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Chapter

Vaccinia Virus-Derived Vectors in Leishmaniases Vaccine Development

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Abstract

Due to an increase in the incidence of leishmaniases worldwide, the development of new strategies such as prophylactic vaccines to prevent infection and decrease the diseases has become a high priority. The development of vaccines against the various species of pathogenic *Leishmania* to humans has been hampered, in part, by the inefficient stimulation of the protective cellular immunity promoted by the administration of purified or recombinant antigens, indicating the need for new approaches. Viral vectors represent an attractive way to deliver and present vaccine antigens that may offer advantages over traditional platforms. Among the most attractive and efficient viral vectors in inducing a cellular immune response, vaccinia virus has been the most used in leishmaniases vaccine trials. The first report of the use of recombinant vaccinia virus (VACV) in the induction of protection against *Leishmania* infection was made in 1993. Since then, several *Leishmania* spp. antigenic subunits were cloned into recombinant VACV. Although highly attenuated poxviral vectors are capable of inducing protective immunity against *Leishmania* spp., their limitation in replicative capacity reduces their potential as compared to replicative vectors. In order to achieve a balance between safety and replication, several VACV strains with intermediate phenotype have been developed.

Keywords: leishmaniases, vaccines, viral vectors, recombinant vaccinia virus, VACV

1. Introduction

Leishmaniases are important neglected tropical diseases (NTD) caused by protozoan parasites from the genus *Leishmania* Ross, 1903, of which more than 20 species are pathogenic to humans. Such parasites are transmitted by about 30 species of infected female sandflies (genus *Phlebotomus* and *Lutzomyia)* [1, 2], and their biological cycle alternates between the amastigote forms (obligatory intracellular), in the mammalian host, and promastigote forms (extracellular), in the vector digestive tract [3]. The diseases present a range of mammalian hosts, such as canids, rodents, marsupials, edentates, and primates, both human and nonhuman. The species that infect humans are distributed in two subgenera: *Leishmania* and *Viannia*, based on the development of the parasites inside the insect vector digestive tracts. Depending on the *Leishmania* species and the host's immune status, leishmaniases present a broad

spectrum of clinical manifestations, which can be divided into two main groups: (I) visceral leishmaniasis (VL) caused by *Leishmania (Leishmania) infantum* (syn. *L. (L.) chagasi)* and *L. (L.) donovani* and (II) tegumentary leishmaniasis (TL), with cutaneous form (CL) caused by *L.* (*L.*) *major*, *L.* (*L.*) *amazonensis*, *L.* (*L.*) *mexicana*, *L.* (*L.*) *aethiopica*, *L. (Viannia) braziliensis*, *L.* (*V.*) *guyanensis,* and *L.* (*V.*) *panamensis* and the mucocutaneous form (MCL) mainly caused by *L. (V.) braziliensis* and *L. (V.) guyanensis*, in the New World, and *L. (L.) aethiopica,* in the Old World [4, 5].

It is estimated that 14 million people are infected worldwide, and 350 million are at risk of infection. Approximately 1.3 million new cases are registered annually [3]. According to the Global Burden of Disease Study (GDB) 2010, about 50,000 people die each year from the diseases, resulting in 3.3 million disability-adjusted life years (DALY) lost [6]. In recent decades, several *Leishmania* species have spread to non-endemic areas [7].

According to the World Health Organization (WHO), leishmaniases are among the emerging and uncontrolled category 1 diseases, and their prevention is based primarily on three parameters: (I) vector control, (II) control of parasitic reservoir animals, and (III) research and development of new vaccine candidates [8]. Spraying of intra- and peri-domiciliary residual insecticides has been crucial in the control of sandflies. However, there is concern about the emergence of vector resistance to dichlorodiphenyltrichloroethane (DDT), especially in highly endemic areas [9]. The chemotherapeutic treatment of infected dogs, the main reservoirs of the parasite in VL, reduces or eliminates symptoms. Yet, many animals are still able to transmit the parasite, remaining the epidemiological risk. Other measures, such as topical insecticides and impregnated collars, are expensive and difficult to implement in national control programs [10]. In the absence of effective strategies, vaccine development is cost-effective in controlling leishmaniases. It is estimated that a vaccine with a 70% efficacy providing protection for 10 years is able to prevent 41–144 thousand CL cases in seven Latin American countries (Bolivia, Brazil, Colombia, Ecuador, Mexico, Peru, and Venezuela) with an inferior cost than the currently recommended treatments. As for VL, even a vaccine that provides protection for only 5 years with a 50% efficacy would still be more economically feasible compared to current treatments [9].

