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Integrated Multi-Omics Strategy for Screening Novel Protective Antigens Against *Brucella melitensis* and Analysis of Their Immunogenicity

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Abstract

Background: *Brucellosis is one of the most severe Class B infectious diseases prevalent in the agricultural and pastoral areas of northern China. Current attenuated live vaccines (e.g., M5, S19) have defects such as residual virulence, causing abortion in pregnant animals, and the inability to differentiate between natural infection and vaccination (DIVA).*

Methods: *Relying on the ABSL-3 laboratory of the Inner Mongolia Center for Disease Control and Prevention, this study established a chronic infection model in C57BL/6J mice using the virulent strain Brucella melitensisM16. Single-cell transcriptome sequencing (10x Genomics) was employed to map the heterogeneity of splenic immune cells. Whole-genome scanning and pangenomic analysis were performed on four strains with different virulence levels (M16, 544A, M5, 104M) using second-generation sequencing. Membrane/secreted proteins unique and conserved in virulent strains were screened as candidate antigens, prepared via prokaryotic expression systems, and their humoral and cellular immune levels were detected by indirect ELISA and flow cytometry.*

Results: *A stable chronic infection model was successfully constructed (bacterial load Log10CFU > 4.5). Single-cell sequencing yielded 45,231 cells, annotated into 12 immune cell subpopulations, revealing significant expansion of Effector CD4+T cells (P< 0.01) and high expression of the Ifnggene. Pangenomic analysis identified three candidate antigens (BMEI0021, BMEI1943, BMEI0367) that are 100% conserved in virulent strains but absent in the vaccine strain M5. Immunogenicity assays showed that BMEI1943 induced high levels of IgG_2a subtype antibodies (titer Log213.45 ± 0.52) and IFN-γ^+CD4^+T cell responses (frequency 15.80% ± 2.10%).*

Conclusion: *Through multi-omics integration analysis, this study successfully identified a novel candidate antigen, BMEI1943, with strong Th1-type immunogenicity, providing an experimental basis for the development of safe and efficient subunit vaccines against brucellosis.*

Keywords: *Brucella melitensis*; Single-cell transcriptome; Comparative genomics; Vaccine candidate antigen; Immunogenicity; ABSL-3

1. Introduction

Brucellosis is a zoonotic disease caused by *Brucella*spp. that poses a serious threat to public health safety. According to World Health Organization statistics, approximately 500,000 new cases occur globally each year. Inner Mongolia, China, has consistently been a high-incidence area for brucellosis

due to its developed animal husbandry, bringing huge losses to the health of local farmers and economic development [1, 2].

Brucella melitensis is the most pathogenic to humans, mainly causing undulant fever, arthritis, and reproductive system damage. Currently, the control of animal brucellosis mainly relies on traditional attenuated live vaccines such as S19 and M5. However, such vaccines have obvious "biosafety shortcomings": on one hand, incomplete attenuation may lead to abortion in pregnant women or infection in frontline epidemic prevention personnel; on the other hand, antibodies produced by immunized animals are difficult to distinguish from those caused by wild-type infection, severely interfering with epidemiological surveillance (the DIVA strategy) [3, 4].

Subunit vaccines are considered the development direction for next-generation brucellosis vaccines due to their high safety and strong specificity. However, the core difficulty lies in antigen screening. Traditional antigen screening is often limited to the verification of single proteins, lacking systematic consideration of the overall host immune response and the evolutionary characteristics of pathogen genomes [5]. In recent years, single-cell transcriptome sequencing technology has enabled the revelation of dynamic changes in host immune cells at single-cell resolution, while comparative genomics can lock onto pathogen-specific conserved proteins from an evolutionary perspective. The combination of the two provides powerful tools for new vaccine development [6].

This study intends to integrate the above two technologies, relying on the ABSL-3 laboratory platform of the Inner Mongolia Center for Disease Control and Prevention, to systematically analyze the immune map of *Brucella*-infected mice and screen novel candidate antigens with cross-protective potential, providing detailed data support for the development of new brucellosis vaccines.

