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IMMUNOMODULATORY IMPACT OF *LEISHMANIA TARENTOLAE* EXTRACELLULAR VESICLES, TOWARDS A NEW PLATFORM FOR VACCINES

By

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ABSTRACT

Sub-unit vaccines use key antigens to stimulate a long-lasting protective immune response; however, they exhibit low immunogenicity and often require an adjuvant for support.

Extracellular vesicles (EVs) are spherical lipid bilayer particles which are released from all cells. It has been previously shown that *Leishmania*, a protozoan parasite, can actively release EVs which can modulate mammalian immune cells. Therefore, we hypothesized that EVs derived from *Leishmania tarentolae*, a non-pathogenic species, may have an immunostimulatory effect towards the development of vaccine adjuvants.

We used a modified SARS-CoV-2 Spike (S) protein as a proof of concept. The full-length S-glycoprotein was produced synthetically, and codon-optimized for the constitutive expression in *L. tarentolae*. EVs derived from *L. tarentolae* were isolated by ultracentrifugation and Fast protein liquid chromatography (FPLC), characterized via Nano tracking analysis (NTA), and Transmission electron microscopy (TEM). Protein expression was demonstrated by western blot.

To evaluate the use of EV as a potential adjuvant *in vitro*, we stimulated bone marrow-derived macrophages (BMM) and bone marrow-derived dendritic cells (BMDC) with EVs expressing S protein (EV-rS), EV which do not express S protein (EV-WT), and recombinant S (rS) protein and then quantified the expression of immunomodulatory cytokine genes 6h post-stimulus by RT-qPCR.

We observed a dose-dependent immunostimulatory effect. Also, rS proteins induced a higher relative cytokine gene expression compared to EV-rS. This effect was overcome by EV-rS only at higher doses in BMDC. Interestingly, we found that EV-rS reduce cytokine expression in BMM when compared with EV-WT and rS in all doses.

This project serves as a baseline for the to exploitation of EVs released by *Leishmania tarentolae* as a vaccine platform, which represents an expression system that can be easily implemented around the world.

Keywords: Extracellular vesicles, sub-unit vaccines, adjuvants, *Leishmania tarentolae.*

RÉSUMÉ

Les vaccins sous-unitaires utilisent des antigènes clés pour stimuler une réponse immunitaire protectrice de longue durée. Cependant, ils présentent une faible immunogénicité et nécessitent le support d'un adjuvant.

Les vésicules extracellulaires (VE) sont des particules formées par une bicouche lipidique sphérique qui sont libérées par toutes les cellules. Il a été démontré que *Leishmania*, un parasite protozoaire, peut activement libérer des EV qui modulent les cellules immunitaires des mammifères. Par conséquent, nous avons émis l'hypothèse que les EV dérivées de *Leishmania tarentolae*, une espèce non pathogène, ont un effet immunostimulant sur le développement d'adjuvants vaccinaux.

Nous avons utilisé la protéine SRAS-CoV-2 Spike (S) comme preuve de concept. La glycoprotéine S pleine longueur a été produite par synthèse et optimisée en codons pour l'expression constitutive dans *L. tarentolae*. Les EV dérivées de *L. tarentolae* ont été isolées par ultracentrifugation et FPLC et caractérisées via NTA, TEM. L'expression des protéines a été démontrée par western-blot.

Pour évaluer le potentiel adjuvant de le VE *in vitro*, nous avons stimulé des macrophages dérivés de la moelle osseuse (BMM) et des cellules dendritiques dérivées de la moelle osseuse (BMDC) avec VE exprimant S (EV-rS), VE qui n'expriment pas S (EV-WT), et S recombinant (rS) et quantification de l'expression de gènes de cytokines après 6h par RT-qPCR.

Nous avons trouvé un effet immunostimulant dose-dépendant. En outre, rS induit une expression relative des gènes de cytokines plus élevée que EV-rS. Cet effet n'a été surmonté par EV-rS qu'aux doses les plus élevées chez les BMDC. Fait intéressant, chez les BMM, nous avons constaté que EV-rS réduisent l'expression des cytokines par rapport à EV-WT et rS à toutes les doses.

Il s'agit d'un projet de base pour exploiter les VE libérées par *Leishmania tarentolae* en tant que plateforme vaccinale, qui est en soi un système d'expression, qui peut être facilement mis en œuvre dans le monde entier.

Mots-clés : Vésicules extracellulaires, vaccins sous-unitaires, adjuvant, *Leishmania tarentolae*

SYNOPSIS/SOMMAIRE RÉCAPITULATIF

Les vaccins sous-unitaires sont basés sur des parties de pathogènes (sous-unités) spécifiquement sélectionnées pour leur capacité à stimuler les cellules immunitaires. Étant donné que ces vaccins ne contiennent pas l'organisme entier, ils présentent une faible immunogénicité car ils manquent de stimulateurs immunitaires innés endogènes nécessaires pour induire une réponse immunitaire adaptative à l'antigène. À cet égard, ces vaccins utilisent des adjuvants comme stimulateurs immunitaires innés pour potentialiser la réponse immunitaire induite par le vaccin. Étant donné que ces vaccins contiennent seulement des molécules purifiées de l'agent pathogène, ils sont incapables de provoquer des maladies, ce qui les rend très sûrs, en particulier pour les personnes immunodéprimées, les personnes âgées et les bébés.

Les adjuvants sont des substances utilisées en combinaison avec un antigène spécifique qui produisent plus d'immunité que l'antigène seul, car ils ont le potentiel d'améliorer l'ampleur, la fonctionnalité, l'étendue et/ou la durabilité des réponses immunitaires adaptatives. Les adjuvants peuvent agir de différentes manières pour déclencher une réponse immunitaire; ils peuvent maintenir et ralentir la libération des antigènes au site d'injection, créant un effet de dépôt; connus sous le nom de systèmes de délivrance, ou ils peuvent agir comme des agonistes des récepteurs immunitaires innés qui fonctionnent comme des modèles moléculaires associés aux agents pathogènes (PAMP, acronyme pour *Pathogen-associated molecular pattern*). Ils sont directement reconnus par les récepteurs immunitaires innés exprimés par les cellules présentatrices d'antigène (CPA) et connus sous le nom de récepteurs de reconnaissance de formes (PRR, acronyme pour *Pattern recognition receptor*), tels que les récepteurs de type Toll (TLR), les récepteurs de type gène-1 inductibles par l'acide rétinoïque (RLR) , les récepteurs de type nod (NLR), le récepteur de type lectine de type C (CLR), les récepteurs de type AIM2 (ALR) et cGAS/STING, et induisent des réponses pro-inflammatoires par l'entremise des cytokines et des interférons (voir tableau 2).

Les cytokines et les chimiokines peuvent recruter des cellules immunitaires au site d'injection, augmentant l'absorption d'antigène par les APC, y compris les macrophages, les cellules B et les cellules dendritiques. Ainsi, en activant les APC et augmentant la capacité de traitement et de

présentation de l'antigène, les APC migreront vers les ganglions lymphatiques drainants pour interagir avec les cellules B ou T spécifiques de l'antigène en fonction des cytokines présentes. Les cellules CD4 + T naïves se différencieront en différents T - auxiliaire (Th) qui peuvent jouer de multiples rôles en tant qu'effecteurs et régulateurs de l'immunité adaptative. À cet égard, ils peuvent activer les cellules B sécrétant des anticorps et/ou les réponses des lymphocytes T CD8+ effecteurs (voir Figure 1).

Les vésicules extracellulaires (VE) sont des particules formées par une bicouche lipidique sphérique d'une taille allant de 20 à 4000 nm, contenant différentes cargaisons telles que des protéines, des acides nucléiques et des lipides. Les VE sont générées et libérées par différents types de cellules, y compris les cellules eucaryotes. Il a été démontré que *Leishmania*, un parasite protozoaire, peut libérer activement des VE qui modulent les cellules immunitaires des mammifères *in vitro* et *in vivo*. Les VE induisent d'importantes réponses pro-inflammatoires telles que la production de cytokines et de chimiokines qui favorisent le recrutement de cellules inflammatoires innées essentielles à la présentation des antigènes aux cellules immunitaires. Par conséquent, les VE agissent comme des adjuvants dans le cadre de la vaccinologie.

A cet égard, nous voulons tirer avantage de *Leishmania tarentolae*, une espèce non pathogène pour l'homme, isolée des lézards et riche en glycoprotéines. Les schémas de glycosylation des protéines de *L. tarentolae* sont étroitement liés à ceux des mammifères. Ceci est important dans divers processus biologiques, tels que la prolifération et la différenciation cellulaires, le développement des organismes, la communication cellulaire, la migration cellulaire et l'immunité. De plus, la glycosylation des protéines est un mécanisme de contrôle de la qualité du statut de réponse des protéines, qui augmente leur demi-vie et régule l'interaction des protéines avec les récepteurs cellulaires. La composition spécifique des glycanes, ou plutôt le résidu de sucre terminal, régule directement la réponse immunitaire. Ceci nous laisse penser que *Leishmania tarentolae* serait un excellent système d'expression pour la production de protéines hétérologues et un bon système de livraison pour ces protéines.

Par conséquent, notre hypothèse est que les VE dérivées de *Leishmania tarentolae* ont un effet immunostimulant et constituent une bonne cible pour le développement de nouveaux adjuvants vaccinaux affinés.

Comme preuve de concept, nous utilisons la protéine Spike du SRAS-CoV-2 (S), une glycoprotéine de fusion de classe I, qui est la principale protéine de surface du virion du coronavirus, essentielle pour la liaison aux récepteurs, la fusion et l'entrée du virus. À cet égard, notre objectif était d'évaluer les propriétés immunomodulatrices de la protéine recombinante Spike (rS) exprimée dans les VE dérivées de *Leishmania tarentolae.*

Pour ce faire, une construction a été produite synthétiquement à partir de la glycoprotéine Spike du SRAS-CoV-2 avec sa pleine longueur. Des modifications ont été apportées au site de clivage S1/S2 pour conférer une résistance aux protéases en mutant le site de clivage de la furine 682-RRAR-685 en 682-GSAS-685. Deux substitutions de proline ont été effectuées pour maintenir l'état de pré-fusion et la séquence a été optimisée en codons pour l'expression constitutive dans la souche P10 de *L. tarentolae*.

L'expression de la protéine S a été obtenue après transfection de la souche P10 de *L. tarentolae* par électroporation à basse tension avec 4,8 µg de plasmide contenant la séquence entière du gène S du virus SRAS-CoV-2 générant des lignées cellulaires stables exprimant la protéine. Pour confirmer l'expression de la protéine S, nous avons effectué un Western blot en utilisant un anticorps anti-His tag. Nous avons observé une bande d'environ 180 kDa, ce qui correspond à la taille de la protéine monomérique, dans les lysats totaux et les protéines sécrétées de *Leishmania tarentolae* exprimant rSpike (LrS) (Figure 8). Cette détection n'a pas été observée dans les protéines de *L. tarentolae* WT (LWT). L'expression de la protéine S n'a pas eu d'impact négatif sur la prolifération et survie du parasite (Figure 9).

Après avoir confirmé que *L. tarentolae* exprime la protéine S, nous avons évalué si leurs VE l'exprimait aussi. Nous avons isolé les vésicules extracellulaires du surnageant de la culture LrS et LWT, en utilisant respectivement FPLC et ultracentrifugation. Pour évaluer la concentration et la taille, nous avons utilisé le système d'analyse des nanoparticules (NTA). Nous avons trouvé que les VE dérivées de LrS et LWT ont une population d'homogénéité avec une taille similaire, ayant une taille moyenne de 113 nm et une concentration de 2,32 x 10^{12} particules/ml pour LrS et pour LWT la moyenne de taille était de 109,6 nm à une concentration de 3,32 x 10^{12} particules/ml (Figure 11). La taille des vésicules extracellulaire a été confirmée par microscopie électronique à transmission (MET). Nous avons déterminé ainsi que la taille des VE varie de 62nm

à 257 nm et nous avons aussi confirmé qu'ils s'agissaient de vésicules rondes avec une bicouche lipidique, qui présentent une morphologie en forme de coupe (Figure 12). Pour confirmer l'expression du rS dans les VE, nous avons utilisé l'anticorps RBD de la protéine spike du coronavirus SRAS dont la taille est d'environ 180 kDa. C'est un anticorps polyclonal de lapin et il a été utilisé à une concentration de 1: 2000. Pour confirmer qu'il s'agit de VE, nous avons utilisé une protéine de surface tenue par une ancre GPI (glycophosphoinositol), la zinc métallo-protéinase GP63, connue pour se trouver au niveau des VE. Ceci nous a permis de détecter les VE de *Leishmania*. Nous avons observé une bande d'une taille de 180 kDa pour rS et une autre bande de 63 kDa pour GP63 sur les VE libérées par les LrS (Figure 13), ce qui confirme que nous avons des VE qui expriment LrS. Deuxièmement, sur les VE libérées par *L. tarentolae* WT (LWT), nous avons seulement observé la bande de 63 kDa qui confirme l'expression du marqueur GP63 des VE de *Leishmania* (Figure 13). Pour comparer l'effet immunomodulateur des VE en tant qu'adjuvants, nous avons purifié la protéine Spike par affinité dans des conditions dénaturantes en utilisant une concentration d'urée de 6M.

Nous avons évalué le potentiel inflammatoire des VE qui expriment la protéine recombinante (EV-rS) dérivée de *L. tarentolae* en quantifiant la production des cytokines. Nous avons stimulé les macrophages (BMM) et les cellules dendritiques dérivées de la moelle osseuse (BMDC) avec des VE dérivées de la souche de type sauvage de *L. tarentolae* (EV-WT) et la souche qui exprime la protéine Spike du SRAS-CoV-2 (EV-rS). Nous avons également utilisé des rS purifiées à partir du surnageant de *Leishmania tarentolae* comme stimulus pour évaluer l'effet de la protéine sans adjuvant, et du LPS comme stimulus témoin positif. Nous avons utilisé 3 doses différentes pour chaque stimulus : 1 µg/ml, 10 µg/ml et 100 µg/ml et nous avons quantifié l'expression relative de l'interleukine-12 (IL-12), de l'interleukine-10 (IL-10), de l'interleukine-6 (IL-6), interleukine-1α (IL-1α), interleukine-1β (IL-1 β), cytokines du gène immunomodulateur du facteur de nécrose tumorale alpha (TNF-α) après 6 h de stimulation par RT-qPCR selon la méthode Livak ou $2^{-\Delta\Delta CT}$.

Les résultats démontrés sur la Figure 17 ont révélé que l'expression génique des cytokines était plus élevée dans les BMM que les BMCD suite à la stimulation par EV-WT et rS. Nous avons également observé que les VE dérivées de *L. tarentolae* ont un effet immunostimulant de manière dose-dépendante, dans les deux populations (BMM et BMDC) comme le montre la Figure 18.

Cependant, lorsque le stimulus a été réalisé avec LrS, nous avons constaté une légère diminution lorsqu'on a utilisé la dose de 100 µg/ml.

Avec les BMDCs, nous avons observé une expression relative plus élevée des gènes de cytokines lorsque nous avons stimulé avec rS à des doses de 1 µg/ml et 10 µg/ml. Cet effet n'a été dépassé que par EV-rS aux doses les plus élevées évaluées (100 µg/ml) (Figure 19), sauf pour la cytokine anti-inflammatoire IL-10, où le stimulus avec rS était plus élevé. Une dose de 10 µg/ml augmente les niveaux de cytokine presqu'au même niveau qu'une dose de 100 µg/ml (à l'exception de l'IL-10) voir Figure 19.

Avec les BMMs, nous avons également découvert que rS stimule les niveaux les plus élevés d'expression des gènes de cytokines, à toutes les doses évaluées. Fait intéressant, nous avons constaté que le stimulus EV-rS réduit l'expression des cytokines par rapport au stimulus EV-WT pour les six cytokines génétiques évaluées, en particulier aux doses 1 et 10 µg/ml. Lorsque nous avons évalué les différences entre les stimuli au sein de chaque dose, nous avons vu que les doses de 1 µg/ml et 10 µg/ml étaient celles engendrant le plus de différences entre les trois stimuli dans toutes les cytokines évaluées (Figure 20). Nous supposons que GP63 et d'autres molécules à la surface des vésicules extracellulaires (VE) de *Leishmania* peuvent provoquer l'effet immunostimulant. À cet égard, il est important d'évaluer le protéome des VE de *Leishmania tarentolae* pour comprendre quelles molécules pourraient agir comme adjuvants.

Ici, nous montrons que *L. tarentolae* peut exprimer des protéines recombinantes hétérologues de haut poids moléculaire telles que Spike du SRAS-CoV-2 (~180kDa), sans altération de sa viabilité. Nous avons réussi à obtenir la protéine Spike exprimée sur les VE libérées par *L. tarentolae*, comme le confirment les analyses TEM et Western blot, ce qui nous permet d'étudier sa capacité à fonctionner comme adjuvant et antigène pour, ultimement, développer une plateforme vaccinale.

