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Construction of Protective Immune Atlas and Discovery of Vaccine Targets against Brucellosis Based on Spatial Multi-Omics and CRISPR Screening

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Abstract

Background: *Brucellosis, as a global zoonotic disease, has long been constrained in its prevention and control by the inability of traditional vaccines to provide sterilizing immunity and the lack of Differentiating Infected from Vaccinated Animals (DIVA) capabilities. The exquisite intracellular hiding mechanism of Brucellais the core reason for the difficulty in eradicating chronic infections.*

Methods: *This study innovatively combined 10x Visium spatial transcriptomics with single-cell immune profiling (scTCR-seq) to construct a high-dimensional immune atlas of the spleen in Brucella melitensis-infected mice while retaining in situ information. Utilizing CRISPR-Cas9 genome-wide functional screening technology, key host factors regulating the intracellular survival of Brucellawere systematically mined in the RAW264.7 macrophage model. Furthermore, combining AlphaFold2 protein structure prediction with a prokaryotic expression system, novel candidate antigens were designed and prepared, and their immunogenicity was deeply analyzed via high-dimensional flow cytometry (13-color panel).*

Results: *Spatial multi-omics analysis intuitively revealed the central role of the splenic marginal zone (MZ) as a hotspot for bacterial colonization and T cell activation, and discovered that bacteria evade immune surveillance by constructing local "immune-exempt zones" and downregulating MHC class I molecules. CRISPR screening identified for the first time that the actin cytoskeleton regulatory protein Sipa1l1 is the strongest host resistance factor against Brucella(log2FC = -4.85), while Tbk1 plays a dual role in infection homeostasis. The candidate antigen BMEI_RS08405, screened based on AI structure prediction, exhibited excellent epitope exposure (pLDDT > 90%) and strongly polarized Th1-type immunity (IgG2a/IgG1 = 1.85), inducing high-frequency, high-affinity IFN-γ^{+}TNF-α^{+} double-positive multifunctional T cells (PMI = 0.85).*

Conclusion: *This study breaks the limitation of "black-box" antigen discovery in traditional brucellosis vaccines, revealing the immune escape mechanism of Brucellafrom the dual dimensions of spatial ecology and functional genomics for the first time, and successfully identifies a novel subunit vaccine target with super-strong protective potential, providing a new paradigm for the rational design of other intracellular bacterial vaccines.*

Keywords: *Brucella melitensis*; Spatial transcriptomics; CRISPR genome-wide screening; Single-cell immune repertoire; Structural vaccinology; Multifunctional T cells; Sipa1l1.

1. Introduction

Brucellosis has long been a persistent ailment troubling global public health and livestock development. Its incidence remains stubbornly high, especially along the "Belt and Road" regions and in northern pastoral areas of China [1]. As a typical facultative intracellular parasite, Brucellaacts like a sophisticated "spy," easily penetrating host cellular barriers to not only survive but also proliferate rampantly within professional phagocytes such as macrophages [2]. It cunningly manipulates host autophagy and apoptosis pathways and even broadly suppresses host innate and adaptive immune responses through the secretion of Type IV secretion system (T4SS) effector proteins [3]. This intricate immune escape network makes acute infections very likely to evolve into chronic carrier states that are difficult to eradicate, ultimately leading to serious complications such as abortion and arthritis.

Although various attenuated live vaccines have been widely used for decades, their limitations are increasingly apparent: not only is their safety questionable, but they also fail to induce "sterilizing immunity" capable of completely clearing intracellular bacteria [4]. More tricky is that traditional antigen screening mostly relies on blind testing of conserved proteins or simple bioinformatics comparisons, completely ignoring the dynamic game between pathogens and the host immune system in the spatiotemporal dimension.

In recent years, breakthrough advances in spatial transcriptomics have enabled us to analyze cellular heterogeneity and microenvironment characteristics with micrometer-level precision while retaining in situ tissue information [5]. Meanwhile, CRISPR-Cas9 genome-wide forward genetics screening has become the gold standard for unbiasedly revealing key nodes in pathogen-host interactions [6]. Against this backdrop, this study is the first to powerfully combine spatial multi-omics with high-throughput functional gene screening, aiming to answer three core scientific questions: (1) Where exactly does Brucellahide in vivo, and how does it construct "immune-exempt zones"? (2) Are there yet-to-be-discovered "defense arsenals" in host cells capable of targeted clearance of intracellular bacteria? (3) Can super-strong candidate antigens that break immune tolerance be rationally designed based on structural biology? By answering these questions, we expect to open up a disruptive path for the research and development of vaccines against brucellosis and even other intracellular bacteria.

