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ALFENAS/MG

2021

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Dissertação apresentada como parte dos requisitos exigidos para obtenção do título de Mestre pelo Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Alfenas - MG. Área de Concentração: Biologia Celular, Molecular e Estrutural das Doenças Agudas e Crônicas.

Orientador: Prof. Dr. Leonardo Augusto de Almeida

ALFENAS/MG

2021

Dados Internacionais de Catalogação-na-Publicação (CIP)
Sistema de Bibliotecas da Universidade Federal de Alfenas
Biblioteca Central – Campus Sede

Oliveira, Karen Cristina
O48e Epitope-based vaccine of a *Brucella abortus* putative small rna target induces protection and less tissue damage in mice. / Karen Cristina Oliveira – Alfenas, MG, 2021.
[51] f.: il. –

Orientador: Leonardo Augusto de Almeida.
Dissertação (Mestrado em Ciências Biológicas) – Universidade Federal de Alfenas, 2021.
Bibliografia.

1. *Brucella abortus*. 2. Vacina. 3. Resposta imune. 4. Vacinologia reversa. 5. Brucelose. 6. Apolipoproteína N-aciltransferase. I. Almeida, Leonardo Augusto de. II. Título.

CDD- 570

Ficha Catalográfica elaborada por Marlom Cesar da Silva
Bibliotecário-Documentalista CRB6/2735

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Aprovada em: 23 de setembro de 2021.

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Documento assinado eletronicamente por **Leonardo Augusto de Almeida**, **Professor do Magistério Superior**, em 24/09/2021, às 10:22, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

Documento assinado eletronicamente por **Fabio Antonio Colombo**, **Professor do Magistério Superior**, em 24/09/2021, às 13:28, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

Documento assinado eletronicamente por **Gilson Costa Macedo**, **Usuário Externo**, em 27/09/2021, às 12:04, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

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Dedico este trabalho à minha mãe, meus irmãos Karla e Cadú e à vovó Nilza (*in memorian*).

AGRADECIMENTOS

À Deus, pela saúde, força, persistência e resiliência concedidas a mim durante esse período para que fosse possível a conclusão mais uma etapa!

Ao professor Leonardo Augusto de Almeida pela orientação e profissionalismo. Agradeço pela paciência e confiança, contribuindo para com o meu amadurecimento, enriquecimento e crescimento profissional, despertando ainda mais o meu interesse pela pesquisa. Obrigada por toda a dedicação, pelos ensinamentos e por me acolher como sua orientada.

Aos amigos do Labiomol, em especial á Natália, Thiago, Ana, Bianca, Jéssica, Caio e Gustavo, por todos os momentos que compartilhamos (inclusive a parceria nas exprimentações e as fugidas pra ir na Don'ana rsrs), fazendo com que os dias se tornassem mais alegres, leves e divertidos. Obrigada por todo o apoio, colaboração, amizade e risadas. Levarei vocês no coração pro resto da vida!

À professora Patrícia Paiva Corsetti e seus orientados Evandro, Thaís e Ana Maria, pelas trocas de conhecimentos e por terem se tornado meus amigos.

Ao Programa de Pós-Graduação em Ciências Biológicas, professores - por todo o conhecimento transmitido, Martha - pela convivência nas disciplinas e durante as reuniões do colegiado e todos os técnicos e funcionários do Departamento de Microbiologia e Imunologia da Unifal: obrigada a todos vocês.

Aos meus familiares, em especial minha Mãe Joelma, meu pai Carlos e meus irmãos Karla e Cadu pelo amor, carinho, incentivo e principalmente por acreditarem no meu sonho.

Ao meu namorado Thiago pela paciência, incentivo, carinho e amor. Obrigada por aturar minhas reclamações, estresses, e principalmente por ser a pessoa que mais acreditou em mim e nos meus sonhos (sonhando e caminhando sempre ao meu lado).

Aos meus quatro melhores amigos da vida, Weverton, Daniel, Fernanda e Taís por estarem sempre do meu lado, fortalecendo minha base e acreditando nos meus sonhos.

À Universidade Federal de Alfenas e ao Programa de Pós-Graduação em Ciências Biológicas pela oportunidade e pelo meu crescimento acadêmico e à todos que contribuíram direta ou indiretamente com o desenvolvimento deste trabalho.

Agradeço, de antemão, a disponibilidade de todos que compuseram minha banca de defesa da dissertação: Prof. Dr. Gilson Costa Macedo, Prof. Dr. Fábio Antônio

Colombo, Prof Dr. Eduardo de Figueiredo Peloso, Prof. Dra. Valéria Quintana Cavicchioli e, novamente, ao Prof. Dr. Leonardo Augusto de Almeida.

À CAPES pela concessão da bolsa de estudos.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) – Código de Financiamento 001.

“Vocês jovens, doutores, cientistas do futuro, não se deixem abalar por um ceticismo estéril, nem se deixem desencorajar pela tristeza de certas horas que as nações passam.”

(Louis Pasteur, 1865)

RESUMO

Brucella abortus é uma bactéria intracelular Gram-negativa que causa uma doença zoonótica chamada brucelose. Emboras as vacinas atualmente disponíveis para a imunização animal possuam potencial imunogênico, essas ainda apresentam muitas desvantagens, causando abortos de grande proporção em fêmeas prenhas e febre ondulante em humanos. Neste contexto, a recente tendência no projeto de novas vacinas contra brucelose, têm se baseado na estratégia de predição de epítopos imunogênicos selecionados por vacinologia reversa. Sendo assim, o objetivo deste estudo foi identificar e avaliar a imunogenecidade de um epítopo vacinal alvo de putativos pequenos RNAs de *B. abortus* mediante a infecção desta bactéria em modelo murino. Foi demonstrado nesse trabalho que pequenos RNAs de *B. abortus* são expressos durante a infecção precoce de macrófagos derivados da medula óssea (BMDMs), sendo identificado uma apolipoproteína N-aciltransferase (Int) como o putativo alvo de maior expressão dos pequenos RNAs. Visto que a apoliproteína N-aciltransferase apresenta diminuição da sua expressão em modelo de BMDMs infectados, um epítopo desta proteína foi racionalmente selecionado por imunoinformática e explorado como candidato a vacinação contra brucelose. Camundongos C57BL/6 imunizados e desafiados com *B. abortus* mostraram menor recuperação no número de bactérias viáveis no fígado, baço e linfonodo axilar quando comparados a camundongos não vacinados. Os camundongos vacinados e infectados apresentaram aumento na expressão de TNF-α , IFN-γ e IL-6, seguido do também aumento na expressão dos genes anti-inflamatórios IL-10 e TGF-β no fígado, justificando a redução no número e tamanho dos granulomas observados. BMDMs estimulados com sobrenadante de esplenócitos de camundongos vacinados e infectados apresentaram marcação para CD86+ mais intensa que os demais estímulos, além de expressarem maior quantidade de iNOS e consequente aumento na produção de NO, sugerindo aumento na capacidade fagocítica e microbicida dessas células em eliminar a bactéria. Em conjunto, os resultados demonstraram que o peptídeo vacinal foi capaz de estimular uma resposta imune protetora em organismos infectados com características sugestivas de predominência do perfil Th1.

Palavras-chave: *Brucella abortus*; Vacina; Resposta imune; Vacinologia reversa; Brucelose; Apolipoproteína N-aciltransferase.

ABSTRACT

Brucella abortus is a Gram-negative intracellular bacterium that causes a zoonotic disease called brucellosis. Although currently available vaccines for animal immunization have immunogenic potential, they still have many disadvantages, causing large-scale abortions in pregnant females and undulating fever in humans. In this context, the recent trend in the design of new vaccines against brucellosis has been based on the strategy of prediction of immunogenic epitopes selected by reverse vaccinology. Therefore, this study aimed to identify and evaluate the immunogenicity of a target vaccine epitope of putative small RNAs of *B. abortus* upon infection of this bacterium in a murine model. It was demonstrated in this work that small RNAs of *B. abortus* are expressed during the early infection of bone marrow-derived macrophages (BMDMs), and an apolipoprotein N-acyltransferase (Int) was identified as the putative target of higher expression of small RNAs. Since the apolipoprotein N-acyltransferase has decreased expression in a model of infected BMDMs, an epitope of this protein was rationally selected by immunoinformatics and explored as a candidate for vaccination against brucellosis. C57BL/6 mice immunized and challenged with *B. abortus* showed lower recovery in the number of viable bacteria in the liver, spleen, and axillary lymph node when compared to non-vaccinated mice. The vaccinated and infected mice showed an increase in the expression of TNF-α, IFN-γ, and IL-6, followed by an increase in the expression of the anti-inflammatory genes IL-10 and TGF-β in the liver, justifying the reduction in number and size of the observed granulomas. BMDMs stimulated with supernatant from splenocytes from vaccinated and infected mice showed more intense CD86+ marking than the other stimuli, in addition to expressing a greater amount of iNOS and consequent increase in NO production, suggesting an increase in the phagocytic and microbicidal capacity of these cells to eliminate the bacteria. Together, the results demonstrated that the vaccine peptide was able to stimulate a protective immune response in infected organisms with characteristics suggestive of a predominance of the Th1 profile.

Keywords: *Brucella abortus*; vaccine; immune response; reverse vaccinology; brucellosis; apolipoprotein N-acyltransferase.

LISTA DE ABREVIATURAS E SIGLAS

BMDM	Bone Marrow-Derived Macrophages
CEUA	Committee on the Ethics of Animal Experiments
CFU	Colony Forming Units
FBS	Fetal Bovine Serum
HLA	Human Leukocyte Antigen
IEDB	Immune Epitope Database
IL	Interleukin
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
NCBI	National Center for Biotechnology Information
NO	Nitric Oxid
PBS	Phosphate-buffered saline
RV	Reverse vaccinology
SLC	Subcellular localization
TGF	Transforming Growth Factor
TNF	Tumor necrosis factor

SUMÁRIO

1 INTRODUÇÃO

O gênero *Brucella* compreende bactérias que apresentam o formato de cocobacilos Gram-negativos, imóveis, não esporogênicos e intracelulares facultativos (TANA *et al*., 2021). Estas bactérias são responsáveis por causar a brucelose, uma zoonose negligenciada amplamente distribuída no mundo, em que qualquer órgão ou tecido do organismo pode ser acometido com incidência variável (JAMIL *et al*., 2020; TIAN *et al*., 2020). Apesar dos esforços feitos para controlar a doença em muitos países (ELFAKI *et al*., 2015), as transmissões dadas por *Brucella* persistem em animais domésticos e, consequentemente, infecções ocorrem frequentemente em humanos. As bactérias do gênero *Brucella* exibem características antigênicas e metabólicas distintas, e são classificadas de acordo com sua preferência pelo hospedeiro animal. Este gênero, atualmente, abrange doze espécies isoladas e nomeadas (HULL, SCHUMAKER, 2018), dentre as quais *B. melitensis*, *B. abortus* e *B. suis* são os principais agentes etiológicos da brucelose humana (EL-SAYED, AWAD, 2018), sendo a *B. melitensis* a mais patogênica e a *B. abortus* a mais difundida no mundo, uma vez que a doença atinge anualmente cerca de 500 mil pessoas em todo o mundo (GŁOWACKA *et al*., 2018). *B. abortus* têm como hospedeiro preferencial os bovinos e mensura-se que 300 milhões das 1,4 bilhões cabeças de gado do mundo estejam infectadas com *B. abortus* (ELFAKI *et al*., 2015; O'CALLAGHAN, 2020).

As principais formas de transmissão de *B. abortus* para os homens é a partir da ingestão de lácteos contaminados não submetidos ao processo de pasteurização (LINDAHL-RAJALA *et al*., 2017), carne crua proveniente de animais infectados (CASALINUOVO *et al*., 2016), pela inalação de aerossóis que contêm o patógeno. Já, entre os animais a contaminação se dá por contato direto com tecidos animais infectados e suas secreções, e pelo hábito dos bovinos de lamber e cheirar animais recém-nascidos ou mesmo fetos abortados (POESTER *et al*., 2013). A brucelose é uma doença sistêmica na qual qualquer órgão ou tecido do organismo pode estar envolvido. Nos animais a brucelose é uma infecção crônica, que pode persistir por toda a vida do hospedeiro. Nas fêmeas, *Brucella* possui tropismo pelo hormônio placentário bovino, o eritritol, levando a lesões nas glândulas uterinas enquanto que, nos machos, a bactéria possui tropismo por hormônios masculinos, como a testosterona, dirigindo-se aos testículos (DORNELES *et al*., 2015; GUTIÉRREZ-JIMÉNEZ *et al*., 2018). Assim, a infecção por *B. abortus* afeta principalmente os órgãos reprodutivos causando aborto e infertilidade. A brucelose

humana raramente é fatal, porém é uma doença severamente debilitante e incapacitante que apresenta tendência à cronicidade e persistência (BYNDLOSS, TSOLIS, 2016; HASANJANI, EBRAHIMPOUR, 2015).

