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The Use of *Leishmania*-Derived Extracellular Vesicles as a Vaccine Platform against Emerging Viral Diseases

Abstract

Extracellular vesicles (EVs) are small membrane-bound "vehicles" responsible for transporting biological materials from source cells to target cells. EVs are thus indirectly capable of inducing changes in the physiological state and behavior of target cells once their contents are successfully released or received. Both prokaryotic and eukaryotic species utilize EVs for a variety of purposes. For example, *Leishmania*, a protozoan parasite, has demonstrated the ability to secrete immunomodulatory EVs. Various studies have shown that it is not these EVs in themselves, but rather the contents of these EVs that are directly involved in the parasite's colonization and replication inside host cells. Although using *Leishmania* as an expression system for recombinant proteins has been explored (investigations have yielded successful and promising results), the use of *Leishmania*-derived EVs is a burgeoning field of research. In fact, considering extant research on EV-based vaccines, substantial potential lies in exploiting *Leishmania*-derived EVs as a novel vaccine platform. Hence, this study aims to discuss the immunomodulatory capabilities of *Leishmania*-derived EVs and their potential application in vaccine development. Lastly, in piecing together the nature of *Leishmania*-derived EVs and the general therapeutic potential of engineered EVs, it is further hypothesized that *Leishmania* may be an effective expression system for EVs that harbour desired viral antigens as a part of more efficient vaccine designs.

Introduction

Over the last 15 years, investigative research in several biological fields of science has developed a great interest in the entities secreted by eukaryotic cells[1]. Extracellular vesicles (EVs), in particular, have garnered significant attention as they are extraordinarily diverse cargo carriers heavily enriched in proteins, lipids and nuclear material[1,2]. Although EVs are involved in various biological processes, these vesicles ultimately mediate regulated cell-to-cell communication for both bacterial and eukaryotic cells[2]. Thus, the communication network created by EVs is seen as highly efficient system for the induction of proximal and distant cellular responses[2].

It is important to note that EVs are not a homogenous population. There exist many different types of EV; in fact, various classification systems were established to create sub-populations based on their biogenesis, release pathway(s), size content and function[2]. Currently, three main subtypes have been identified: microvesicles (MVs), exosomes and apoptotic bodies. However, despite identifying these three distinct groups, current technological methods still struggle with accurate differentiation[3].

Within the growing body of literature on EVs, a select number of studies focus on the production of EVs in pathogenic protozoan parasites. Research has demonstrated that parasites are typically highly sensitized to their environments; as such, they are intrinsically capable of modulating host responses to evade elimination or neutralization tactics[4]. Initial studies proposed that soluble parasite factors were solely responsible for this form of parasite-to-host communication. However, recent evidence has suggested that parasite-derived EVs are key players in mediating these processes. In particular, the immuno-modulatory capabilities of *Leishmania*, a zoonotic protozoan parasite, present a highly unique parasite-to-host dynamic[3]. Initially demonstrated by Silverman and colleagues in 2008, it has since become well-established that various *Leishmania* species secrete exosomes carrying active proteins and RNAs that may impede macrophage signalling and anti-microbicidal activities[4–6]. However, the full role, effects and biotechnological potential of these protozoan EVs remains largely under-appreciated.

On the other hand, other forms of EV research have focused on utilizing them as a delivery mechanism. As described, EVs possess a remarkable ability to transmit large amounts of biological material, therefore making them befitting candidates for drug or vaccine use[1]. In fact, success with EV-based therapies has already been documented in anti-cancer research. For example, exosomes derived from tumours have demonstrated the ability to induce anti-tumor responses through the delivery of immuno-stimulatory factors[7]. There exists a high degree of biocompatibility with this line of therapy, as the exosomes are derived from cultured tumours[7].

Ultimately, the rising interest in EVs stems from their unique therapeutic potential as a safe and biocompatible alternative to other traditional and novel avenues in immunotherapy[7]. Currently, studies have shown that EVs derived from excised tissue samples will not induce any detrimental genotoxic, hematological or immunological effects when re-introduced into the organism of origin[8,9]. It is important to understand that an EV itself is not inherently immunogenic. As EVs are derived from endogenous tissues, the body is already conditioned to tolerate them. Similarly, pathogen or exogenously derived EVs will typically mimic a host's tissues to prevent detection[8]. Hence, the introduction of an EV alone (without any alterations) should not trigger inflammation or detrimental consequences[8]. The stimulatory effects of EV-based therapies are truly dependent on the contents of the EV and their regulated release at the appropriate targets[7]. Thus, in the context of vaccines, engineered EVs represent a flexible and safe strategy to create a treatment that is free of attenuated viruses or virus-like particles (VLPs)[7].