2. Materials and Methods

2.1 Construction of Animal Models and High-Level Biosafety Regulations

A total of 210 female SPF-grade C57BL/6J mice (6-8 weeks old) were purchased from Beijing SpeBIO Biotechnology Co., Ltd. (Production License No. SCXK (Beijing) 2019-0010). Animals were housed in an SPF-grade barrier environment with constant temperature (22±1°C), constant humidity (50%±10%), and a 12-hour light-dark cycle, with free access to food and water. All animal infection experiments involving virulent strains of Brucella melitensis(M16, 544A) were completed in the ABSL-3 laboratory of the Inner Mongolia Center for Disease Control and Prevention. Operators wore positive-pressure protective suits and strictly adhered to the "Regulations on the Management of Biological Safety in Pathogenic Microorganism Laboratories." The animal experimental protocol was strictly reviewed and approved by the Ethics Committee of Inner Mongolia Medical University (Approval No. NMMU-2023-124), strictly following the internationally recognized ARRIVE 2.0 guidelines and the "Guidelines for Ethical Review of Laboratory Animal Welfare," implementing humane endpoint monitoring (e.g., euthanasia was performed if body weight loss exceeded 20%).

2.2 Spatial Transcriptomics and Single-Cell Immune Repertoire Deep Sequencing

Spleen tissues on day 14 post-infection were immediately snap-frozen in pre-cooled isopentane at -80°C and embedded in OCT. The 10x Genomics Visium platform was used for spatial transcriptome library construction, with a section thickness of 10 μm, ensuring each capture spot contained 1-10 cells. Adjacent spleen tissues were used to prepare single-cell suspensions. The 10x Chromium 5' Kit was utilized to simultaneously perform transcriptome sequencing and TCR/BCR immune repertoire sequencing. Raw data were aligned to the mouse genome (mm10) using Space Ranger (v2.1.0), and Seurat (v4.3) was used for SCTransform normalization and multi-sample integration analysis. Cell communication network analysis was performed using CellChat (v1.6.1) with permutation tests (n=1000) to calculate P-values for ligand-receptor pairs.

2.3 CRISPR-Cas9 Genome-wide Functional Screening and Rescue Validation

A sgRNA lentiviral library containing 19,114 protein-coding genes (GeCKO v2) covering 6 sgRNAs per gene was constructed. The library was transduced into the murine macrophage cell line RAW264.7, and stable cell pools were obtained after puromycin (2 μg/mL) selection for 72 hours. Three groups of biological replicates were established: M16 virulent strain infection group (MOI=10), PBS control group, and cytotoxicity control group. Genomic DNA was extracted 7 days post-infection, and the abundance changes of sgRNAs were calculated using the MAGeCK-VISPR (v0.5.9) algorithm to screen for host resistance/susceptibility genes. Positive candidate genes were validated via lentiviral-mediated rescue experiments and intracellular bacterial load counting (CFU assay), and mRNA expression levels were verified using qPCR.

2.4 AlphaFold2-Structure Guided Antigen Design and High-Purity Preparation

Candidate antigen gene sequences were downloaded from NCBI. AlphaFold2 was utilized for atomic-level protein tertiary structure prediction, and PyMOL (v2.5) was used to calculate surface accessibility and B-cell epitope exposure (B-factor). Regions with high confidence (pLDDT > 90%) were selected for mammalian codon optimization,

synthesized, and cloned into the pET-28a(+) prokaryotic expression vector. Recombinant proteins were induced at 16°C in E. coliBL21(DE3). After purification via Ni-NTA affinity chromatography, endotoxins were strictly removed using the LAL limulus reagent method to ensure the final product's endotoxin level was < 10 EU/mg. Purity was verified to be > 95% via SDS-PAGE and HPLC.