Por ser um microrganismo intracelular com tropismo principalmente por células do sistema mononuclear fagocitário, a resposta à *Brucella* spp. envolve toda a gama do sistema imunológico, desde a imunidade inata à imunidade adaptativa. A resposta protetora eficaz contra a infecção por *B. abortus* requer a ativação de linfócitos TCD4+ e TCD8+, produção de citocinas e produtos do perfil de resposta Th1 característico contra bactérias intracelulares, como IFN-γ e TNF-α, além da ativação de mácrofagos e células dendríticas (JEZI *et al*., 2019). Considerando os mecanismos de virulência de *B. abortu*s, aliados ao fato da bactéria estar localizada intracelularmente, a antibioticoterapia torna-se particularmente pouco eficaz nesses casos (GLOWACKA *et al*., 2018). Levando em consideração tais aspectos, a vacinação dos animais caracteriza a principal medida preventiva com capacidade de conferir proteção efetiva e duradoura contra a infecção por *B. abortus* (CARVALHO *et al*., 2020).

No mercado veterinário atual, as cepas S19 (linhagem lisa) e RB51 (linhagem rugosa) têm sido usadas para controlar brucelose bovina em todo o mundo. No entanto, ambas cepas apresentam riscos potenciais para os animais e humanos (DE OLIVEIRA *et al*., 2021). Nos animais, a vacinação utilizando as cepas atenuadas vivas S19 e RB51 pode ocasionar a reversão de virulência, provocando abortos, artropatias, orquite e infertilidade, além de induzir a produção de anticorpos, o que interfere no diagnóstico sorológico da doença (GRUPTA *et al*., 2020; LALSIAMTHARA, LEE, 2017). Ademais, ambas as vacinas são secretadas no leite e podem infectar humanos em contato direto e causar abortos em animais grávidas (FRANC *et al*., 2018). Para humanos, a exposição acidental às vacinas contra brucelose caracteriza uma importante fonte de contaminação, uma vez que essas são patogênicas, podendo causar diversas morbidades, dentre elas: febre ondulante provocada devido a episódios de bacteremia seguido por novo foco de infecção, artrite, endocardite, osteomielite e complicações neurológicas (BALDI, GIAMBARTOLOMEI, 2013; FRANC *et al*., 2018). Até então, não há nenhuma vacina destinada a imunização humana contra brucelose, apenas animal.

Atualmente, objetivando desenvolver uma vacina segura e melhorada contra a brucelose, numerosos estudos tem sido realizados para entender os mecanismos de imunidade protetora de *Brucella* no modelo murino (CARVALHO *et al*., 2016; GHASEMI *et al*., 2014) e também nos hospedeiros naturais (NOL *et al*., 2016; OLSEN

et al., 2015). Nesse contexto, a vacinologia reversa tem sido utilizada com uma importante ferramenta moderna capaz de predizer *in silico* antígenos de *Brucella* com propriedades imunogênicas, capazes de promover resposta humoral e celular com produção de citocinas de resposta Th1, Th2 e Th17 (GOMEZ *et al*., 2013), e permitindo o estudo de novas vacinas de *Brucella* (VISHNU *et al*., 2015). A aquisição de conhecimentos genômicos, proteômicos, tecnologias em vacina e técnicas de DNA recombinante tem possibilitado um número crescente de pesquisas com vacinas mais seguras (ESCALONA *et al*., 2017), entretanto, apenas alguns antígenos isolados demonstram imunidade protetora importante *in vivo* (ABKAR *et al*., 2015; JAIN *et al*., 2014). Sabe-se que as respostas imunológicas do tipo Th1 contra a infecção de *Brucella* são melhores estimuladas por vacinas de cepas vivas atenuadas ou cepas mutantes (CARVALHO *et al*., 2016). Embora a vacinologia reversa seja um método ainda não aplicado em vacinais comerciais contra *Brucella* spp., a mesma provou ser útil na identificação de antígenos protetores contra outra patógens importantes, por exemplo, o meningococo do sorogrupo B (PIZZA *et al*., 2000), caracterizando a eficácia dessa ferramenta.

Dada a relevância da bactéria *B. abortus* sendo causadora de uma zoonose de importânica médico-sanitária e estimulados pela necessidade de novas estratégia vacinais mais seguras, o objetivo deste trabalho foi identificar e avaliar a imunogenecidade de um epítopo vacinal alvo de putativos pequenos RNAs de *B. abortus* mediante a infecção desta bactéria em modelo murino.

CAPÍTULO 1

ARTIGO: Epitope-based vaccine of a *Brucella abortus* putative small RNA target induces protection and less tissue damage in mice

Epitope-based vaccine of a *Brucella abortus* putative small RNA target induces protection and less tissue damage in mice

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Abstract

Brucella spp. are Gram-negative, facultative intracellular bacteria that cause brucellosis in humans and animals. Currently available live attenuated vaccines against brucellosis still have drawbacks. Therefore, subunit vaccines, produced using epitope-based antigens, have the advantage of being safe, cost-effective and efficacious. Here, we identified *B. abortus* small RNAs expressed during early infection with bone marrow-derived macrophages (BMDMs) and an apolipoprotein N-acyltransferase (Int) was identified as the putative target of the greatest expressed small RNA. Decreased expression of Int was observed during BMDM infection and the protein sequence was evaluated to rationally select a putative immunogenic epitope by immunoinformatic, which was explored as a vaccinal candidate. C57BL/6 mice were immunized and challenged with *B. abortus*, showing lower recovery in the number of viable bacteria in the liver, spleen, and axillary lymph node when compared to non-vaccinated mice. The vaccinated and infected mice showed the increased expression of *TNF-α*, *IFN-γ*, and IL-6 following expression of the anti-inflammatory genes *IL-10* and *TGF-β* in the liver, justifying the reduction in the number and size of the observed granulomas. BMDMs stimulated with splenocyte supernatants from vaccinated and infected mice increase the CD86+ marker, as well as expressing greater amounts of iNOS and the consequent increase in NO production, suggesting an increase in the phagocytic and microbicidal capacity of these cells to eliminate the bacteria.

Keywords: *Brucella abortus*; vaccine; immune response; reverse vaccinology; brucellosis; apolipoprotein N-acyltransferase

1 Introduction

Brucellosis is a global zoonotic infectious disease caused by bacteria of the genus *Brucella*. The disease is a serious public health threat worldwide, particularly in developing countries of Central Asia, Africa, South America, and the Mediterranean region (1). Brucellosis affects mammals, causing abortion and infertility in affected animals. This infection can spread from animals to humans, mainly via the ingestion of unpasteurized milk or dairy products and, to a lesser extent, via direct contact with infected animals (2). In humans, brucellosis can cause a severe febrile disease with various clinical complications ranging from mild to severe symptoms including undulant fever, joint pain arthritis, endocarditis, and meningitis (3-5). The genus *Brucella* includes Gram-negative facultative intracellular bacteria from Alphaproteobacteria, and, currently, the genus consists of 12 species that are classified based on their host preferences (6). Although several *Brucella* species are potentially zoonotic agents, *Brucella melitensis*, *Brucella abortus*, and *Brucella suis* are considered the most pathogenic *Brucella* species that have a serious impact on public health and the livestock industry (7,8), with *B. abortus* being the most widespread throughout the world, according to the World Health Organization (WHO) (9,10). Since brucellosis is the most common zoonotic disease worldwide and has become a serious concern in recent years (11), the strategy used to control brucellosis depends mainly on the massive vaccination of domestic animals to prevent the disease from spreading to healthy animals and humans (12,13). Almost all vaccines against *Brucella* spp. are live attenuated strains with extensive global use but with various drawbacks, such as pathogenicity to humans and residual virulence in animals, which can cause abortion, orchitis, and infertility (14-16). Moreover, it is difficult to differentiate infected animals from vaccinated animals by serological tests. These drawbacks have prompted several research groups to attempt the development of safer vaccines.

Subunit vaccines have promising applications with the advantage of being safe, cost-effective, and efficacious. During the past two decades, various antigens have been extracted from *Brucella*, such as Omp19, Omp25, L7/L12, P39, SodC, InpB, AsnC, and TF (17-24). These available antigens have been shown to provide protection against *Brucella* infection by reducing the organ's bacterial load in mice. While such findings are highly promising, subunit vaccines using known antigens cannot provide the levels of protection conferred by live attenuated vaccines (25). Further investigation is needed to identify novel antigens, and increase vaccine efficacy. In contrast to the conventional vaccine development that requires cultivation and extensive empirical screening, reverse vaccinology (RV) is an interesting *in silico* approach to identify protective antigens using pathogen genomic data (26-29). RV has been implemented to identify protective antigens of numerous pathogens, including *B. abortus* (30-32).

The main goal of this study was to screen potential antigens in the genome of *B. abortus* using RV as a search strategy and subsequently evaluating the immunogenic capacity of the peptide in an animal model. We used an *in silico* methodology to select epitopes candidates based on their biological characteristics strongly associated with protective antigenicity from putative targets of small RNAs expressed in infected BMDMs. From these predictions, a transmembrane epitope of apolipoprotein N-acyltransferase was selected for efficacy verification in a mouse model showing promising results to be used as an epitope-based vaccine against brucellosis that may induce robust immunity against the bacterium.

2 Material and methods

2.1 Ethics statement

This study was carried in strict accordance with the Brazilian laws 6638 and 9605 in Animal Experimentation. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Federal University of Alfenas (CEUA 16/2020).

2.2 Mice, cell culture and bacteria

The strain C57BL/6 mice aged 6–8 weeks were purchased from the Federal University of Minas Gerais animal facility (UFMG, Belo Horizonte, Brazil). Bone marrow cells were obtained from femora and tibia of mice and they were grown in bone marrow-derived macrophages (BMDMs) as

previously described by our group [33]. *B. abortus* virulent strain 2308 was obtained from our own laboratory collection. They were grown in *Brucella* broth medium (BD Pharmingen, San Diego, CA, USA) for 3 days at 37°C.

2.3 BMDM infection with *B. abortus*

BMDMs were infected with virulent *B. abortus* strain 2308 at a multiplicity of infection of 100:1. Bacteria were centrifuged onto macrophages at 400 × g for 10 min at 4°C and then cells were incubated for 30 min at 37°C under 5% CO_2. Macrophages were extensively washed with HBSS to remove extracellular bacteria and incubated for an additional 90 min in medium supplemented with 100 µg/mL gentamycin to kill extracellular bacteria. Thereafter, the antibiotic concentration was decreased to 10 µg/mL. Thirty minutes after infection, BMDMs were washed three times with HBSS before processing following homogenization with 100µl of LS TRIzol® reagent Invitrogen (Waltham, Massachusetts, EUA) for total RNA isolation.

2.4 Small RNA sequencing and bioinformatics identification

The construction and sequencing of a strand-specific small RNA (15–50 nt) library was conducted by FASTERIS SA (Plan-les-Ouates, Switzerland), based on the Illumina® TruSeq® Small RNA Library Prep Kit for Illumina HiSeq 2000 sequencing (Illumina Inc., San Diego, CA, USA). To remove adapter sequences from small RNA raw reads of sequencing, the Cutadapt tool was used [34] and sequencing quality was analyzed using the Trimmomatic V0.32 tool (35). Reads were mapped to the *B. melitensis* biovar *Abortus* (strain 2308) genome using the Bowtie program (36) to report the best alignment for each read allowing a maximum of one replacement per alignment. Using BedTool, reads mapped at alignment were analyzed to determine the depth of coverage across the genome determining the hotspots areas of sRNAs (37). Coverage with more than 50 reads and areas with a distance of less than 50bp were joined as the same hotspot area.

