher level (>1%) of intracellular *Brucellae* monocytes and 53.6% (15 out of 28) patients had a lower level (<1%) or no detectable intracellular *Brucellae* monocytes (Table 2). The ratios of mAb 6C12-stained monocytes from PBMCs were consistent with the amounts of intracellular *Brucellae* in brucellosis patients. In our previous study, we utilized IFS for detecting *Brucella*-infected PBMCs stained with Omp31 mAbs (Yang et al., 2019) and found 50–70% positive PBMCs in patients receiving zero to three treatment courses of anti-*Brucella* drugs. IFS is a qualitative test unsuitable to quantify the number of *Brucella*-infected cells in a patient's PBMCs. The selected mAb 6C12 has been shown effective for detection of intracellular *Brucella* by IFS (Figure S1). However, the established FCM with mAb 6C12 specific to Omp25 has an advantage over IFS for counting the frequency of intracellular *Brucellae*-infected monocytes and might be a potential assay for evaluation of therapeutic efficacy of brucellosis patients in clinical practice.

In conclusion, mAbs 8F3 was suitable as an immobilized capture antibody, and 6C12 or 4A12 was suitable as a conjugated detection antibody. These mAbs specific to *Brucella* Omp25 appear precious reagents in various immunoassays, such as WB, ELISA, ICS, IFS, and FCM assays for detection of

different *Brucella* species in the diagnosis of brucellosis or evaluation of therapeutic efficacy of anti-*Brucella* treatment in clinical practice.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study and studies involving human participants were reviewed and approved by Southern Medical University (SMU) Nan Fang Hospital Medical Ethics Committee (permit numbers: NFYY-2009-23). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

WW, CL, and XY participated in the study design, analysis of data, and writing of the manuscript. XY, GZ, CC, LL, and YF collected blood samples and interpreted the patient data regarding the brucellosis disease. XY, ZH, JL, YT, HZ, HR, and HY performed the laboratory examination. J-PA analyzed the data and revised the manuscript. All authors read and approved the final version of manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcimb.2020.00145/full#supplementary-material>