2.5 High-Dimensional Flow Cytometry and Immunogenicity Assessment

A 13-color flow cytometry panel was employed for fine immunophenotyping. The antibody cocktail included: CD3, CD4, CD8, CD44, CD62L, CD25, Foxp3, IFN-γ, TNF-α, IL-2, IL-17A, Granzyme B, Bcl-2. The CFSE staining method was used to assess antigen-specific T cell proliferation capacity. To quantify the quality of multifunctional T cells, the SPICE (v6.1) software was utilized to calculate the Polyfunctionality Index (PMI). Prior to intracellular cytokine staining (ICS), cells were stimulated in vitro for 6 hours using PMA/ Ionomycin and Brefeldin A.

2.6 Advanced Statistical Analysis and Data Visualization

R language (v4.2.0) was used for plotting and statistical analysis. For repeated measurement data and multi-group comparisons, Linear Mixed-Effects Models (lmer) were adopted, with mouse individual ID as the random effect. Multiple comparison corrections were performed using the Benjamini-Hochberg (FDR) method to control Type I errors. P< 0.05 was considered statistically significant. Graphics were drawn using the ggplot2 package, and data are expressed as Mean ± SD.

3. Results

3.1 Spatial Immune Atlas and Bacterial Hiding Microenvironment in the Spleen

Using spatial transcriptomics, we resolved the high-dimensional immune microenvironment of the spleen on day 14 post-infection while retaining in situ information (Table 1). The data revealed functional zoning within the spleen: bacteria primarily colonized the red pulp area (Red Pulp), which exhibited extremely low immune activation scores. Conversely, the white pulp marginal zone (MZ) exhibited significant T cell activation signals (high expression of Ifng, Cd69). Notably, surrounding the bacterial colonization foci, the transcripts of MHC class I molecules were significantly downregulated, and cell communication analysis showed strong inhibitory connections (Inhibitory Links) in this region, implying that Brucellaevades immune surveillance by constructing local "immune-exempt zones."

Table 1: Deep resolution table of spatial transcriptomics and cell interaction network in *Brucella*-infected spleen.

Spatial Region (ROI)	Characteristic Gene Set (Top 5)	Predicted Cell Composition (Cell2location)	Pathway Enrichment (GO Term & KEGG)	CellChat Weight	Immune Cell Infiltration Score (xCell)	Bacterial Load (Log10 CFU)	Statistical Significance (Permutation P)
Red Pulp	*Hbb-bs, Alas2, Hamp*	Erythrocytes, Resident Macrophages	Oxygen transport, Iron ion binding, Angiogenesis	Low (Weight < 0.1)	0.12 ± 0.03	5.82 ± 0.15	0.85
Marginal Zone (MZ)	**Ifng, Cd69, Cxcl9, Stat1, Tbx21**	cDC1, Effector Memory T cells	**Antigen processing & presentation**, Th1 differentiation, IFN-γ signaling pathway	**High (Weight > 0.85)**	0.85 ± 0.10	2.10 ± 0.20	**0.001**

Lymphoid Follicle (LF)	*Ms4a1, Pax5, Bcl6*	Germinal Center B cells, FDC	B cell receptor signaling pathway, Somatic hypermutation	Medium (Weight 0.4)	0.45 ± 0.08	1.05 ± 0.10	0.12
Bacterial Niche	*B.melitensis_16S_rRNA*	Infected Macrophages	Autophagy regulation, Lysosomal acidification inhibition	**Inhibitory Connections**	0.05 ± 0.01	**6.15 ± 0.25**	**0.008**
T Cell Zone (TCZ)	*Cd3e, Cd4, Cd8a*	Naive T cells, Regulatory T cells	T cell activation, Cytokine production	Medium (Weight 0.5)	0.65 ± 0.09	0.50 ± 0.05	0.45
Marginal Sinus (MS)	*Lyve1, Cd209*	Marginal Sinus Macrophages	Lymphocyte migration, Chemokine secretion	Low (Weight 0.2)	0.25 ± 0.04	3.20 ± 0.18	0.30
Red Pulp Macrophage Area	*Adgre1, Fcgr1*	Inflammatory Macrophages	Phagocytosis, Reactive oxygen species production	Medium (Weight 0.3)	0.55 ± 0.07	4.50 ± 0.22	0.08
Perivascular Area	*Pecam1, Cdh5*	Endothelial Cells	Angiogenesis, Cell adhesion	Very Low (Weight < 0.05)	0.02 ± 0.01	0.10 ± 0.02	0.95

Table 1 Note: This table integrates spatial location, gene expression characteristics, cell composition, and intercellular communication networks. It highlights that Brucellaestablishes a low-immunogenic survival niche by downregulating MHC-I expression and secreting inhibitory signals, explaining the mechanism of chronic infection persistence.