The first leishmaniases vaccination attempts, named leishmanization, were based on the observation that an individual cured of a cutaneous lesion became refractory to reinfection [7, 8]. In leishmanization, the infectious lesion material, later replaced by the cultured parasites, was used in the inoculation of uninfected individuals. This method was interrupted due to a number of factors, including quality control, persistence of the parasite in the body, the emergence of the HIV virus in the 1980s, and ethical reasons [11].

The first generation of vaccines emerged from leishmanization and comprises heat or phenol-killed promastigote forms associated with different adjuvants, including BCG (*Mycobacterium bovis,* bacillus Calmette-Guérin) and irradiated or attenuated live promastigotes. However, the standardization of vaccines derived from parasites in culture hinders their register by the competent national institutions [7, 8]. Human vaccination using dead strains of *Leishmania* spp. dates back to the late 1930s was a pioneering strategy among Brazilian scientists. Phase III clinical trials conducted in Ecuador and Colombia utilized a Brazilian vaccine called Leishvacin®, composed of *L. amazonensis* killed promastigotes in association with BCG adjuvant, which demonstrated safety but low efficacy [10, 11]. After a period of 4 years of commercial production by Bioquímica do Brasil (BIOBRÁS, Brazil), Leishvacin® is now only produced in a nonindustrial way in research laboratories for clinical assays. The vaccine is also accepted as an immunotherapeutic agent with or without association with Glucantime® (Rhône

Poulenc Rorer, France), for the treatment of resistant individuals or for the ones Glucantime® induces high toxicity. Of late, three forms of vaccines consisting of *L. major*, *L. amazonensis*, and *L. Mexicana* were evaluated by first-generation vaccines of human clinical trials [12].

The second generation of vaccines includes purified or recombinant *Leishmania spp.* proteins [8]. In Brazil, in 2003 and 2006, respectively, two second-generation vaccines against canine visceral leishmaniasis (CVL), Leishmune® (Fort Dodge, Brazil) and Leish-Tec® (Hertape Calier, Brazil), were registered. Leishmune® is composed of a purified fraction of the fucose-mannose ligand (FML) isolated from *L. donovani* promastigotes, associated with the saponin adjuvant. Their formulation has been shown to be safe, protective, and highly immunogenic for dogs, in addition to being able to prevent the transmission of CVL [13]. However, since November 2014, the vaccine has been suspended for manufacturing and marketing due to noncompliance with the complete requirements of the Ministério da Agricultura, Pecuária e Abastecimento (MAPA, Brazil) for phase III studies on vaccine efficacy (NOTA TÉCNICA N° 038/2014/DFIP/DAS). As for Leish-Tec®, it is composed of the *L. donovani* recombinant A2 protein associated with the saponin adjuvant. A2 is a highly expressed surface protein in the amastigote form of *L. donovani* and was the first virulence factor identified in *Leishmania* spp.; such protein is necessary for the survival of the parasite in the mammalian host and is involved in the visceralization of the pathogen during infection [14]. Dogs immunized with Leish-Tec® and experimentally infected by *L. infantum* were able to develop a partially protective immune response against CVL, presenting positive parasitism in the bone marrow 9 months after the challenge [15]. In Europe, the first CVL vaccine registered and commercially available in 2011 was LiESP/QA-21, named CaniLeish® (Virbac, France), a second-generation vaccine composed of *L. infantum* excreted/secreted recombinant proteins (LiESP) associated with a highly purified fraction of *Quillaja saponaria* saponin (QA-21) as an adjuvant [16]. Clinical trials in dogs vaccinated with CaniLeish® and experimentally infected by *L. infantum* demonstrated, after 1 year, reduced parasite load, specific cellular immune response, and decreased chance of relapses [17]. Another vaccine currently commercialized in Europe is LetiFend®, whose active principle is a recombinant chimeric protein, named Protein Q, composed by the fusion of five epitopes of the acidic ribosomal proteins LiP2A, LiP2B, LiP0, and the histone H2A of *L. infantum*. The efficacy of vaccination in a large-scale dog population demonstrated that LetiFend® is a novel, safe, and effective vaccine for the active immunization of noninfected dogs from 6 months of age in reducing the risk of developing clinical visceral leishamaniasis after natural infection with *L. infantum* [18].