This study aims to conduct a systematic review of the immune-modulatory capabilities of *Leishmania*-derived EVs and their potential use as a novel

vaccine candidate against emerging viral diseases. This will be achieved by summarizing extant research on the molecular mechanisms of *Leishmania*-derived EV immunomodulation and the successes and challenges in EV use. It is rationalized that because *Leishmania*-derived EVs possess unique immunomodulatory capabilities, they promise a solution that may combat overly aggressive immune responses (i.e., Cytokine Response Syndrome) against novel viral diseases. Moreover, due to the inherent biocompatibility of EVs, they may prove to be a highly effective and safer alternative to other vaccine candidates.

Methods

This review was conducted using PubMed and Google Scholar with search terms ('Extracellular vesicles' AND 'Leishmania'), ('Leishmania' AND 'viral vaccin*'), and ('Extracellular vesicle' AND 'viral vaccin*'). Initial searches returned 92 articles which were then screened via their abstracts for relevance to the topic and for providing background information. Priority was given to primary research articles published after 2019, following the last major review on extracellular vesicles by Dong et al. in 2019. The remaining papers were then assessed for relevancy by reading titles and abstracts. For further details on the selection criteria applied during the screening process, see Table 1. Overall, the literature search supporting this review sought to find sources that could address the following questions: "(1) Why might *Leishmania* EVs serve as a better vaccine platform than current vaccine designs? (2) How can *Leishmania* EVs be modified so that they can be used to induce protective immunity against emerging viral diseases?" Ultimately, 25 papers were deemed relevant and included in this review.

Table 1. Screening Criteria for the Selection of Articles

Component	Inclusion criteria	Exclusion criteria
Title	Mentions EVs and/or (more specifically) *Leishmania* EVs	
	Mentions or implies the use of *Leishmania* as an expression system	
	Implies the use of *Leishmania* EVs in a biotechnology capacity/context	
Abstract	Mentions of the subsequent immune response following exposure to *Leishmania* EVs	
	Mentions of the immunemodulation capacities of *Leishmania* via their production of EVs	
	Identifies the factors that typically make *Leishmania* EVs virulent	
	Use of EVs as a (potential) viral vaccine platform	Use of EVs as a diagnostic tool
	Peer-reviewed and published in an academic journal	

Results

Immunomodulation conducted by *Leishmania*-derived EVs

Leishmania possess strategies to evade or subvert the effector mechanisms of macrophages[8]. The inhibition of macrophage activity is largely attributed to the counteractivity of a diverse range of *Leishmania*-derived virulence factors. Various studies have revealed that two key factors, lipophosphoglycan (LPG) and GP63, in particular, are crucial to orchestrating the interaction between the parasite and host (Figure 1)[8].

LPG is a highly abundant *Leishmania* surface molecule known to inhibit the mounting of robust anti-parasitic responses—thereby promoting parasitic survival within macrophages. Infection studies conducted with LPG-defective species of *Leishmania* revealed a considerable decrease in intracellular parasitic colonization and replication, increased expression of cellular nitric oxide synthase (iNOS) and enhanced Mitogen Activated Protein Kinase (MAPK) activation compared to their wild-type counterparts. Prior work with infection models has clearly established that the activity of iNOS to produce intracellular NO is vital to the elimination of *Leishmania*[10]. Authors, Prive and Descoteaux, have hypothesized that LPG preferentially inactivates MAPK to interfere with intracellular signalling, thus resulting in a cascade of effects across the host's ability to resist infection. Although other signal transducers (such as c-JUN N-terminal kinase and ERK 1/2) were identified as targets of LPG, the full extent of the downstream effects remains unclear[3,9].