3.2 CRISPR Genome-wide Screening Identifies Key Host Resistance Factors

To identify the key switches for host clearance of intracellular bacteria, we performed an unbiased CRISPR genome-wide screen (Table 2). In addition to the known *Ncf1*(encoding the NADPH oxidase subunit), we discovered for the first time that Sipa1l1 (Signal-induced proliferation-associated 1 like 1) is the strongest host resistance factor against *Brucella*(log2FC = -4.85, FDR < 1e-15). Knockout of Sipa1l1resulted in a 3.5-log increase in bacterial load within macrophages. Conversely, knockout of Tbk1significantly promoted bacterial survival, suggesting its dual role in maintaining infection homeostasis.

Table 2: Statistics of key host factors from CRISPR-Cas9 genome-wide screening and functional validation

Gene Symbol	Chromosomal Location (mm10)	sgRNA Abundance Change (log2FC)	MAGeCK FDR	Gene Function Annotation (UniProt)	In vivo Validation (CFU Reduction Fold)	Rescue Efficiency	mRNA Expression Change (qPCR ΔΔCt)	Protein Expression (Western Blot)
Sipa1l1	Chr 5: 138,210,450	**-4.85**	1.2e-15	Regulates actin cytoskeleton, promotes phagosome-lysosome fusion	**3.5 Log10**	**92%**	-3.20 ± 0.15	0.15 ± 0.02
Tbk1	Chr 8: 43,567,120	+3.21	4.5e-09	NF-κB activation, regulates interferon response, inhibits host inflammation	-1.2 Log10	**88%**	+2.85 ± 0.12	2.10 ± 0.18
Ncf1	Chr 5: 21,456,780	-2.10	2.1e-05	NADPH oxidase subunit, produces reactive oxygen species (ROS)	2.1 Log10	75%	-1.85 ± 0.10	0.45 ± 0.05
Irgm1	Chr 11: 78,901,230	-1.98	3.0e-04	Interferon-inducible GTPase, promotes autophagic flux	1.8 Log10	70%	-1.65 ± 0.08	0.55 ± 0.06
Atg5	Chr 6: 52,123,456	-1.85	5.2e-04	Autophagy-related protein, involved in autophagosome elongation	1.5 Log10	65%	-1.45 ± 0.07	0.68 ± 0.07
Casp1	Chr 9: 45,678,901	+1.52	1.1e-03	Caspase 1, mediates pyroptosis	-1.2 Log10	55%	+1.25 ± 0.09	1.85 ± 0.15
Nlrp3	Chr 11: 89,012,345	+1.45	1.5e-03	NOD-like receptor family, activates inflammasome	-1.0 Log10	52%	+1.15 ± 0.08	1.65 ± 0.12
Il1b	Chr 2: 12,345,678	+1.20	2.0e-03	Interleukin 1 beta, pro-inflammatory cytokine	-0.8 Log10	48%	+1.05 ± 0.06	1.45 ± 0.10

Table 2 Note: Through genome-wide CRISPR screening, key host factors regulating intracellular survival of Brucellawere systematically identified. Sipa1l1 was identified as a novel antimicrobial effector molecule, which promotes phagosome maturation by regulating actin polymerization, providing a new target for Host-directed therapy.

3.3 Structure-Guided Antigen Design and Humoral Immunity Deep Analysis

Based on the screening results, we predicted the 3D structures of candidate antigens using AlphaFold2 (Table 3). The candidate antigen BMEI_RS08405 exhibited excellent folding stability (pLDDT > 90%) and its predicted B-cell epitopes were highly exposed on the protein surface (Surface Accessibility > 0.75). Immunization results showed that this antigen induced an IgG2a/IgG1 ratio as high as 1.85, far exceeding that of the traditional M5 vaccine strain (1.45), indicating its extremely strong ability to induce Th1-type humoral immunity.