Ce travail a permis l'évaluation initiale et mis en lumière les optimisations nécessaires pour les prochaines étapes vers l'utilisation de VE comme plate-forme vaccinale. Nous avons commencé par l'évaluation du rôle immunomodulateur de l'antigène dans les VE dans le but d'affiner notre système. À cet égard, nous suggérons d'améliorer la façon dont nous quantifions les quantités

d'échantillon utilisées pour stimuler les cellules afin d'avoir la même quantité de protéines pour chaque stimulus. Nous avons optimisé la procédure d'isolement pour obtenir les VE exprimant Spike par FPLC. Néanmoins, ces VE représentent une source de protéines qui sont incluses dans le total de protéines mesurées, et n'auront donc pas la même quantité de protéine Spike que l'échantillon rS a. En ce sens, il est recommandé de faire une analyse par Western blot avec différentes quantités, de comparer par densitométrie et d'utiliser les quantités ajustées à chaque échantillon. Une autre stratégie pour régler ce problème pourrait être de trier les VE par nanoFACS. D'autre part, il pourrait être intéressant d'améliorer l'expression de rS sur les VE. Notre approche comprend l'utilisation d'un peptide signal (SP) de la phosphatase acide sécrétée de *L. mexicana* (LMSAP1) qui ajoute un peptide signal à notre protéine à sécréter. Cependant, comme nous l'avons vu avec l'immunogold (Figure 14), tous les VE n'avaient pas la nanoparticule d'or, ce qui signifie que ce ne sont pas toutes les VE libérées par *L. tarentolae*-rS qui expriment rS.

En conclusion, nous démontrons que *Leishmania tarentolae* libère de vésicules extracellulaires fortement pro-inflammatoires qui ont le potentiel d'être utilisées comme adjuvant et comme véhicule pour la production et l'administration d'antigènes. Ce système pourrait en effet être développé comme étant efficace, facile à manipuler et abordable. Celui-ci pourrait être très bénéfique pour la recherche en laboratoire dans le monde entier.

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1 INTRODUCTION

1.1 VACCINES

Surviving infectious diseases was the trigger for immunological studies. This resulted in the development and use of rudimentary vaccines even before there existed any knowledge of how vaccines work. For instance, "variolation" (referred to then as "inoculation") traces back to the mid-1500s in Asia and was used to protect people against smallpox. This procedure consisted of inserting pus from pustules of smallpox infected individuals into scratches made on the skin surface of an uninfected person to confer protection (Flemming, 2020). Years later, in 1796, the physician Edward Jenner formally tested the hypothesis that cowpox infection could prevent severe disease from smallpox infection in humans. His hypothesis was proven right by variolating a young boy with material from lesions of cows affected with cowpox and subsequently injecting him with the smallpox virus. Thus, the boy neither became infected nor had symptoms, demonstrating that such a procedure confers protection against smallpox. This then became to be known as "vaccination", given the bovine heritage, and this is known as the first well-described attempt at immunization (Clem, 2011; Pollard and Bijker, 2021).

Thus, it was shown by Asians with variolation, and Jenner with vaccination, that inoculating biological products (antigens from a pathogen) into host induced an immune response capable of conferring protection against infection and/or disease on subsequent exposure to the pathogen. These first examples of "vaccines" demonstrates their capacity of activating the immune system to respond and remember a pathogenic antigen after prior exposure. Therefore, vaccines confer protection in different ways from preventing infection (big aim) to reducing disease severity or rate of hospitalization (Pollard and Bijker, 2021). This milestone has transformed public health and has contributed to the increase of our lifespan by eliminating many infectious diseases that devastated mankind (Rappuoli et al., 2011). For example, broad vaccination coverage at the population level led to a marked reduction in the impact of some diseases such as diphtheria, measles, mumps, whooping cough, rubella (German measles), polio, and tetanus, which at some point collectively claimed millions of lives. Moreover, vaccination has also resulted in the disappearance of other diseases, such as smallpox and rinderpest (Greenwood, 2014; Owen et al., 2014).

Recently, we have witnessed the impact of the SARS-CoV-2 vaccines, which have prevented over 14.4 million deaths from COVID-19 in 185 countries and territories between Dec 8, 2020, and Dec 8, 2021, according to a recent study (Watson et al., 2022).

There are several approaches used in vaccine development that can be divided broadly into: i) whole microbe vaccines, ii) subunit vaccines, iii) nucleic acid vaccines; whose main characteristics are summarized inTable 1.

1.1.1 Whole microbe vaccines

Traditional vaccines consist of a killed or a live-attenuated pathogens that cannot cause disease. Live-attenuated vaccines contain a weakened version of a living pathogen, that can still trigger a protective immune response similar to the one observed in natural infections, but which does not cause disease (Zhou et al., 2022). However, there are restriction in the use of such vaccines in immune-suppressed individuals given the ability of attenuated pathogens to replicate in an uncontrolled manner in such people (Pollard and Bijker, 2021). On the other hand, vaccines containing killed pathogens are more stable and are often combined with adjuvants to elicit a stronger immune response. Another astonishing type of vaccine that is worth mentioning in this section is the viral vector-based vaccine. They use recombinant viruses (either replication competent or not) to express desired antigens to target the pathogen of interest. The stimuli caused by the viral vector together with the expression of the pathogen antigen mimics natural infection and leads to the induction of a strong humoral and cellular immune response without the assistance of an adjuvant. The disadvantage of this type of vaccine is the presence of pre-existing immunity to the viral vector used, which can be overcome by using vectors from other similar species.

1.1.2 Subunit vaccines

Subunit vaccines are based on specific parts (subunits) of a pathogen which are selected for their ability to stimulate immune cells. Subunits vaccines are mostly composed of proteins, sugars, or a conjugation between both of these molecules (Zhou et al., 2022), but they can also be based on toxins secreted from a pathogenic organism (toxoid vaccines), (Pollard and Bijker, 2021), virus-like particles (VLP), and extracellular vesicles-based vaccines. Since these vaccines do not contain the whole pathogen, they exhibit low immunogenicity because they lack the endogenous

innate immune stimulators required to induce an adaptive immune response against the antigen (Aoshi, 2017).

In this regard, these vaccines use adjuvants as an innate immune stimulator to potentiate the vaccine-induced immune response. Since these vaccines contain purified molecules from the pathogen, they are incapable of causing disease, which makes them very safe, especially for immunocompromised people, the elderly and babies (Rappuoli et al., 2011), their main risk may be caused by the adjuvant, which enhances their immunogenicity (Zhou et al., 2022).

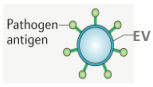
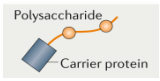
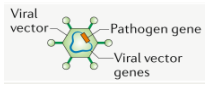
1.1.3 Nucleic acid vaccines

Nucleic acid vaccines are composed of DNA or RNA sequences encoding a target antigen. The genetic information for the antigen is delivered instead of the antigen itself to the recipient's host cells in which the antigen is then expressed. This event potentially induces humoral and cellular immune responses. Those vaccines are versatile, quick, and easy to adapt to large-scale production, as seen with the SARS-CoV-2 mRNA-based vaccines, which entered into clinical testing 2 months after the SARS-CoV-2 genome was sequenced (Corbett et al., 2020). Nonetheless, there are some limitations for DNA-based vaccines, the main one being the delivery of the genetic payload to the nucleus of an antigen-presenting cell, which must be done by using an electroporator, thus limiting their use. The main disadvantage of RNA-based vaccines is their stability that depends on storage in frozen conditions (Krammer, 2020; Kumar et al., 2018; Pollard and Bijker, 2021; Sheridan, 2021).

1.2 ADJUVANTS

Adjuvants were first described in 1925 as substances used in combination with specific antigen that elicit stronger immunity than the antigen alone (Awate et al., 2013; Turley and Lavelle, 2022) as they have the potential to enhance the magnitude, functionality, breadth and/or durability of adaptive immune responses. Different compounds have been used as adjuvants including mineral salts, microbial products, emulsions, saponins, cytokines, polymers, microparticles, and liposomes (Awate et al., 2013) Table 2.

Table 1 Types of Vaccine and principal characteristics

Type of vaccine	Advantages	Limitations	Licensed vaccines using this technology	First introduced
Live attenuated (weakened or inactivated) 	Strong immune responses No adjuvant required Cost-effective	Biosafety issue Risk of reversion to virulence Time-consuming development	Measles, mumps, rubella, yellow fever, influenza, oral polio, typhoid, Japanese encephalitis, rotavirus, BCG, varicella zoster	1798 (smallpox)
Killed whole organism 	Stable and no risk of reversion Strong antibody response Cost-effective	Biosafety issue, Usually requires adjuvants, Weak cellular immune response	Whole-cell pertussis, polio, influenza, Japanese encephalitis, hepatitis A, rabies	1896 (typhoid)
Toxoid 	No risk of infection and reversion Stable and long lasting Less susceptibe to change in temperature, humidity and light [a]	Adjuvants and several doses need it. Local reactions at the vaccine site are more common [a]	Diphtheria, tetanus	1923 (diphtheria)
Subunit (purified protein, recombinant protein, polysaccharide, peptide) 	No risk of infection and reversion Fewer side effects Easy antigen modification	Low immunogenicity Requieres adjuvants High cost	Pertussis, influenza, hepatitis B, meningococcal, pneumococcal, typhoid, hepatitis A	1970 (anthrax)
Virus-like particle 	No risk of infection and reversion Fewer side effects Good antibody response	Complicated manufacturing process Requieres adjuvants High cost	Human papillomavirus	1986 (hepatitis B)
Outer membrane vesicle /Extracellular Vesicles 	No risk of infection and reversion Inherent immunostimulatory properties [b]	The immune response may dominantly target the EVs instead of Ag of interest [b]	Group B meningococcal	1987 (group B meningococcal)
Protein–polysaccharide conjugate 	Cost-effective Affinity maturation Induction of memory B cells Long-lived antibody production Improved immune responses in infants	Same carrier use may increase the chance of immune interference Maternal immunization with conjugate vaccines may affect infant immunization responses [c]	Haemophilus influenzae type B, pneumococcal, meningococcal, typhoid	1987 (H. influenzae type b)
Viral vectored 	Strong immune responses Various viral vectors Large-scalable	Pre-existing immunity against the vector	Ebola	2019 (Ebola)
Nucleic acid vaccine DNA 	Rapid development and production Stable in room temperature High producibility	Low immunogenicity Requires a delivery device (electroporator or jet-injector)	Equine West nile virus, tyrosinase for melanoma canine, two salmon vaccine: hematopoietic necrosis virus and alphavirus subtype[d], and SARS-CoV-2	2005 (West Nile Virus) Veterinary use[d] 2021 (SARS-CoV-2)
RNA 	Cell-free Rapid development and production Good immunogenicity	Unstable High cost Requieres low temperature storage	SARS-CoV-2	2020 (SARS-CoV-2)

Adapted from (Lee et al., 2021; Pollard and Bijker, 2021).

Schematic representation of different types of vaccines against pathogens. The text indicates against which pathogens certain vaccines are licensed and when each type of vaccine was first introduced. Abbreviations: BCG, *Mycobacterium bovis* bacillus Calmette–Guérin; EV, Extracellular Vesicle; SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2; [a](Baxter, 2007); [b](Wallis et al., 2019); [c](Bröker et al., 2017); [d](Sheridan, 2021)

Adjuvants function in different ways to elicit an immune response. For example, they can sustain and slowly-release antigens at the site of injection, creating a depot effect; known as *delivery systems*. Table 2 shows there are adjuvants of many natures, such as microspheres, nano- or microparticles, polymers, emulsion, liposomes and immune-stimulating complexes that are used to encapsulate the antigen and deliver it to a specific cell (Araujo Correa et al., 2022).

On the other hand, adjuvants can act as *innate immune receptor agonists* that function as pathogen-associated molecular patterns (PAMPs). They are directly recognized by innate immune receptors expressed in antigen-presenting cells (APC) known as pattern recognition receptors (PRR), such as Toll-like receptors (TLRs), retinoic acid-inducible gene-1 -like receptors (RLRs), nucleotide oligomerization domain-like receptors (NLRs), C-type lectin-like receptor (CLR), AIM2-like receptors (ALRs), and cGAS/STING, which induce proinflammatory cytokine and interferon responses (Aoshi, 2017) (Table 2).

Table 2 Classification of adjuvants according to their main mechanism of action.

Adjuvant Groups	Types of Adjuvants
Delivery systems	
Mineral Salts	Aluminium salts
Emulsions	Freund's adjuvants
	MF59
	AS03
Microparticles	Virus-like particles
	Virosomes
	PLA/PLGA
Immune Potentiators	
TLR1/2 agonists	L-pampo, MALP-2, Pam2CSK4 and Pam3CSK4
TLR3 agonists	Poly(I:C) (polyinosinic:polycytidylic acid)
	Poly-ICLC
TLR4 agonists	Monophosphoryl lipid A (MPL)
TLR5 agonists	Flagellin
TLR7/8 agonists	Imiquimod (R837; 1-(2-methylpropyl)-1H-imidazo [4,5-c]quinolin-4-amine) and resiquimod (R848, 4-amino-2-(etoximetil)-a,a-dimethyl-1H-imidazo [4,5-c]quinoline-1-ethanol)
TLR9 agonists	CpG ODNs
Combined adjuvants	AS01 and AS02
	AS04
Mucosal adjuvants	Cholera toxin (CT)
	Heat-labile enterotoxin (LTK3 and LTR72)
	Chitosan

Modified from (Facciolà et al., 2022)**.**

In turn, cytokines and chemokines can recruit immune cells at the site of injection increasing antigen uptake by APCs, including macrophages, B-cells, and dendritic cells, thus activating APCs and increasing their antigen processing capacity and presentation. In consequence, the APCs migrate to the draining lymph nodes to interact with antigen-specific B- or T-cells (Awate et al., 2013; Coffman et al., 2010; Facciolà et al., 2022). Depending on the cytokines present, naïve CD4+T-cells will differentiate into different T-helper (Th) subset that can play multiples roles as an effector and regulator of adaptive immunity (Turley and Lavelle, 2022). In this regard, they can activate antibody-secreting B-cells and/or effector CD8+ T-cell responses (Figure 1).

Additionally, some adjuvants can also induce damage in host cells, causing them to release several factors classified as damage-associated molecular pattern (DAMP) (e. g. DNA and RNA), which subsequently activate innate immune receptors, which then trigger inflammation and induce an adaptive immune response (Aoshi, 2017; Turley and Lavelle, 2022). Some of the substances such as lipids, mineral salts, or polymers particles are part of the adjuvants that induce damage-associated molecular patterns (Aoshi, 2017).

The innate immune system is pivotal to adjuvant function as we described above, however, it is important to know the molecular and cellular mechanisms they elicit to promote antibodies and T-cell responses. In this regard, improving or designing new adjuvants which can functions as carriers and potent immune response activators that promote durable antibodies and cellular immune responses is most important in the future of vaccine development (Turley and Lavelle, 2022). An important mode of action for such adjuvants is to prolong antigen exposure to dendritic cells (DCs) to induce their maturation. These mature DCs are extremely effective in activating antigen-specific T-cells, which is a pre-requisite for the establishment of a potent and long-lasting cellular immunity (Ho et al., 2018).

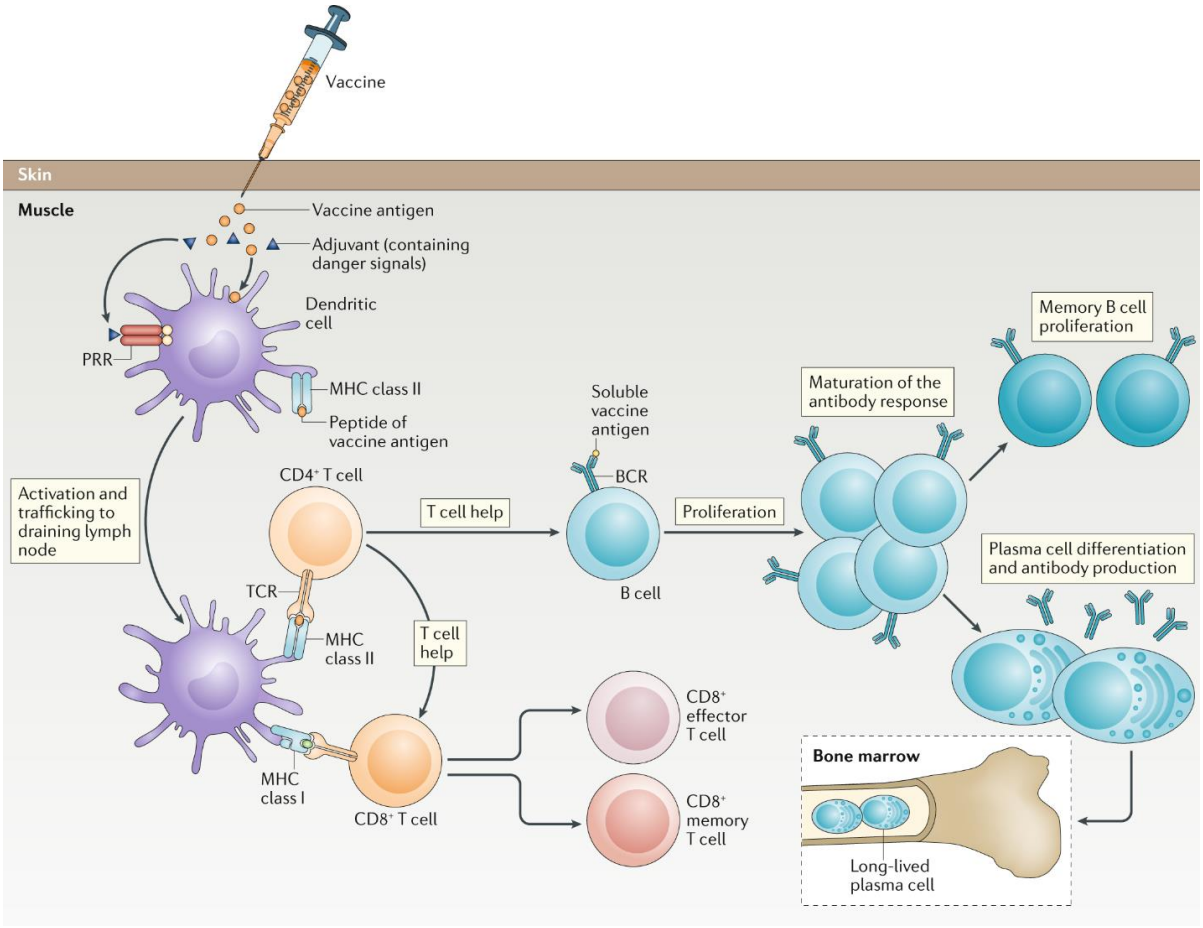


Figure 1 The generation of an immune response to a vaccine

Reproduced from (Pollard and Bijker, 2021). The immune response following immunization with a conventional protein antigen. The vaccine is injected into the muscle and the protein antigen is taken up by dendritic cells, which are activated through pattern recognition receptors (PRRs) by danger signals in the adjuvant, and then trafficked to the draining lymph node. Here, the presentation of peptides of the vaccine protein antigen by MHC molecules on the dendritic cell activates T-cells through their T-cell receptor (TCR). In combination with signaling (by soluble antigen) through the B-cell receptor (BCR), the T-cells drive B-cell development in the lymph node. Here, the T-cell-dependent B-cell development results in maturation of the antibody response to increase antibody affinity and induce different antibody isotypes. The production of short-lived plasma cells, which actively secrete antibodies specific for the vaccine protein, produces a rapid rise in serum antibody levels over the next 2 weeks. Memory B-cells are also produced, which mediate immune memory. Long-lived plasma cells that can continue to produce antibodies for decades travel to reside in bone marrow niches. CD8+ memory T-cells can proliferate rapidly when they encounter a pathogen, and CD8+ effector T-cells are important for the elimination of infected cells.