Similarly, GP63 is a Zinc-dependent metalloprotease also expressed at high levels on the surface of the promastigote form of *Leishmania* species[11]. Classically, it is thought that once the parasite is phagocytosed by a host cell, GP63 would be released and spread across the cytosol of the infected cell. From there, GP63 cleaves several of host cell proteins involved in the regulation of microbicidal functions[3,11]. Projects such as that of Joshi and colleagues have utilized *L. major* GP63 KO mutants and have observed decreased virulence both *in vitro* and *in vivo*[10,12]. Interestingly, despite both factors initially being described as surface proteins, proteomic studies conducted within the past decade have revealed that *Leishmania*-derived EVs contain high quantities of both factors[3,8]. These findings appear to be consistent across a number of *Leishmania* species, including *L. donovani* and *L. braziliensis*[11]. Besides LPG and GP63, the contents of *Leishmania*-derived EVs also include proteins such as elongation factor-1α (EF-1α), fructose-1,6-biphosphate aldolase (FBA), HSP70 and HSP90 as well as small noncoding RNAs (Figure 1)[8,12].

Furthermore, it has been observed that infected cells or cells stimulated by parasitic components can also release EVs of their own (Figure 1). These EVs contain messenger RNA (mRNA), small noncoding RNAs, chromosomal DNA, mitochondrial DNA and major histocompatibility complexes (MHC) (Figure 1)[8]. Although much remains unknown about the exact downstream effects of these molecules, it is presumed that they may influence the behavior of proximal leukocytes[11]. These findings have ultimately contributed to the ever-growing theory that *Leishmania* and their EVs possess highly effective immunomodulatory capabilities that hold great biotechnological potential[8].

Use of *Leishmania* as an expression system for engineered EVs

Advancements in gene cloning technology over the past couple of decades have made it feasible to express therapeutic proteins and agents in a variety of hosts[13]. However, it remains a considerable challenge to find an appropriate expression system that will produce substantial and quality yields of the protein of interest cost-effectively[13]. Of the various prokaryotic and eukaryotic systems explored, non-pathogenic *Leishmania* species have stood out as candidates with high potential[13]. Among these species, *Leishmania*

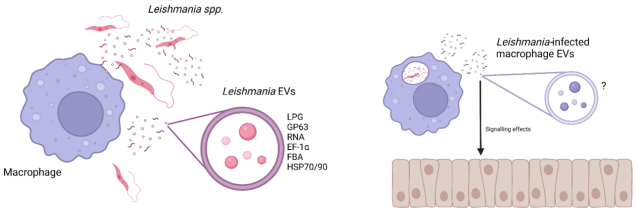

Figure 1. Contents of *Leishmania*-derived EVs contribute to their colonization and replication in macrophages. Proteomic studies of the EVs secreted by various species of *Leishmania* have revealed that they contain molecules such as LPG, GP63, RNAs, elongation factor 1 (EF-1α), and heat shock protein (HSPs). Further investigations of the molecular behavior of LPG and GP63 have demonstrated that they are directly responsible for inhibiting intracellular microbicidal features of the macrophage. Macrophages that have been infected by *Leishmania* are known to also secrete EVs. Contents of these EVs include RNAs, DNA and MHCs. (*Adapted from Zauli et al.*)[12] (Created with Biorender.com)

tarentolae is one of the more attractive species as it can be cultured in inexpensive media at 26 °C and in large batches with a fairly short generation time (approximately 5–6 hours)[13–15]. Studies conducted in the late 2000s have shown that *L. tarentolae* can produce high yields of recombinant proteins. Since then, this non-pathogenic species has become widely used for gene manipulation, gene targeting, gene function studies and the generation of live vaccines[13]. The broad range of post-translation modification mechanisms, such as mammalian-type N-glycosylation has made *L. tarentolae* a considerably compatible expression system[15].

It was initially unclear whether *L. tarentolae* was among the species of *Leishmania* that secreted EVs. This uncertainty stemmed partly from the heavy focus on pathogenic species of *Leishmania*, given the widely recognised role of EV secretion in supporting parasitic survival and pathogenesis[14]. However, a recent study by Shokouhy and colleagues revealed through electron microscopy-captured images that *L. tarentolae* secretes EVs, and that these EVs contain GP63[14]. Moreover, when examining the effects of *L. tarentolae*-derived EVs on THP1 macrophages, the observed increase in pro-inflammatory cytokine levels suggested that despite being non-pathogenic, *L. tarentolae*-derived EVs will still induce an immune response[14].