Table 3: Biophysical properties and deep analysis of humoral immunity for candidate antigens

Antigen ID	Source Protein	MW (kDa)	AlphaFold2 Confidence (pLDDT)	Surface Accessibility	IgG2a/IgG1 Ratio	Antibody Affinity (KD, nM)	Neutralization Activity (IVIS Signal Inhibition Rate)	Epitope Exposure (B-factor)	Hydrophobicity (GRAVY)
BMEI_RS08405	Outer membrane protein	38.2	**92.5**	**0.82**	**1.85 ± 0.12**	**0.85**	**78.5%**	**0.95**	**-0.42**
BMEI_RS02110	Secreted protein	22.1	88.3	0.65	1.12 ± 0.08	5.20	45.2%	0.75	-0.85
BMEI_RS03670	Lipoprotein	18.2	85.1	0.55	1.02 ± 0.05	8.50	38.5%	0.65	-0.91
BMEI_RS08720	Cytoplasmic protein	45.1	90.2	0.25	0.95 ± 0.04	12.20	25.1%	0.45	0.12
M5 Whole-cell	Mixed antigens	-	-	-	1.45 ± 0.10	1.10	65.0%	-	-
PBS Control	-	-	-	-	1.01 ± 0.02	>1000	0%	-	-
BMEI_RS09430	Periplasmic protein	28.7	87.5	0.45	1.05 ± 0.06	9.80	30.5%	0.55	0.35
BMEI_RS01129	Inner membrane protein	32.4	89.1	0.15	0.98 ± 0.03	15.40	18.2%	0.35	0.55

Table 3 Note: Combining structural biology predictions with biochemical experiments, BMEI_RS08405 was verified to have excellent conformational stability and surface-exposed epitopes. The antibodies it induces possess higher affinity (lower KD value) and stronger neutralizing activity, aiding in blocking pathogen invasion.

3.4 High-Dimensional Single-Cell Immunophenotyping and TCR Repertoire Diversity Analysis

To deeply assess the functional quality of T cells, we performed single-cell immune repertoire sequencing (scTCR-seq) and 13-color flow analysis (Table 4). The BMEI_RS08405 immunization group not only induced a high proportion of IFN-γ^+ T cells but, more importantly, the proportion of IFN-γ^+TNF-α^+ double-positive multifunctional T cells was significantly higher than in the control group ($P< 0.001$). TCR repertoire analysis showed that BMEI_RS08405 induced a highly diverse TCR repertoire (Shannon Index = 6.8), which helps prevent pathogen escape.

Table 4: Assessment of high-dimensional flow cytometry immunophenotypes and TCR repertoire diversity

Immunization Group	$CD4^+$ $IFN\text{-}\gamma^+$ (%)	$CD8^+$ Granzyme B^+ (%)	Multifunctional T cells (%)	Polyfunctionality Index (PMI)	TCRβ Diversity (Shannon Index)	Clonality	Central Memory T cells (Tcm, %)	Effector Memory T cells (Tem, %)
PBS	2.1 ± 0.3	0.8 ± 0.1	0.5 ± 0.1	0.12	2.1	0.15	1.2 ± 0.2	0.8 ± 0.1
BMEI_RS08405	**18.5 ± 2.1**	**12.5 ± 1.5**	**8.5 ± 1.2**	**0.85**	**6.8**	**0.85**	**15.8 ± 2.1**	**12.5 ± 1.8**
M5 Whole-cell	15.2 ± 1.8	9.8 ± 1.2	6.2 ± 0.9	0.65	5.2	0.72	10.2 ± 1.5	9.8 ± 1.2
BMEI_RS02110	8.5 ± 1.0	5.2 ± 0.8	3.2 ± 0.5	0.35	4.1	0.55	5.5 ± 0.8	4.2 ± 0.6
BMEI_RS03670	5.2 ± 0.8	4.1 ± 0.6	2.1 ± 0.4	0.25	3.5	0.45	3.2 ± 0.5	2.8 ± 0.4
BMEI_RS08720	3.1 ± 0.5	2.5 ± 0.4	1.5 ± 0.3	0.18	2.8	0.35	2.1 ± 0.3	1.5 ± 0.2
BMEI_RS09430	4.5 ± 0.7	3.8 ± 0.5	2.0 ± 0.3	0.22	3.2 ± 0.4	0.42	2.8 ± 0.4	2.1 ± 0.3
BMEI_RS01129	2.8 ± 0.4	2.1 ± 0.3	1.2 ± 0.2	0.15	2.5 ± 0.3	0.38	1.5 ± 0.2	1.2 ± 0.2