1.3 EXTRACELLULAR VESICLES

The designation 'extracellular vesicles' was first suggested in 2011 as a collective term to describe lipid bilayer-enclosed cell-derived particles (György et al., 2011) which range in sizes from 30 to 5,000 nm (Atkin-Smith and Poon, 2017; Verweij et al., 2021; Willms et al., 2018), that can be released from any cells types. These characteristics have been shown to be a conserved feature throughout evolution from bacteria to higher eukaryotic organisms such as humans and plants (Deatheragea and Cooksona, 2012).

EV secretions were initially described as a way to eliminate cellular waste components with no important biological significance (El Andaloussi et al., 2013). However, current knowledge suggests that these vesicles enclose different components such as lipids, proteins, carbohydrates and nucleic acids, creating a heterogeneous population that plays an important role in intercellular communication (Battistelli and Falcieri, 2020; Huda and Nurunnabi, 2022), pathogenesis (acting as viral factors) (Atayde et al., 2015; Barbosa et al., 2018; Castelli et al., 2019; Gabriel et al., 2021; Gioseffi et al., 2020), progression in different diseases (Lenassi et al., 2010; Todd and Tripp, 2020; Van Niel et al., 2018; Xu et al., 2020) and even play a role in immune surveillance (Huda and Nurunnabi, 2022).

Based on the current knowledge of their biogenesis, EVs can be broadly divided into exosomes (EXOs), microvesicles (MVs), and apoptotic bodies (APOs) (Figure 2).

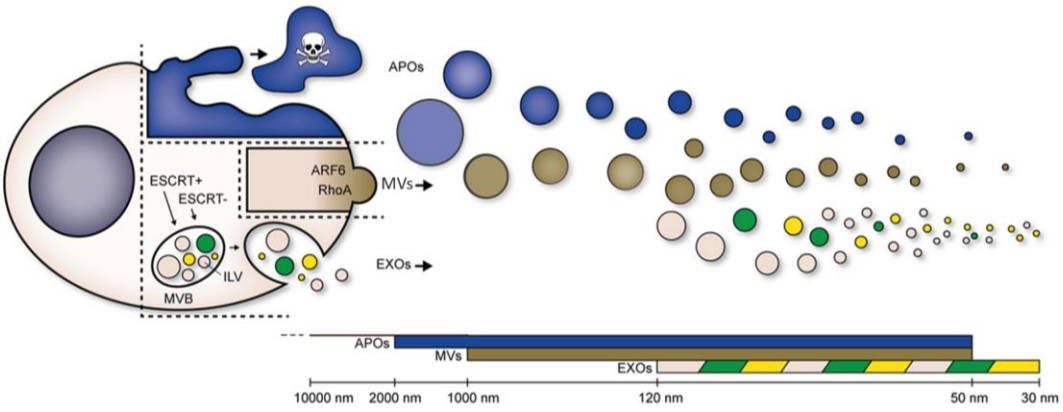


Figure 2 Classification and biogenesis of Extracellular Vesicles.

Modified from (Willms et al., 2018). Cells release heterogeneous populations of EVs with overlapping sizes. APOs (blue) are released by cells undergoing apoptosis. MVs (brown) are derived directly from the plasma membrane, ARF6 and RhoA are key players in the biogenesis of MVs. EXOs (pink) are derived from intracellular endosomal compartments. ILVs form within MVBs and are subsequently released upon fusion of MVBs with the plasma membrane. Both ESCRT-dependent (ESCRT+) and -independent (ESCRT−) pathways are involved in biogenesis of EXOs. Unique subpopulations of EXOs (as indicated by green and yellow EVs) have been identified. Abbreviations: MVB, multivesicular body; ILV, intraluminal vesicle; ESCRT+, endosomal sorting complex required for transport-dependent; ESCRT, endosomal sorting complex required for transport-independent; ARF6, ADP-ribosylation factor 6; RhoA, Ras homolog gene family, member A; EVs, extracellular vesicles; APOs, apoptotic bodies; MVs, microvesicles; EXOs, exosomes.

1.3.1 Exosomes

The term exosome (which is not to be confused with the exosome complex involved in RNA degradation) was initially used for vesicles of an unknown origin released from a variety of cultured cells and having 5´nucleotidase activity (Van Niel et al., 2018). Subsequently, the term exosome was adopted to refer to membrane vesicles derived from inward budding of the inner endosomal membrane to form multivesicular bodies, which are released upon fusion with the plasma membrane. These vesicles range in size from 30 to 150 nm and can contain different cargos such as proteins, nucleic acids, and lipids (Devhare and Ray, 2017; Van Niel et al., 2018; Willms et al., 2018).

Exosomes biogenesis is driven either by the endosomal sorting complex required for transport (ESCRT) machinery, which is responsible for protein sorting and intraluminal vesicle (ILV) formation, but can also take place in the absence of the ESCRT machinery, in a process where tetraspanin proteins appear to be particularly important (Willms et al., 2018). This independent mechanism influences the formation of differently sized ILV subpopulations within MVBs and ultimately contribute to exosome heterogeneity.

1.3.2 Microvesicles

Microvesicles represent a population of non-apoptotic EVs which originate by the outward budding and fission of the plasma membrane and the subsequent release of vesicles into the extracellular space (Van Niel et al., 2018). In this process, there are different actors: on the one hand, there is a required increase in the cytosolic Ca^{2+} level, which leads to changes in the transbilayer lipid distribution and membrane blebbing by means of alterations in the activity of the enzymes flippase, translocase, and scramblase.

On the other hand, there is the ADP-ribosylation factor 6 (ARF6), and Ras homolog gene family member A (RhoA), which are regulators of MV budding and regulate the actin dynamics and reorganization of the actin cytoskeleton to allow the MV formation. MVs range in size from 50 to 1,000 nm in diameter (Willms et al., 2018).

1.3.3 Apoptotic bodies

Apoptotic bodies (APOs) are an EV population with a diameter of 500 to 5,000 nm (Battistelli and Falcieri, 2020; Verweij et al., 2021). This population is released by apoptotic cells through outward blebbing and fragmentation of the cell membrane. Those EVs may have a variety of cellular components such as micronuclei, chromatin remnants, portions of cytosol, degraded proteins, DNA fragments, or even intact organelles (Battistelli and Falcieri, 2020), and they can even package and sort RNA and DNA into separate different APO subpopulations (Willms et al., 2018). After being released into the extracellular space, they can be phagocytosed by macrophages, parenchymal cells, or neoplastic cells and degraded within phagolysosomes, thus preventing secondary necrosis without cytokine production (Battistelli and Falcieri, 2020).

1.4 EXTRACELLULAR VESICLES AS VACCINES

The different EV populations have varying highly dynamic and heterogeneous contents and membrane compositions, which depend on the cellular source, state and environmental conditions (Yáñez-Mó et al., 2015). Those features play an important role, as they provide an important immunostimulatory structure that can mediate the immune response (Jesus et al., 2018). In this regard, EVs released from immune cells are well studied as they can carry major histocompatibility complex molecules (MHC) I and MHC II, co-stimulatory, and adhesion molecules, which can directly stimulate CD8+ and CD4+ T cell clones (Devhare and Ray, 2017; Jesus et al., 2018). This idea received further support with the demonstration that vaccination of mice with tumor peptide-pulsed dendritic cells (DCs) exosomes primes tumor-specific cytotoxic T-lymphocytes and suppresses the tumor growth in a T-cell dependent manner (Zitvogel et al., 1998). In this sense, the strategy of using EVs from *in vitro* pulsed DCs has been used not only in anti-tumour immunotherapy but also as immunization against infectious diseases. Beauvillain and collaborators (Beauvillain et al., 2007) found that mice immunized with EVs collected from splenic dendritic cells pulsed *in vitro* with *Toxoplasma gondii*-derived antigens induced protective responses against infection with the parasite when adoptively transferred to mice and this

immunization works as an effective vaccine preventing congenital toxoplasmosis when given to mice before pregnancy (Beauvillain et al., 2009). In *Mycobacterium*, mice vaccinated with EVs derived from macrophages that were *Mycobacterium bovis* BCG-infected or pulsed with protein from *M. tuberculosis* culture filtrate were protected from a low-dose aerosol *M. tuberculosis* infection. The EVs induced antigen-specific IFN-γ and IL-2-expressing CD4+ and CD8+ T-cell responses in naïve mice or mice previously vaccinated with *M. bovis* BCG (Cheng and Schorey, 2013). This EV-vaccine application can cause a similar Th1 immune response but a more limited Th2 response than the Bacillus Calmette–Guérin (BCG)-vaccine, providing better protective immunity.

Other strategies to use EVs as a vaccine focus on EVs released by the pathogen or infected cells. Thus, according to the intrinsic nature of EVs, they can express virulence factors in their membrane that may act as PAMPs, consequently contributing to the activation of immune cells. Given these characteristics, EVs have been used as vaccination agents which facsimile the pathogen they are derived from (Alaniz et al., 2007; del Cacho et al., 2016; Montaner-Tarbes et al., 2018; Pal et al., 2020). For instance, the immunogenicity of extracellular vesicles from bacteria such as *Neisseria meningitis* serogoup B, has already been harnessed in two licensed vaccines: Bexsero®, developed by Novartis, and VA-MENGOC-BC®, developed by the Finlay institute in Cuba (Acevedo et al., 2014; Bai et al., 2011). Recently, more advances have been made on other bacteria such *Salmonella,* whose EVs possess important proinflammatory and antigenic properties similar to the parent bacteria and these EVs have the intrinsic combination of antigens and adjuvant properties required of an effective non-replicating complex vaccine to stimulate Salmonella immunity (Alaniz et al., 2007). This was also found for immunization against *Staphylococus aureus* infections where *S. aureus*-derived EVs induce adaptive immunity of antibody and T cell responses (Choi et al., 2015).

Another example of benefits that EVs provide is marked in studies which use probiotic bacteria released EVs therapeutic purposes. This was shown by Morishita and collaborators who demonstrated that EVs from *Bifidobacterium longum*, *Clostridium butyricum*, and *Lactobacillus plantarum WCFS1* stimulated innate immune cells to produce TNF-α and IL-6 suggesting those may be used as vaccine adjuvants (Morishita et al., 2021). In another study, Jesus and collaborators used LPS-induced EVs produced in the human monocytic THP-1 cell line as adjuvants co-administered with Hepatitis B antigen (HBsAg). They observed that LPS-induced EVs

enhanced the cellular immune response as illustrated by the increased IFN-γ concentrations but were not able to evoke a stronger specific immune response against HBsAg (Jesus et al., 2018).

Extracellular vesicles have been used as vehicles to express immunomodulatory pathogen antigens. As one example, Kuate and collaborators (Kuate et al., 2007) demonstrated that 293T cell-derived EVs expressing the Spike protein from SARS virus can trigger a humoral response, including neutralizing antibodies, which show to be highly immunogenic in mice. Polak and collaborators show that EV expressing Spike from SARS-CoV2 evoke a bigger humoral response, but this does not provide neutralizing capability, nor an effective T-cell response compared to an adenoviral vector DNA vaccine coding for Spike. Nonetheless, when they used EVs expressing Spike as a booster after priming with the DNA Spike-EVs, they obtained both types of protective responses (Polak et al., 2020), which was also observed by a different group (Kuate et al., 2007). It is important to note that these studies do not use adjuvants and since the EVs used in the assay were obtained from human cells, these vesicles would be expected to contain human proteins, which might be targeted by the murine immune response.

Interestingly, it has been shown that *Leishmania,* a protozoan parasite, can actively release EVs which modulate mammalian immune cells both *in vitro* and *in vivo* (Atayde et al., 2015). Therefore, there is a possibility that such vesicles may be used in vaccine design. It is known that not all *Leishmania* parasites are pathogenic to humans, a fact which can potentially provide an excellent EV platform that could be exploited to create EV based vaccines expressing antigens of different pathogens. However, limited information is available regarding the use of *Leishmania* derived EVs in vaccine design, which is a topic that we will unveil further down in the scope of the main objective of this thesis.

1.5 *LEISHMANIA TARENTOLAE*

L. tarentolae is a unicellular eukaryotic protozoan lizard parasite isolated from the gecko species *Tarentola mauritanica* (Raymond et al., 2012) and *Tarentola annularis* (de Oliveira et al., 2019), which belongs to the class of Kinetoplastida, order Trypanosomatida, and subgenus *Sauroleishmania* (Klatt et al., 2019; Raymond et al., 2012).

1.5.1 Life cycle

The life cycle of *L. tarentolae* is complex and digenetic, like their pathogenic counterparts, involving two species – *Sergentomya spp*. as the sand fly vector and the geckos as vertebrate hosts (Figure 3).

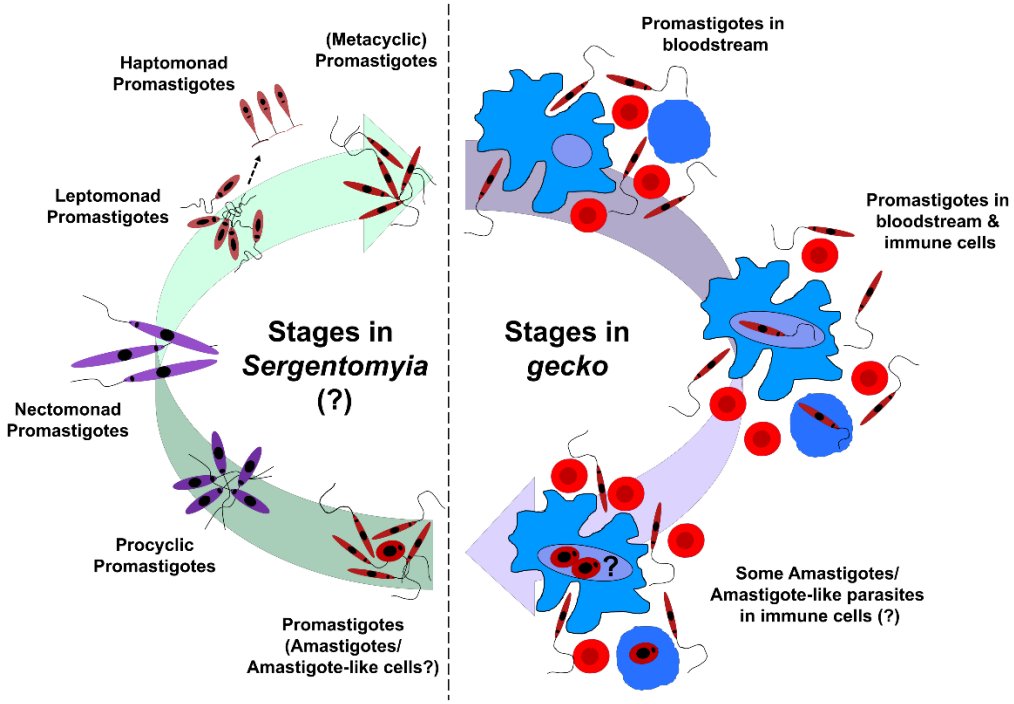


Figure 3 Life cycle of *Leishmania tarentolae*.

