

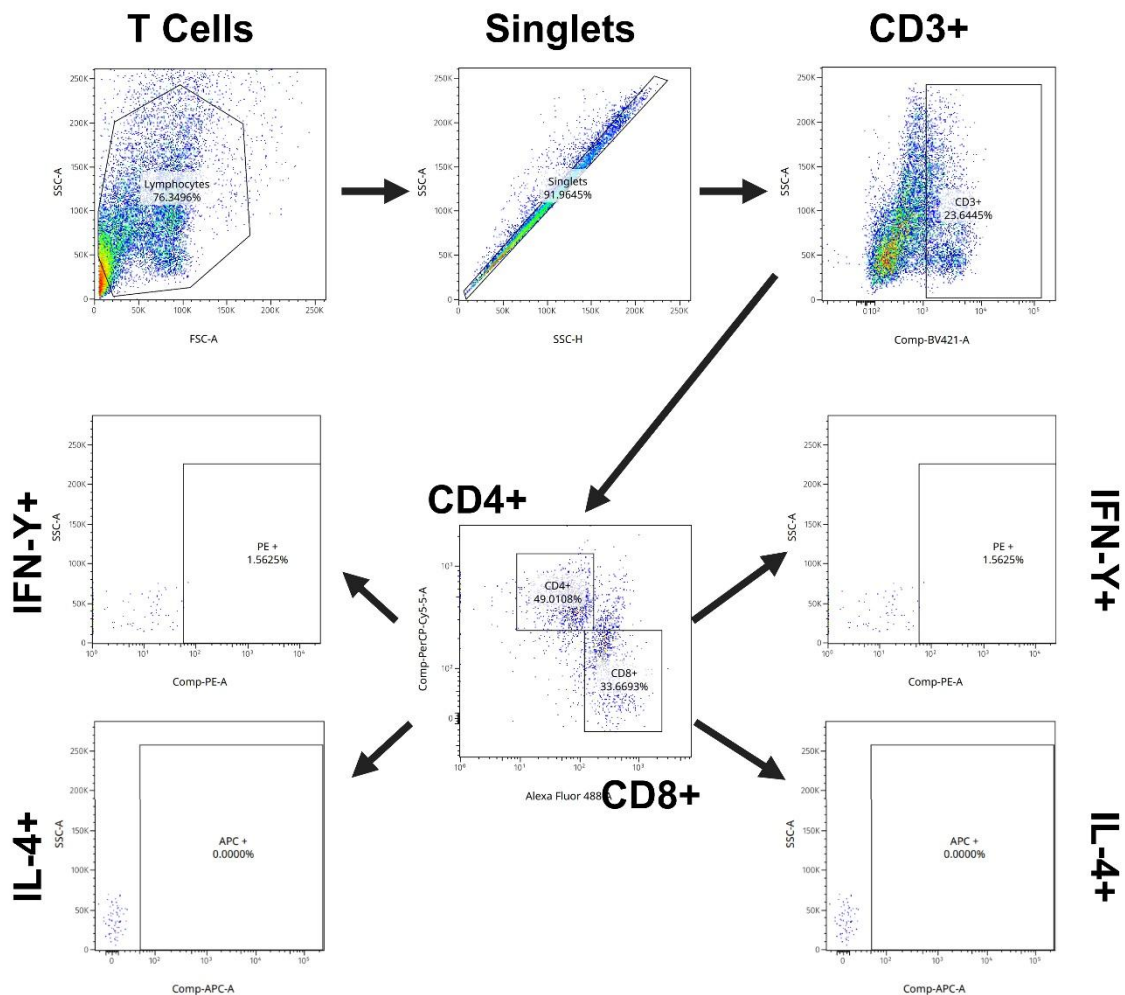


Figure S1. Gating strategy used in FlowJo for the analysis of intracellular cytokine production by flow cytometry. Cells were first gated on the lymphocyte population according to FSC-A and SSC-A parameters. Singlets were then selected to remove doublets and cell aggregates. CD3⁺ cells were identified from the singlet population and further subdivided into CD4⁺ and CD8⁺ T-cell populations. Intracellular IFN- γ ⁺ and IL-4⁺ events were subsequently quantified within each T-cell subset. Representative dot plots show the sequential gating workflow used to define cytokine-producing CD4⁺ and CD8⁺ T cells.