Likewise A2, FML, LiESP, and Protein Q, several other *Leishmania*-derived antigens have already been identified as immunogenic based on T cell clones, due to its abundance and specific location in the parasite, by screening of expression libraries against human- and dog-infected sera [19] or by reverse vaccinology [20, 21]; and their efficacy has been thoroughly evaluated in preclinical and clinical trials. However, to date, there is no effective vaccine against the different clinical forms of human leishmaniases, despite the progress of the vaccines against CVL. The development of vaccines against the various species of pathogenic *Leishmania* to humans has been hampered, in part, by the inefficient stimulation of the protective cellular immunity promoted by the administration of purified or recombinant antigens. The third generation of leishmaniases vaccines is based on coding DNA, including recombinant microorganisms used as gene expression vectors [22].

Among the possible vaccine vectors, the most promising are those based on recombinant viruses, capable of expressing heterologous proteins directly

within the cells of the host organism, likewise in natural infection. Vaccines based on viral vectors represent a highly versatile platform for the development of vaccines. Viral genomes can be manipulated to express any target antigen and consistently carry relatively large transgene insertions [23]. Moreover, among the advantages of using recombinant viruses as vaccine vectors is the fact that viruses have evolved as the most efficient organisms in infecting cells. After 10 minutes of infection, more than 95% of certain viruses can be found inside host cells. Another advantage is that viral proteins can play as powerful adjuvants. Besides, viruses can infect antigen-presenting cells (APC), avoiding cross-presentation. Lastly, some recombinant viruses can be lyophilized and stored without the need for special refrigeration equipment [22]. Considering the recombinant viruses most commonly used as vaccine vectors, there are already established high-throughput and large-scale production processes, aiming to use this technology in the context of pandemics [23]. Vaccinia virus is one of the most attractive and efficient vectors [22] and widely used in leishmaniases vaccine trials, which is the focus of the present study.

2. Immunology of leishmaniases

Resistance to infection by *Leishmania* spp. is mediated by both innate (macrophages, neutrophils) and adaptive (T cells) immunity. Macrophages are the main cells of the mononuclear phagocytic system parasitized by *Leishmania* spp., despite the fact that neutrophils are among the first cells recruited to contain the parasite at infection site [19]. A protective immunity against all forms of leishmaniases depends on the elimination of parasites by activated macrophages. Paradoxically, *Leishmania* spp. use the phagocytic function of macrophages as a strategy of internalization and replication within phagolysosomes. In this way, macrophages play both as host cells and as effector cells that attack parasites. Internalization of *Leishmania* spp. by host cells induces the production of pro-inflammatory cytokines involved in the elimination of parasites [11]. Activation of macrophages is firstly mediated by Toll-like receptors (TLR), subtypes of pattern recognition receptors (PRR) that play as the first line of defense against parasites, activating NFκB (nuclear factor "kappa-light-chain enhancer" of activated B cells) and resulting in the production of pro-inflammatory cytokines, such as interleukin-12 (IL-12) and tumor necrosis factor (TNF). Also part of the innate immune response is the NOD-like receptors, which are cytosolic PRR essential in the detection of intracellular pathogens. Together, the signaling cascades of TRL and NOD regulate the inflammatory and apoptotic responses of infected cells [24].