Reproduced from (Klatt et al., 2019). A sand fly (Sergentomyia) takes a blood meal from a gecko and thereby ingests promastigotes (major form) and some amastigotes/amastigote-like cells (minor form) of *L. tarentolae*. Inside the gut of the sand fly, immune cells burst, and the phagocytized part of Leishmania is released. Promastigotes colonize the lumen of the cloaca, the intestine (hindgut), and rarely, the blood of the sand fly. Although not proven, it is possible that promastigotes (and amastigotes) of *L. tarentolae* undergo several transformations inside the sand fly, through the stages of procyclic, nectomonad, leptomonad, haptomonad, and finally, metacyclic promastigotes. Metacyclic promastigotes are the gecko-infective stage, and they are transferred back to a gecko during a new blood meal. Inside the gecko, promastigotes mainly live free in the blood. A small part is probably phagocytized by immune cells (monocytes and macrophages). Phagocytosed promastigotes might transform back into amastigotes/ amastigote-like cells, and the life cycle repeats. Immune cells are represented in blue, and erythrocytes are represented in red.

Female sand flies ingurgitate *L. tarentolae* during blood ingestion and the parasite develops extracellularly into flagellated promastigotes in the hindgut of sand flies. In the proboscis of the sand fly, promastigotes then proliferate and are introduced into the gecko either through sand fly bites or via ingestion of the infected insect (Klatt et al., 2019; Lai et al., 2019).

L. tarentolae is rarely observed in the amastigotes stage in lizards. Instead, it exists predominantly as promastigotes in the bloodstream and in the lumen of the cloacae and intestine of the gecko. Nonetheless, amastigotes can be found infrequently either free or inside monocytes (Raymond et al., 2012).

1.5.2 *Leishmania tarentolae* as an expression system

L. tarentolae is building a pathway to becoming a first-choice eukaryote expression system for producing recombinant proteins with homogenous N-glycans. Its principal characteristics include the fact that it is a unicellular eukaryote and a non-pathogenic organism. This combines easy handling with a eukaryotic protein folding and modification machinery. Additionally, rapid culture growth in either a brain–heart infusion (BHI) medium or a yeast soybean based complex medium (Terrific broth) leads to reduced production costs down to levels comparable with *E. coli* (de Oliveira et al., 2019; Lai et al., 2019). Its serum-free growth environment is also advantageous to prevent viral or prion contaminations, making products generally safe for biomedical applications (Lai et al., 2019). Together with glycosylation potential, characteristic features such as RNA editing, arrangement of genes in tandem arrays, polycistronic RNAs and trans-splicing, and regulation of gene expression at the post-translational modification (PTM) level make *L. tarentolae* suitable expression platform (Basile and Peticca, 2009; Khan et al., 2017).

The most significant advantage of these parasites is the capacity to perform PTM similar to those in mammals (de Oliveira et al., 2019), such as phosphorylation and glycosylation (Lai et al., 2019). Glycans are known to play essential roles in various biological processes, such as cell proliferation and differentiation, organism development, cell communication, cellular migration, and immunity (Schön et al., 2020). In addition, their work as a quality control for the folding status of proteins allows them to properly develop their function and increase their half-life. For example, in protein interaction with cell receptors, the specific glycan composition or rather the terminal sugar residue directly regulates the immune response (Lai et al., 2019). This feature makes *L. tarentolae* a better option than their expression system counterparts, as was summarized in Table 3.

Table 3 Expression system characteristics

	Bacteria	Insect	Yeast	Plant	Mammalian	*L. tarentolae*
Supplement for growth	Not necessary	Not necessary	Not necessary	Not necessary	Animal-based serum	Hemin
Costs	Very low	High	Low	Low	High	Low to moderate
Growth rate	Fast (division time 20–30 minutes)	Slow (division time 18–24 h)	Fast (division time 2–4 h)	Slow (division time 16 h)	Slow (division time 24 h)	Moderate (division time 6–9 h)
Up scaling	Easy	Fair, suitable for batch production only	Easy	Easy	Not easy	Fair
Protein yield	High	Low to high	Low to high	Low to high	Moderate to high	Moderate
Protein quality	Poor, formation of inclusion bodies, biased codon usage	Good, properly folded	Fair, truncated protein	Good, properly folded	Good, properly folded	Good, properly folded
Glycosylation	No	Nonmammalian type, lack of multiantennary glycans	High mannose, nonmammalian type	Nonmammalian type, contain xylose	Yes, highest similarity to humans	Mammalian-type (missing sialic acid only)
End products contamination risk	Endotoxins	Low	Low	Low	Prion, Virion	Low

Reproduced From (Lai et al., 2019)

It has been reported that *L. tarentolae* produce O-glycans (Klatt et al., 2013) and biantennary N-glycans (Breitling et al., 2002; Klatt et al., 2013), including galactose and fucose residues only lacking the terminal sialic acids (N-acetyl-neuraminic acid) (Breitling et al., 2002) (Figure 4). Nonetheless, using genetic engineering, its absence can be overcome by integrating the trans-sialidase (TS) gene, an enzyme of the *Trypanosoma* species, which can help transfer sialic acids to endogenous glycoproteins and can further humanize the *L. tarentolae* glycosylation profile (Khan et al., 2017). This makes this parasite a striking candidate for the use as an expression system to produce recombinant proteins that require special characteristics such as stability, solubility, immunogenicity, serum half-life and more important, proteins which are biologically active (Breitling et al., 2002; Khan et al., 2017; Niimi, 2012).

The structures of N-linked oligosaccharides from various pathogenic *Leishmania* and *Trypanosoma* species have been well-investigated because they have been implicated in parasite virulence and the N-glycosylation pattern is highly variable between the species and life stages (Niimi, 2012). Although *L. tarentolae* is not a pathogenic parasite for humans, it does possess several virulence factors from the pathogenic *Leishmania* species, such as cysteine protease B (CPB), lipophosphoglycan 3 (LPG3), and the leishmanolysin GP63, but it lacks the major virulence factor amastigote-specific protein A2 (Raymond et al., 2012). A2 has been demonstrated to play a role in parasite virulence and visceralization capability (Lai et al., 2019).

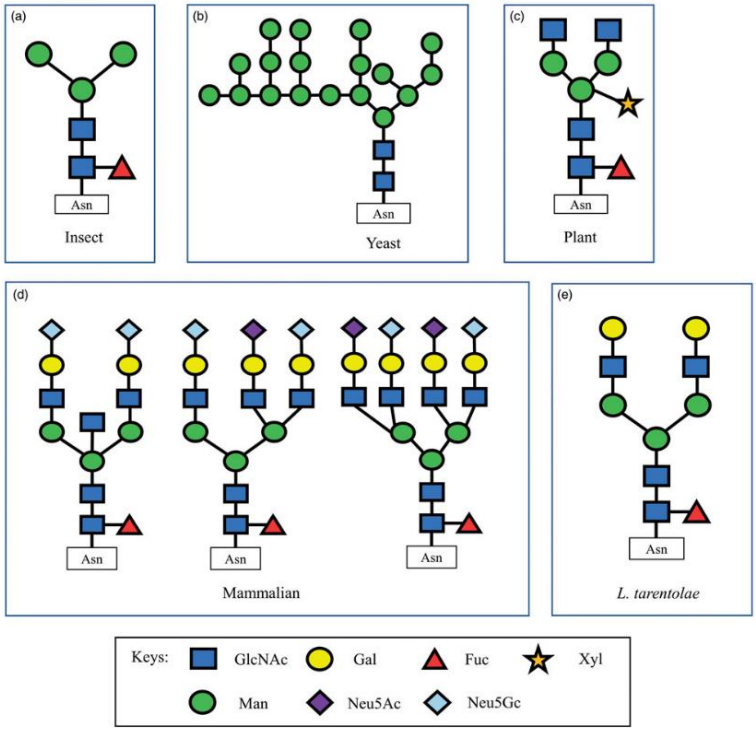


Figure 4 N-linked glycosylation pattern derived from different hosts

Reproduced from (Lai et al., 2019) (a) Insect: insect cells produce simple paucimannose N-glycan without complex side chains. (b) Yeast: N-glycan in yeast is highly branched and mannose rich. (c) Plant: N-glycan in plants has b-1,2-xylose which is plant-specific and immunogenic to humans. (d) Mammalian cell lines: mammalian N-glycan is similar to humans, consisting of complex bi-, tri-, and tetra-antennary structures. The structure comprises a core heptasaccharides, two mannose arms, and the addition of fucose (Fuc), galactose (Gal), bisecting N-acetylglucosamine (GlcNAc) (i.e., GlcNAc attached to base mannose of trimannosyl core), N-acetylneuraminic acid (Neu5Ac), and N-glycolylneuraminic acid (Neu5Gc) in different combinations, depending on the cell line and cell culture conditions. (e) *L. tarentolae*: glycosylation in *L. tarentolae* is highly similar to mammalian-type N-glycosylation but is devoid of the terminal sialic acid.

1.5.3 Leishmania and extracellular vesicles

Leishmania species are able to release extracellular vesicles that have an immunomodulatory effect to orchestrate changes in the host environment to ensure successful infection. Some studies have shown different modulator effects that vary according to the species (Castelli et al., 2019; Nogueira et al., 2020; Silverman et al., 2010) or may vary according to the type of cells used (Barbosa et al., 2018). Indeed, EVs have been shown to have an effect on the inflammatory recruitment of neutrophils and macrophages to the injection site, which favors parasite replication (Hassani et al., 2014) and results in exacerbated pathology in footpad infections when co-injected with *L. major* metacyclic parasite (Atayde et al., 2015).

Recently, researchers have studied the effects of treatment with EVs derived from *L. tarentolae* and *L. major* prior to infection with a pathogenic species, *L. major,* and found an increase in IFN-γ, TNF-α, and IL-1β production when pre-treating with EVs derived from *L. tarentolae* compared with EVs derived from *L. major.* Additionally, they found a decrease in parasite load in *L. tarentolae* EV-treated *Leishmania*-infected macrophages. Nonetheless, when they assessed the effect of EVs derived from *L. tarentolae* alone, they failed to exert a statistically significant effect on cytokine production (Shokouhy et al., 2022).

Besides EVs, there is a plethora of studies using *L. tarentolae* as a live vaccine (Ansari et al., 2019; Breton et al., 2005; Majidiani et al., 2021; Salari et al., 2020; Varotto-Boccazzi et al., 2022), virus-like particle vaccine (Bolhassani et al., 2015; Czarnota et al., 2020; Panasiuk et al., 2021) and subunit vaccines (Fischer et al., 2016; Grzyb et al., 2016; Pion et al., 2014; Salari et al., 2021). These studies show that *L. tarentolae* efficiently targets dendritic cells and lymphoid organs, enhancing antigen presentation and consequently influencing the magnitude and quality of T-cell immune responses. In murine models, this parasite induced the production of Th1-type cytokines (Salari et al., 2021, 2020) even in the absence of adjuvants (Breton et al., 2005; Majidiani et al., 2021), and lead to the production of neutralizing antibodies when used against viruses which make it a good platform for vaccine and/or antigen delivery.

Based on this, we hypothesized that EVs derived from the *L. tarentolae* P10 strain have an immunostimulatory effect which could be used for the development of vaccine adjuvants. As a proof of concept, we use the Spike protein from the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) as an antigen, given the current pandemic context we are in and since viral glycosylation is strongly dependent on the host system selected for virus propagation and/or protein expression, choosing a viral protein to test our platform for human use will show the advantages of *L. tarentolae* as a vaccine platform.

1.6 SARS-COV-2 SPIKE PROTEIN

β-coronaviruses are a family of positive-strand enveloped RNA viruses that include the SARS-CoV-2, which spread to humans in late 2019 and caused a worldwide pandemic (Ghosh et al., 2020; Lu et al., 2020).

SARS-CoV-2 Spike (S) protein is a structural protein, a class I fusion transmembrane glycoprotein that forms homotrimers, and is the major surface protein of the SARS-CoV-2 virion. It is essential for receptor binding, fusion, and virus entry (Corbett et al., 2020). Therefore, it determines the range of susceptible hosts and cell tropism and is a great inducer of the host's immune response (Eslami et al., 2022; Li, 2016; Planchais et al., 2022).

The S protein comprises two domains; the S_1 domain, which contains the receptor-binding domain (RBD) responsible for binding to the ACE2 receptor on the host cell, and the S_2 domain, which includes the fusion machinery enabling the fusion of the host and viral membranes (Figure 5) (Li, 2016; Walls et al., 2020; Wrapp et al., 2020a). S is proteolytically cleaved by furin or TMPRSS2 proteases between the S_1 and S_2 domains to activate the S protein. The RBD in the S_1 domain contributes to the viral attachment through the stabilization of the prefusion state and allows the S_2 domain to interact with the host membrane, mediating the fusion between the host cell and the virus once cleaved by host proteases at the S_2'. This follows an irreversible conformational change (Figure 5) which allows the virus entry into susceptible cells (Walls et al., 2020).

Since S is exposed on the virion surface and mediates the entry into host cells, it is the principal target of neutralizing antibodies upon infection and the focus of therapeutic and vaccine antigens (Liu et al., 2020; Walls et al., 2020; Wrapp et al., 2020a). Thus, we will use the S protein as a model to test our hypothesis.

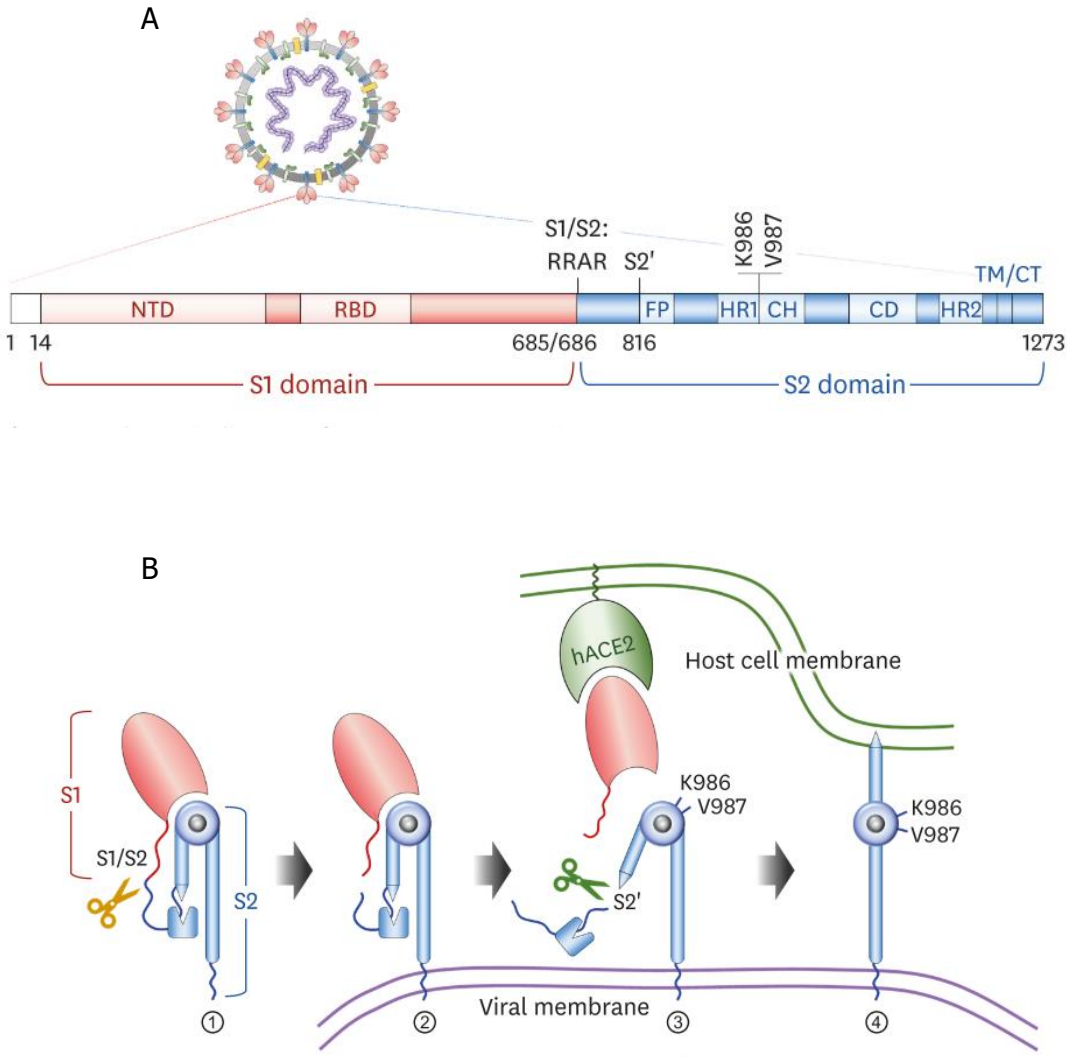


Figure 5 Schematic diagram of a SARS-CoV-2 S protein and proteolytic activation

Adapted from (Lee et al., 2021) A) Schematic diagram of SARS-CoV-2 S protein; B) Proteolytic activation of S. S protein is expressed as a single polypeptide and cleaved by a furin-like protease into S1 and S2 (①). The two fragments exist in a metastable prefusion conformation on the viral membrane (②). Upon binding of S1 to hACE2, a TMPRSS2 protease cleaves the S2' site. The proteolytic cleavage triggers a conformational change in S2 and S1 dissociates from S2 (③). Finally, S2 undergoes an irreversible 'jack-knife transition' into a stable post fusion structure (④). Abbreviations: CD, connector domain; CH, central helix; CT, cytoplasmic domain; HR1, heptad repeat 1; HR2, heptad repeat 2; NTD, N-terminal domain; S1/S2, S1/S2 protease cleavage site; S2', S2 protease cleavage site; TM, transmembrane domain. TMPRSS2, transmembrane protease serine subtype 2.