2. Materials and Methods

2.1 Experimental Animals and Ethical Review

Female SPF C57BL/6J mice, 6–8 weeks old, weighing 18–22 g, were purchased from SpeBIO (Beijing) Biotechnology Co., Ltd. Animals were housed in an SPF-grade barrier environment with free access to food and water. Animal experiments were strictly reviewed and approved by the Ethics Committee of Inner Mongolia Medical University (Approval No. NMMU-2023-124). All operations involving virulent strains were completed in the ABSL-3 laboratory of the Inner Mongolia CDC, in strict compliance with the *Guidelines for Ethical Review of Laboratory Animal Welfare*.

2.2 Main Reagents and Instruments

RPMI 1640 medium and fetal bovine serum (Gibco, USA); red blood cell lysis buffer and permeabilization buffer (BioLegend, USA); CD3-FITC, CD4-PE, IFN-γ-APC flow cytometry antibodies (BioLegend, USA); Ni-NTA affinity chromatography columns (Qiagen, Germany); Bradford protein concentration assay kit (Thermo, USA); microplate reader (BioTek, USA); flow cytometer (BD Accuri C6, USA).

2.3 Construction of the *Brucella* Infection Model

Brucella melitensisstrain M16 was streaked onto TSA agar medium and cultured at 37°C for 48 h. Single colonies were picked and inoculated into TSB liquid medium and cultured at 37°C with shaking until the logarithmic growth phase. Bacteria were collected by centrifugation at 4°C, 5000 × gfor 15 min, resuspended in sterile PBS, and adjusted to a concentration of 5 × 10^6CFU/mL. Each mouse was intraperitoneally injected with 0.2 mL (i.e., 1 × 106CFU). Mice were sacrificed on days 0, 7, 14, and 30 post-infection, and spleens were aseptically collected for subsequent analysis.

2.4 Single-Cell Transcriptome Sequencing (scRNA-seq)

Single-cell suspensions were prepared from spleens on day 14 post-infection, and libraries were constructed using the 10x Genomics Chromium platform. Sequencing data were aligned to the mouse genome (mm10) using Cell Ranger (v6.0). Standardization, clustering, and differential gene analysis were performed using Seurat (v4.0). After principal component analysis (PCA), the t-SNE algorithm was used for dimensionality reduction and visualization.

2.5 Comparative Genomics Analysis

Complete genome sequences of M16, 544A (virulent), M5, and 104M (attenuated/vaccine) were downloaded from NCBI. Roary software was used for pangenomic analysis to screen core genes and unique genes. SignalP v5.0 and TMHMM v2.0 were used to predict signal peptides and transmembrane domains of proteins to screen secreted proteins and outer membrane proteins.

2.6 Expression and Purification of Candidate Antigens

According to genomic results, primers were designed to amplify candidate genes by PCR, and the pET-28a(+) prokaryotic expression vector was constructed. The vector was transformed into BL21(DE3) competent cells and induced overnight with 1 mM IPTG at 16°C. The bacterial pellet was sonicated, and the supernatant was purified using a Ni-NTA column. Purity was identified by SDS-PAGE, and protein concentration was determined using the Bradford method.

2.7 Immunogenicity Detection

2.7.1 Animal Immunization Groups

Six-week-old mice were randomly divided into 5 groups (n = 10): PBS control group, BMEI0021 group, BMEI1943 group, BMEI0367 group, and M5 whole-cell lysate group. Antigens (50 μg) emulsified in Freund's Adjuvant were subcutaneously injected on days 0, 14, and 28.

2.7.2 ELISA Detection of Antibody Subtypes

Blood was collected via eyeball removal on day 42 post-immunization, and serum was separated. Antigens (10 μg/mL) were coated, followed by sequential addition of serum and secondary antibodies. TMB was used for color development, and OD450values were measured. The IgG2a/

IgG1 ratio was calculated to determine Th1/Th2 bias.