Reactive oxygen, nitrogen, and nitric oxide (NO) species, induced by IL-12, are the main responsible for the macrophages leishmanicidal activity. NO is produced from the metabolism of L-arginine, in a reaction catalyzed by the inducible nitric oxide synthase (iNOS). Cytokines such as interferon gamma (IFN-γ) and TNF-α stimulate iNOS expression, while IL-4 and IL-10 inhibit its expression, turning macrophages refractory to leishmanicidal activity [23, 24].

Dendritic cells (DC) also belong to the mononuclear phagocytic system and play as a link between innate and adaptive immune responses. DC are recruited to the site of infection by cytokine/chemokine released by infected macrophages and neutrophils. The ability of DC to present antigens through MHC (major histocompatibility complex) classes I and II induces the stimulation of *Leishmania*-specific $CD8^+$ and $CD4^+$ T cells, respectively, which are essential in acquiring *Leishmania* spp. resistance [19].

$CD4^+$ T cells play a crucial role in the protective immunity against *Leishmania* spp. due to the production of various cytokines associated with parasite resistance, such as IFN-γ and TNF-α [25]. The use of murine models in leishmaniases preclinical vaccine trials allowed the identification of two subtypes of $CD4^+$ T cells, which produce and secrete cytokines capable of inducing different effector functions. The studies that used as basis the model of *L. major* infection, established in BALB/c mice and proposed by Sacks *et al*. [26], defined the Th1/Th2 paradigm of resistance/susceptibility to infection and the role of cytokines such as IL-12 and IL-4 in the development of Th1 and Th2 cells subtypes, respectively [25, 27]. Generally, CL-causing *Leishmania* species require a Th1-type immune response pattern for cure in murine models [28]. Protective immunity in visceral infection is also related to the Th1 response pattern and occurs in the presence of macrophage-activating cytokines, such as IL-12 and IFN-γ, and by the formation of hepatic granulomas, structures capable of containing infection through the action of the mononuclear phagocytic system cells, which are activated by IFN-γ [29]. However, unlike the disease caused by *L. major*, the dichotomy of the Th1/Th2 immune response profile is not evident in VL murine models [30]. The susceptibility phenotype in VL murine seems to be more related to the inability to develop an effective Th1 response than in the elaboration of an exacerbated Th2 response [31]. The mechanisms involved in the differentiation of naïve $CD4^+$ T cells in the Th1 and Th2 phenotypes are not yet well known, and several factors influence the resistance or susceptibility to leishmaniases, including host genetic variations, genetic variations between species and parasite strains, as well as the size of inoculum, and number of *Leishmania* spp. infective forms received by the host through the phlebotomine bite [24].

Although *Leishmania* spp. reside within phagolysosomes of mononuclear phagocyte system cells, mainly macrophages, their antigens can be presented via MHC class I to $CD8^+$ T cells by cross-presentation [32]. The production of cytokines and the cytotoxic activity of $CD8^+$ T cells contribute to the completion of *Leishmania* spp. infection. It was initially believed that $CD8^+$ T cells performed effector function only during reinfection by parasites. However, studies have shown that they are also crucial in controlling primary infection by inducing the Th1 profile of immune response through the production of IFN-γ [11]. In addition to the production of cytokines, $CD8^+$ T cells also participate in the control of infection through cytotoxic mechanisms, such as the production of granzyme and perforin [8, 33].

The wide variety of cytokines and effector mechanisms involved in the immune responses induced by various species of *Leishmania* clarifies the complexity of leishmaniases. However, murine models of *Leishmania* spp. are able to mimic several aspects of human disease, being the main source of knowledge about the immunology of leishmaniases and the tool most used in the evaluation of efficacy in preclinical vaccine trials [11].