2.5 Bioinformatic and reverse vaccinology

To assess the putative targets of those small RNAs highly expressed by *B. abortus* during infection, one hundred coding genes were obtained using the intersect BedTool to indicate the overlap between small RNAs and coding genes (37). As the overlaps showed the possibility of affecting more than 50% of the *B. abortus* coding genes, which genes contain the hottest hotspots (i.e., with the highest average coverage) was evaluated. The proteins from the putative target coding genes were filtered by assigning selection criteria according to biological characteristics: (1) subcellular location (SCL) using Psortb v3.0.2 program (38), (2) the presence of a signal peptide using SignalP 5.0 Server tool (39), and (3) the presence of transmembrane helices and exposed regions using three tools: TMPRED, SOSUI 1.11, and TMHMM 2.0 (40-42). The obtained proteins were then analyzed for biological function using an online tool UniProt Knowledgebase (UniProtKB) and only one of the 7 was selected [43]. The selected protein apolipoprotein N-acyltransferase (WP_002965220.1) was used to identify epitopes composed of fifteen amino acids present in the extracellular portion of Int. The extracellular portion of the surface-associated target protein was subjected to the sequential mapping of epitopes predicted to bind tightly to major histocompatibility complex (MCH) class II, using the tools NetMHCII 2.2, SYFPEITHI, and RANKPEP (44-46). To obtain the best candidates of putative immunogenic epitopes from Int, using Multalin 5.4, the obtained epitopes were aligned, and the linear amino acid sequence including approximately 15 amino acids that presented a repeats sequence was selected (47). For the epitope antigenicity test, the VaxiJen 2.0 server was used with the 0.5 and “probable antigen” cutoff (48). For the allergenicity test, the server “AlgPred: Prediction of Allergenic Proteins and Mapping of IgE Epitopes” was used with the hybrid method that consists of using the following five tools available on the server: SVMc, IgE, epitope, ARPs BLAST, and MAST (49). For the epitope similarity analysis, the Protein BLAST tool available on the NCBI platform was used (50). For the physical and chemical properties test, the ExPASy server ProtParam tool was used with a cutoff of 40 for structure stability values (51). The pipeline of bioinformatic analysis is depicted and summarized in Figure 1.

2.6 Real-time RT-PCR for apolipoprotein N-acyltransferase expression

Thirty-minute *B. abortus*-infected BMDMs or the exponential growth of *B. abortus* in *Brucella* broth medium total RNA were extracted using TRIzol reagent (Invitrogen). Reverse-transcription of 100 ng from total RNA was performed using random primers according to the Illustra™ Ready-To-Go RT-PCR Beads kit (GE Healthcare, Buckinghamshire, UK). Real-time RT-PCR was conducted in a final volume of 10 µL containing the following: SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), with cDNA as the PCR template and primers to amplify specific fragments corresponding to specific gene targets: IntF: 5`-CTGATGTGATTGTCTGGCCG-3`, IntR: 5`-CCTGAGGTGTCGATTCCAGT-3`, *Brucella* 16S F: 5`- TCTCACGACACGAGCTGACG -3`, *Brucella* 16S R: 5`- CCTGAGGTGTCGATTCCAGT -3`. The PCR reaction was performed using the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA), with the following cycling parameters: 60°C for 10 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min, and a dissociation stage of 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec, and 60°C for 15 sec. All data are presented as relative expression units after normalization to the *Brucella* 16S gene. PCR measurements were conducted in triplicate. The differences in the relative expression were analyzed by Student's *t* test with a two-tailed distribution ($p < 0.05$ indicates statistical significance).

2.7 Int structural modeling and epitope-MHCII docking

The Int protein from *Brucella* was retrieved via the NCBI database, followed by molecular modeling by homology via WebServer Phyre², which uses the Markov model for the best possible global alignments to generate the most accurate protein in its main function (52). After modeling, the epitope under investigation from the protein was obtained, and the preparation was performed using the MGL tool, in which missing atoms were corrected and water molecules were removed (53). The same preparation was performed for MHCII molecules found on the PDB server (Protein Data Bank) (54). For docking execution, both files were converted to the PDBQT format required by AutoDock Vina (55), using the Openbabel tool (56). The gridbox was generated around the active sites of the recovered MHCII proteins. To visualize the interactions between epitope-MHCII in 2D and 3D diagram format, the Ligplot+, and Pymol tools were used, respectively (57,58).

2.8 Mice experiments

To assess the ability of vaccinal peptide to induce immune responses, 6–8-week-old C57BL/6 mice were contained in cages on a 12:12 light/dark cycle and fed *ad libitum* with standard rodent diet and no water restrictions. Mice were randomly separated into four treatment groups ($n = 5$): (I) vaccinated and infected with *B. abortus*, (II) vaccinated and non-infected with *B. abortus*, (III) non-vaccinated and infected with *B. abortus*, and (IV) non-vaccinated and non-infected with *B. abortus*. Mice were immunized intraperitoneally with a prime and two boosts (0, 7, 14 days) of vaccine formulation containing 10 µg of peptide in phosphate-buffered saline (PBS), combined with complete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO, USA) at day 0 and incomplete Freund's adjuvant at days 7 and 14. On day 21, mice were infected intraperitoneally with *B. abortus* at a dose of $1x10^6$ CFU/animal. Bacterial loads in the spleen, liver, and axillary lymph node from individual animals were homogenized in PBS, serially diluted 10-fold, and plated on *Brucella* broth agar (Difco, BD-Pharmingen, San Diego, CA). Plates were incubated at 37°C, and the CFU were counted after 3 days as previously described (59). The experimental design is represented in Figure 2.

2.9 Histopathology and immunohistochemistry assays

The medial lobes of the mice liver were collected, fixed in 10% buffered formaldehyde solution, dehydrated, diaphanized, and embedded in paraffin. Four-micrometer-thick tissue sections were stained with hematoxylin and eosin (H&E). Digital images were captured and digitized with the AxioVision LE software (Carl Zeiss, Oberkochen, Germany); all of the sample fields were photographed for histopathological evaluation. The histopathological changes were analyzed by Image Pro-PlusR 4.5 software (Media Cybernetics Inc., Silver Spring, MD, USA). The total number and size of granulomas present in histological liver sections was determined using an Axiophot

microscope (Carl Zeiss, Oberkochen, Germany) with a 40x objective lens. Immunohistochemistry was performed as previously described (60). Briefly, liver sections were hydrated and incubated with 10% hydrogen peroxide in PBS for 30 min. After being washed with PBS, slides were transferred to a humid chamber at room temperature, incubated with 25 mg/ml of skim milk for 45 min, and then incubated with a primary antibody for 30 min. For immunolabeling, diluted (1:5,000) serum from a rabbit experimentally inoculated with *B. abortus* S19 strain was used as polyclonal anti-*B. abortus* antibody. Then, tissue sections were washed with PBS, incubated with secondary antibody for 20 min, washed again with PBS, and incubated for 20 min with streptavidin-peroxidase from a commercial kit (LSAB + kit; Dako Corporation, Carpinteria, CA). The reaction was revealed using 0.024% diaminobenzidine (DAB; Sigma), and sections were counterstained with Mayer's hematoxylin.

2.10 Measurement of NO into splenocyte culture supernatants

Spleens cells from C57BL/6 mice under treatment obtained after maceration were treated with ACK buffer (0.15 M NH4Cl, 1.0 mM KHCO3, 0.1 mM Na2EDTA, pH 7.2) to lyse red blood cells. After that, the cells were washed with saline (NaCl 0.8%, wt/vol) and suspended in RPMI 1640 (Gibco, Carlsbad, Calif) supplemented with 2 mM L-Glutamine, 25 mM HEPES, 10% (vol/vol) heat-inactivated FBS (Gibco, Carlsbad, Calif), penicillin G sodium (100 U/mL), and streptomycin sulfate (100 µg/mL). *Spleen* cells ($1x10^6$) were cultured in 200µL *culture* medium and incubated at 37°C with 5% CO_2. The supernatant of splenocyte cultures was collected after 24h and nitric oxide (NO) measurement was performed according to the Griess method (61).

2.11 Analysis of surface markers CD86 and CD11b by fluorescence microscopic

BMDMs were plated on imaging slides (µ-Slide 12-well, glass bottom, Ibidi GmbH, Munich, Germany), followed by stimulation with splenocytes supernatant. The cells were then washed three times with PBS and incubated with the anti-CD16/32 antibody (BD Biosciences, San Jose, CA) for 2 hours to block nonspecific bonds. The cells were then incubated with anti-CD86 and anti-CD11b, followed by staining with FITC-conjugated and PE-conjugated (BD Biosciences), respectively, overnight at 4°C. The slides were washed with PBS and the nuclei were stained with 150 ng/mL 40,6-diamino-2-phenylindole (DAPI; Thermo Scientific) for 1 hour. All images were captured using a Nikon Eclipse 80i fluorescence microscope (Melville, New York, U.S.A). Image J software was used to analyze the markings obtained for the nucleus (blue fluorescence), CD11b+ cells (green fluorescence), and CD86+ cells (red fluorescence).

2.12 Real-time RT-PCR for pro and anti-inflammatory cytokines expression

Liver and spleen macerate as well as BMDMs stimulated with splenocytes supernatant from the four experimental groups were homogenized with TRIzol reagent (Invitrogen) to isolate total RNA. Reverse-transcription of 1 µg total RNA was performed using Illustra™ Ready-To-Go RT-PCR Beads (GE Healthcare, Buckinghamshire, UK). Real-time RT-PCR was conducted in a final volume of 10 µL containing the following: SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), with cDNA as the PCR template and primers to amplify specific fragments corresponding to specific gene targets (Table 1). The PCR reaction was performed with ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA), using the following cycling parameters: 60°C for 10 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min, and a dissociation stage of 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec, 60°C for 15 sec. All data are presented as relative expression units after normalization to the β-actin gene. PCR measurements were conducted in triplicate. The differences in the relative expression were analyzed by analysis of variance (ANOVA) followed by Tukey's test ($p < 0.05$ denotes statistical significance).

2.13 Statistical analysis

Graphs were created and data analysis was performed using GraphPad Prism 8 software (San Diego, CA, USA), using one-way ANOVA or two-way ANOVA (Bonferroni *post hoc* test). Values <0.05 were considered statistically significant.

3 Results

3.1 High-throughput sequencing identifies the expression of small RNAs of *B. abortus* during infection in macrophages

In the small RNA libraries from infected macrophages, we observed that 7.26% of all mapped sequences belonged to the *B. abortus* genome (Table 2). Knowing this, using the Bowtie software, it was seen that the small RNAs were found to be distributed in both chromosomes of the bacteria. By analyzing the depth of coverage of the sequences, we identified a total of 3954 regions of broad mapping of small RNAs in the genome of *B. abortus*, 2694 in chromosome I and 1260 in chromosome II. However, in local data of the bacterial genome, the presence of three peaks (hotspots) was observed, with two in chromosome I and one in chromosome II, constituted by small RNAs being mapped in the same position of the genome and large quantities (Supplementary Figure 1). With that, these three hotspots were selected and we next concentrated our analysis on selecting the small RNAs that were mapped multiple times in these hotspots. In total, we identified one hundred small RNAs with this characteristic which were then submitted to further analysis to detect the target mRNA in NCBI.

3.2 Apolipoprotein N-acyltransferase (BAB1_2158) is a putative target of *B. abortus* sRNAs and its expression is diminished during earlier time of BMDM infection

After the sequences were obtained from NCBI, the surface-associated proteins were selected by our SCL prediction tools. In this phase, 10 proteins were included, making up the final list of surface-associated proteins. The exposure of extracellular structures is an attraction for the immune system in the recognition of antigens and, therefore, the topology predictive tools selected seven proteins composed of extracellular domain amino acids. All selected extracellular portions were composed of at least 35 amino acids. To gain more insight into the biological functions of these 7 proteins, an analysis was performed to identify biological patterns associated with antigens. Of the 7 proteins analyzed, all were inferred by homology in UniProtKB, and a few are those reviewed regarding their biological function (Table 3). The information obtained by the Gene Ontology (GO) project showed that only two of these proteins would be involved in the biosynthesis process of the bacterial structure, yet one of them had a smaller number of exposed amino acids. The other, in addition to exposing 277 amino acids in the extracellular portion, also participates in the bacterial lipopolysaccharide biosynthesis process, becoming the target protein for further analyses. The other proteins analyzed had a transmembrane transport function, in addition to mediating cellular vesicle fusion processes.

Therefore, apolipoprotein N-acyltransferase (BAB1_2158) was selected for the further analysis of differential expression during BMDM infection. Knowing that small RNAs were expressed in large quantities by the bacterium during intracellular infection by *Brucella*, we investigated the levels of mRNA expression of Int in samples of intracellular and extracellular growth in a model of BMDM infection with *B. abortus*. The result of differential expression analysis showed that during BMDM infection, the bacteria decreased the Int gene expression when compared to an exponential extracellular growth model (Figure 3). Taking into account the results of the *in silico* analysis, the negative expression of the Int coding gene may be related to a post-transcriptional gene regulation process used by the bacteria to repress the expression of this protein during intracellular infection, thus establishing its replicative niche in the host cell.