2 HYPOTHESIS

EVs derived from *L. tarentolae* P10 strain have an immunostimulatory effect towards the development of vaccine adjuvants.

3 OBJECTIVE

Our objective is to evaluate the immunomodulatory properties of recombinant S (rS) protein expressed in EVs derived from *L. tarentolae.*

3.1 SPECIFIC OBJECTIVES:

3.1.1 Engineering and characterization of EVs derived from *L. tarentolae* to express rS.

3.1.2 Evaluation the inflammatory response of EVs expressing rS *in vitro*

4 METHODOLOGY

4.1 ETHICS STATEMENT

All animal handling was performed in accordance with the protocols 1806–01 and 1806–02, which were approved by the *Comité Institutionel de Protection des Animaux* of the INRS-Centre Armand-Frappier Santé Biotechnologie. These protocols respect procedures on animal practice as instructed by the Canadian Council on Animal Care, described in the Guide to the Care and Use of Experimental Animals.

4.2 EXPRESSION VECTORS AND MOLECULAR CLONING

The full-length SARS-CoV-2 Spike glycoprotein was derived from the genomic sequence of the isolated virus "severe acute respiratory syndrome coronavirus 2 Wuhan-Hi-1" released in January 2020 (number MN908947). This sequence was codon-optimized for *L. tarentolae* expression with some modifications in the S1/S2 cleavage site to confer protease resistance by mutating the furin cleavage site (682-RRAR-685) to 682-GSAS-685. Two proline substitutions were done to maintain the pre-fusion state at amino acid position K986 – V987 (Figure 6) and a signal peptide cleavage (SPCS) site was added to the cDNA sequence right after the SalI site (Annex 8.1). The cDNA sequence was synthetically produced by BioBasic and cloned into the pUC57 plasmid using SalI and KpnI (total size of 6316 bp).

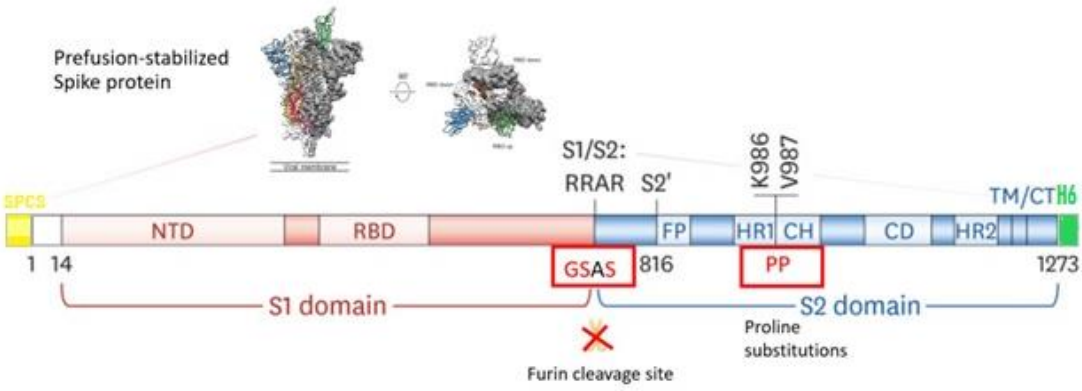


Figure 6 Schematic diagram of Spike modification

Adapted from (Lee et al., 2021; Wrapp et al., 2020b). Substitution of K986 and V987 by two prolines and mutation in the S1/S2 cleavage site to prevent the S protein from changing into a post-fusion conformation, resulting in enhanced immunogenicity and efficacy of COVID-19 vaccines. Abbreviations: SPCS, Signal peptide cleavage site; NTD, N-Terminal Domain; RBD, Receptor Binding Domain; FP, Fusion Peptide; TM, Transmembrane domain; CT, Cytoplasmic Domain; H6, Hexa Histidine.

The codon-optimized Spike gene was linearized with SwaI and subcloned into the pLEXSY-hyg2.1 vector (Jena Bioscience, Jena, Germany), for a constitutive expression which integrates into the chromosomal 18S rRNA (ssu) locus of *L. tarentolae*. Genes were transcribed by the RNA polymerase I of the host cells under the control of a chromosomal ribosomal promoter. The Spike protein was designed to have secretory expression by the fusion of its ORF to the *Leishmania* signal peptide (SP) coding region present on the vector and contains a hexa-histidine tag at its C-terminus for protein identification and purification purposes (Figure 7).

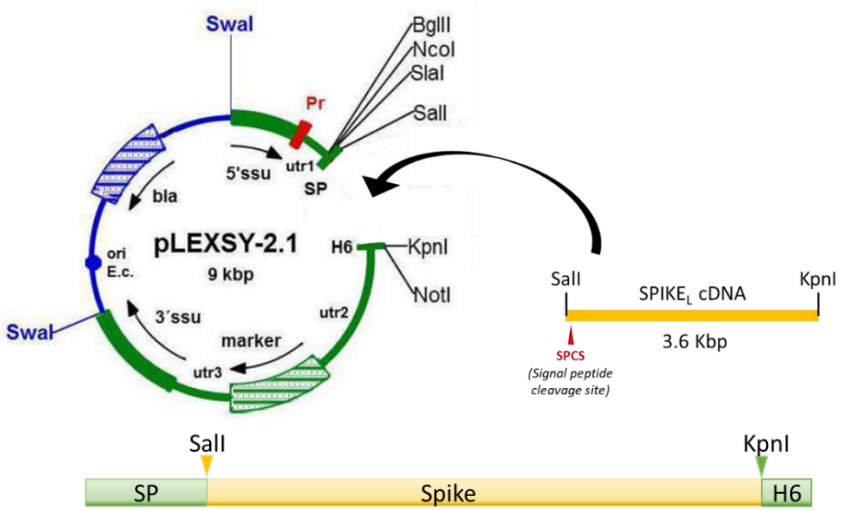


Figure 7 Sub-cloning S in pLEXSY-2.1 vector

Adapted from (Jena Bioscience GmbH, 2016). Map of the pLEXSY-2.1 vector family with cloning sites for the Spike genes (3.6 Kbp). 5´ssu and 3´ssu are regions for homologous recombination into the host chromosome following linearization of the pUC57 plasmid with SwaI. Pr is a Leishmania ribosomal promoter in front of the vector expression cassette. utr1 derived from 0.4k-IR of *L. tarentolae* aprt, utr2 from 1.4k-IR camCB and utr3 from 1.7k-IR are optimized non-translated gene-flanking regions providing the splicing signals for posttranscriptional mRNA processing for expression of S and hyg genes in the *L. tarentolae* host P10. SP designates the signal peptide of *L. mexicana* secreted acid phosphatase LMSAP1, SPCS the signal peptide cleavage site and H6 the hexa-Histidine stretch.

4.3 ENGINEERING THE *LEISHMANIA TARENTOLAE* P10 STRAIN

Following construction of the S protein expression vector in *Escherichia coli*, the *L. tarentolae* strain P10 was transfected with the linearized plasmid by electroporation.

4.3.1 Electroporation

Parasites at a density of 6 x 10^7 cells/ml that looked vital and drop-like in shape under the microscope were used. Cells were centrifuged 3 min, 2000g at room temperature and then half the supernatant's volume removed. The pellet was resuspended in the remaining medium to a concentration of 10^8 cells/ml and kept it on wet ice for 10 min. In parallel, two different amounts of the S expression vector (4.8µg and 10µg) dissolved in 50 µl of dH_2O. 350 µl of the pre-chilled cells were added to the tubes with plasmid DNA. After mixing, the volume was transferred to a chilled electroporation cuvette (2mm diameter). We use the low voltage electroporation protocol employing a Gene Pulser II (Bio-Rad Laboratories) which consist of electroporation at 450V, 450 µF and monitoring of pulse time (ca. 5-6 msec). Then, the electroporated promastigotes, were put on ice for exactly 10 min, followed by transfer to 10 ml Brain Heart Infusion (BHI) medium supplemented with hemin and PenStrep in a ventilated TC flask. Cells were incubated overnight at 26°C as static suspension culture before polyclonal selection.

4.3.2 Polyclonal selection

A polyclonal selection in suspension culture was used to select the transgenic parasites. As soon as the 10 ml overnight cultures obtained from the transfection experiments started to get slightly turbid (10^7 cells/ml; approx. 20 h after electroporation), hygromycin 100 µg/ml was added and the incubation continued as static suspension culture at 26 °C. The status of the hygromycin-containing cultures was followed microscopically until cells electroporated without DNA (negative control) began to die, and cells electroporated with the Spike gene showed motility, with a drop-like shape and grown as a "cloudy" suspension culture.

4.4 PARASITE CULTURING CONDITIONS

Selected parasites were grown in a BHI (BD Bacto) medium supplemented with hemin (5 µg/ml), penicillin 50,000 U/L, streptomycin 50 mg/L (Life Technologies) at 26 °C in darkness under aerated conditions containing hygromycin (100 µg/ml) selection antibiotic in static suspension cultures.

4.5 EVALUATION OF THE TARGET PROTEIN

4.5.1 Analysis of intracellular expression

We used 0.5 ml aliquots from culture supernatants. The cells were centrifuged 7 min at 2600g. The pellet was resuspended in 0.2 ml of Lysis buffer (1% de NP-40, 500 mM Tris-HCl (pH 7,5), 150 mM NaCl, 1mM EDTA (pH8),1,5 mM EGTA, 1 mM Na_3VO_4, 50 mM NaF, 10 mM $Na_4P_2O_7$, 10 mM 1,10-Phenanthroline (Sigma) and Protease inhibitors), mixed well and passed through a needle to help disrupt cells, membranes and DNA. After incubation at -80 ºC for at least 30 min, samples were quantified and keep it at -20 ºC to posterior use.

4.5.2 Analysis of secreted protein expression

The cell culture supernatants were concentrated 100x with trichloroacetic acid (TCA) as follows: Cells were pelleted from 10 ml of culture 10 min at 2600 g and 8 ml of sterile-filtered supernatant were added to 2 ml of 50% ice-cold TCA to yield a final concentration of 10%. The sterile filtration of supernatants prior to TCA precipitation was done to avoid carry-over of cells and was left on ice for 30 min before centrifugation for 15 min at 15,000g and 4 °C. After removing the supernatant, the pellet was resuspended in 1 ml of 80% acetone and transferred to an Eppendorf tube (to remove residual TCA). After an additional centrifugation for 15 min at 15,000g and 4 °C, the supernatant was aspirated, and the pellet was resuspended in a final volume of 80 µl lysis buffer to posterior use.

4.5.3 SDS-PAGE and Western blotting

Expression of the target protein was confirmed by Western blot analysis. Proteins were loaded on 7.5% bis-acrylamide gel in denatured conditions. After electrophoresis, semi-dry transfer of proteins onto PVDF membranes was performed. Membranes were blocked for 1 h in a 5% Bovine serum albumin (BSA) solution (5% BSA/TBS/0.01% Tween20) and incubated overnight at 4°C with the following primary antibodies: SARS-CoV-2 (2019-nCoV) Spike RBD Antibody, rabbit polyclonal 1:2000 (Sinobiological), Monoclonal mouse GP63 Antibody 1:10 (Button et al., 1993; Macdonald et al., 1995), or Anti-Penta His mouse polyclonal antibody at 1:1,000 dilution (Qiagen). After washing three times with TBS-Tween buffer, the horseradish peroxidase (HRP)-conjugated IgG anti-rabbit 1:5,000 (Sigma-aldrich A0545) and the horseradish peroxidase (HRP)-conjugated IgG anti-mouse 1:5,000 (Sigma-aldrich A4416) were added to the blot, respectively,

and incubated under shaking at room temperature for 1 h. Following this, the membranes were washed with TBS-Tween four times and are incubated for 3.5 min in ECL solution (GE Healthcare, RPN2106) before detection by chemiluminescence.

4.6 EXTRACELLULAR VESICLES ISOLATION

Cell culture medium was harvested from parasites expressing the S protein or not, which were grown for 3 to 4 days until they reached a density of 9 x 10^6 parasites/ml, centrifuged two-times at 3,000 rpm for 7 min. This was followed by addition of protease inhibitor and 1,10-Phenanthroline (Sigma) and filtration through a 0.22 µm membrane filter before following the procedure described in (Vucetic et al., 2020). Briefly, the supernatant was ultracentrifugated at 100,000g and 4 °C for 70 min, and the pellet was washed two times with exosome buffer (137 mM NaCl, 20 mM HEPES, pH 7.5). The EV pellet was resuspended in approximately 150-200 µl exosome buffer and the samples were aliquoted. Samples were stored at -80 °C.

4.6.1 Enrichment of extracellular vesicles expressing spike

Recombinant strains expressing the S protein were cultured for 3 to 4 days until they reached a concentration of 9 x 10^6 parasites/ml, centrifuged two times at 3,000 rpm for 7 min before the addition of EDTA-free protease inhibitor and 1,10 Phenanthroline (Sigma) and followed by filtration through a 0.22 µm membrane filter. The medium was passed through 5 ml High-performance immobilized metal affinity chromatography (IMAC) HisTrap HP His tag protein purification columns (Cytiva) for His-tag recombinant protein purification in a fast protein liquid chromatography system (ÄKTA FPLC Systems, GE Healthcare). The column was equilibrated with (50 mM NaH_2PO_4, 300 mM NaCl pH 8), then the fractions were obtained using elution buffer (50 mM NaH_2PO_4, 300 mM NaCl 500mM Imidazole pH 8).

4.7 SPIKE PROTEIN PURIFICATION

S protein purification was done as describe above with a modification in the equilibrium buffer with the denaturing agent Urea as follow (Urea 6M, 50 mM NaH_2PO_4, 300 mM NaCl pH 8), then the fractions were obtained as described above using elution buffer (50 mM NaH_2PO_4, 300 mM NaCl 500mM Imidazole pH 8). The samples were then dialyzed, and a western blot was run to confirm the protein expression.

4.8 TRANSMISSION ELECTRON MICROSCOPY

The transmission electron microscopy (TEM) analysis was performed at the INRS-Armand-Frappier Santé Biotechnologie Service de Microscopie Electronique using EVs derived from *L. tarentolae* expressing S or not. Grids were placed on a parafilm and a 10 µl drop of EVs suspension was deposited for 20 min. Using a piece of bibulous paper, grids were dried and then immersed in a drop of UA5% (Uranyl Acetate) in 50% ethanol for 1 min. After the grids were dried again, the imaging was performed on a Hitachi H7100 transmission electron microscope with an AMT Camera (XR-100) at 75kV.

4.9 IMMUNOGOLD STAINING

For immunogold labeling, EVs derived from *L. tarentolae* expressing the S protein or not were fixed and embedded as follows: fixation in 0.1% glutaraldehyde + 4% paraformaldehyde in a cocodylate buffer at pH 7.2 overnight, and dehydration by successive passage through 25, 50, 75 and 95 % solutions of acetone in water for 30 minutes each, followed by immersion in two changes of pure acetone for at least 30 minutes each. Then the sample was immersed for 16-18 hours in SPURR (TedPella): acetone (1:1) and embedded by immersion in two successive baths of SPURR (TedPella) for at least 2 hours each, cut into small pieces and placed in BEEM capsules (TedPella) which were filled with SPURR mixtures and let stand at room temperature for 18 hours. After this, the filled capsules were placed at 60°C for 24 hours to polymerize the resin before the samples were cut using an ultramicrotome system (Leica UC7) and put onto a Formvar (EMS) and carbon covered (Leica ACE600) nickel 200 mesh grids (EMS). After cutting the sample with the ultramicrotome, samples were placed on nickel grids treated with sodium metaperiodate and blocked with 1% BSA in PBS. Grids were then incubated with rabbit polyclonal SARS-CoV-2 (2019-nCoV) Spike RBD Antibody 1:2000 (Sinobiological) primary antibody, washed, and incubated in 20 nm anti-rabbit gold particle-conjugated secondary antibody (Abcam). After washing, samples were contrasted with uranyl acetate and lead citrate and subsequently visualized using a Hitachi H7100 transmission electron microscope.

4.10 NANOPARTICLE TRACKING ANALYSIS

The concentration and size distribution of EVs were measured using nanoparticle tracking analysis (NTA) on a Nanosight NS300 instrument (Malvern Instruments Ltd., Malvern, UK). Fractions were diluted 1000- to 4000-fold in water. Each sample was captured in triplicates for 30s with the camera level set to 14 and a detect threshold of 5. The data were analyzed using the NTA software (version 3.4 build 3.4.003).

4.11 MAMMALIAN CELL CULTURE

Female 129 BL/6 mice at 6- to 12-weeks of age were used to isolate and differentiate macrophages and dendritic cells. Bone marrow was isolated from the femur and tibia of mice. Bone marrow-derived macrophages (BMM) were differentiated for 7 days in complete DMEM (containing L-glutamine (Life Technologies), 10% v/v heat inactivated fetal bovine serum (FBS) (Life Technologies), 10 mM HEPES (Bioshop) at pH 7.4, and penicillin-streptomycin (Life Technologies)) supplemented with 15% v/v L929 cell-conditioned medium (LCM) as a source of macrophage colony-stimulating factor-1 at days 3, 5 and 7. To render the BMM quiescent prior to experiments, cells were transferred to tissue culture-treated 24-well plates at day 8 for 24 hours in complete DMEM without LCM (Descoteaux and Matlashewski, 1989). The cells were kept in a humidified 37˚C incubator with 5% CO_2. The number of macrophages used per well was $8x10^5$.