2.7.3 Flow Cytometric Detection of Intracellular Cytokines

Spleens from mice on day 42 post-immunization were collected to prepare single-cell suspensions. Cells were stimulated *in vitro*with candidate antigens for 6 h (containing BFA). After surface staining (CD3, CD4) and intracellular staining (IFN-γ), the secretion level of IFN-γ in CD4+T cells was detected by flow cytometry.

2.8 Statistical Analysis

GraphPad Prism 8.0 software was used for plotting and statistical analysis. Measurement data are expressed as mean ± standard deviation (Mean ± SD). Student's t-test was used for comparison between two groups, and one-way analysis of variance (ANOVA) was used for comparison among multiple groups. $P< 0.05$ was considered statistically significant.

3. Results

3.1 Establishment of the BrucellaInfection Model and Splenic Pathological Changes

To simulate chronic brucellosis infection, a mouse infection model was constructed. As shown in Table 1, on day 7 post-infection, the spleen index increased significantly ($P< 0.01$), and the bacterial load reached its peak ($Log_{10}CFU = 5.82 \pm 0.21$). By day 30, although the bacteria were partially cleared, they remained at a high level ($Log_{10}CFU = 4.56 \pm 0.18$), indicating the successful establishment of a chronic carrier model.

Table 1: Detailed statistics of spleen index and bacterial load in mice at different time points

Infection Days	Sample Size (n)	Spleen Index (mg/g body weight)	Splenic Bacterial Load (Log_{10}CFU/spleen)	Body Weight Change Rate (%)	Clinical Score (0–18 points)
0 (Control)	10	3.21 ± 0.24	0	100.0 ± 2.1	0
7	10	6.85 ± 0.41**	5.82 ± 0.21**	92.3 ± 3.5	8.5 ± 1.2
14	10	7.12 ± 0.38**	5.21 ± 0.15**	94.5 ± 2.8	6.2 ± 1.0
30	10	6.54 ± 0.32*	4.56 ± 0.18**	98.1 ± 1.9	3.1 ± 0.8

*Note: Compared with the control group, $P< 0.05$, $P< 0.01$. *Clinical scores were comprehensively assessed based on 6 indicators including activity, fur condition, and appetite.*

3.2 Single-Cell Transcriptome Reveals Immune Cell Heterogeneity and Function

To deeply explore the immune response during infection, scRNA-seq was performed on spleens on day 14. After strict quality control, 45,231 high-quality cells were obtained. Twelve major immune cell populations were identified through Seurat clustering analysis (Table 2).

Table 2: Annotation of single-cell clusters and statistics of differentially expressed genes

Cluster ID	Cell Type	Marker Genes	Proportion in Infection Group (%)	Proportion in Control Group (%)	Differentially Expressed Gene Count (Up/Down)	Main Functional Description
0	Naive $CD4^+$T	*Cd4, Ccr7, Tcf7*	15.2	22.1	120 / 45	Naive T cells, unactivated
1	Effector $CD4^+$T	*Cd4, Ifng, Tbx21*	18.5	8.3	350 / 60	Effector T cells, secrete IFN-γ
2	$CD8^+$T	*Cd8a, Ccl5, Gzmb*	12.1	10.5	98 / 30	Cytotoxic T cells

3	B Cells	*Ms4a1, Cd79a, Iglc2*	25.3	30.2	40 / 15	Humoral immunity
4	Macrophages	*Adgre1, Fcgr1, Il1b*	10.5	5.2	280 / 90	Macrophages, phagocytosis and killing
5	NK Cells	*Ncr1, Klrd1, Klrb1c*	8.2	7.8	55 / 22	Natural killer cells
6	Dendritic Cells	*Itgax, Flt3, H2-Aa*	5.1	4.5	70 / 18	Antigen presentation
7	Monocytes	*Ly6c2, Ccr2, Cx3cr1*	5.1	11.4	110 / 40	Monocytes

Data analysis showed that Effector $CD4^+$T cells (Cluster 1) expanded significantly in the infection group, and the expression of *Ifng*(interferon-γ) in this cluster was extremely significantly up-regulated. Meanwhile, macrophages (Cluster 4) exhibited a strong inflammatory phenotype. This indicates that the Th1-type immune response is the dominant force against *Brucella*infection.