Since the results indicate a possible mechanism of gene expression control by the bacterium in stressful intracellular environments, we were searching for exposed immunogenic epitopes in the extracellular amino acid sequence of Int. The results obtained indicated approximately 60 epitopes that showed a strong binding affinity for HLA molecules (HLA-DRB1 0101, HLA-DRB1 0301, HLA-DRB1 0401, HLA-DRB1 0701, HLA-DRB1 1101, and HLA-DRB1 1501). In this stage, the

predictor tools selected epitopes composed of exactly 15 amino acids. When subjected to alignment with the extracellular portion of the protein, the most promiscuous epitopes formed a conserved region; from this region, the epitope "AIPYILESTPQALAH" was selected. To identify the position of the selected putative immunogenic epitope in the Int, we performed molecular modeling, showing the selected epitope highlighted in red with transmembrane helices indicated in the model in green, as indicated in Figure 4. When submitted to *in silico* screening tests, the epitope was shown to be antigenic in addition to showing 100% similarity with sequences present only in the apolipoprotein N-acyltransferase of *B. abortus*. To increase the confidence of using the selected epitope in future *in vitro* and *in vivo* evaluations, allergenicity was tested using the AlgPred software, characterizing it as non-allergenic. Although when analyzing the physicochemical properties, the epitope was shown to be unstable, since it did not reach the minimum cut-off value for stability, it had a relatively good half-life in mammalian cells (Table 4); that is, despite its instability in the medium, it is suggestive that when it binds to the MHCII cleft, it can present the desired stability.

3.3 Molecular docking between the selected putative immunogenic epitope from Int and MHC-II shows high probabilities of interaction between them

Although not all structures of MHC-II were found in the database (IEDB), we used the structure of 3 available proteins: HLA-DRB1:0101 (PDB: 5NI9), HLA-DRB1:0401 (PDB: 5V4M) and HLA-DRB1:1501 (PDB: 5V4N). When performing the molecular docking of the "AIPYILESTPQALAH" epitope, the results showed great interaction energy of all alleles based on the AutoDock Vina software (Table 5). To represent the molecular docking between the epitope and the MHC-II allele, we chose the interaction with the highest interaction power pointed out by the software. The epitope in question interacted very well with the MHCII of the HLA-DRB1:0101 allele, having the best interaction values, with an energy of -8.1 $Kcal.mol^{-1}$ and presenting a total of 9 hydrogen bonds between the epitope and the MHC, with two bonds involving the amino acid HIS259 of the MHC with the amino acids LEU303 at a distance of 3.09Å and with the amino acid ALA304 at a distance of 3.26Å from the epitope. One link of amino acid ASN260 of MHC with amino acid HIS305 was 3.21Å from the epitope, one link of amino acid GLN242 of MHC with amino acid PRO293 showed a distance of 3.09Å from the epitope, two linkages involving amino acid TYR238 of MHC with the amino acid ILE292 was 2.91Å away and the link with the amino acid ALA291 was at a distance of 3.00Å from the epitope; also, a link of the amino acid LYS249 of the MHC with the amino acid GLU297 was at a distance of 3.06Å and finally two bonds involving amino acids TYR208 and HIS191 of the MHC, showed distances of 2.90Å and 3.24Å, respectively, with amino acid SER298 of the epitope (Figure 5A and B). In summary, the peptide was shown to have a great ability to interact with MHC-II slits, especially with the HLA-DRB1:0101 allele, showing excellent interaction with 9 of the 15 amino acids of the epitope. Although minor binding strengths have been identified, this does not preclude the possibility of interaction between peptide-alleles.

3.4 Immunization of mice with epitope-based vaccine provides protects against *B. abortus* infection

After immunization, vaccinated and unvaccinated mice were challenged by intraperitoneal infection with *B. abortus*; after seven days, organs were collected to determine bacterial loads. In the spleen, liver and axillary lymph node of mice vaccinated with the peptide, fewer viable bacteria were recovered when compared to the control group (Figure 6).

To evaluate the potential use of the epitope-based vaccine, the protection level induced in mice against virulent challenge infection was assessed. The degree of vaccine efficacy in C57BL/6 mice was determined by subtracting the mean CFU/organ recovered from mice after vaccination and challenged from the mean CFU/organ recovered from non-vaccinated but challenged control mice. At this time, it was seen that the presence of vaccinal peptide in the animal organism triggered a higher degree of protection against infection, approximately 1.20/0.80/0.84-log in spleen, liver, and

axillary lymph node, respectively (Table 6). This result showed that the RV-selected epitope provided significant protection to C57BL/6 mice against *B. abortus*.

3.5 Unvaccinated mice showed greater liver damage when infected with *B. abortus*

B. abortus infection is associated with the formation of focal granulomatous lesions in the spleen, liver, and lymphoid tissues of both humans and rodents, starting 1–2 weeks post-infection (62). To determine the characteristics of liver pathology upon vaccination with peptide during *B. abortus* infection, we performed the histopathological analysis of liver tissue from vaccinated and unvaccinated C57BL/6 mice and, challenged with *B. abortus*. Infection with *B. abortus* resulted in the formation of hepatic granulomas in both groups (Figure 7A and C). In morphometric analysis, a greater number of granulomas was observed in the tissue of animals that were not vaccinated with the peptide and challenged by bacteria compared to the vaccinated group (Figure 7G). The same result was seen when analyzing the area, which was greater in granulomas from unvaccinated animals (Figure 7H). Histopathological lesions during *Brucella* infection usually are associated with the bacterial load. To determine the relationship between granuloma formation and the bacteria present in the granuloma, we performed immunohistochemistry to immunolabel *B. abortus*. Figures 7E and 7F showed the presence of *B. abortus* in the granulomatous lesions presented in the liver of vaccinated and infected mice and non-vaccinated and infected mice, respectively. The detection of intralesional bacteria confirms that the inflammatory lesions described in this study are due to systemic *B. abortus* infection. No observable lesions were found in tissues from uninfected mice (Figure 7B and D).

3.6 Vaccination induces positive expression of pro- and anti-inflammatory cytokines in animals infected with *B. abortus*

The liver is the most commonly affected organ in patients with active brucellosis (63), which is why liver macerates from the four experimental groups were collected for evaluation of the expression of the anti-inflammatory genes *IL-10* and *TGF-β*. In the differential expression analysis, an increase in these cytokines was observed in vaccinated and infected C57BL/6 mice when compared to the other groups (Figure 8A-B). Therefore, the reduction in liver pathology can be attributed to a decrease in the number of viable bacteria and an increase in anti-inflammatory cytokines, resulting in a consequent reduction in liver damage. Concomitantly, the splenic tissue was evaluated and the results obtained showed that all groups, except for the control, showed upregulation of the proinflammatory cytokine-coding genes *INF-γ*, *TNF-α*, and *IL-6*, which are characteristic of inflammation (Figure 8C-E), but the vaccinated and infected group stood out due to the increased expression when compared to other groups. Likewise, it was seen that IL-10 (Figure 8E) is up-regulated in this group, suggesting that vaccination induces an attempt to control the inflammatory process generated by systemic infection with *B. abortus*.

3.7 BMDM's from C57BL/6 mice stimulated with supernatant splenocytes from the vaccinated and infected group showed higher expression of CD86

In vivo analysis results indicate the activation of an adaptive immune response. Knowing this, we tried to understand the mechanisms by which a more efficient immune response activation process against the bacteria occurs. BMDMs were evaluated for the expression of co-stimulatory molecules after being stimulated with the splenocyte supernatant. The results obtained by fluorescence microscopy show that BMDMs stimulated with the supernatant from the spleen of vaccinated and infected animals were more activated, due to intense CD86 labeling (Figure 9A), and that they even expressed a higher level of *iNOS* expression, the gene that stimulates NO production (Figure 9B). To confirm this result, the measurement of NO was performed in the splenocyte supernatant used to stimulate BMDMs. The results obtained showed the greater production of NO by supernatant from the vaccinated and infected group, that is, a product that suggestively activated the BMDMs more intensely (Figure 9C), which may reveal an increased phagocytic and microbicide capacity to eliminate the bacteria.

4 Discussion

The development of subunit vaccines to protect against brucellosis is crucial to avoid the disadvantages of the used live attenuated vaccines RB51 and S19 against *B. abortus* (64,65). New vaccines will be designed according to immune responses during a natural infection in animal models and the identification of intracellular and cell surface immunodominant components of *Brucella* spp. (66-69). Using RV, it has been shown that the genome of *B. abortus* contains approximately 80 genes encoding putative lipoproteins that have diverse functionalities, including pathogenic processes (70,71). Knowing this, recent studies have shown that some bacterial cell surface proteins can provide significant protection against *Brucella*, such as L7/L12 (72), Omp19, Omp31 (73), BP26 (74), and Omp25 (75,76), which have been shown to be immunodominant antigens that stimulate host immunity and trigger a protective response against infection in a mouse model. Bacteria of the genus *Brucella* can live, replicate and persist within phagocytes using their mechanisms to evade the immune system and establish their replicative niche within the host cell (77). In this context, previous studies suggest that small RNAs may be directly related to the timely gene expression of virulence factors in a variety of pathogenic bacteria such as *Listeria* and *Yersinia* (78,79). Considering that the expression of small bacterial RNAs allows changes in the host cell phenotype, and knowing that these small RNAs act on gene activation and repression, we evaluated the capacity that a rationally predicted epitope of Int, a target protein of small RNAs expressed on large scale by the bacteria during macrophage infection, would have to activate protective immune responses during infection by *B. abortus* in a murine model.

Since *Brucella* species are equipped with a variety of well-organized immune evasion strategies to establish chronic infections, including the use of small non-coding RNAs (80,81), we performed a detailed analysis from data from the sequencing of small RNAs expressed during the infection of BMDMs with *B. abortus*, showing that 7.26% of the small RNAs were mapped in the bacterial genome. This was consistent with previous reports that identified expression levels of similar small *B. abortus* RNAs during the infection of murine macrophages (82). Casewell et al. reported that *B. abortus* small RNAs, abcR1 and abcR2 play essential roles in pathogenicity and chronic infection, resulting in a significant decrease in intracellular survival in a mouse model and in macrophages (83). Another group identified 129 small RNAs of *Brucella* that play significant roles in diverse biological processes, ranging from physiology to virulence, as well as in host-pathogen interaction (84). These reports shed light on the importance of small non-coding RNAs in *Brucella* immunity, pathogenesis, and intracellular survival, modulating the host's immune response. Here, we selected a non-allergenic but antigenic epitope with a good half-life in mammals, yeast, or *E. coli*, from a putative target of a more highly expressed *Brucella* sRNA. Rationally, we also take account of the structural and functional aspects of this target meeting the epitope "AIPYILESTPQALAH" of apolipoprotein N-acyltransferase (Int). Although other proteins were predicted as possible targets of *Brucella* sRNA in our analyses, this one, in particular, stood out due to its biological function and strong epitope MHCII-interact capacity. Reportedly, Int functionally constitutes *Brucella*'s outer membrane and plays a crucial role in bacterial LPS biosynthesis (70,71,85). Interestingly, one of the main virulence factors of *Brucella* identified so far is its non-canonical LPS (86,87) which exhibits favorable properties for the bacterium, including low endotoxicity, high resistance to degradation in macrophages, and protection against immune responses (88-90). The differential expression analysis of this protein-coding gene was performed in intracellular growth samples in a BMDM model infected with *B. abortus*, showing a drastic reduction in expression, corroborating our hypothesis that there is a post-transcriptional gene regulation process used by the bacterium to repress the expression of Int during the early time of infection, which can favor the bacteria permanence and replication in the host cell. Therefore, in this study, in an unprecedented way, we evaluated the capacity of a specific Int epitope selected by RV to induce an immune response in a murine model infected with *B. abortus*. The candidate epitope-based vaccine was able to trigger protective immune responses, when the amount of viable *B. abortus* in the liver, spleen, and axillary lymph nodes of vaccinated and unvaccinated mice when

challenged intraperitoneally by this pathogenic bacterium was evaluated. It was observed that immunization considerably decreased the recovery of *B. abortus* in the tissues evaluated and induced mean systemic protection of 0.94 logs when compared to unvaccinated animals. Other evaluated *Brucella* antigens behaved similarly; for example, the recombinant Omp16 and Omp19 and the encapsulated recombinant liposome Omp25 induced protection comparable to S19 in vaccinated mice after challenge (17,18,91). In addition, the Omp28 subunit vaccine increased resistance against the *B. abortus* challenge by inducing a CD4+ Th1 response, that protects against infection, but at a lower level than live attenuated vaccines (92). Corroborating our results, other studies, when analyzing the level of splenic CFU, showed that the levels of protection in animals immunized with RB51 averaged 0.91 logs [93,94], suggesting that the vaccine peptide in this study induces levels of splenic protection similar to the live attenuated RB51 vaccine.