Bone marrow-derived dendritic cells (BMDC) were differentiated for 7 days in complete RPMI (Life Technologies), supplemented with 10% v/v heat inactivated fetal bovine serum (FBS) (Life Technologies), 10 mM HEPES (Bioshop) at pH 7.4, and penicillin-streptomycin (Life Technologies) and 10% X63 source of granulocyte-macrophage colony-stimulating factor (GM-CSF). At day 3 the medium was refreshed. At day 7 non-adherent cells were harvested in RPMI supplemented with FBS 10% and 5% x63. The cells were kept in a humidified 37˚C incubator with 5% CO2. The number of cells used per well was $8x10^5$.

4.12 *IN VITRO* STIMULUS

BMM or BMDC were kept in a humidified 37˚C incubator with 5% CO2, stimulated for 6h with 1, 10, and 100µg/ml of extracellular vesicles expressing the S protein or not, or purified S protein. In addition, cells treated with 100ng/ml of LPS were used as a positive control, and unstimulated cells

as a negative control. Then, the cells were washed with HBSS two times and RLT lysis buffer from the RNeasy kit (Qiagen) was added. The samples were kept at -80°C until the RNA extraction was done.

4.13 GENE EXPRESSION ANALYSIS

RNA extractions from BMM and BMDC were done with the RNeasy® Mini Kit (Qiagen) following the manufacturer's instructions. 500ng of RNA was reverse transcribed to cDNA using the iScript cDNA synthesis Kit (Bio-Rad laboratories).

cDNAs were analyzed using Agilent Technologies Stratagene Mx3000P or QuantStudio3 Applied biosystems qPCR for IL-6, IL-10, IL-12, TNF-α, IL-1α, IL-1β genes using ribosomal protein S29 as housekeeping gene. The $2^{\wedge\Delta\Delta Ct}$ method was applied to compare the relative expression between the target gene and the housekeeping gene.

4.14 STATISTICAL ANALYSIS

The statistical analyses were performed using GraphPad Prism 6. Statistical differences between the evaluated groups were done with one-way ANOVA, then each group was compared using an unpaired t-test. Each experiment was performed in triplicates and a p value $p<0.05$ was considered as statistically significant.

5 RESULTS

5.1 EXPRESSION OF THE CONSTITUTIVE SPIKE PROTEIN IN *LEISHMANIA TARENTOLAE*

Expression of the S protein was achieved after transfection of *L. tarentolae* strain P10 by electroporation at low voltage with two different amounts, 4.8 µg and 10 µg, of plasmid containing the entire sequence of the S gene of SARS-CoV-2 virus. Both generated stable cell lines expressing the protein. We chose the sample electroporated with 4.8 µg of plasmid to work with. To confirm the expression of the S protein, we performed a western blot using an anti-His tag antibody. A band of approximately 180 kDa, which corresponds to the size of the recombinant monomeric protein, was visible in the total lysates and the secreted proteins of the *L. tarentolae* expressing rSpike (LrS) (Figure 8), nonetheless, in *L. tarentolae* WT (LWT) we did not find the band corresponding to S protein, as expected.

Furthermore, we confirmed that expression of the S protein by the parasite does not have a negative impact on their fitness by evaluating their viability (Figure 9). Parasites expressing Spike do not show a statistical difference in culture density as compared to parasites that do not express the protein. Both were kept under the same conditions and were not used for more than 3 months. Each new batch of parasites thawed was evaluated for spike production.

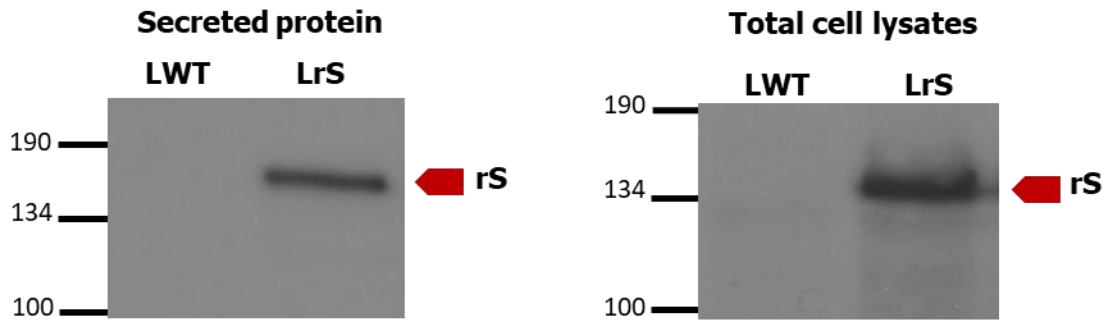


Figure 8 Evaluation of the LrSpike protein expression.

To confirm Spike expression, we used *Leishmania* supernatant to evaluate the secretory expression, and total lysates to evaluate intracellular expression of rS protein using α-Penta-His antibody, taking advantage of the his-tag in the protein. The protein ladder is in kDa. Abbreviations: rS: recombinant Spike protein, LWT: *Leishmania tarentolae* WT, LrS: *Leishmania*-expressing rS.

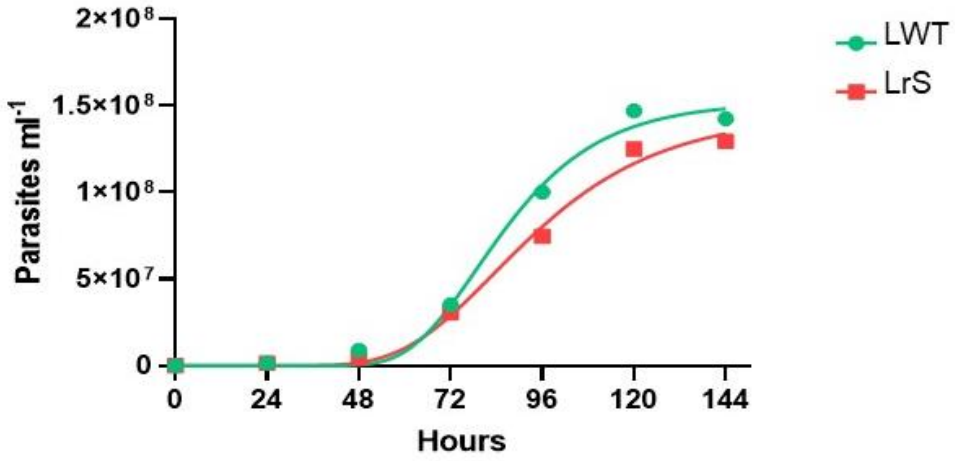


Figure 9 Viability of parasite expressing LrS protein compared with LWT parasites.

The viability of parasites was evaluated by counting the density of each culture daily for one week. Abbreviations: LWT: Leishmania tarentolae WT, LrS: *Leishmania*-recombinant Spike protein.

We evaluated the optimal harvest time and culture conditions (static or agitated) to increase the amount of recombinant Spike produced in *L. tarentolae.* In this regard, we evaluated LrS expression by western blot after 48 h and 72 h in static and agitated conditions at 26 °C. We also introduced a shift in the temperature at 37 °C for 4 h prior to harvesting the parasites with the objective of increasing EVs release, since a temperature shift serves as a stressor to make the parasite secrete more EVs (Silverman et al., 2010). We found LrS expression at 48 h and 72 h in static condition at 26 °C was optimal for the secreted LrS protein production. We observed that in agitated conditions, the parasite density doubled compared to static conditions for 48 h, however, at 72h the parasite decreased by 38%. Interestingly, we did not find detectable expression of LrS by the His-tag antibody when the parasites were kept in agitated conditions (Figure 10). In addition, when we evaluated the temperature shift, we found the same amount of LrS at 48h in static conditions. However, we did not find LrS at the 72h time point. In conclusion, we determined the static culture condition is optimal for LrS protein expression. Depending on the parasite density, either 48h or 72h can be used without a temperature shift.

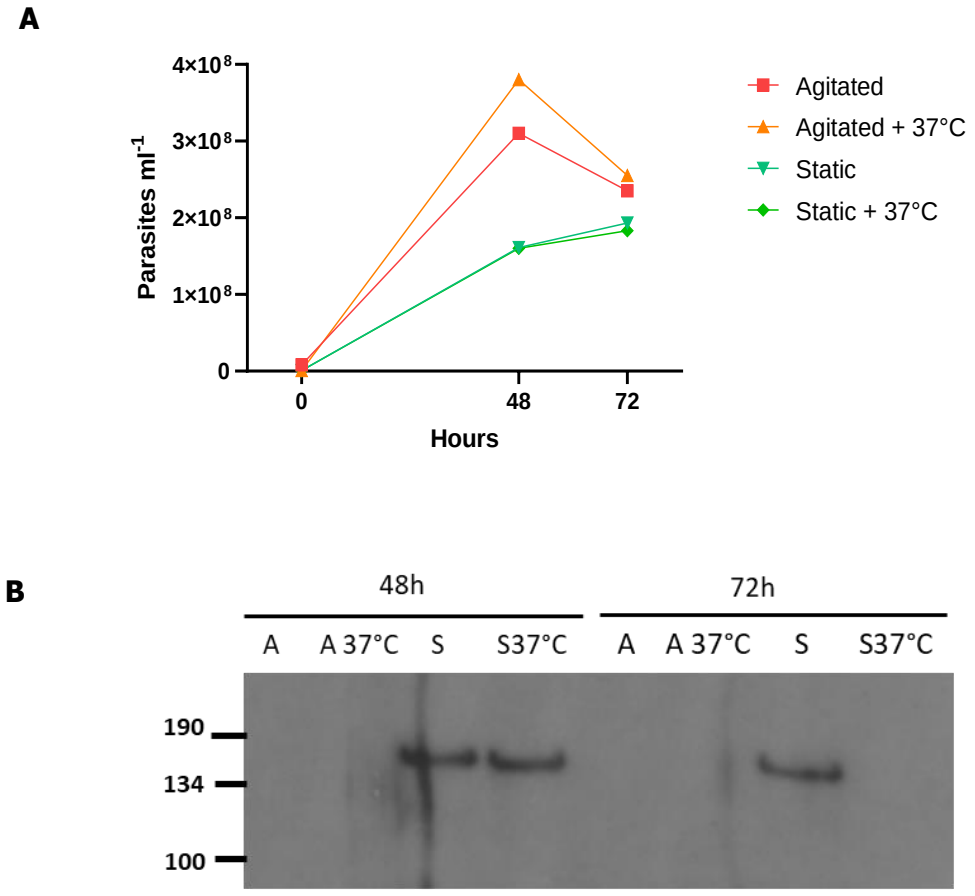


Figure 10. Optimal condition for LrS expression.

To find the optimal conditions, parasites were grown in static and agitated conditions at 26 °C with or without a temperature shift to 37 °C for 4 h. A) Density of the LrS parasites under different conditions; B) Western blot against anti-his-tag to detect LrS. Protein ladder is in kDa. Abbreviation: S, static; A, agitated; h, hours; C, Celsius.

5.2 EXTRACELLULAR VESICLES CHARACTERIZATION

As the characterization of extracellular vesicles is important in research proposing a biological effect, we isolated our EVs from the supernatant of LrS and LWT cultures using ultracentrifugation. To assess their concentration and size, we used the Nanoparticle Analysis system (NTA). We found that EVs derived from LrS and LWT have a homogeneous population with similar size, having a mode of 113nm and a concentration of 2.32 x 10^{12} particles/ml for LrS while for LWT the mode of size was 109.6nm and the concentration was 3.32 x 10^{12} particles/ml (Figure 11).

The size of vesicles was confirmed by transmission electron microscopy (TEM) which revealed sizes varying from 62 nm to 257 nm and we confirmed them to be round vesicles with a lipid bilayer. They present a cup-shaped morphology resulting from the dehydration phenomena during the sample preparation which is frequently mistaken as a typical feature of exosomes (Figure 12). To confirm the expression of the rS in the EV, we use rabbit polyclonal SARS Coronavirus spike RBD antibody 1:2,000, which revealed a band at approximately 180 kDa. To confirm they are actually EVs, we use a GPI-anchored protein which works as a marker for *Leishmania*'s EVs, the zinc metallo-proteinase GP63. We obtained a band at approximately 180 kDa for rS and another band at 63 kDa for GP63 on the EVs released by the LrS (EV-rS) (Figure 13), confirming that the EVs indeed express rS. Secondly, EVs released by LWT (EV LWT) only showed the band at 63 kDa, which confirms the expression of the *Leishmania* EVs marker GP63 (Figure 13).

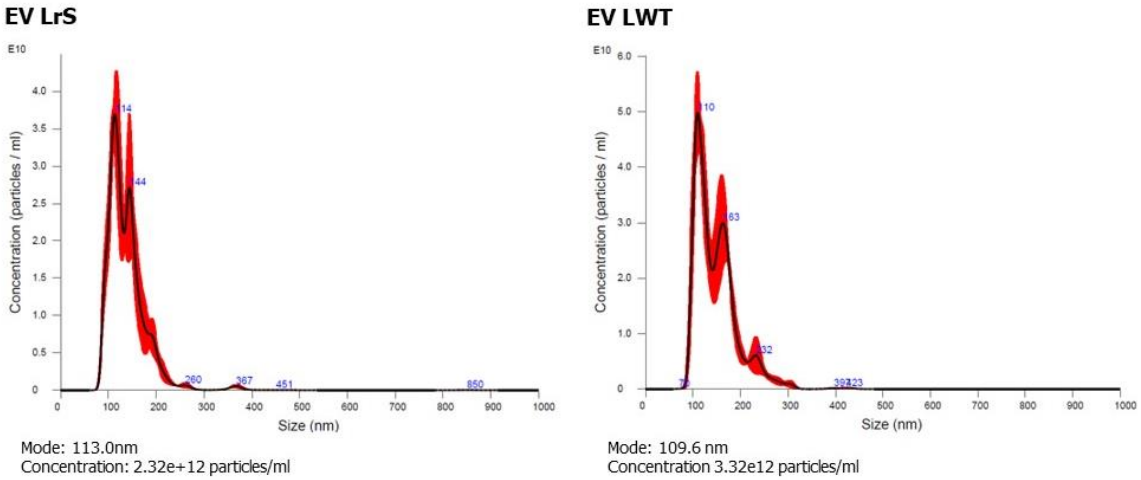


Figure 11 Concentration and size distribution

EVs were obtained by ultracentrifugation of supernatant culture of *L. tarentolae* expressing rS and *L. tarentolae* WT, the concentration and size distribution was measured using nanoparticle tracking analysis (NTA) in on a Nanosight NS300. Abbreviation: *L. tarentolae* EVs expressing rS (EV LrS) or not (EV LWT)

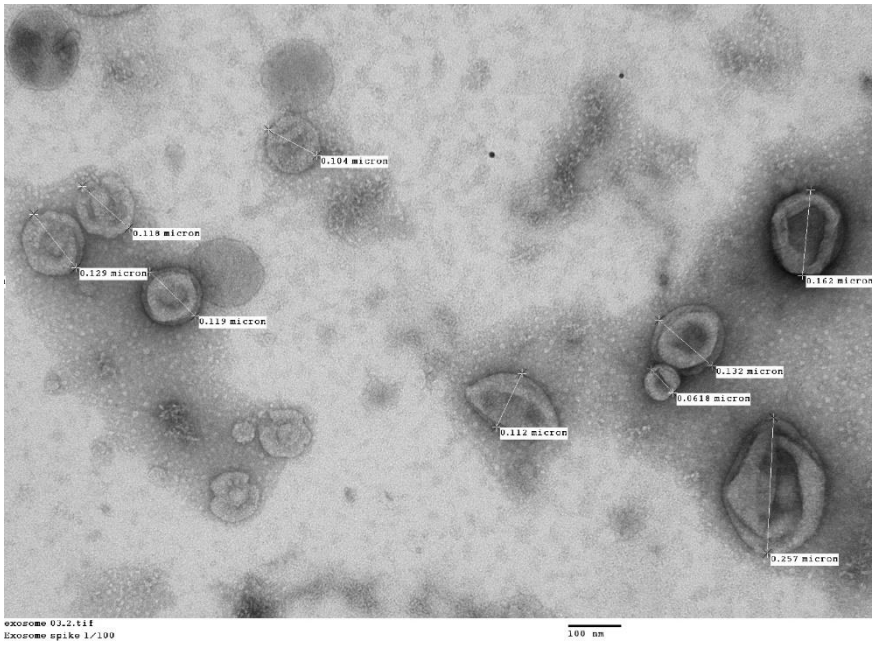


Figure 12 Representative TEM imagen of EV released by *L. tarentolae* expressing rS

EV were obtained by ultracentrifugation of culture supernatant of *L. tarentolae* expressing rS. TEM images were recorded at a voltage of 75 kV, 40000x magnification with a Hitachi H7100.