It should be noted that the current research continues to elucidate the pathways involved in protein sorting and loading for EV secretion[14]. Reports indicate that only 5–9% of all proteins present in *Leishmania* EVs contain a signal peptide. Hence, it is speculated that unconventional mechanisms may be involved[14].

EV-based vaccine proposals against novel/(re-)emerging viral diseases

Currently, four major vaccine technologies and platforms are used extensively for the production of antiviral vaccines: (i) RNA vaccines (delivered through lipid-based nanoparticles) (ii) adenovirus-based viral vectors (iii) subunit vaccines (typically produced in eukaryotic expression systems) and (iv) inactivated or attenuated viruses[15,16]. However, the inherent limitations of these vaccine platforms are exacerbated in the face of novel and complex viruses. Namely, traditional expression or production systems may lack the appropriate post-translation modification mechanisms to enhance the biocompatibility of recombinant protein components. Furthermore, the lifespan of certain vaccinal components may be shorter than what is desired or needed[16,17]. Additionally, the delivery of immunogenic stimulants and antigens to the appropriate tissues may greatly impact the efficacy of the vaccine[18]. Considering these concerns, a new interest in the use of EVs as a vaccine platform has emerged[19]. Not only can EVs induce a strong immune response by guiding cell communication, but they can be engineered to display specific viral antigens for the activation of lymphocytes[19].

Moreover, in the context of viral infections, it has been reported that CD63+/CD81+ EVs loaded with viral peptides are released from monocytes to induce the production and release of interferon-γ from CD8+ T cells in an antigen-specific manner[19]. As such, research has sought to exploit this biological feature and construct vaccine designs based on antigen-loaded EVs. In two critical studies, Montaner-Tarbes and colleagues demonstrated that CD63+/CD81+ EVs loaded with porcine respiratory and reproductive syndrome virus (PRRSV) antigens were generated during the natural progression of the disease[20]. Moreover, intramuscular administration of these antigen-loaded EVs into healthy hosts successfully elicited a specific IgG immune response. Further investigations demonstrated that even high doses of these EVs did not trigger clinical symptoms associated with PRRSV[20].

However, as not all viruses appear to induce the production of antigen-loaded EVs, ongoing research has proposed EV engineering as an alternative approach. EV engineering involves artificially adding antigens of interest into or onto these vesicles, thus mimicking the previously described antigen-loaded EVs. Currently, greater success has been achieved by directly modifying cells to produce EVs loaded with the antigens of interest[19]. This is opposed to the lower degree of success seen amongst attempts to load pre-existing EVs with antigens through methods such as electroporation. When utilizing a cell-based approach, researchers will transduce cells to produce an EV-specific protein fused to the antigen of interest. In this respect, Anticoli et al., have managed to create cell-derived EVs embedded with various viral antigens such as HPV E7, Ebola VP24/VP40/NP, Influenza NP, West Nile NS3 and hepatitis C virus (HCV) NS3[21]. Subsequent testing of these EVs in intramuscular vaccines induced a specific and enhanced immune response from CD8+ T cells without inducing detrimental over-inflammation[21].

Overall, with growing evidence demonstrating their potency and safety, several biotech companies have already begun to design virus and adjuvant-free vaccines against Chikungunya, Zika, Dengue, West Nile and coronaviruses[19]. In particular, through the recent global outbreak of the novel coronavirus, Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV2), a great deal of interest in creating an EV-based vaccine against SARS-CoV2 has developed. As the highly conserved viral Spike (S) protein is primarily responsible for viral entry into host cells, researchers have altered and stripped it to its core domains for embedding onto EVs[22]. However, among the four following examples leading in this field of research, the actual construction of the EV varies slightly from company to company[19]. Alternatively, some companies have chosen to combine the EV-based vaccine approach with aspects of the DNA-based vaccine approach.

Notably, the company Capricor Therapeutics has achieved success in transfecting HEK293 cells with vectors expressing the four structural SARS-CoV2 proteins to induce the production of EVs carrying all four viral antigens in their native configuration (Figure 2A) Although the S protein mediates viral entry, previous work has shown that immunization with multiple proteins allows for greater range in the modulation of the immune response.