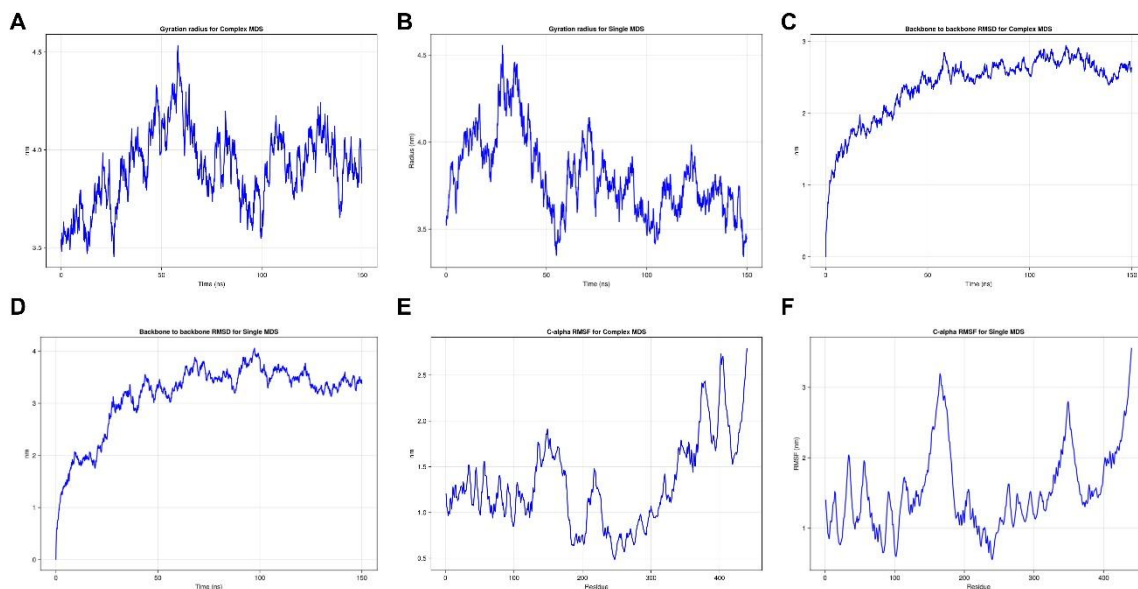


Figure S2. Molecular dynamics simulation analysis of the RecoV-TLR4 complex and RecoV alone. (A) Radius of gyration (Rg) of the RecoV-TLR4 complex during molecular dynamics simulation. (B) Radius of gyration (Rg) of RecoV alone during molecular dynamics simulation. (C) Backbone RMSD of the RecoV-TLR4 complex over the simulation time. (D) Backbone RMSD of RecoV alone over the simulation time. (E) C α RMSF profile of the RecoV-TLR4 complex, showing residue-level flexibility during the simulation. (F) C α RMSF profile of RecoV alone, showing residue-level fluctuations along the sequence.

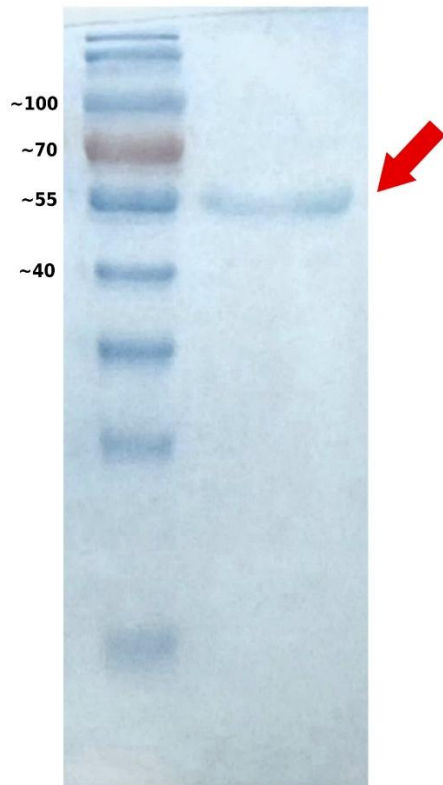


Figure 3. SDS-PAGE analysis of the purified recombinant vaccine construct. The red arrow indicates the main protein band (~50 kDa), consistent with the theoretical molecular weight of 44.4 kDa.