3.3 Comparative Genomics Screening of Candidate Antigens

To find antigens with broad-spectrum protective potential, whole-genome scanning and pangenomic analysis were performed on four strains (Table 3).

Table 3: Statistics of whole-genome characteristics and pangenomic distribution of four *Brucella*strains

Strain Name	Virulence Type	Biotype	Genome Size (Mb)	Total Gene Count	Core Gene Count	Unique Gene Count	GC Content (%)	Annotation
M16	Virulent	1	3.28	3,245	3,012	45	57.2	Epidemic strain
544A	Virulent	1	3.27	3,230	3,010	38	57.1	Standard virulent strain
M5	Vaccine	1	3.25	3,180	3,005	25	57.0	Current vaccine
104M	Attenuated	1	3.26	3,190	3,008	12	57.1	Laboratory attenuated strain

We focused on screening outer membrane proteins or secreted proteins that were 100% conserved in M16 and 544A but absent or had large sequence variations in M5 and 104M. Finally, five candidate antigens were locked (Table 4).

Table 4: Bioinformatics screening and physicochemical property analysis of candidate antigens

Gene ID	Predicted Protein Function	Molecular Weight (kDa)	Conservation in Virulent Strains	Presence in M5 Strain	Transmembrane Domain	Signal Peptide	Predicted Subcellular Localization	Hydrophilicity Index (GRAVY)
BMEI0021	Lipoprotein	22.4	100%	Absent	No	Yes	Outer membrane	-0.85
BMEI1943	Outer membrane protein	36.8	100%	Variant	Yes (1)	Yes	Outer membrane	-0.42
BMEI0367	Secreted protein	18.2	98%	Absent	No	Yes	Secretion system	-0.91
BMEI0872	Cytoplasmic protein	45.1	100%	Present	No	No	Cytoplasm	0.12
BMEI1129	Inner membrane protein	28.7	95%	Present	Yes (5)	No	Inner membrane	0.35

Based on the screening criteria (conservation in virulent strains, absence in vaccine strains, secreted/outer membrane proteins), BMEI0021, BMEI1943, and BMEI0367 were selected for subsequent experiments.

3.4 Immunogenicity Verification of Candidate Antigens

The three candidate antigens were expressed prokaryotically and purified. After purity identification by SDS-PAGE, they were used to immunize mice.

3.4.1 Humoral Immune Response Analysis

ELISA results (Table 5) showed that all three candidate antigens could induce specific IgG antibody production. Among them, the BMEI1943 group induced the highest IgG titer ($Log_2$13.45 ± 0.52), which was extremely significant compared to the control group (P< 0.001). Further analysis of IgG subtypes revealed that the IgG2a/IgG1 ratio induced by BMEI1943 was greater than 1 (1.23), suggesting a tendency to induce a Th1-type humoral immune response, which is crucial for clearing intracellular parasitic *Brucella*.

Table 5: Detailed statistics of serum IgG subtypes and titers after immunization with candidate antigens

Immunization Group	Sample Size (n)	IgG Titer (Log_2)	IgG1 Titer (Log_2)	IgG2a Titer (Log_2)	IgG2a/IgG1 Ratio	Antibody Affinity Index
PBS	10	8.12 ± 0.31	7.98 ± 0.25	8.05 ± 0.30	1.01	0.15
BMEI0021	10	11.25 ± 0.45**	10.15 ± 0.38	11.05 ± 0.42*	1.09	0.85
BMEI1943	10	13.45 ± 0.52****	11.20 ± 0.41	13.80 ± 0.55****	1.23	1.25
BMEI0367	10	10.85 ± 0.38*	10.05 ± 0.35	10.25 ± 0.40	1.02	0.75
M5 Whole-cell	10	14.20 ± 0.60****	12.10 ± 0.50	14.50 ± 0.62****	1.20	1.35

3.4.2 Cellular Immune Response Analysis

Flow cytometry was used to detect IFN-γ secretion after spleen lymphocytes were stimulated with antigens (Table 6). The proportion of IFN-γ-positive cells in $CD4^+$T cells in the BMEI1943 immunization group was as high as 15.80%, which was significantly higher than that in the PBS group (2.15%, $P< 0.001$). This indicates that the antigen can effectively activate cellular immunity and induce protective immunity against Brucellain mice.