Capricor Therapeutics has also formulated a second vaccine design based on an alternative mRNA approach. Having designed mRNAs for a modified S protein, a full-length N protein, and soluble fragments of the M and E proteins, researchers at Capricor Therapeutics and John Hopkins University then fused these to a Lamp1 mRNA transcript for optimal antigen presentation in the context of MHC (Figure 2B). This strategy is grounded on the well-established knowledge that Lamp1 undergoes intracellular degradation into smaller peptides by the MHC pathway in antigen-presenting cells[22]. When the proposed vaccine was administered intramuscularly into mice at various concentrations, the animals showed a concentration-dependent antibody response for both S and N proteins[22].

The Ciloa Company has also developed an EV-based vaccine against SARS-CoV2 called CoVEVax (Figure 2C). Similar to Capricor Therapeutics, the EVs are derived from transfected HEK293 cells. Although little public information exists on how Capricor Therapeutics guides the translocation of the viral proteins onto the surface of the EV during its genesis, Ciloa has described CoVEVax to utilize their patented EV-sorting peptide, CilPP[22]. Unlike the Capricor Therapeutic design, Ciloa has chosen to only include a modified version of the S protein (Figure 2C)[22]. Testing of this vaccine proposal has elicited adequate levels of specific IgG production and enhanced IFN-γ production (Figure 3A and 3B)[22].

A third biotech company, Codiak BioSciences, has also presented a similar vaccine to that of Ciloa's. Named the exoVACC, the proposed design uses a proprietary engEX platform (built around a central scaffolding protein called PTFGRN) to preferentially sort and display the viral S protein on EVs (Figure 2D)[19,23]. As of July 2022, Codiak BioSciences had partnered with the Coalition for Epidemic Preparedness Innovations (CEPI), to continue with preclinical studies as exoVACC had demonstrated the ability to stimulate a broad immune response comprising of both humoral and cell-mediated immunity[23]. Unfortunately, Codiak BioSciences, on March 27, 2023, news was released that Codiak BioSciences has filed for bankruptcy and will no longer be continuing with the development of exoVACC[24].

Finally, Versatope Therapeutics has proposed an EV-based vaccine that uniquely uses the nano-sized vesicles sourced from bacterial cells (Figure 2E). Known as Outer Membrane Vesicles (OMVs), they possess a high degree of similarity to human EVs. Using an OMV-anchoring protein called cytolysin A (ClyA), Versatope has engineered OMVs to display a modified version of the viral S protein (Figure 2E)[25]. Although previous studies using OMVs have demonstrated success in eliciting adequate immune responses against the H1N1 influenza virus and MERS-CoV, there remain concerns over the biosafety of bacterial-derived OMVs[26].

Evidence of the efficacy of EV-based vaccination

Of the various proposals described above, some have demonstrated promising results in their ability to elicit an appropriate cell-mediated and humoral immune response. Of the five proposals, the Ciloa Company's design has directly demonstrated great capacity in promoting viral antigen-specific IgG antibodies and IFN-γ production in pre-clinical trials[19]. When testing their proposal *in vivo*, Ciloa utilized a two-component vaccine comprised of the DNA that encodes for the fused S protein-EV complex ($DNA^{S\text{-}EV}$) and the S-EVs themselves[22]. Prior *in vitro* testing demonstrated that the inclusion of the $DNA^{S\text{-}EV}$ allows for *in situ* production of the desired EV[22]. Ciloa predicted that *in situ* production would lead to the genesis of a more robust form of immunological memory[22].

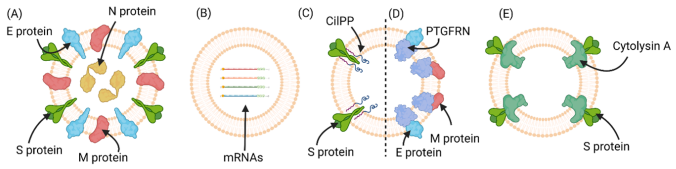

Figure 2. EV-based vaccine designs from various biotechnology companies. (A) By transfecting HEK 293 cells, Capricor Therapeutics have successfully created EVs embedded with/containing S, M, E and N proteins of SARS-CoV2 in their native configurations. (B) Capricor Therapeutics has also successfully loaded EVs with mRNAs for full-length S protein, as well as modified M, E and N proteins of SARS-CoV2. (C) The Ciloa Company has successfully created an EV that displays a modified version of the SARS-CoV2 S protein by using a proprietary EV-sorting peptide called CilPP. (D) Similarly, Codiak BioSciences has created an EV that displays various SAR-CoV2 proteins that have been sorted into the EV using the scaffold protetin, PTGFRN. (E) Using a bacterial OMV, Versatope Therapeutics has created an EV that displays Spike protein by fusing it the OMV-anchoring protein cytolysin A(ClyA). (*Adpated from Sabanovic et al.*)[19] (Created with BioRender.com)