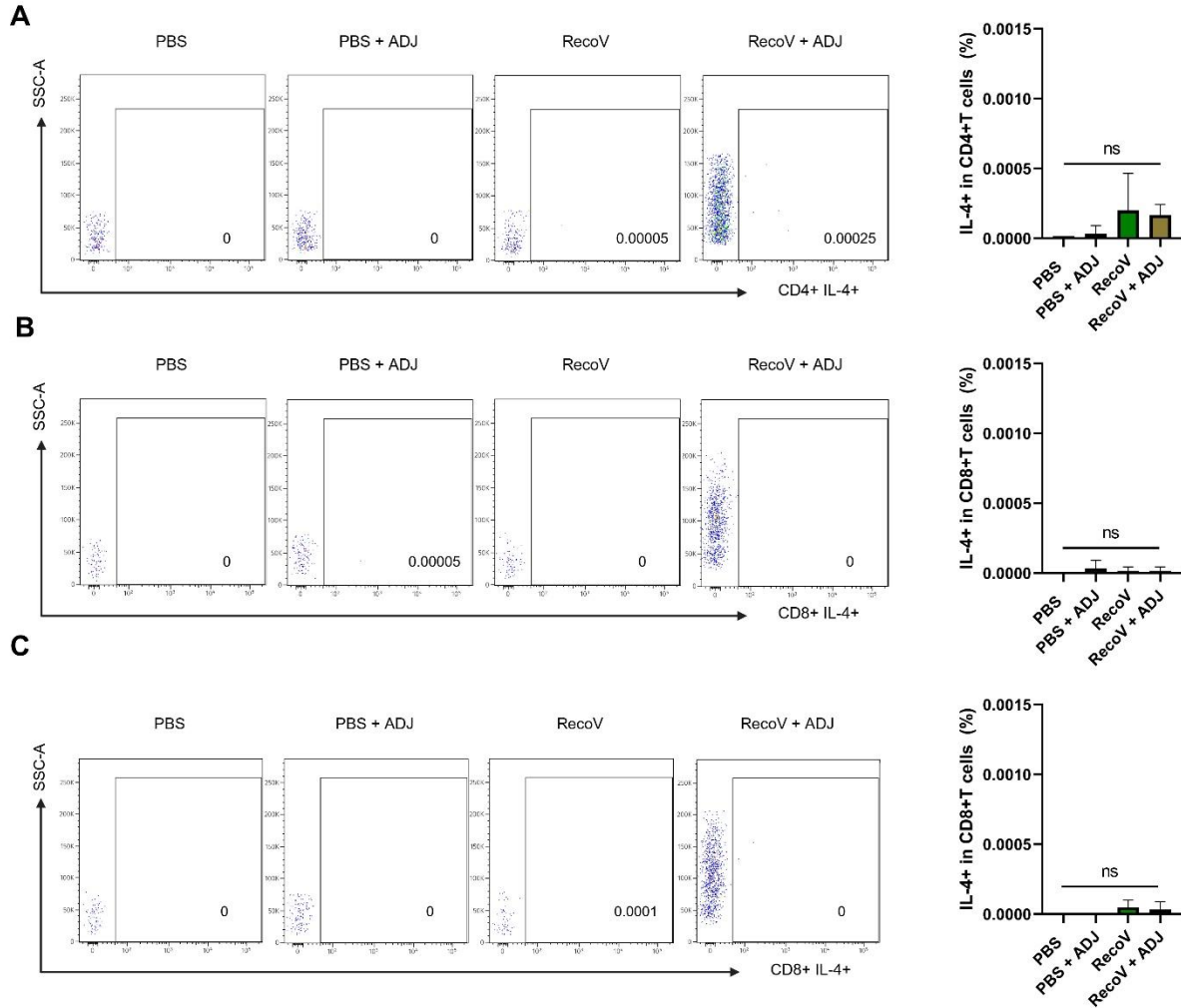


Figure S4. Limited induction of IL-4-producing T cells following RecoV immunization. Representative flow cytometry dot plots and quantification of intracellular IL-4 production in splenic T-cell subsets from immunized BALB/c mice after ex vivo stimulation. (A) Frequency of CD4⁺IL-4⁺ cells after stimulation with RecoV. (B) Frequency of CD8⁺IL-4⁺ cells after stimulation with total *B. abortus* proteins. (C) Frequency of CD8⁺IL-4⁺ cells after stimulation with RecoV. Percentages shown correspond to cytokine-positive cells relative to total acquired events. Data are presented as mean $\pm$ SEM. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple-comparison test; ** $P < 0.01$; ns, not significant.

Table S1: Proteins from *Brucella abortus* 2308 detected in EXOI, based on Álvarez et al., 2025.

Protein ID	Protein Name	Post-infection incubation time (h)	
		8	24
Q2YLI5	ATP synthase subunit alpha	✓	✓
Q2YLE6	ATP synthase subunit beta	✓	✓
Q2YP67	Bacterial extracellular solute-binding protein, family 1	✓	✓
Q2YMJ1	Modulator of DNA gyrase	✓	
Q2YRQ2	Outer membrane protein assembly factor BamA	✓	
Q2YLP2	Chaperone protein ClpB	✓	
Q2YQV2	Chaperone protein DnaK	✓	
Q2YIJ3	Chaperonin GroEL	✓	✓
Q2YIJ2	Co-chaperonin GroES	✓	
Q2YLR6	Outer membrane lipoprotein Omp19	✓	