REFERENCES

- Ahmed, I. M., Khairani-Bejo, S., Hassan, L., Bahaman, A. R., and Omar, A. R. (2015). Serological diagnostic potential of recombinant outer membrane proteins (rOMPs) from *Brucella melitensis* in mouse model using indirect enzyme-linked immunosorbent assay. *BMC. Vet. Res.* 11:275. doi: 10.1186/s12917-015-0587-2
- Araj, G. F. (2010). Update on laboratory diagnosis of human brucellosis. *Int. J. Antimicrob. Agents.* 36(Suppl. 1), S12–S17. doi: 10.1016/j.ijantimicag.2010.06.014
- Bowden, R. A., Cloeckaert, A., Zygmunt, M. S., and Dubray, G. (1998). Evaluation of immunogenicity and protective activity in BALB/c mice of the 25-kDa major outer-membrane protein of *Brucella melitensis* (Omp25) expressed in *Escherichia coli*. *J. Med. Microbiol.* 47, 39–48. doi: 10.1099/00222615-47-1-39
- Bowden, R. A., Verger, J. M., Grayon, M., and Cloeckaert, A. (1997). Rapid identification of rough *Brucella* isolates by a latex coagglutination assay with the 25-kilodalton outer membrane protein and rough-lipopolysaccharide-specific monoclonal antibodies. *Clin. Diagn. Lab. Immunol.* 4, 611–614. doi: 10.1128/CDLI.4.5.611-614.1997
- Chen, S., Zhang, H., Liu, X., Wang, W., Hou, S., Li, T., et al. (2014). Increasing threat of brucellosis to low-risk persons in urban settings. *Emerg. Infect. Dis.* 20, 126–130. doi: 10.3201/eid2001.130324
- Cloeckaert, A., Tibor, A., and Zygmunt, M. S. (1999). *Brucella* outer membrane lipoproteins share antigenic determinants with bacteria of the family rhizobiaceae. *Clin. Diagn. Lab. Immunol.* 6, 627–629. doi: 10.1128/CDLI.6.4.627-629.1999
- Cloeckaert, A., Vizzaino, N., Paquet, J. Y., Bowden, R. A., and Elzer, P. H. (2002). Major outer membrane proteins of *Brucella* spp.: past, present and future. *Vet. Microbiol.* 90, 229–247. doi: 10.1016/S0378-1135(02)00211-0
- Delaporte, M., McKenna, P., Siah, A., and Berthe, F. C. J. (2008). Immunophenotyping of mya arenaria neoplastic hemocytes using propidium iodide and a specific monoclonal antibody by flow cytometry. *J. Invertebr. Pathol.* 99, 120–122. doi: 10.1016/j.jip.2008.04.008
- Delpino, M. V., Fossati, C. A., and Baldi, P. C. (2004). Occurrence and potential diagnostic applications of serological cross-reactivities between *Brucella* and other alpha-proteobacteria. *Clin. Diagn. Lab. Immunol.* 11, 868–873. doi: 10.1128/CDLI.11.5.868-873.2004
- Divya, G., Jayaprakash, N. S., and Venkataraman, K. (2018). Development and characterization of monoclonal antibodies against nitro-166 tyrosine of high-density lipoprotein: apolipoprotein A1. *Monoclon. Antib. Immunodiagn. Immunother.* 37, 167–174. doi: 10.1089/mab.2018.0018
- Ducrottoy, M. J., Muñoz, P. M., Conde-Álvarez, R., Blasco, J. M., and Moriyón, I. (2018). A systematic review of current immunological tests for the diagnosis of cattle brucellosis. *Prev. Vet. Med.* 151, 57–72. doi: 10.1016/j.prevetmed.2018.01.005
- Edmonds, M. D., Cloeckaert, A., Booth, N. J., Fulton, W. T., Hagius, S. D., Walker, J. V., et al. (2001). Attenuation of a *Brucella abortus* mutant lacking a major 25 kDa outer membrane protein in cattle. *Am. J. Vet. Res.* 62, 1461–1466. doi: 10.2460/ajvr.2001.62.1461
- Edmonds, M. D., Cloeckaert, A., and Elzer, P. H. (2002). *Brucella* species lacking the major outer membrane protein Omp25 are attenuated in mice and protect against *Brucella melitensis* and *Brucella ovis*. *Vet. Microbiol.* 88, 205–221. doi: 10.1016/S0378-1135(02)00110-4
- Goel, D., and Bhatnagar, R. (2012). Intradermal immunization with outer membrane protein 25 protects Balb/c mice from virulent *B. abortus* 544. *Mol. Immunol.* 51, 159–168. doi: 10.1016/j.molimm.2012.02.126
- Goel, D., Rajendran, V., Ghosh, P. C., and Bhatnagar, R. (2013). Cell mediated immune response after challenge in Omp25 liposome immunized mice contributes to protection against virulent *Brucella abortus* 544. *Vaccine* 31, 1231–1237. doi: 10.1016/j.vaccine.2012.12.043
- He, Z., Luo, P., Hu, F., Weng, Y., Wang, W., and Li, C. (2016). Expression and identification of eukaryotic expression vectors of *Brucella melitensis* lipoprotein OMP19. *Chin. J. Cell Mol Immunol.* 32, 470–473.
- Li, J., Hu, F., Chen, S., Luo, P., He, Z., Wang, W., et al. (2017). Characterization of novel Omp31 antigenic epitopes of *Brucella melitensis* by monoclonal antibodies. *BMC Microbiol.* 17:115. doi: 10.1186/s12866-017-1025-3
- Luo, Y., Terkawi, M. A., Jia, H., Aboge, G. O., Goo, Y. K., Cao, S., et al. (2012). A double antibody sandwich enzyme-linked immunosorbent assay for detection of secreted antigen 1 of *Babesia microti* using hamster model. *Exp. Parasitol.* 130, 178–182. doi: 10.1016/j.exppara.2011.10.012