To test the vaccine's efficacy, the study administered various alterations of the proposed design, alongside the original design, to generate comparative data. The first of the experimental groups received the original vaccine design containing both $DNA^{S\text{-}EV}$ and S-EVs; the second group received injections containing $DNA^{S\text{-}EV}$ and the S protein itself; and finally, the last group received injections containing only S-EVs[22]. To then quantify the subsequent immune responses, sera samples were drawn from each group and their antibody concentration levels were measured using an indirect ELISA[22]. Results demonstrated that mice immunized with PBS possessed no circulating antibodies (Figure 3A). ELISA results from the $DNA^{S\text{-}EV}$ and S-EV group and the $DNA^{S\text{-}EV}$ and S protein group demonstrated a moderate humoral response, while results obtained from the S-EVs-only group demonstrated the highest antibody titers (Figure 3A)[22]. However, to ensure that the vaccine could elicit more than antibody production (i.e. the vaccine could stimulate pro-inflammatory cytokine production), a separate ELISA was performed to quantify the concentration of IFN-γ in the extracted sera samples. Unlike the results obtained when quantifying antibody titers, the S-EV-only group demonstrated the lowest IFN-γ concentrations (Figure 3B)[22]. Hence, it was concluded by Ciloa that both components, the $DNA^{S\text{-}EV}$ and the S-EVs, are necessary to induce a proper and protective immune response.

Ciloa's success in creating an EV-based vaccine against SAR-CoV2 that elicits an appropriate and desirable immune response (the production of virus-specific antibodies) is extremely promising to the broader field of EV-based therapeutic research. Further research is needed to determine vaccine kinetics and the longevity of the induced immunity before testing can proceed to stages such as clinical trials.

Discussion

With great potential for a diverse range of clinical applications, the study of EVs has become increasingly popular. Ultimately used as a form of cell-to-cell communication, their ubiquity amongst prokaryotic and eukaryotic cell types contributes greatly to their high degree of biocompatibility and flexibility[19]. Of the various applications currently under investigation, substantial attention has fallen on the development of EV-based vaccines against viral diseases. EVs can be modified to present viral antigens, thus mimicking the appearance of a viral particle without the need for pathogenic substances. Furthermore, as several viral vaccines are based on viral vectors, particularly adenoviruses, one of the primary limitations is natural, pre-existing immunity against these vectors[22]. Pre-existing immunity often implies that the individual's immunological memory will induce an anti-adenovirus immune response, thus overriding and hampering any immune response against the virus of interest[22]. Due to the localization of memory lymphocytes in the periphery, immunological memory will respond far faster than any attempt to generate a novel immune response[27]. In the context of emerging viruses, one of the major concerns is the threat of continued re-infections in the general population, thereby driving a virus to become endemic. As seen with the influenza virus, a combination of its enhanced mutation rate and the population's lack of long-lasting immunity has necessitated yearly vaccination campaigns—which may be less feasible for emerging viruses that are of greater immunogenicity[22]. EV-based vaccines overcome these problems. By preserving the native (or near-native) configuration of viral antigens for display on the EV, the elicited immune response is not only more robust, but studies have shown that its administration leads to enhanced B cell receptor (antibody) crosslinking and circulation as well as memory CD8+ T cell expansion and survival[22].

Albeit, that is not to say that EV-based vaccines do not introduce a new set of challenges. Although their ubiquity provides a great deal of flexibility for modifications to resolve biocompatibility issues, a single body of EVs generated from a single cell type may still be a heterogeneous population.