3. Activation mechanisms of the immune response by recombinant viruses

The mammalian immune system has evolved to the efficient recognition of intruder viruses, being able to activate potent innate and adaptive immune responses (see **Figure 1**). Depending on the nature and replication strategy of the viral genome, several PRR are involved in the innate immune response to the recombinant virus (see **Figure 1**). Receptors for nucleic acids include TLR3, TLR7, TLR8, and TLR9 in the endosome, as well as cytosolic RNA/DNA sensors such as RIG-I (retinoic acid inducible gene I), MDA5 (melanoma differentiation-associated

gene 5), and cGAS (cyclic GMP-AMP synthase). After binding to the viral genome, these receptors signal via the NFκB and MAPK (mitogen-activated protein kinase) pathways, resulting in the induction of pro-inflammatory cytokines and chemokines. Viral vectors that induce inflammation generally play as "self-adjuvanted." A second effect of endosomal TLR signaling is the activation of interferon regulatory factor (IRF) 3 and IRF7, transcription factors necessary for the expression of the type I interferon (IFN-I) genes: IFN-α and IFN-β [34]. IFN-I induces the maturation of APC (see **Figure 2**), especially DC, by stimulating the expression of co-stimulatory molecules such as CD80, CD86, and CD40, which in turn, lead to an efficient DC homing to secondary lymphoid organs and the antigens presentation to $CD4^+$ and $CD8^+$ T cells. IFN-I also promotes the cross-presentation of viral antigens processed on the DC endosomes to $CD8^+$ T cells [35].

While first-generation (killed or attenuated parasites) or second-generation (purified or recombinant proteins) vaccines are capable of inducing an intense humoral immune response, they are inefficient in activating cellular immune response based on cytotoxic $CD8^+$ T cells (CTL). Recombinant viral vectors, however, have the specificity of inducing an intense expression of heterologous proteins, encoded in the transgene, inside infected cells [22]. Activation of CTL requires the expression of the pathogen proteins in the cytosol APC, as well as the binding of the antigen to the MHC class I molecules [36]. The immune response based on $CD8^+$ T cells is initiated by the generation of peptides from their protein precursors cleaved in the cellular proteasome. After cleavage, the resulting peptides are complexed to TAP (transporter associated with antigen processing) and transported from the cytosol