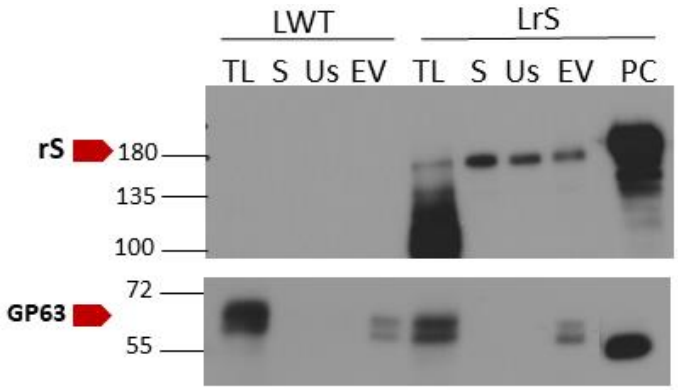


Figure 13 EV characterization by Western blot.

EVs isolated from supernatant by ultracentrifugation of *L. tarentolae* expressing rS (LrS) and *L. tarentolae* that does not express rS (LWT) were evaluated for the expression of rS and GP63. The latter one was used as an EV marker. As control, we used the total lysate, the supernatant, and the supernatant from the ultracentrifugation. Abbreviations: LWT, *L. tarentolae* WT*;* LrS, *L. tarentolae* expressing rS; TL, Total lysated; S, secreted; Us, ultracentrifugation supernatant; EV, Extracellular Vesicles; PC, Positive Control rS; rS, recombinant Spike; GP63, *Leishmania* EV marker.

To confirm the presence of LrS in EVs, we used immunogold labeling against rS for electron microscopy (Figure 14). Here, we found a heterogenous population in which not all the EVs were expressing the nanogold tag that binds LrS. In this regard, we enriched and purified the EV expressing LrS by exploiting the His tag on rS. We use immobilized metal affinity chromatography (IMAC) and elution with imidazole to purify the rS protein.

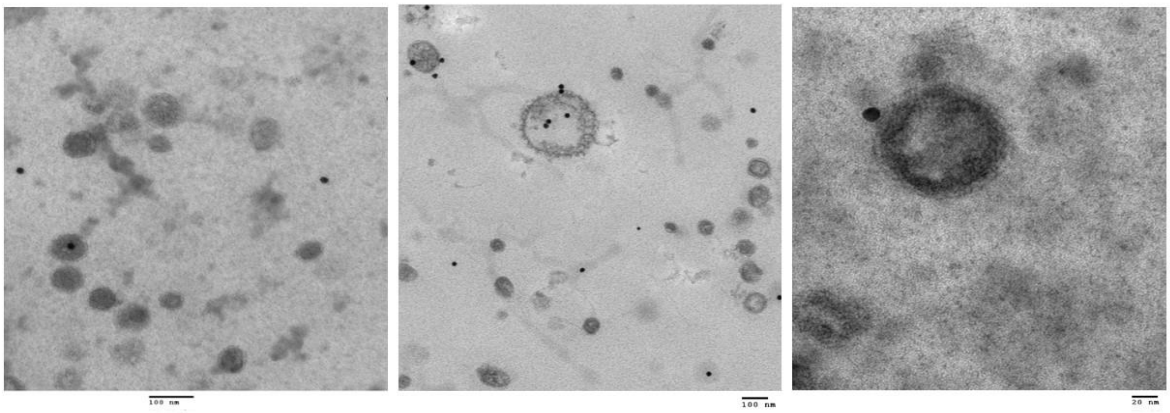


Figure 14 Immunogold against α-SARS-CoV-2.

Immuno-electron microscopy image of a representative EV derived from *Leishmania tarentolae* expressing rS (20 nm nanoparticles). TEM images were recorded at a voltage of 75 kV, 40,000x magnification in left and central panel, 100,000x magnification in right panel.

We confirmed and pooled the fractions expressing LrS and dialyzed to remove the imidazole, then characterized again by WB and NTA analysis. As shown in Figure 15, we first confirmed that we have EVs by detecting the expression of GP63 (band at around 63kDa) and that these EVs express LrS with a band at around 180kDa. The NTA analysis showed a more heterogenic population compared to the ultracentrifugation methodology, with a mean 139.7 nm, and mode of 100.7nm in size, keeping 90% of EVs below 210.9nm (D90) (Figure 15). Having thus characterized our isolates, we used this EV-rS to evaluate their inflammatory potential on BMDCs and BMDMs.

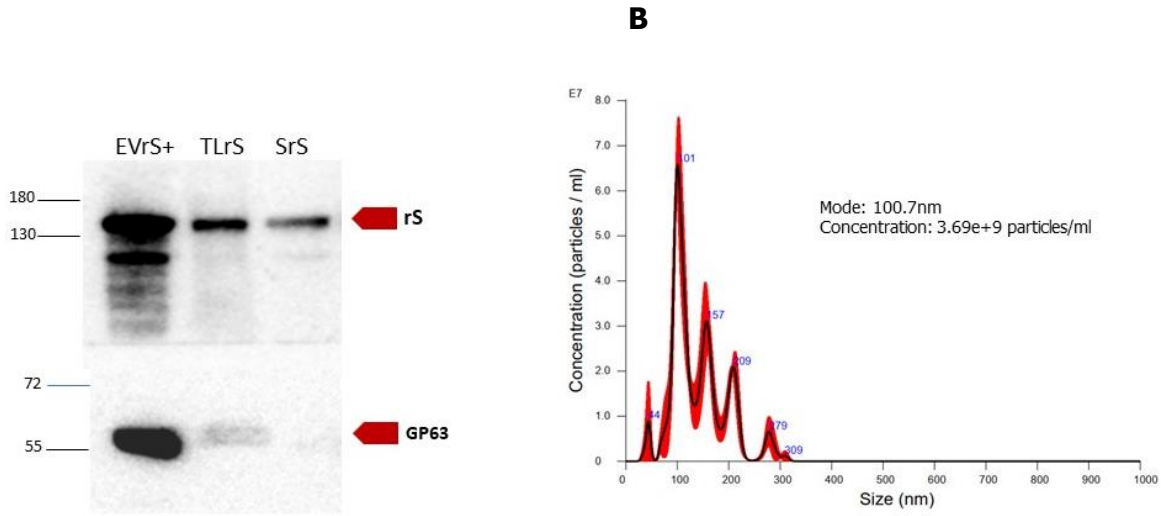


Figure 15 His-tag EV enrichment.

Supernatant from *Leishmania tarentolae* expressing rS was used to isolate and enrich the EVs expressing rS by FPLC and characterized by WB and NTA analysis. Rabbit polyclonal SARS-CoV-2 (2019-nCoV) Spike RBD Antibody 1:2000 was used to identify rS and mouse monoclonal GP63 Antibody 1:10 was used as an EV marker, panel left. To corroborate the EV size NTA analysis was done, panel right. Abbreviations: EVrS, EV expressing recombinant Spike; TL, Total lysate from *L. tarentolae* expressing rS; SrS, secreted protein from *L. tarentolae* expressing rS; rS, recombinant Spike; GP63, Leishmania EV marker.

5.3 PURIFICATION OF SPIKE PROTEIN

To compare the immunomodulatory effect of EVs as adjuvants, IMAC was used under denaturing conditions at urea 6M. After affinity purification, urea and imidazole were rinsed by dialysis, and the samples were analyzed by Western blot using α-SARS-CoV-2 (2019-nCoV) Spike RBD and α-GP63 antibodies. The α-GP63 antibody was used as a control to verify the purity of the recombinant Spike protein. This sample was then used for immunostimulatory assays.

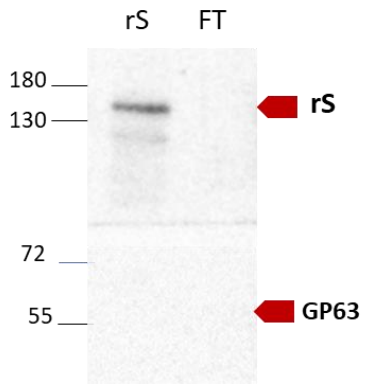


Figure 16 Western blot for recombinant Spike

Culture supernatant of *Leishmania tarentolae* expressing rS was used to purify the rS protein under denatured conditions using FPLC. Rabbit polyclonal SARS-CoV-2 (2019-nCoV) Spike RBD Antibody 1:2,000 was used to identify rS and mouse monoclonal GP63 Antibody 1:10 was used as an EV marker. Abbreviation: rS, recombinant Spike; FT, Flow-through; GP63, Leishmania EV marker.

5.4 INFLAMMATORY POTENTIAL OF EV DERIVED FROM *LEISHMANIA TARENTOLAE*

Vaccines mimic pathogen infections and our aim was to develop an EV-platform for vaccine production with the dual function of expression of antigen candidates and adjuvant stimulus. Our first step was to assess the *in vitro* inflammatory potential of the EVs expressing recombinant Spike (EV-rS) derived from *L. tarentolae* in terms of cytokine production on two different antigen presenting cells (APC); macrophages (MΦ) and dendritic cells (DC), given their role in the activation of the adaptative immune system and in enhancing antigen presentation by MHC (Araujo Correa et al., 2022; Awate et al., 2013).

To evaluate the adjuvant potential of EV *in vitro*, we stimulated BMM and BMDC - since they reflect more the *in vivo* environment - with EVs derived from both the wild-type strain of *L. tarentolae* (EV-WT) and the same strain engineered to express SARS-CoV-2 spike protein (EV-rS). We also used rS purified from *L. tarentolae* supernatant as a stimulus to evaluate the effect of the protein without adjuvant, and LPS as a positive control stimulus due to it being a PAMP and hence having ability to increase the production of pro-inflammatory cytokines. We used three different doses for each stimulus: 1 µg/ml, 10 µg/ml, and 100 µg/ml and quantified the relative expression of interleukin-12 (IL-12), interleukin-10 (IL-10), interleukin-6 (IL-6), interleukin-1α (IL-1α), interleukin-1β (IL-1 β), tumor necrosis factor (TNF) immunomodulatory gene cytokines after 6 h by RT-qPCR following the Livak method or $2^{-\Delta\Delta CT}$.

The results shown in Figure 17 reveal that both EV-WT and rS induced a higher expression of gene cytokines in BMM (square in blue) compared to BMDC (square in red). However, when stimulated with EV-rS, the expression varied depending on the dose, although IL-12 has a higher relative expression in BMDC (Figure 17).

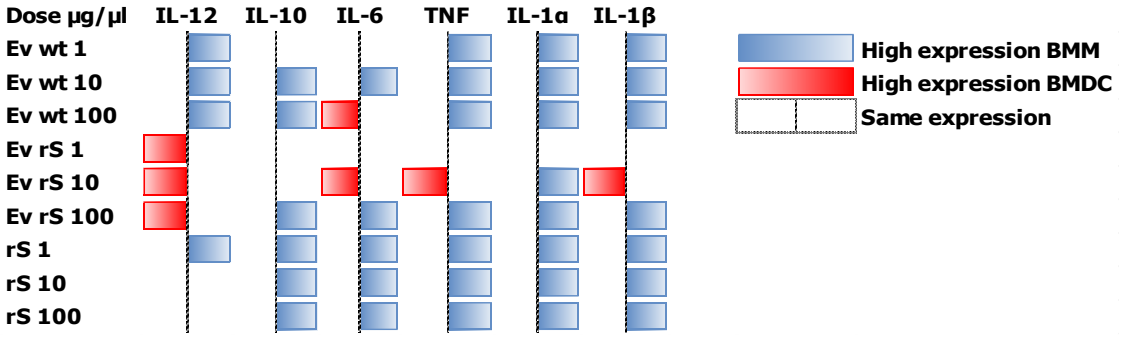


Figure 17 Heat map of differential expression in cytokines

BMM and BMDC were stimulated with different doses of EVs expressing rS (EV-rS) or not (EV-WT) and recombinant Spike alone (rS) for 6h. The cytokine gene expression was determined using RT-qPCR. The heat map was built using the highest $2^{-\Delta\Delta CT}$ value for each cytokine either on BMM or BMDC. When the $2^{-\Delta\Delta CT}$ value was higher on BMM it was represented as blue, when the $2^{-\Delta\Delta CT}$ value was higher on BMDC, it was represented as red. When they showed a similar expression, no colors were used.

We also observed that EVs derived from *L. tarentolae* display an immunostimulatory effect in a dose-dependent manner in both populations, as shown in Figure 18. However, when stimulated with rS, we found a slightly decreased effect at a dose of 100 µg/ml.

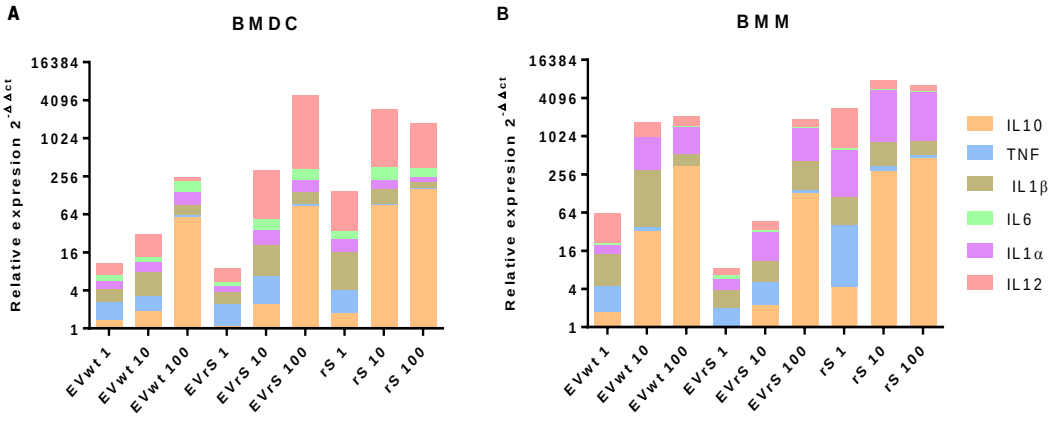


Figure 18 Dose-dependent effect in BMDC and BMM

The inflammatory potential of EVs expressing recombinant S (EV-rS) was evaluated by quantifying the expression of cytokine genes such as IL-6, IL-10, IL-12, TNF, IL-1α, IL-1β *in vitro* in bone marrow-derived macrophages (BMM) and bone marrow-derived dendritic cells (BMDC) after 6h stimulus with EV-rS, EV which does not express S (EV-WT), and recombinant S (rS). In this way, we evaluated the adjuvant potential of leishmania EVs expressing rS by RT-qPCR.

In BMDC, we observed higher relative cytokine gene expression when we stimulated with rS at 1 µg/ml and 10 µg/ml doses. This effect was overcome by EV-rS only at the highest doses evaluated (100 µg/ml) (Figure 19), except in the case of the anti-inflammatory cytokine IL-10, where the stimulus with rS yielded higher expression. When the 1 µg/ml dose of EV-WT and EV-rS was used, no statistically significant difference was observed. A dose of at least 10 µg/ml was needed to see a difference between those two stimuli, which, on the other hand, increases cytokine levels almost to the same level as the 100 µg/ml dose, excluding IL-10. This dose also gave rise to statistical differences between the three stimuli in all the gene cytokines evaluated in Figure 19. Therefore, it would be interesting to evaluate an intermediate dose between 10 and 100 µg/ml to check for differences between groups and to evaluate the "adjuvant" effect we saw with the 100 µg/ml dose. After obtaining these results, we could use them for assays in *in vivo* model.

In BMM, we also found that rS stimulates the highest levels of cytokine gene expression at all doses evaluated. Interestingly, we found that an EV-rS stimulus reduces cytokine expression of the six cytokine genes evaluated compared to an EV-WT stimulus, especially at doses of 1 and 10µg/ml.

When we evaluated the differences between the three stimuli within each dose, we saw the highest differences at the 1µg/ml and 10 µg/ml doses in all the cytokines evaluated. Nonetheless, at the 100µg/ml dose, the EVs induce similar relative expression in IL-12, IL-6, IL-1α and IL-1β cytokine genes. Notably, when we evaluated the IL-1β cytokine gene, all the stimuli had a similar relative expression at 100 µg/ml dose (Figure 20).