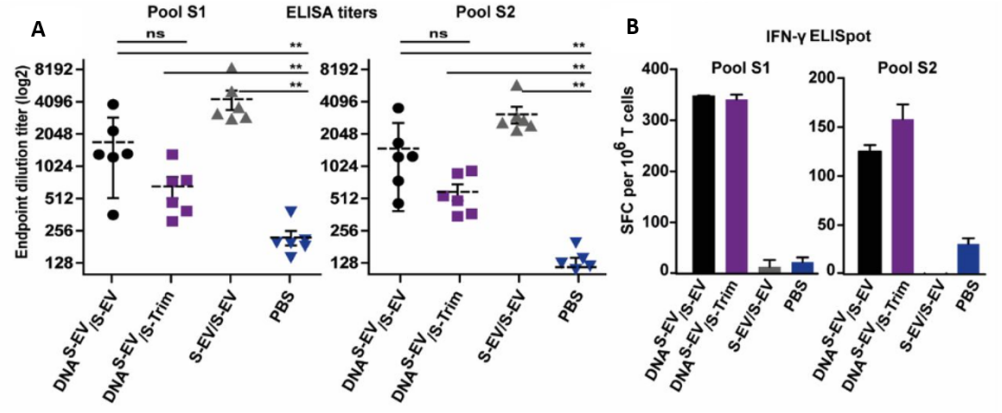

Figure 3. Quantified immune responses after immunizing mice with the Ciloa Company's proposed EV-based vaccine design[22]. (A) Using an ELISA to quantify the levels of IgG antibodies specific to the SARS-CoV2 S protein complex (designed and constructed by Ciloa), it was demonstrated that three injections of S-EVs alone would elicit the greatest production of antigen specific antibodies[22]. (B) Conversely, when quantifying the levels of IFN-γ produced following immunization, mice injected with S-EVs alone did not demonstrate increased IFN-γ levels; instead, mice immunized with a combination of DNAS-EV and S-EVs/the S protein complex itself were observed to produce greater levels of IFN-γ. Hence the Ciloa Company has concluded that both are necessary for a protective and effective vaccine[22].

Moreover, this issue is further exacerbated by the lack of efficient technological methods to identify the contents of and sort through a large population of EVs[3,19]. However, ongoing research has already begun to formulate solutions. Studies focused on the identification of increasingly specific EV sub-types, and the standardization of EV-based fluorescence-activated "cell" sorting (FACS) protocols are gradually closing this technical gap[3,28].

In light of this, this review proposes that exploiting the immunostimulatory properties of *Leishmania*-derived EVs for EV-based vaccine designs should be explored further. Since these *Leishmania*-derived EVs are immunogenic by themselves but not directly responsible for pathogenesis, engineering *Leishmania* models to produce 'pre-loaded' EVs may be an effective improvement to current EV-based vaccine designs[8]. Given the success of Ciloa's vaccine in eliciting the production of desirable antigen-specific antibodies, there is strong evidence that using EV-based vaccine design may be worthwhile to research. Furthermore, as it has been shown that *Leishmania* expression systems can be temporally efficient, cost-effective and compatible with mammalian-derived recombinant proteins, relying on *Leishmania* expression systems may resolve issues with the upscaling of vaccine production[13]. Current industry-standard practices for the production of recombinant EVs use either a yeast-based expression system or transfected immortalized cell lines[29–31]. Although most yeast species produce glycosylated proteins, these end-products are not fully humanized, which results in downstream issues concerning compatibility when employed in a therapeutic context for humans[30]. Efforts have been made to genetically alter existing yeast species using CRISPR/Cas9 systems to create new strains with enhanced abilities to produce humanized protein products, but further research is needed to assess whether this would make the overall workflow more efficient while maintaining adequate accuracy[30]. Additionally, the use of transfected immortalized cell lines (such as HEK293 cells) for EV production presents unique challenges during the purification stage. As the EVs released by transfected cells share similar size and biophysical characteristics as the lentivirus (LV) used to transfect, EVs and LVs are often co-purified together[31]. The lingering presence of LVs in the final EV-based vaccine product would be considered an "impurity" that could potentially induce off-target immune responses[31].

Altogether, given the works reviewed and described in this review, the natural biocompatibility, immunogenicity, and low-cost nature of *Leishmania*-derived EVs make them a preferable vaccine platform. Moreover, emerging studies have already begun tapping into the biotechnological potential of *Leishmania* EVs for potential vaccines against emerging viral diseases. This review foresees and suggests that further research into how *Leishmania* can be exploited as an expression system could facilitate greater growth for the prospects of EV-based vaccines.

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