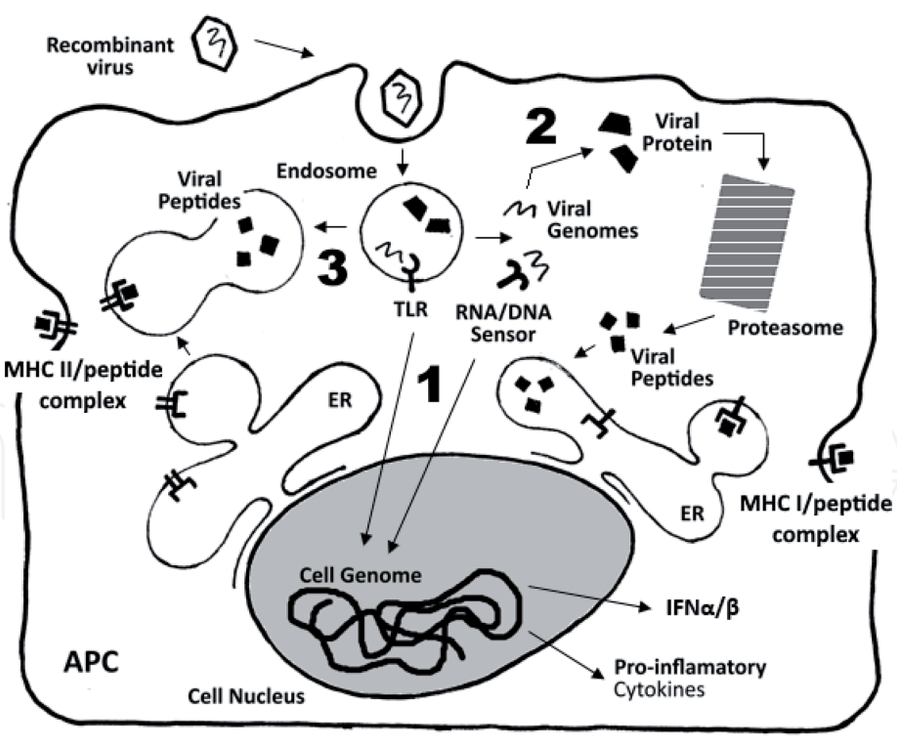

Figure 1.
*Mechanisms of immune activation by recombinant virus as a vaccine. The recombinant viruses inside the endosome release their genome into the cytoplasm of an antigen-presenting cell (APC). (1) If the viral genome gets exposed inside endosome rather than being released into the cytoplasm, it is sensed by toll-like receptors (TLR). Once inside the cytoplasm, the viral genome is amplified and detected by cytoplasmic sensors of viral nucleic acids ("RNA/DNA sensor"). Both pathway signals, through common pathways, will result in the transcriptional activation of pro-inflammatory cytokines but also in type I interferon (IFN-α/β) production. (2) Simultaneously, the viral genomic will be expressed, leading to synthesis of viral proteins. Cytosolic proteins are proteolytically digested and delivered to nascent major histocompatibility complex (MHC) class I chains in the endoplasmic reticulum (ER). (3) The recombinant viruses inside the endosome are degraded to yield peptide fragments that can associate with MHC class II molecules. *This image has not been previously published.*

into the endoplasmic reticulum (ER), where the interaction between the peptide and the MHC class I molecule occurs (see **Figure 1**). Subsequently, the peptide/MHC I complex is transported to the cell surface, and the epitope can be presented and recognized by $CD8^+$ T cells [34]. $CD8^+$ T cells recognize the antigenic peptides of endocytosed microorganisms, producing cytokines such as IFN-γ, which activate infected phagocytes to extinguish microorganisms (cytotoxic mechanism) and stimulate inflammation (see **Figure 2**).

In addition to the $CD8^+$ T cell epitopes, other important epitopes are those responsible for the induction of immune response by $CD4^+$ T cells. Viral proteins ("self-adjuvanted") or heterologous antigens fused to the viral capsid structural proteins may activate immune responses based on $CD4^+$ T cells. Viral protein or heterologous proteins fused to the virus are processed inside endosomal/lysosomal vesicles, and the resulting peptides bind to MHC class II molecules (see **Figure 1**). The peptide/MHC II complex is presented on the surface of APC to $CD4^+$ T cells. Vaccine viral vectors composed of these epitopes may induce memory $CD4^+$ T cells potentially capable of being activated by the body's natural exposure to the pathogen [22]. The differentiation of $CD4^+$ T cells in the Th1 subtype occurs in response to microorganisms, including viruses, which infect or activate APC. Activated Th1 cells secrete IFN-γ, among other cytokines. IFN-γ acts in the APC to stimulate the destruction of microorganisms (see **Figure 2**). If the heterologous proteins expressed by the recombinant viral vectors present associated signal-peptide (SP), they have the potential capacity to be surface and/or secreted proteins. When the destination of these proteins is the mitochondria or the secretory pathway, their displacement usually requires the presence of N-terminal sequences capable of being recognized by the cellular transport machinery. SP are responsible for targeting the proteins to the ER and, later, to the cell secretory pathway. Thus, these proteins may be anchored to the cytoplasmic membrane or secreted [37] and recognized by B cells, activating the production of specific antibodies (see **Figure 2**).