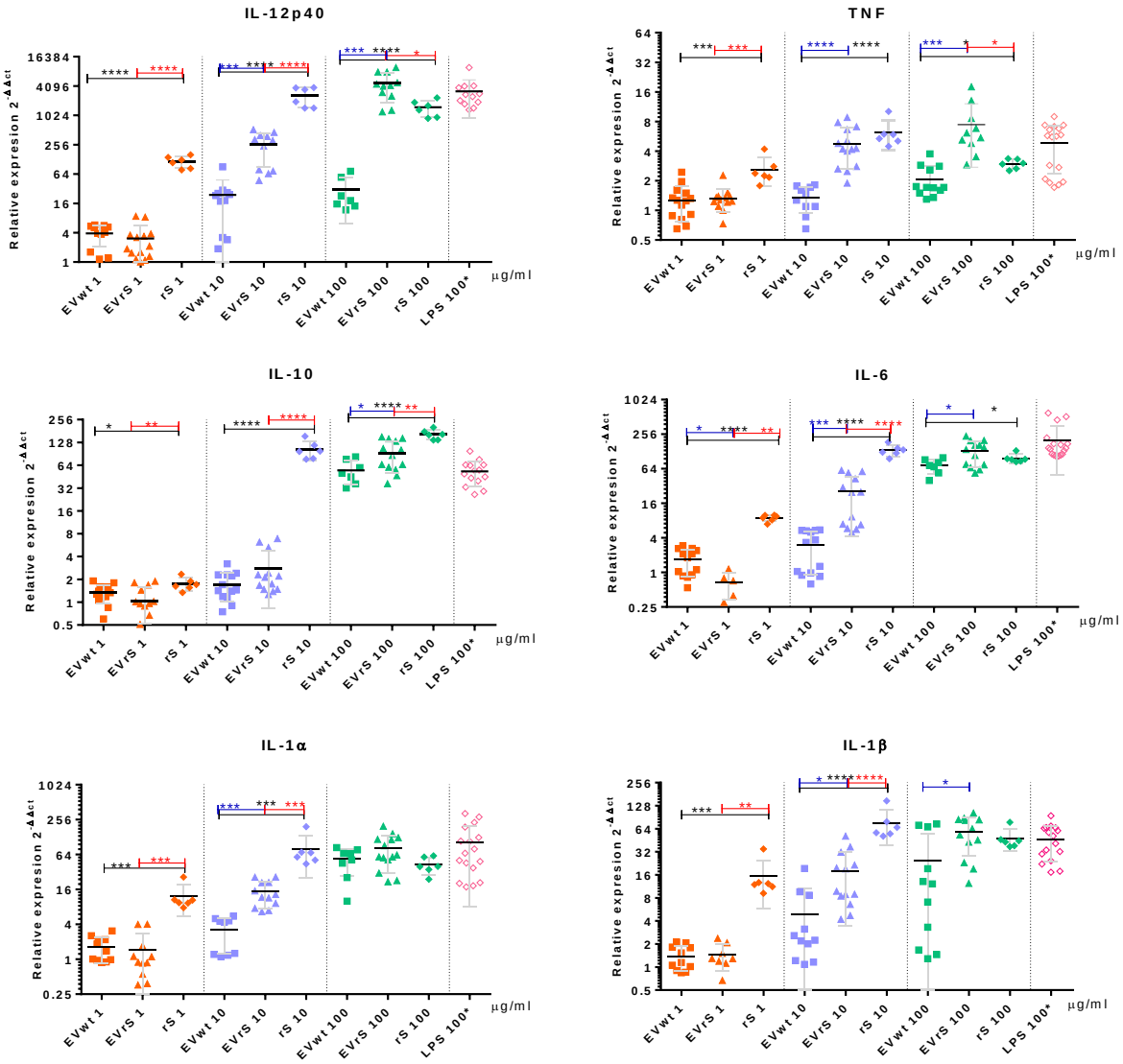


Figure 19 Spike has an immunostimulatory effect in BMDC

The inflammatory potential was evaluated by quantifying the expression of cytokine genes in bone marrow-derived dendritic cells (BMDC) after 6h stimulus with the different stimuli, EVs expressing recombinant S (EV-rS), EVs which does not express S (EV-WT), recombinant S (rS), LPS as a positive control, and normalized to the Rps29 gene as a reference gene and expressed as fold increase to non-stimuled control BMDC, at three different doses of stimuli, 1, 10, and 100 µg/ml. Statistical differences between the groups were evaluated using one-way ANOVA, then each stimulus was compared using a two-tailed, unpaired Student's t-test for each dose. Each experiment was performed in triplicates and a p value of p<0.05 was considered statistically significant. Abbreviations: p value 0.01 to 0.05*, p value 0.001 to 0.01 **, p value 0.0001 to 0.001*, <0.0001****, LPS 100* ng/ml.**

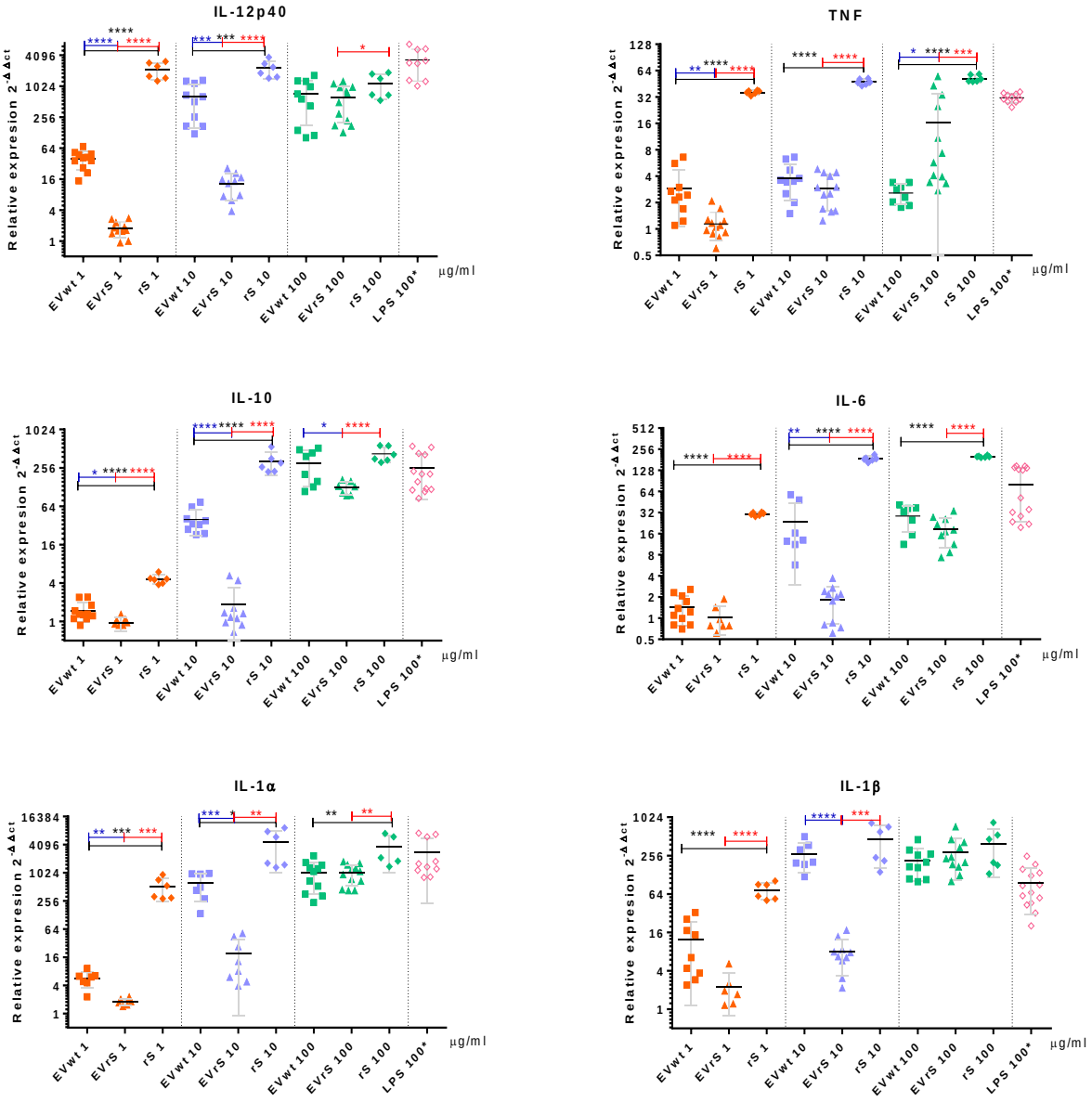


Figure 20 Spike has an immunostimulatory effect in BMM

The inflammatory potential was evaluated by quantifying the expression of cytokine genes in bone marrow-derived macrophages (BMM) after 6h of treatment with the different stimuli, EVs expressing recombinant S (EV-rS), EVs not expressing S (EV-WT), recombinant S (rS), and LPS as a positive control, and normalized to the Rps29 gene as a reference gene and expressed as fold increase compared to non-stimulated control BMM, at three different stimuli doses of 1, 10, and 100 µg/ml. Statistical differences between the groups were evaluated with one-way ANOVA, then each stimulus was compared using a two-tailed, unpaired Student's t-test for each dose. Each experiment was performed in triplicates and a p value of p<0.05 was considered statistically significant. Abbreviations: p value 0.01 to 0.05*, p value 0.001 to 0.01 **, p value 0.0001 to 0.001*, <0.0001****, LPS 100* ng/ml.**

6 DISCUSSION

To stimulate adaptive immunity, a vaccine requires a pathogen-specific immunogen as well as an adjuvant. The adjuvant stimulates the innate immune system without inducing systemic inflammation and provides the second signal for T cell activation (Teijaro and Farber, 2021). Therefore, choosing an adjuvant is as important as choosing an antigen for vaccine development. It has been shown that EVs do not require adjuvants since the EVs themselves already contain a plethora of microorganisms components, for example, pathogen-associated molecular patterns which lead to the activation of innate and adaptive immune components (Choi et al., 2015; Jesus et al., 2018; Pérez-Cabezas et al., 2018). Given these characteristics, we studied the EVs of *L. tarentolae*, which express GP63 zinc metallo-proteinase, one of the major surface molecules and virulence factors in *Leishmania* implicated in the control of host microbicidal function and regulation of immune response (Arango Duque and Descoteaux, 2015; Guay-Vincent et al., 2022). It is presumed that GP63 and other molecules on the surface of *Leishmania* EVs may provoke effective immune responses similar to adjuvants, activating the immune response, and building a bridge toward an adaptive immune response. Since EVs act as a mirror from the cells they are derived from (Olivier and Fernandez-Prada, 2019) and studies have already shown that a proper activation is induced when the entire parasite is engulfed by macrophages and dendritic cells, consistent with an increase in the expression of molecules involved in antigen presentation (HLA-DR II and CD83 expression) and CD4+ T cell stimulation (CD80 expression) (Breton et al., 2005; Varotto-Boccazzi et al., 2022), they may serve as a natural adjuvant for vaccination purposes.

Here we show that *L. tarentolae* can express heterologous high molecular weight recombinant proteins such as SARS-CoV-2 Spike (approximately 180kDa), as was shown in Figure 8, without alteration of its viability (Figure 9). We were able to express the S protein on the EVs released by *L. tarentolae*, as confirmed by TEM and western blot analysis (Figure 14 and Figure 15), which allows for the study of its capacity to work as an adjuvant and antigen to develop a vaccine platform.

In this study, we use two different methodologies to isolate EVs, ultracentrifugation and affinity chromatography, the latter with the aim of enriching the population of EVs expressing LrS by taking advantage of its His-tag and evaluating the EV-rS set under more uniform conditions compared to EV-WT and rS counterparts. In this manner, we evaluated the immunomodulatory impact of EV stimuli in BMM and BMDC in terms of cytokine production. We found that EVs

potently stimulate innate proinflammatory responses from these professional APCs (Figure 19 and Figure 20) with a high relative gene expression of pro-inflammatory cytokines (IL-12, TNF, IL-1α, IL-1β, IL-6), and anti-inflammatory cytokines (IL-10) on both cell types in a dose dependent manner (Figure 18). This potent stimulus was also observed in the rS alone, which induced a higher relative gene expression of pro- and anti-inflammatory cytokines.

Contrary to the immunostimulatory potential shown by our EVs derived from *L. tarentolae*, a recent study of EVs derived from *L. tarentolae* used to stimulate THP-1 MΦ failed to exert any statistically significant effect on the pro-inflammatory cytokine production, including IFN-γ,TNF-α, IL-6 and IL-1β (Shokouhy et al., 2022). Nonetheless, in the same study, THP-1 MΦ pre-treated with EVs derived from *L. tarentolae* and then infected with *L. major* showed increased levels of IFN-γ, TNF-α, and IL-1β and a decrease in IL-6, compared to the reference group i) THP-1 MΦ pre-treated with EVs derived from *L. major* and infected with *L. major* and the control group, ii) THP-1 MΦ infected with *L. major* alone (Shokouhy et al., 2022).

The use of *L. tarentolae* as a live vaccine has shown its ability to target DC and secondary lymphoid organs, and produce a Th1 response (Breton et al., 2005; Shokouhy et al., 2022; Varotto-Boccazzi et al., 2022), contrary to their pathogenic *Leishmania* species counterparts, which tend to skew the immune response toward Th2, producing an alternative M2 macrophage activation which allows multiplication and parasite survival (Martínez-López et al., 2018; Varotto-Boccazzi et al., 2022). However, recently, Varotto-Boccazzi and collaborators found that *L. tarentolae* does not entirely polarize towards a distinctive Th1 or Th2 profile, which agrees with our preliminary results. Nonetheless, they saw a moderate immune polarization on the Th1 side by the expression of IL-2, IL-12, IFN-γ and transcriptional factors (STAT1 and STAT4) when they infected DC with *L. tarentolae* expressing the SARS-CoV-2 full-length spike protein as an antigen (Varotto-Boccazzi et al., 2022). In our model, there was no such effect since EV-WT and EV-rS induced both pro- and anti-inflammatory cytokine gene expression.

This work reports the initial evaluation towards the construction of a new vaccine platform and considerations for the next steps. We started with the evaluation of the immunomodulatory role of the antigen in the EVs with the aim of refining our system. First of all, a potential limitation of this study is that we did not confirm the presence of the integrated DNA in the transfected parasites. Hence, we cannot know where and how the transfected DNA is located in the

engineered parasite. Our validation was based in the expression of the exogenous protein by Western blot using an anti-His tag antibody and an anti-SARS-CoV2 Spike protein antibody.

Our approach to express the protein on the EVs was via use of a signal peptide (SP) from *L. mexicana secreted acid phosphatase* (LMSAP1) which has a signal peptide for the rS protein to be secreted. Nonetheless, since EVs are secreted by different pathways, we do not know if the SP increases the availability and accessibility of the S protein on *Leishmania* EVs. Our immunogold staining was performed on an unenriched sample (Figure 14), where not all EVs stained with the gold nanoparticle, which means that not all EVs released by *L. tarentolae* also expressed LrS. In this regard, we cannot confirm the location of the S protein on EVs. This constitutes a limitation in our work, which must be addressed to consequently improve the expression of LrS on the EVs. Considering this, a way to improve the availability and accessibility of the S protein on the surface of *Leishmania* EVs could be by fusion of the S protein with the TM of GP63, a marker of *Leishmania* EVs. This is similar to the approach followed by Kuate and collaborators, who used the SARS full-length spike with the TM of vesicular Stomatitis Virus-glycoprotein (VSV-G) to be expressed in a membrane-bound form, thus improving the antigen expression on EVs (Kuate et al., 2007). Other methodologies have been utilized to express antigen on the surface of EVs, such DSPE-PEG-NHS linker use, which was conjugated to the receptor-binding domain (RBD) of spike protein (Wang et al., 2022). In both works antigen was expressed on EVs and these EVs showed neutralizing capability without using adjuvants.

Additionally, an important aspect to improve is the way we quantify the amounts of sample used to stimulate the cells to make sure that same amount is used for each stimulus. We improved the isolation procedure to get EV expressing Spike by FPLC. Nonetheless, those EVs represent a source of different proteins which are included in our quantification process. Furthermore, the amount of rS on each EV varies. In this sense, it is recommended to do a blot with different amounts, compare them by densitometry and use the relative amounts for each sample. Another strategy to address this issue could be use of an ELISA method to quantify the antigen standard and total protein from EVs and/or sorting the EVs by NanoFACS to ascertain the number of antigen(s) attached to individual EVs (Morales-Kastresana et al., 2019).

It may be convenient to evaluate an intermediate dose between 10 and 100 µg/ml to see if we observe differences between groups and stimulation of an adjuvant effect given the results on BMDC; however, increasing the stimulus dose could also improve the results in both cell types,

since we only observed an adjuvant effect at 100 µg/ml dose on BMDC. In this sense, higher doses have been shown to prolong antigen exposure, enhancing the magnitude, quality, and persistence of antibody responses by the creation of a depot-effect (Turley and Lavelle, 2022). For example, for CD8+ T-cell responses, the size of the initial antigen dose appears to be key, rather than the duration of exposure. For instance, the success of vaccines such as yellow fever in terms of durable humoral and CD8+ T-cell responses is attributed to sustained and high antigen expression, in addition to DC activation via multiple TLRs and RLRs (Akondy et al., 2015). Interestingly, mRNA vaccines display high but transient antigen expression and when expressing the RBD of SARS-CoV-2 spike, they elicit robust antigen-specific CD8+ T-cells but short-lived neutralizing antibody responses in humans. Therefore, it seems possible that the incapacity of current adjuvants in licensed vaccines to evoke long-term protection or CD8+ T-cells may be explained by transient and low antigen levels, respectively, rather than an insufficient innate activation (Turley and Lavelle, 2022).

In the future, we plan to evaluate the stimuli in an animal model to assess the induction of specific antibody production against rS and EV-rS and neutralizing antibodies which are SARS-CoV-2 specific, assess the activation of APC and their capacity to activate T-cells and confirm the glycosylation pattern of rS in terms of immunogen integrity.

7 CONCLUSION

In summary, we demonstrated that *L. tarentolae* releases proinflammatory EVs that have the potential to be used as an adjuvant and as a vehicle for antigen production and delivery. They could indeed be developed as an efficient, easy-to-handle, and cheap system that could be highly beneficial for laboratory research around the world.

8 ANNEXES

8.1 SPIKE SEQUENCE CODON OPTIMIZED FOR LEISHMANIA

SARS-CoV2 Spike glycoprotein (MN908947) codon-optimized for Leishmania tarentolae expression.

On red, SalI cut site; blue, mutation on furin cleavage site; green, Proline substitution; pink, KpnI cut site

>g|tcgacgctggcgccagcgtcgtgaacctgacgacgcgcacgcagctgccgccggcgaacacgaacagcttcacg
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