The characteristic pathological manifestation of *B. abortus* infection is granulomatous inflammation associated with bacterial load (95). In this study, we detected a significant reduction in the number and size of granulomas in the livers of animals vaccinated with the peptide compared to unvaccinated animals, suggesting that the vaccination induced an effective inflammatory immune response in this tissue. This condition is well defined, whereas, as infection in mice infected with *Brucella* progresses, the granulomas progressively decrease in size and number after 2–3 weeks of infection (62). In parallel, we saw that the reduction of pathology in vaccinated mice was accompanied by an increase in the expression of *IL-10* and *TGF-β* in the liver of infected animals. Although the impact of IL-10 on *Brucella* persistence and the establishment of chronic infection through macrophage modulation has been previously demonstrated using IL-10-deficient mice (96,97), our findings provide evidence that the increased expression of *IL-10* is related to an immunoregulatory mechanism dampening excessive Th1 responses (98). In this context, it is noted that the absence of IL-10 results in severe pathological changes in different bacterial infections (98,99). Here, we can speculate that the reduction in liver pathology in immunized and infected animals may have been mediated by an increase in anti-inflammatory cytokines, and attributed to a previous reduction in the number of viable bacteria.

Cell-mediated immunity is considered critical for the protective immune response against facultative intracellular pathogens (100,101). Our results showed that the Int epitope induced the enhanced production of TNF-α and IFN-γ in spleen cells, suggesting that there is some induction of a Th1-type immune response by the vaccine peptide. In particular, IFN-γ is essential for immune protection against *Brucella* infection that induces more polarization toward Th1 cells (102,103). In addition, functional TNF-α has been shown to link the proinflammatory response and adaptative immune response in *Brucella*-infected mice (104). High levels of IL-6 were produced by the splenocytes of immunized and infected mice. It was recently shown that IL-6 is required for the induction of IFN-γ and TNF-α by infected splenocytes, in addition to promoting the differentiation of CD8+ T cells, indicating a protective role for IL-6 against *B. abortus* that parallels the type of Th1 immunity response (105,106). Similarly, significant levels of IL-10 in immunized mice were also detected in spleen cells. These results are consistent with the scenario seen in liver tissue, suggesting that an inflammatory immune response has already occurred to the point of generating an anti-inflammatory response with infection control characteristics. In this context, the balance between the production of pro- and anti-inflammatory cytokines appears to be crucial for the host's ability to eradicate the infection (97).

Since immunization triggered an effective adaptive immune response *in vivo*, the activation of BMDMs when stimulated with supernatant from splenocytes from treated animals was evaluated in vitro. It was observed that the supernatant from vaccinated and infected animals secreted cellular components capable of stimulating macrophages more intensely in the BMDMs compared to the other groups, even showing that these cells expressed higher levels of iNOS, and the consequent increased production of NO, an important cell signaling molecule involved in infectious diseases and the death of intracellular pathogens (107), justifying here the reduction in the number of viable bacteria recovered from the vaccinated group. *In vivo*, IL-6, TNF-α, and CD80/CD86 are required for activation of the interferon gamma-producing CD4+ Th1 and CD8+ cytotoxic T cells, a protective response induced by the host against brucellosis (81,103,108,109). Although our study

did not assess the predominant subset of T cells in the immune response, it is believed that the bactericidal and phagocytic function of macrophages to eliminate the bacteria was mainly enhanced by the secretion of IFN-γ and TNF-α, since these cytokines showed high expression levels in tissues from immunized animals, suggesting that the Int subunit vaccine predominantly induced an effective Th1 profile response and triggered protection against *B. abortus* infection (Figure 10).

The information gathered shows that the bioinformatics is a strong approach for vaccine candidate discovery as it offers a faster, cheaper, and safer method to identify potential vaccine targets when compared with traditional laboratory identification methods, particularly when dealing with risk group 3 microorganisms such as *Brucella*. Here, we provide an RV strategy that was able to identify a *B. abortus* antigen that is found to be strongly associated with bacterial virulence. Thus, immunization with the peptide vaccine had a significant effect on protection against murine infection, inducing an immunoprotected response; therefore, it is plausible to assume that this antigen can form a solid basis for designing an efficient and safe vaccine against animal brucellosis.

6 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.

7 Ethical Statement

This study was conducted in strict accordance with the Brazilian laws 6638 and 9605 in Animal Experimentation. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Federal University of Alfenas (CEUA 16/2020).

8 Author Contributions

KO and LA performed study design. RS, PC, and SO involved in contribution of study materials. KO, GB, LA, and EN provided guidance for analytical tools and performed bioinformatic analysis. KO and NS performed acquisition and collection of data *in vitro* and *in vivo*. KO and LA were involved in manuscript preparation. All authors contributed to the article and approved the submitted version.

9 Funding

This study was supported in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brasil (CAPES) (Finance Code 001) and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (Grant 864/14).

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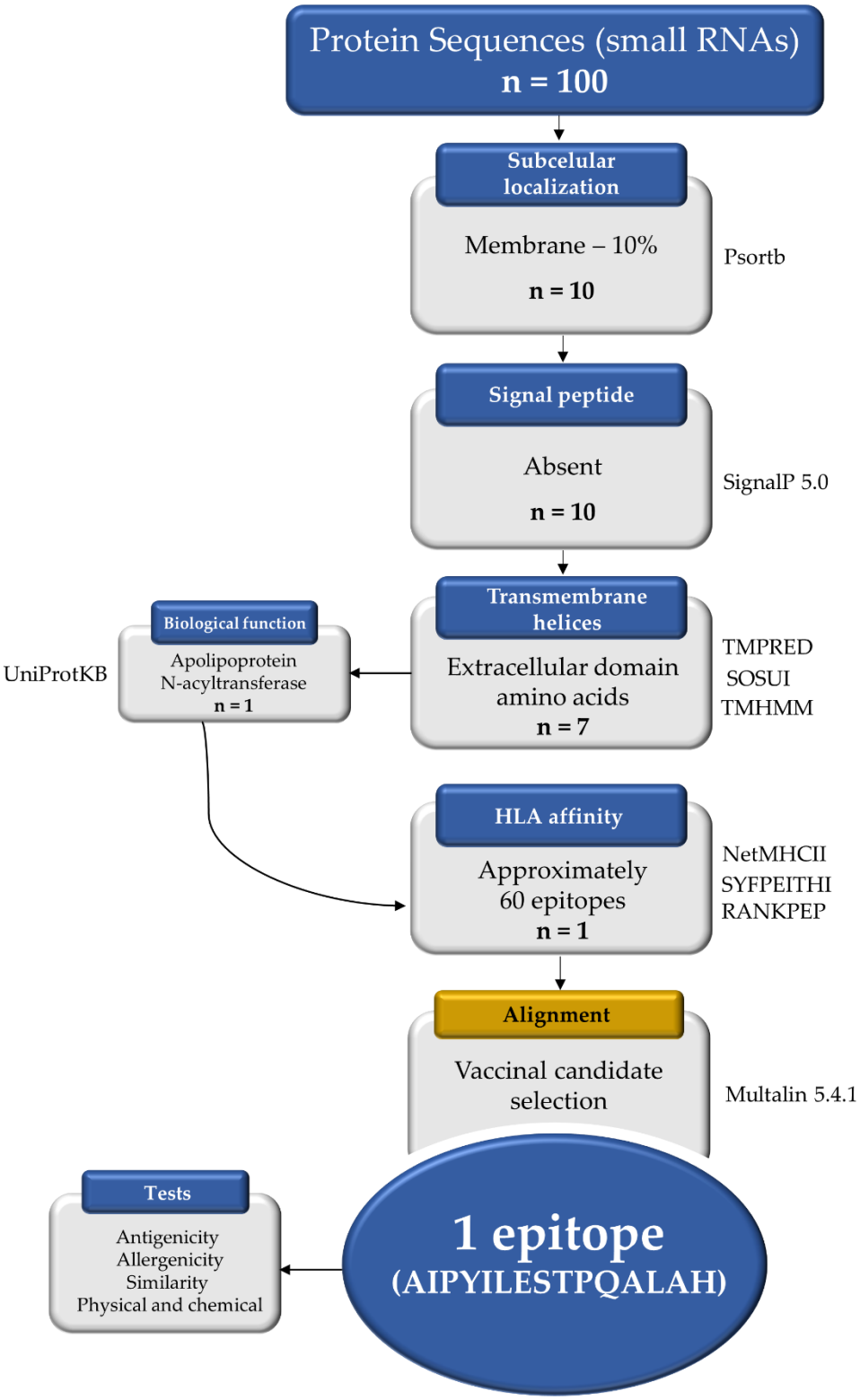
9 Supplementary Material

The Supplementary Material for this article can be found online:

10 Data Availability Statement

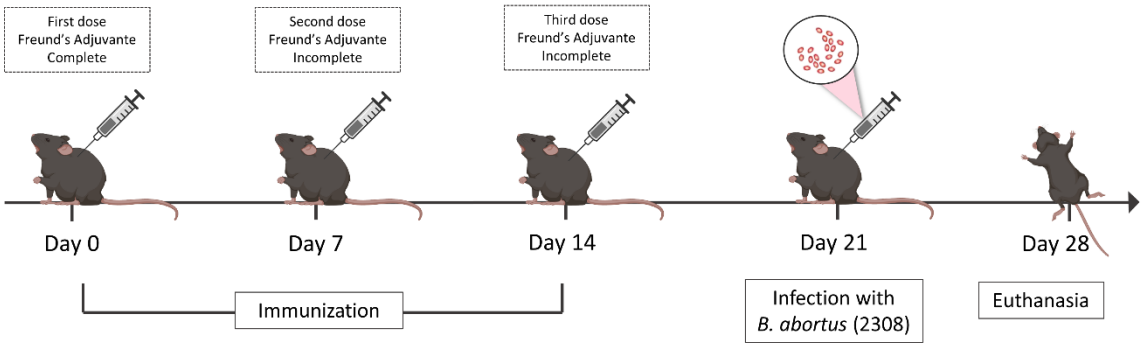
All datasets generated for this study are included in the article/Supplementary Material.

FIGURE AND TABLES LEGENDS



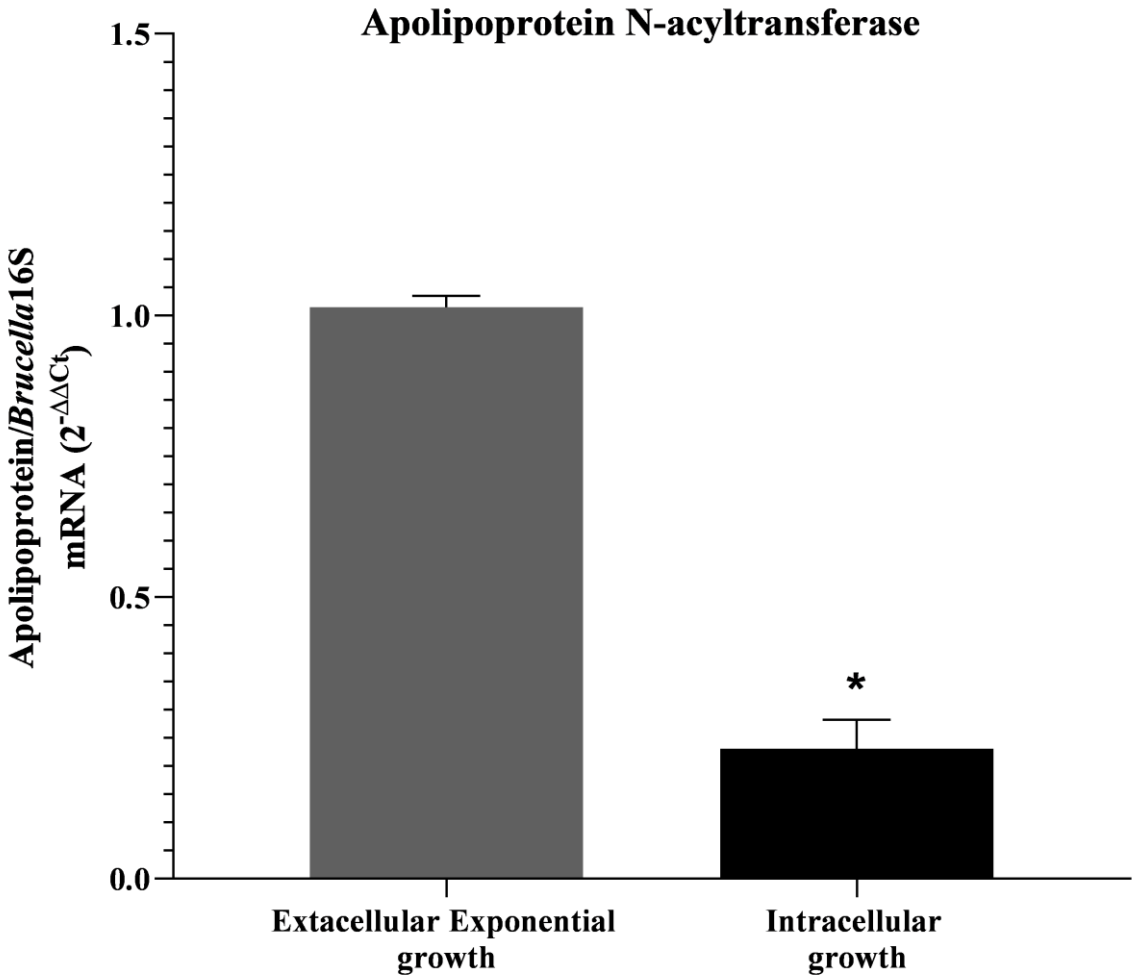
881

882 **Figure 1.** Reverse vaccinology protocol workflow applied in this study to select a vaccinal epitope candidate.
883 The one hundred protein sequences were obtained from the NCBI and ten proteins were localized in the
884 plasma membrane by the Psortb tool. The TMPRED, SOSUI, and TMHMM tools identified 7 proteins with
885 extracellular amino acid domain, but when analyzing the biological functions in the UniProtKB, only one
886 was selected. Approximately 60 epitopes from the extracellular portion showed strong binding by HLAs
887 according to the tools NetMHCII, SYFPEITHI, and RANKPEP. Using the Multalin tool the alignment was
888 performed and the most promiscuous candidate among the epitopes was selected. n= number of proteins.