Q2YMY7	Porin Omp2b	✓	
Q2YQH3	Peptidoglycan-associated lipoprotein	✓	
Q2YIY2	Periplasmic binding protein/LacI transcriptional regulator	✓	
Q2YKV9	Superoxide dismutase [Cu-Zn]	✓	✓
Q2YRD7	Tol-Pal system protein TolB	✓	
Q2YM08	Elongation factor Tu	✓	
Q2YPK5	Invasion protein B homolog BAB1_0368	✓	
Q2YRV0	Isocitrate dehydrogenase [NADP]		✓

Q2YK12	DUF1269 domain-containing protein		✓
Q2YKI4	Bacterioferritin		✓
Q2YK18	Probable acid stress chaperone HdeA		✓
Q2YLS0	Succinate-CoA ligase [ADP-forming] subunit beta		✓

Table S2: Homology analysis of *B. abortus* proteins with host proteomes using BLASTp.

Protein ID	Protein Name	<i>Bos taurus</i>	<i>Mus musculus</i>	<i>Homo sapiens</i>	Selection criterion
Q2YLI5	ATP synthase subunit alpha	71.57%	71.63%	71.77%	Does not meet criteria
Q2YLE6	ATP synthase subunit beta	81.40%	82.55%	77.39%	Does not meet criteria
Q2YP67	Bacterial extracellular solute-binding protein, family 1	No hit	No hit	No hit	Meets criteria
Q2YMJ1	Modulator of DNA gyrase	No hit	No hit	No hit	Meets criteria
Q2YRQ2	Outer membrane protein assembly factor BamA	No hit	No hit	No hit	Meets criteria
Q2YLP2	Chaperone protein ClpB	34.16%	34.93%	34.52%	Meets criteria
Q2YQV2	Chaperone protein DnaK	67.09%	67.26%	67.09%	Does not meet criteria
Q2YIJ3	Chaperonin GroEL	54.05%	54.80%	54.32%	Does not meet criteria