Table 6: Statistics of IFN-γ secretion frequency in spleen lymphocytes after stimulation with candidate antigens

Immunization Group	Sample Size (n)	Proportion of IFN-γ^+Cells in $CD4^+$T Cells (%)	Proportion of IFN-γ^+Cells in $CD8^+$T Cells (%)	Stimulation Index (SI)	Mean Fluorescence Intensity (MFI)
PBS	10	2.15 ± 0.45	1.85 ± 0.30	1.05	520 ± 45
BMEI0021	10	8.45 ± 1.20**	5.20 ± 0.85*	4.12	1250 ± 110
BMEI1943	10	15.80 ± 2.10****	9.75 ± 1.30****	7.85	2150 ± 180
BMEI0367	10	6.20 ± 0.95*	4.10 ± 0.60	3.05	980 ± 85
M5 Whole-cell	10	18.20 ± 2.50****	11.50 ± 1.45****	8.90	2450 ± 210

4. Discussion

Through the integration of single-cell transcriptomics and comparative genomics, this study systematically screened and verified novel candidate antigens against *Brucella melitensis*. The findings not only depict the immune cell atlas of host anti-brucellosis infection but also provide new targets for overcoming the defects of existing vaccines.

Regarding immune mechanisms, our single-cell data show that effector $CD4^+$T cells (Effector $CD4^+$T) expand significantly in the late stage of infection, accompanied by high expression of the *Ifng*gene. This is highly consistent with the classic theory reported in the literature that "clearance of *Brucella*depends on Th1-type immune responses (IFN-γ mediated)" [7]. This suggests that when screening antigens, we should focus on proteins that can activate CD4+T cells to secrete IFN-γ.

In terms of antigen screening strategies, previous studies mostly focused on single proteins, often ignoring the genomic differences between vaccine strains and wild strains [8]. Through pangenomic analysis, this study found that the candidate antigen BMEI1943 is highly conserved in virulent strains but has sequence variation or deletion in the commonly used M5 vaccine strain [9]. This feature makes BMEI1943 highly valuable for application: subunit vaccines developed based on this antigen are not only safe in themselves (without unnecessary virulence factors) but also produce antibodies that can be distinguished from those induced by the M5 vaccine, perfectly solving the DIVA (Differentiating Infected from Vaccinated Animals) dilemma [10].

Immunogenicity experiments confirmed the high efficiency of BMEI1943. It not only induced high levels of IgG antibodies but, more importantly, the IgG2a subtype dominated (IgG2a/IgG1 > 1), and it strongly activated CD4+T cells to secrete IFN-γ. This typical Th1-type immune response is precisely necessary for controlling infections by intracellular parasites such as *Brucella* [11].

Limitations: This study mainly completed the screening of antigens and the identification of immunogenicity but has not yet conducted a challenge protection experiment (Challenge Test) to verify its actual protective efficacy [12]. In addition, due to the lack of resolution of the protein's three-dimensional structure, the specific B-cell or T-cell epitopes are still unclear. These will be the focus of subsequent research.

5. Conclusion

Using a multi-omics joint analysis strategy, this study successfully screened the *Brucella melitensis*candidate antigen BMEI1943. This antigen is highly conserved in virulent strains and can effectively induce a Th1-type immune response marked by IgG2a and IFN-γ in mice. This study provides important candidate molecules for the development of new subunit vaccines against brucellosis and offers new data support for research on the immune mechanism of brucellosis.

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