889

890 **Figure 2.** Experimental design of in vivo analysis. C57BL/6 mice were vaccinated three times with intervals
891 of seven days between doses, the first dose being in the presence of complete Freund's adjuvant and the other
892 doses using the same incomplete adjuvant. Seven days after the last immunization the mice were challenged
893 with the virulent strain 2308 of *B. abortus* and on day twenty-eight the animals were euthanized to obtain a
894 spleen, liver and axillary lymph node.



895

896 **Figure 3.** Decreased in the gene expression of apolipoprotein N-acyltransferase is shown in an intracellular-
897 growing model. Differential expression analysis of apolipoprotein N-acyltransferase in model of
898 extracellular and intracellular growth was measured by real-time RT-PCR. Statistically significant
899 differences relative to the extracellular exponential growth are represented by an asterisk (*P <0.05).

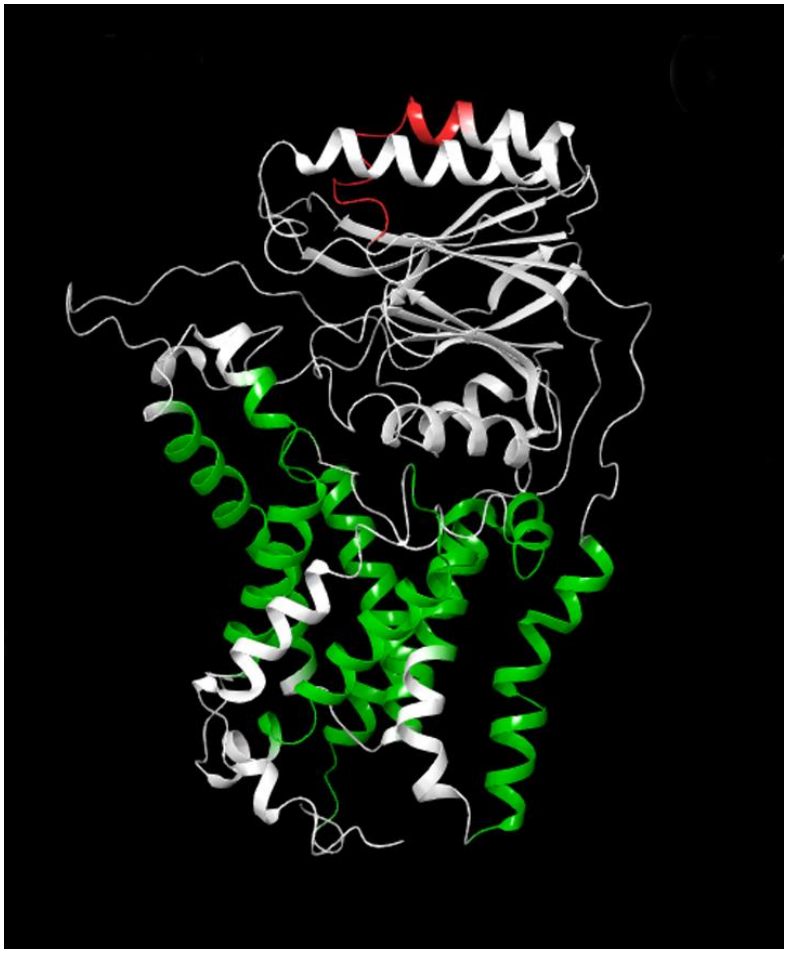


Figure 4. Position identification of selected epitope in apolipoprotein N-acyltransferase 3D. Molecular modeling by homology for the apolipoprotein N-acyltransferase. Protein is represented in white; the selected epitope is highlighted in red, and the transmembrane helices are indicated in the models in green.

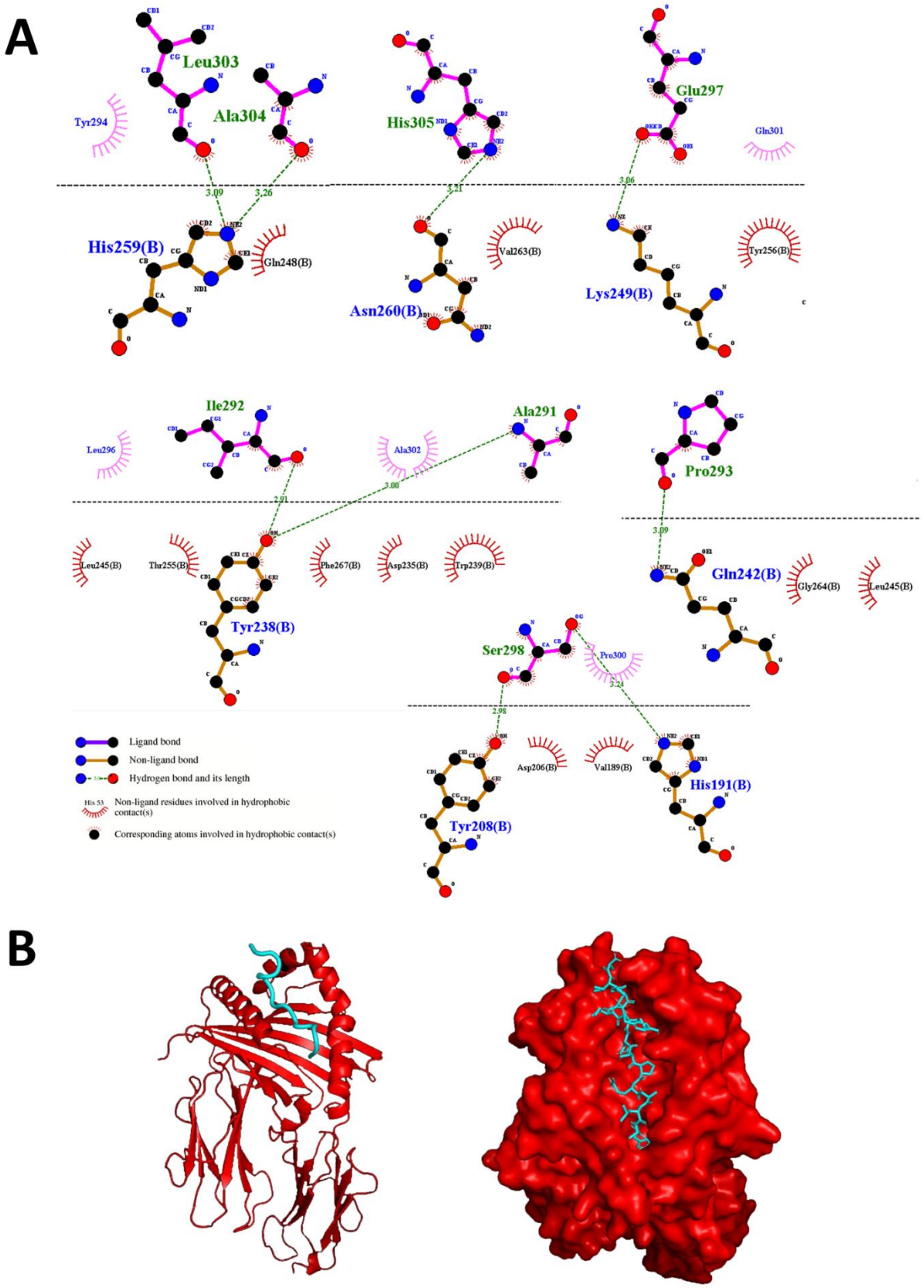


Figure 5. Interaction between the selected epitope from apolipoprotein N-acyltransferase and MHC-II allele. (A) 2D diagram of the best interaction between the HLA-DRB1:0101 allele and the epitope, representing above the dotted line epitope amino acids and below line MHCII amino acids and (B) 3D diagram of the interactions.

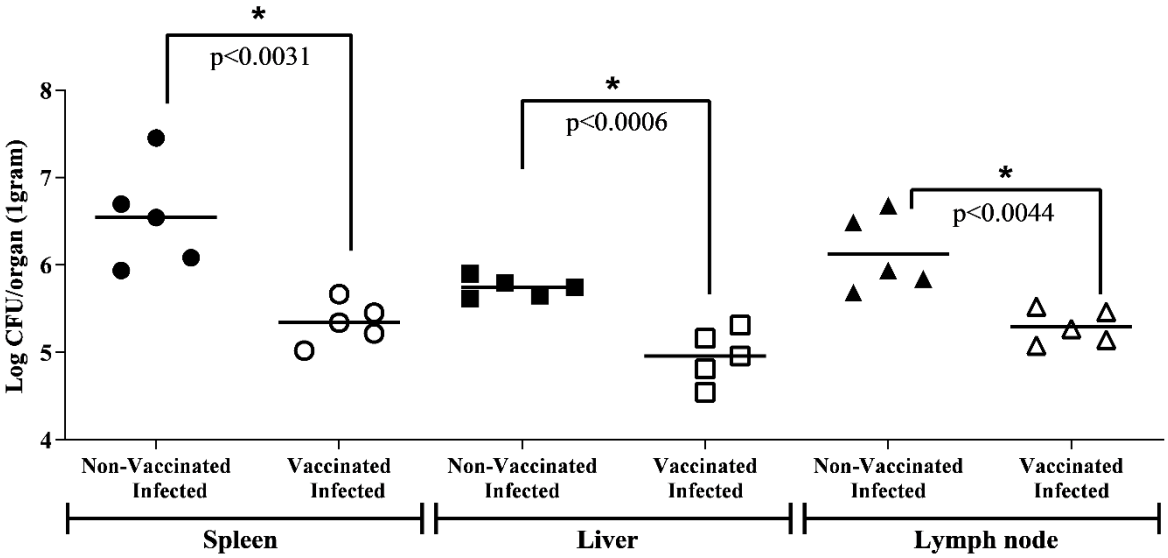
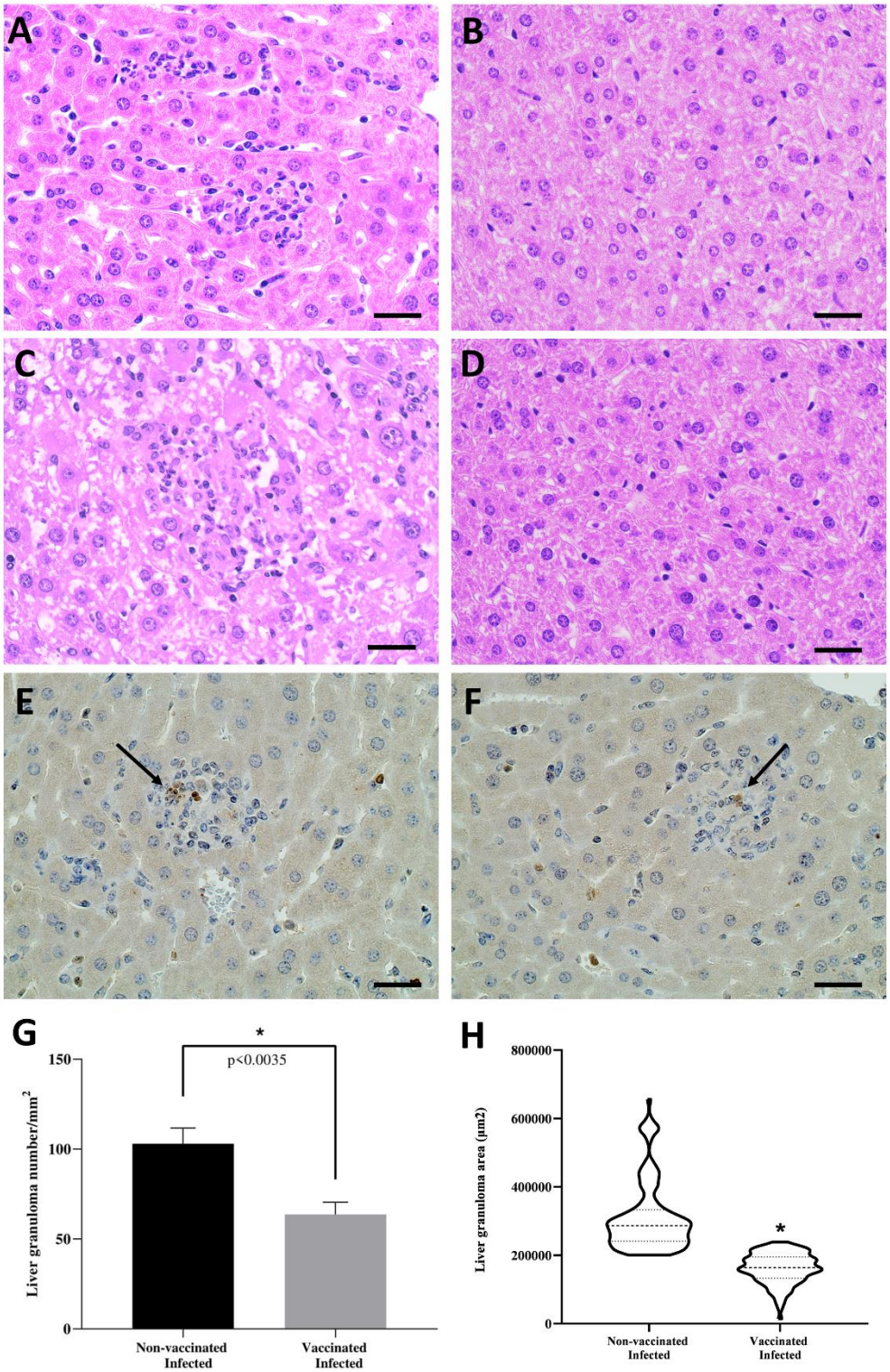