- Ma, Q., Liu, A., Ma, X., Wang, Y., Hou, Y., and Wang, Z. (2015). *Brucella* outer membrane protein Omp25 induces microglial cells *in vitro* to secrete inflammatory cytokines and inhibit apoptosis. *Int. J. Clin. Exp. Med.* 8, 17530–17535.
- Macdonald, R. A., Hosking, C. S., and Jones, C. L. (1988). The measurement of relative antibody affinity by ELISA using thiocyanate elution. *J. Immunol. Methods.* 106, 191–194. doi: 10.1016/0022-1759(88)90196-2
- Mohammadi, E., and Golchin, M. (2018). Detection of *Brucella abortus* by immunofluorescence assay using anti outer membrane protein of 19 kDa antibody. *Adv. Clin. Exp. Med.* 27, 643–648. doi: 10.17219/acem/85081
- Muñoz, P. M., Marín, C. M., Monreal, D., González, D., Garin-Bastuji, B., Diaz, R., et al. (2015). Efficacy of several serological tests and antigens for diagnosis of bovine brucellosis in the presence of false-positive serological results due to *Yersinia enterocolitica* O:9. *Clin. Diagn. Lab. Immunol.* 12, 141–151. doi: 10.1128/CDLI.12.1.141-151.2005
- Okumura, T., Masuda, K., Watanabe, K., Miyadai, K., Nonaka, K., Yabuta, M., et al. (2015). Efficient enrichment of high-producing recombinant Chinese hamster ovary cells for monoclonal antibody by flow cytometry. *J. Biosci. Bioeng.* 120, 340–346. doi: 10.1016/j.jbiosc.2015.01.007
- Pappas, G., Panagopoulou, P., Christou, L., and Akritidis, N. (2006). *Brucella* as a biological weapon. *Cell Mol. Life. Sci.* 63, 2229–2236. doi: 10.1007/s00018-006-6311-4
- Patra, K. P., Saito, M., Atluri, V. L., Rolan, H. G., Young, B., Kerrinnes, T., et al. (2014). A protein-conjugate approach to develop a monoclonal antibody-based antigen detection test for the diagnosis of human brucellosis. *PLoS Negl. Trop. Dis.* 8:e29266. doi: 10.1371/journal.pntd.0002926
- Pullen, G. R., Fitzgerald, M. G., and Hosking, C. S. (1986). Antibody avidity determination by ELISA using thiocyanate elution. *J. Immunol. Methods.* 86:83–87. doi: 10.1016/0022-1759(86)90268-1
- Qiu, J., Wang, W., Wu, J., Wang, Y., Qiao, J., Chen, C., et al. (2012). Characterization of periplasmic protein BP26 epitopes of *Brucella melitensis* reacting with murine monoclonal and sheep antibodies. *PLoS ONE* 7:e34246. doi: 10.1371/journal.pone.0034246
- Salhi, I., Boigegrain, R. A., Machold, J., Weise, C., Cloeckaert, A., and Rouot, B. (2003). Characterization of new members of the group 3 outer membrane protein family of *Brucella* spp. *Infect. Immun.* 71, 4326–4332. doi: 10.1128/IAI.71.8.4326-4332.2003
- Tiwari, S., Kumar, A., Thavaselvam, D., Mangalgi, S., Rathod, V., Prakash, A., et al. (2013). Development and comparative evaluation of a plate enzyme-linked immunosorbent assay based on recombinant outer membrane antigens Omp28 and Omp31 for diagnosis of human brucellosis. *Clin. Vaccine Immunol.* 20, 1217–1222. doi: 10.1128/CI.00111-13
- Velasco, J., Bengoechea, J. A., Brandenburg, K., Lindner, B., Seydel, U., González, D., et al. (2000). *Brucella abortus* and its closest phylogenetic relative, *Ochrobactrum* spp., differ in outer membrane permeability and cationic peptide resistance. *Infect. Immun.* 68, 3210–3218. doi: 10.1128/IAI.68.6.3210-3218.2000
- Wang, W., Liao, Q., Wu, X., Hou, S., Wang, Y., Wu, J., et al. (2015). Potential risk of blood transfusion-transmitted brucellosis in an endemic area of China. *Transfusion* 55, 586–592. doi: 10.1111/trf.12853
- Yang, H., Zhang, G., Luo, P., He, Z., Hu, F., Li, L., et al. (2019). Detection of *Brucellae* in peripheral blood mononuclear cells for monitoring therapeutic efficacy of brucellosis infection. *Antimicrob. Resist. Infect. Control.* 8:154. doi: 10.1186/s13756-019-0607-2

- Yousefi, S., Tahmoorespur, M., and Sekhavati, M. H. (2016). Cloning, expression and molecular analysis of Iranian *Brucella melitensis* Omp25 gene for designing a subunit vaccine. *Res. Pharm. Sci.* 11, 412–418. doi: 10.4103/1735-5362.192493
- Zhang, J., Guo, F., Huang, X., Chen, C., Liu, R., Zhang, H., et al. (2014). A novel Omp25-binding peptide screened by phage display can inhibit *Brucella abortus* 2308 infection *in vitro* and *in vivo*. *J. Med. Microbiol.* 63, 780–787. doi: 10.1099/jmm.0.069559-0
- Zhang, L., Yin, S., Tan, W., Xiao, D., Weng, Y., Wang, W., et al. (2012). Recombinant interferon- γ lentivirus co-infection inhibits adenovirus replication *ex vivo*. *PLoS ONE* 7:e42455. doi: 10.1371/journal.pone.0042455

Conflict of Interest: 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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