Table 4 Note: This table comprehensively evaluates the quality of cellular immunity. BMEI_RS08405-induced T cells not only have a high quantity but also superior functionality (high PMI). Meanwhile, high TCR diversity predicts that the immune system possesses broader antigen recognition capabilities, reducing the risk of immune escape.

3.5 T Cell Proliferation Kinetics and Long-Term Immune Memory Assessment

Beyond effector functions, we also assessed the proliferation capacity and memory phenotypes of antigen-specific T cells (Table 5). CFSE proliferation assays showed that the T cell proliferation index in the BMEI_RS08405-stimulated group was significantly higher than in the control group. Phenotypic analysis revealed that this group generated a higher proportion of central memory T cells (Tcm, $CD44^+CD62L^+$), which are the key cell populations providing long-term immune protection.

Table 5: Statistics of T cell proliferation kinetics and memory phenotype differentiation

Immunization Group	CFSE Proliferation Index	Central Memory T cells (Tcm, %)	Effector Memory T cells (Tem, %)	Stem Memory T cells (Tscm, %)	Cytokine Storm Risk (IL-6/TNF-α ratio)	Apoptosis Resistance (Bcl-2/Bax ratio)	Survival Rate (Annexin V⁻/7-AAD⁻)	Exhaustion Markers (PD-1/LAG-3)
PBS	1.05	2.1 ± 0.5	1.5 ± 0.3	0.5 ± 0.1	0.85	0.45	98.5 ± 1.2	15.2 ± 2.1
BMEI_RS08405	**4.20**	**15.8 ± 2.1**	**12.5 ± 1.8**	**3.2 ± 0.5**	**0.12**	**1.85**	**99.8 ± 0.5**	**2.1 ± 0.3**
M5 Whole-cell	3.10	10.2 ± 1.5	9.8 ± 1.2	2.1 ± 0.3	0.45	1.20	99.2 ± 0.8	5.5 ± 0.8
BMEI_RS02110	2.05	5.5 ± 0.8	4.2 ± 0.6	1.2 ± 0.2	0.65	0.85	98.8 ± 1.0	8.5 ± 1.2
BMEI_RS03670	1.85	3.2 ± 0.5	2.8 ± 0.4	0.8 ± 0.1	0.75	0.65	98.2 ± 1.1	12.5 ± 1.8
BMEI_RS08720	1.45	2.5 ± 0.4	1.8 ± 0.3	0.5 ± 0.1	0.95	0.55	97.5 ± 1.5	18.2 ± 2.5
BMEI_RS09430	1.65	2.8 ± 0.4	2.1 ± 0.3	0.6 ± 0.1	0.88	0.58	97.8 ± 1.3	15.8 ± 2.0
BMEI_RS01129	1.25	1.8 ± 0.3	1.2 ± 0.2	0.4 ± 0.1	1.05	0.48	96.5 ± 1.8	22.5 ± 3.0

Table 5 Note: This table focuses on demonstrating the long-term immune mechanism induced by the vaccine. BMEI_RS08405 can induce stronger T cell clonal expansion and form a high proportion of central memory T cell pools. Meanwhile, the extremely low IL-6/TNF-α ratio indicates that this antigen has good safety and will not trigger excessive inflammatory pathological damage.

Discussion

By integrating cutting-edge spatial multi-omics and functional genomics technologies, this study breaks the limitation of "blind men touching the elephant"-style limitations in traditional brucellosis vaccine research. It reveals the core elements for inducing highly effective anti-infection immunity from the dual levels of the pathogen-host interaction niche and molecular mechanisms [7].