Figure 6. Protective efficacy after three rounds of immunization. For protection evaluation, vaccinated mice were challenged with 1×10^6 CFU/bacteria. One-week post-challenge, organs were collected to determine bacterial loads to assess protection efficacy. A smaller number of viable bacteria was identified in vaccinated mice, indicating great protection efficacy. Data points were individual values of CFU determinations (n = 5) and analyzed using a student's t test. *$P < 0.05$ relative to the non-vaccinated and infected group.



915

916 **Figure 7.** Histopathology analysis, immunohistochemistry, and morphometric of hepatic tissue of *B. abortus*
917 infected C57BL/6 mice. (A) Representative of hematoxylin- and-eosin-stained sections of hepatic tissue
918 from mice vaccinated and infected, (B) vaccinated and non-infected, (B) unvaccinated and infected, and (D)
919 non-vaccinated and non-infected. Immunohistochemistry sections of hepatic tissue from mice vaccinated
920 and infected (E) and non-vaccinated and infected (F) mice containing the *B. abortus* inside the granuloma.
921 The graphs analyze the granulomas of liver tissue sessions that were sequentially captured in terms of
922 number (G) and area (H). Statistically significant differences relative to the non-vaccinated group are
923 represented by an asterisk (**P* <0.05). The arrows indicate the *B. abortus* within the granuloma. Scale bars:
924 50 µm.

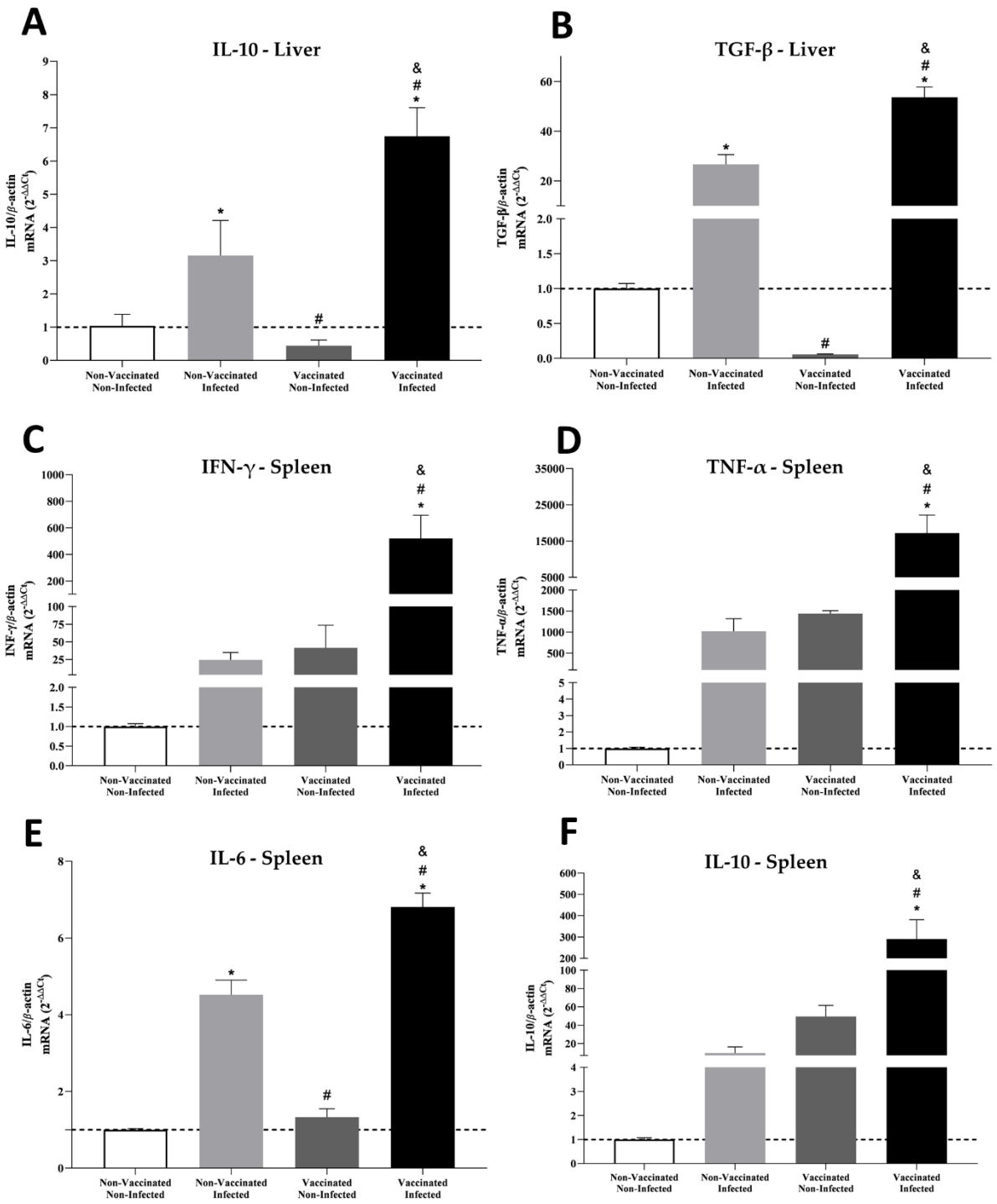
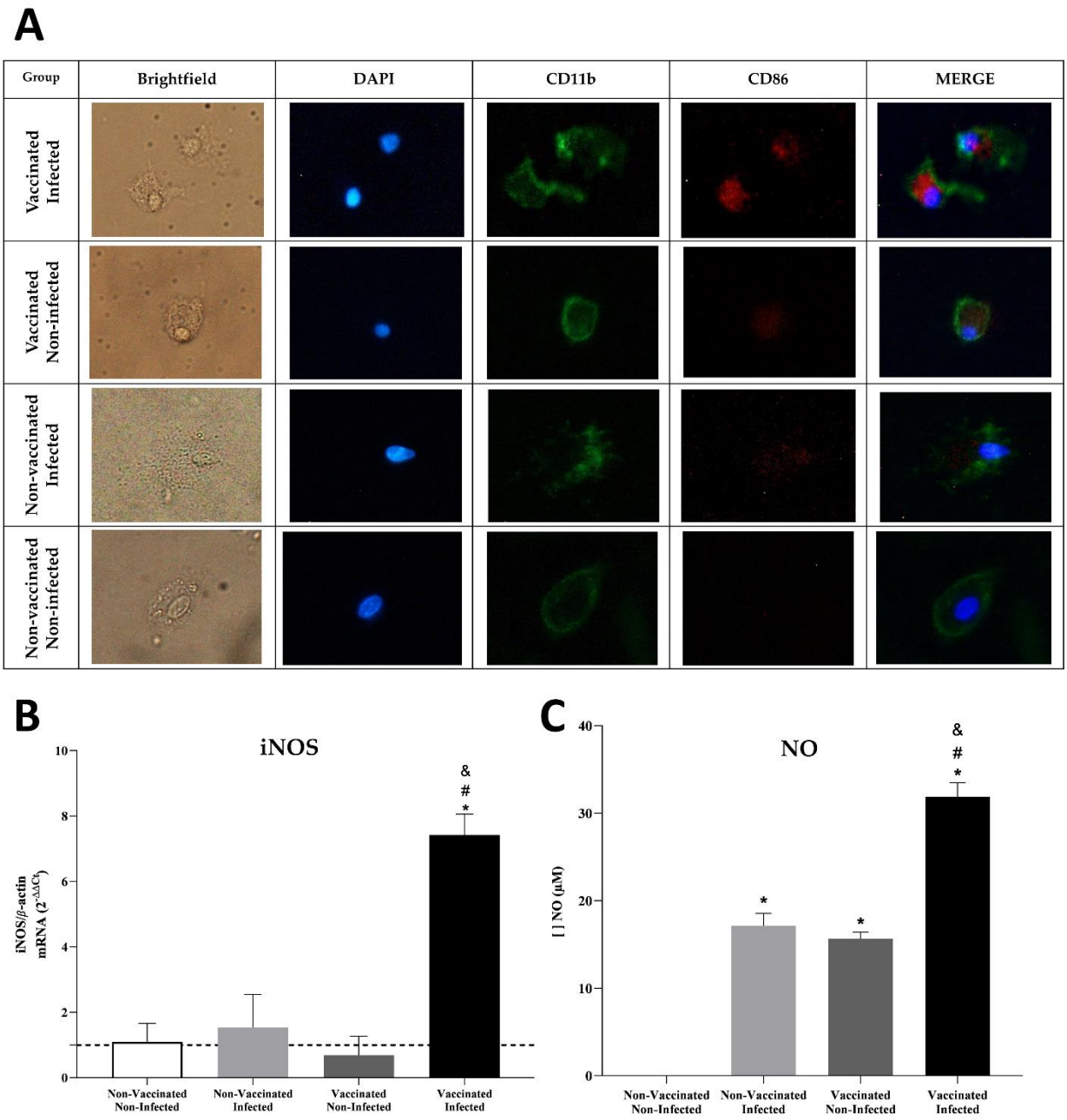


Figure 8. Liver and spleen showed increased expression of pro and anti-inflammatory cytokine in mice vaccinated and infected with *B. abortus*. Differential expression analysis of (A) IL-10 and (B) TGF-β from the liver tissue and (C) IFN-γ, (D) TNF-α, (E) IL-6, and (F) IL-10 from the spleen of C57BL/6 mice from the four experimental groups evaluated in this study. Transcript levels were measured by real-time RT-PCR. Error bars represent the mean ± SD of samples assayed in triplicate. **P* < 0.05 relative to the non-vaccinated and non-infected group. #*P* < 0.05 relative to the non-vaccinated and infected group. &*P* <0.05 relative to the vaccinated and non-infected group.



933

934 **Figure 9.** *In-vitro* analysis showed an increased phagocytic and microbicide capacity in BMDM's. (A)
935 Fluorescence microscopy shows activation of BMDM's by the CD86 molecule after being stimulated with
936 supernatant from the spleen of C57BL/6 animals. (B) Differential expression analysis of iNOS in stimulated
937 BMDM's was measured by real-time RT-PCR and (C) NO dosage by splenocyte supernatant using Griess
938 method. Error bars represent the mean ± SD of samples assayed in triplicate. **P* < 0.05 relative to the non-
939 vaccinated and non-infected group. #*P* < 0.05 relative to the non-vaccinated and infected group. &*P* <0.05
940 relative to the vaccinated and non-infected group. Scale bars: 20 µm.