Q2YIJ2	Co-chaperonin GroES	No hit	41.76%	40.66%	Does not meet criteria
Q2YLR6	Outer membrane lipoprotein Omp19	No hit	No hit	No hit	Meets criteria
Q2YMY7	Porin Omp2b	No hit	No hit	No hit	Meets criteria
Q2YQH3	Peptidoglycan-associated lipoprotein	No hit	No hit	No hit	Meets criteria
Q2YIY2	Periplasmic binding protein/LacI transcriptional regulator	No hit	No hit	No hit	Meets criteria
Q2YKV9	Superoxide dismutase [Cu-Zn]	No hit	No hit	No hit	Meets criteria
Q2YRD7	Tol-Pal system protein TolB	No hit	No hit	No hit	Meets criteria
Q2YM08	Elongation factor Tu	58.82%	57.76%	57.36%	Does not meet criteria
Q2YPK5	Invasion protein B homolog BAB1_0368	No hit	No hit	No hit	Meets criteria
Q2YRV0	Isocitrate dehydrogenase [NADP]	70.10%	70.83%	70.59%	Does not meet criteria
Q2YK12	DUF1269 domain-containing protein	No hit	No hit	No hit	Meets criteria
Q2YKI4	Bacterioferritin	No hit	No hit	No hit	Meets criteria
Q2YK18	Probable acid stress chaperone HdeA	No hit	No hit	No hit	Meets criteria
Q2YLS0	Succinate-CoA ligase [ADP-forming] subunit beta	47.54%	46.17%	47.27%	Does not meet criteria

Table S3. Antigenicity prediction of non-homologous *Brucella abortus* proteins.

Protein ID	Protein Name	AntigenPRO	VaxiJen v2.0	Classification
Q2YP67	Bacterial extracellular solute-binding protein, family 1	0.800794	0.4845	Antigenic
Q2YMJ1	Modulator of DNA gyrase	0.583971	0.5745	Antigenic
Q2YRQ2	Outer membrane protein assembly factor BamA	0.78953	0.5762	Antigenic
Q2YLP2	Chaperone protein ClpB	0,308211	0.4342	Non-antigenic
Q2YLR6	Outer membrane lipoprotein Omp19	0.931412	0.6547	Antigenic
Q2YMY7	Porin Omp2b	0.882412	0.6551	Antigenic
Q2YQH3	Peptidoglycan-associated lipoprotein	0.836304	0.55	Antigenic
Q2YIY2	Periplasmic binding protein/LacI transcriptional regulator	0.733967	0.4505	Antigenic
Q2YKV9	Superoxide dismutase [Cu-Zn]	0.895077	0.8047	Antigenic
Q2YRD7	Tol-Pal system protein TolB	0.793408	0.5553	Antigenic
Q2YPK5	Invasion protein B homolog BAB1_0368	0.888563	0.7603	Antigenic
Q2YK12	DUF1269 domain-containing protein	0.405402	0.4933	Non-antigenic
Q2YKI4	Bacterioferritin	0.405402	0.4933	Non-antigenic
Q2YK18	Probable acid stress chaperone HdeA	0.845077	0.8047	Antigenic

Table S4. Physicochemical parameters of the multi-epitope vaccine construct

Parameter ^a	Values
Number of amino acids	440
Molecular weight (Da)	44,418.52
Theoretical pI	5.53
Estimated half-life	30 h (mammalian reticulocytes, <i>in vitro</i>), > 20 h (yeast, <i>in vivo</i>), >10 h (<i>E. coli</i> , <i>in vivo</i>)
Inastibility index	38.18 (classified as stable)
GRAVY (grand average of hydropathicity)	-0.239 (hydrophilic)
Solubility prediction (SolPRO)	0.907794

^a Physicochemical properties were predicted using the ProtParam tool from the ExPASy server. Solubility prediction was performed using the SolPro server.

Table S5: Estimated binding-free energy for the vaccine construct–TLR4 complex.

Energy contributions	Vaccine construct–TLR4 complex (kcal/mol)
Van de Waals energy	-8661.79
Electrostatic energy	-81770.88
Gas phase energy	-12274.46
Solvation energy	-15874.47