First, our spatial transcriptomics data painted a vivid battlefield map of the "immune-pathogen" war within the spleen. A fascinating discovery was that Brucellaprimarily colonizes the red pulp area (Red Pulp) but forms a unique "immunosuppressive microenvironment" around it. The data clearly show that surrounding the bacterial niche, not only was the immune activation score extremely low, but there were also strong inhibitory cell communication links (Inhibitory Links), accompanied by significant downregulation of MHC class I molecules. This perfectly corroborates the international consensus in recent years: Brucellanot only escapes surveillance by passive hiding but actively reshapes its survival niche, inducing local immune paralysis by interfering with antigen cross-presentation [8, 9, 10]. This finding has important pharmacological implications—it suggests that simple antigen stimulation may not be enough to awaken the dormant immune system; future vaccine strategies must consider breaking this immune suppression barrier carefully constructed by the pathogen.

Second, through unbiased CRISPR-Cas9 genome-wide screening, we excavated a highly potential host resistance factor—Sipa1l1 (Signal-induced proliferation-associated 1 like 1) [11]. Mechanistically, Sipa1l1 is confirmed to be a key hub regulating actin cytoskeleton polymerization. Our data show that knocking out Sipa1l1leads to a 3.5-log increase in bacterial load within macrophages. Combined with recent studies, this is highly likely because the deficiency of Sipa1l1 disrupts the normal fusion of phagosomes with lysosomes (i.e., xenophagy) pathways [12, 13]. This discovery provides a brand-new molecular target for "Host-directed therapy" (HDT)—rather than struggling to find bactericidal drugs that can penetrate cell membranes, it is better to enhance the innate clearance capacity of host cells to "kill people with a borrowed knife." Additionally, we locked down the dual role of Tbk1in infection, adding new evidence to understanding how intracellular bacteria delicately balance host inflammatory responses to avoid excessive immunopathological damage [14].

At the level of vaccine antigen design, this study completely abandoned the traditional "sequence alignment" strategy and prospectively introduced the concept of AlphaFold2-based structural vaccinology [15]. The candidate antigen BMEI_RS08405 we screened not only has extremely high folding stability (pLDDT > 90%) but also has its key B-cell epitopes highly exposed on the protein surface. This natural conformational advantage enables it to induce high-quality antibodies that combine high affinity (KD = 0.85 nM) with high neutralizing activity [16].

The core protective mechanism lies in the reprogramming of cellular immunity. Our high-dimensional flow cytometry and scTCR-seq data consistently indicate that BMEI_RS08405 can break conventional immune tolerance and induce high-frequency IFN-γ^{+}TNF-α^{+} double-positive multifunctional T cells (Multifunctional T cells) [17]. Multiple studies in top immunology journals in recent years have repeatedly emphasized that when fighting stubborn intracellular bacterial infections such as tuberculosis and leishmaniasis, multifunctional T cell subsets capable of secreting multiple effector cytokines simultaneously have much stronger bactericidal activity and tissue penetration power than cells secreting single factors [18]. Furthermore, this antigen also prompts the body to establish a highly diverse TCR repertoire (Shannon Index = 6.8). This broad antigen receptor diversity is like equipping the immune system with a full set of "weapon libraries," greatly reducing the risk of immune escape caused by antigenic variation [19].

Limitations and Future Directions:

Although this study achieved exciting breakthroughs in mouse models, the road to translation from rodents to large animals (such as sheep) and even humans still needs to bridge gaps. In the future, we will use humanized mouse models and CRISPR-edited large animal models, combined with Organoid technology, to further confirm the cross-species protective efficacy of BMEI_RS08405 in complex immune systems. Meanwhile, exploring the combination strategy of host-targeted agonists against Sipa1l1 and BMEI_RS08405 is expected to become the ultimate solution for eradicating chronic brucellosis [20, 21].

5. Conclusion

In summary, this study not only mapped the finest in vivo spatial immune atlas of Brucellainfection to date, clarifying the key ecological niches for bacterial hiding and immune escape, but also discovered the key host defense checkpoint Sipa1l1 through genome-wide screening. Based on this, we successfully developed the disruptive candidate antigen BMEI_RS08405 capable of inducing extremely strong Th1-type multifunctional T cell responses using an AI-driven structural biology strategy. This research not only provides a highly translational subunit vaccine candidate target for brucellosis but also sets a new golden paradigm for the rational design of vaccines against other stubborn intracellular bacteria globally.

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