941

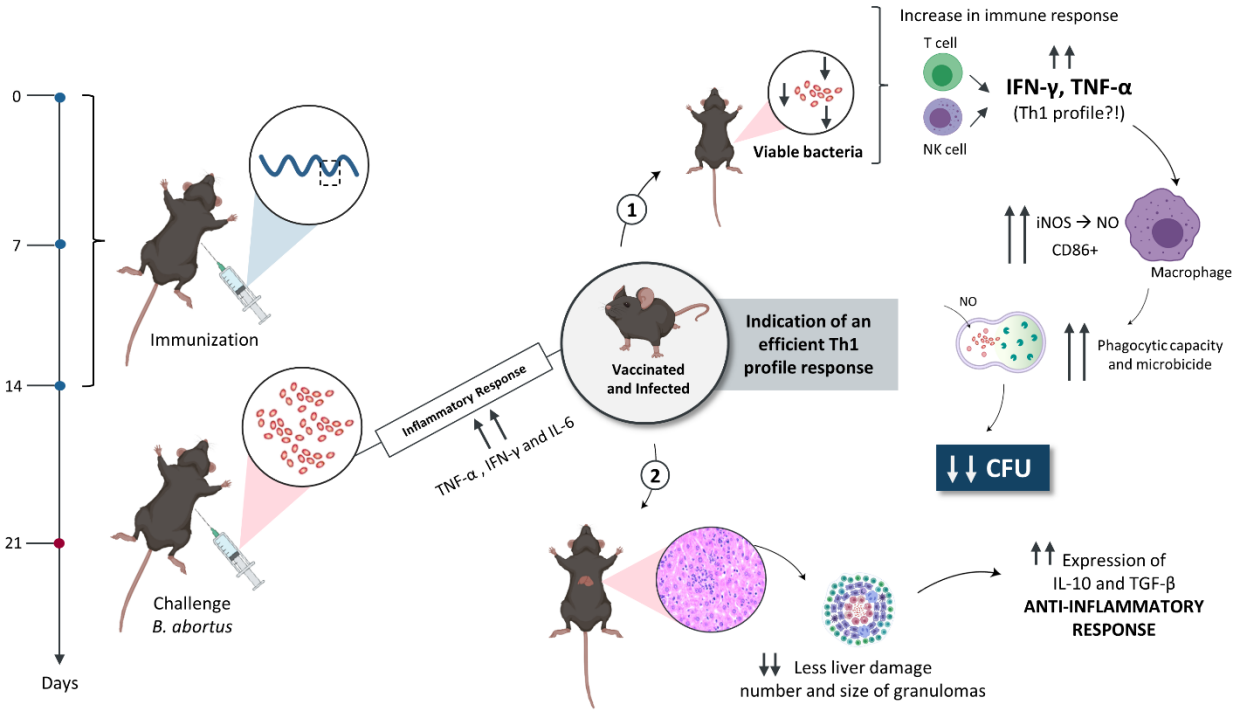


Figure 10. Model proposed in this study suggesting optimal efficacy of the rationally predicted vaccine peptide. After being immunized and challenged, C57BL/6 mice were more resistant to infection by *B. abortus*, with less systemic recovery of viable bacteria and reduced tissue damage, mediated by an anti-inflammatory response. When evaluating the immune response profile, the predominance of characteristic components of the Th1 profile was observed, such as IFN-γ and TNF-α, suggesting that the vaccine peptide stimulates a profile mediated by CD4+ Th1 lymphocytes to secrete specific components such as IFN-γ, that mediates the production of NO and acts directly on infected macrophages, enhancing their microbicidal and phagocytic capacity and controlling the spread of systemic infection by this bacterium. Created with BioRender.com.

Table 1. Primers used in this study.

Primer	Forward Sequence	Reverse Sequence
β-actina	5'-AGGTGTGCACTTTTTATTGGTCTCAA-3'	5'-TGTATGAAGGTTTGGTCTCCCT-3'
TNF-α	5'-CATCTTCTCAAAATTCGAGTGACA-3'	5'-TGGGAGTAGACAAGGTACAACCC-3'
IFN-γ	5'-TCTGGAGGAACTGGCAAAG-3'	5'-TTCAGACTTCAAAGAGTCTGAGG-3'
IL-10	5'-GGTTGCCAAGCCTTATCGGA-3'	5'-ACCTGCTCCACTGCCTTGCT-3'
IL-6	5'-CCAGGTAGCTATGGTACTCCAGAA-3'	5'-GATGGATGCTACCAAACTGGA-3'
TGF-β	5'-TGACGTCACTGGAGTTGTACGG-3'	5'-GGTTCATGTCATGGATGGTGC-3'
iNOS	5'-CAGCTGGGCTGTACAAACCTT-3'	5'-CATTGGAAGTGAAGCGTTTCG-3'

959 **Table 2.** Data obtained after mapping of reads.

	***B. abortus* infected BMDMs**	**%**	**Control noninfected BMDMs**	**%**
Processed Reads	35.713.113		46.516.010	
Reads mapped in the *B. abortus* (S2308) genome	2.593.062	7.26%	17.269	0,04% of the total
Reads mapped in the *Mus musculus* (GRCm38) genome	33.120.051	92.74%	46.498.741	99,96% of mapped reads

960

961 **Table 3.** List of proteins predicted to have transmembrane helices and respective biological functions.

Protein ID (NCBI)	**Length (aa)**	**Single-line annotation (NCBI)**	**Biological function (UniProtKB)**	**Gene**
WP_002968965.1	386	Lipase	Pathogenesis and negative regulation of endosome organization, and vesicle fusion	BAB2_0654
WP_002964284.1	270	Phosphatidate cytidylyltransferase	CDP-diacylglycerol biosynthetic process	BAB1_1179
WP_002965220.1	532	Apolipoprotein N-acyltransferase	Lipoprotein biosynthetic process	BAB1_2158
WP_002971227.1	430	Xanthine/uracil/vitamin C permease family	Transmembrane transporter activity	BAB2_0578
WP_002971267.1	510	Amino acid permease	Transmembrane transporter activity	BAB2_0864
WP_002966986.1	412	MFS transporter superfamily	Transmembrane transporter activity (carbohydrate)	BAB1_1882
WP_002964796.1	400	OPGC	Transmembrane transporter activity (sugar)	BAB1_1718

962

963 **Table 4.** Similarity, antigenicity, allergenicity, and physical-chemical properties of selected epitope.

Sequence: AIPYILESTPQALAH									
Similarity	**Allergenicity**	**Antigenicity**	**MW[a]**	**pI[b]**	**Stability**	**Half-life Reticulocytes**	**Half-life Yeast**	**Half-life *E. coli***	**IgE epitope**
100%	No	0.7800	1623.87	5.24	40.27	4.4h	>20h	>10h	No

964 [a] MW, molecular weight; [b] isoelectric point.
965

966 **Table 5.** Molecular docking results according to AutoDock Vina software.

MHC-II	**Connection Power (Kcal.mol-1)**	**Hbonds**	**Non-ligand residues involved in hydrophobic contacts MHC**	**Non-ligand residues involved in hydrophobic contacts epitope**
5NI9	-8,1	9	11	5
5V4M	-6,6	5	8	6
5V4N	-7,1	7	10	3

967

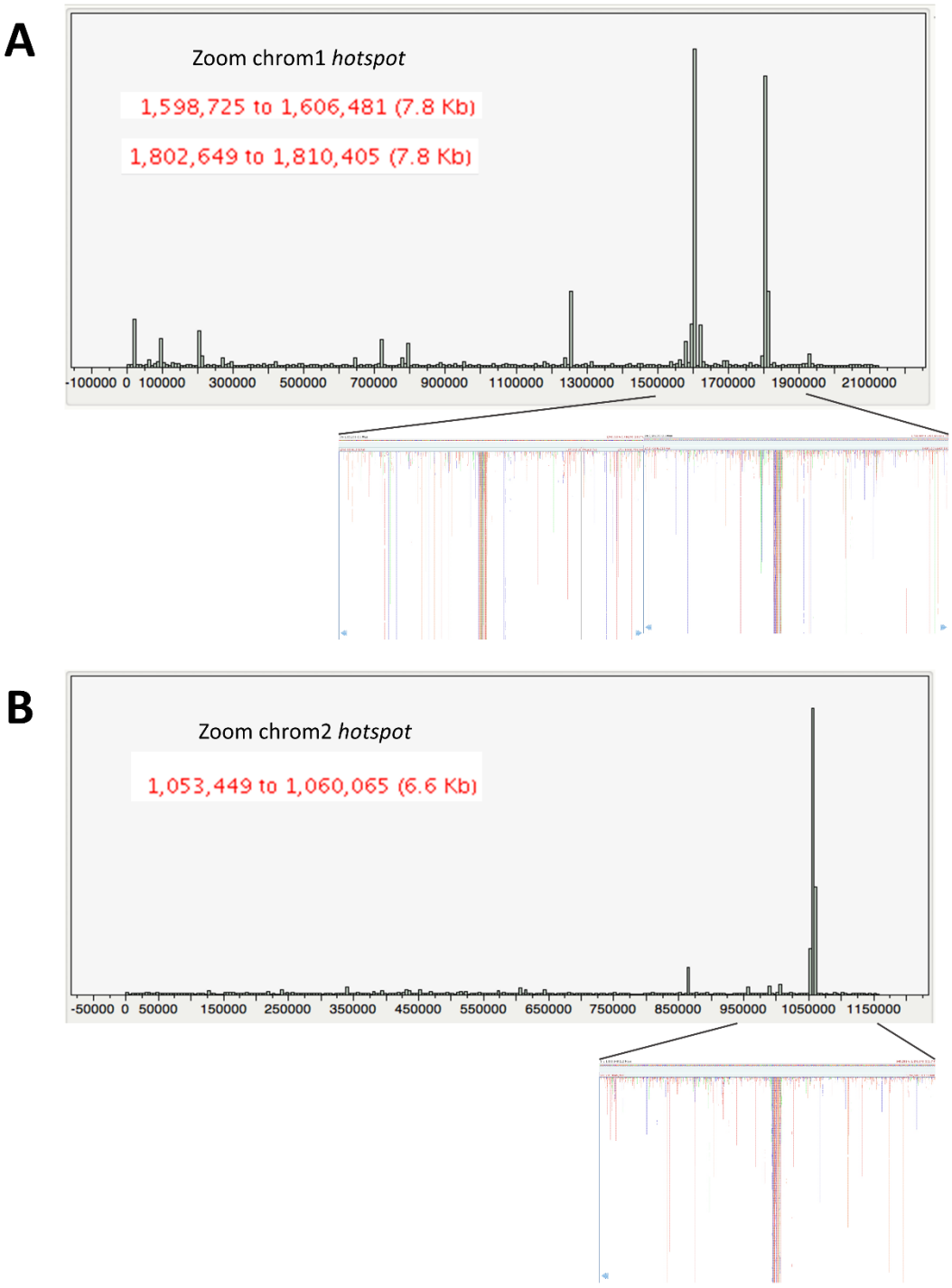
968

969

970 **Table 6.** Protective efficacy conferred by vaccinal peptide against *B. abortus* infection.

Group (n = 5)	Log_{10} CFU (Spleen)[a]	Log Protection	Log_{10} CFU (Liver)	Log Protection	Log_{10} CFU (Lymph node)	Log Protection
Vaccinated	5.34 ± 0.28*	1.20	4.96 ± 0.34*	0.80	5.30 ± 0.19*	0.84
Non-vaccinated	6.54 ± 0.63	-	5.74 ± 0.13	-	6.13 ± 0.43	-

971 Protection units of vaccinated group are compared with that of non-vaccinated with Student's t-test, $*P < 0.05$ is statistically
972 significant.
973 [a] CFU, colony-forming units.
974
975
976



977

978 **Supplementary Figure 1.** Arrangement of hotspots along the genome of *B. abortus* bacteria expressed
979 during macrophage infection. (A) On chromosome I of *B. abortus* there is the formation of two hotspots
980 characterizing regions of intense mapping of small RNAs and (B) on chromosome II there is the formation
981 of only one hotspot.

2 CONCLUSÃO

A abordagem *in silico* empregada neste estudo permitiu identificar de forma racional um epítopo potencialmente antigêncio e a partir dele contruir uma vacina de subunidade bem determinada. A imunização com a vacina de peptídeo apresentou efeito protetor significativo contra a infecção murina por *B. abortus*, induzindo uma resposta imunoprotetora e reduzindo as danos em tecidos acometidos pela infecção. Embora não tenha sido avaliado o subconjunto de células T predominantes na resposta imune, os resultados obtidos frente ao perfil imunológico de animais vacinados e infectados, sugere que a vacina de subunidade de apoliproteína N-aciltransferase induziu predominantemente uma resposta de perfil Th1 eficaz e desencadeou proteção contra a infecção por *B. abortus*. Portanto, é plausível supor que esse antígeno possa se tornar um forte candidato vacinal para o desenvolvimento de uma vacina melhorada, segura e eficiente contra a brucelose animal.

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