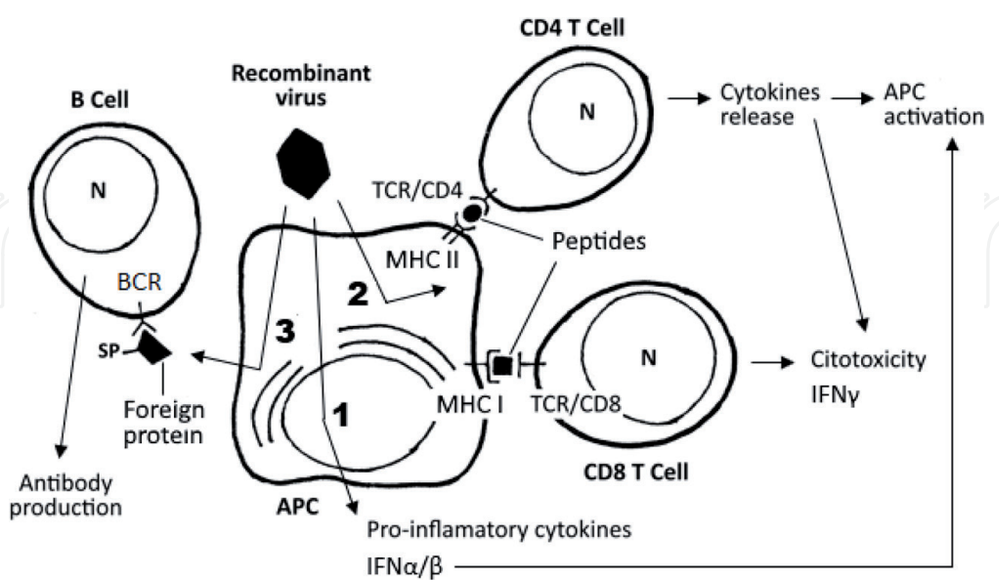

Figure 2.
*Effector functions of innate and adaptive immune cells responses induced by recombinant virus infection. (1) The viral genome stimulates endosomal TLR or RNA/DNA cytosolic sensors, triggering signaling cascades that lead to the production of pro-inflammatory cytokines, IFN-I, and APC activation. (2) Heterologous proteins are available for antigen-processing pathways, and the resulting peptides are bound to the MHC class I or II molecules, favoring the presentation of the antigens to $CD8^+$ or $CD4^+$ T cells, respectively. (3) If the heterologous proteins present associated signal-peptide (SP), they can be led to the cellular secretory pathway and activate B cells. APC, antigen-presenting cell; BCR, B cell receptor; MHC, major histocompatibility complex; N, cell nucleus; TCR, T cell receptor; TLR, toll-like receptors. *This image has been previously published.*

4. Leishmaniases experimental vaccines based on vaccinia virus-derived vectors

Although almost every viral genome can be manipulated in order to acquire heterologous protein expression capacity in host cells, not all viruses are as effective in doing so. Some types have been shown to be more efficient than others in the induction of cellular immune response, with vaccinia virus being one of the most attractive and efficient vector [22] and widely used in leishmaniases vaccine trials.

The vaccine virus (VACV or VV) is a member of the family *Poxviridae*, genus Orthopoxvirus, able to replicate in cells of several species of vertebrates, both *in vitro* and *in vivo*. The virus is the etiologic agent of smallpox. However, VACV does not have a natural reservoir nowadays and is considered, almost exclusively, a laboratory virus [22]. The vaccinia virus has an approximate size of 200 nm in diameter and 300 nm in length, and its genome consists of a segmented linear double-stranded DNA (dsDNA) of 130–300 kb. Highly attenuated strains, such as modified vaccinia virus Ankara (MVA) or NYVAC, are able to accommodate large segments of exogenous DNA (>20–25 kb) in their genome, constituting excellent expression vectors. Among the main characteristics that make them excellent vaccine vectors are (I) thermostability, low cost, and easy manufacture/administration; (II) gene expression in the cytoplasm of cells; (III) ability to induce humoral and cellular immune responses to heterologous antigens and may exhibit long-term immunity after a single inoculation; (IV) and its genome flexibility, which allows loss or deletion of much of the DNA for transgene insertion without, however, losing infectivity. In addition, in the global population, the prevalence of vector immunity is low due to the discontinuation of smallpox vaccination in the 1970s after its eradication [38].

4.1 Construction of a recombinant vaccinia virus by homologous recombination

The construction of recombinant viral vectors requires adaptation of the gene of interest for expression in host cells. In many cases, this requires intracellular recombination steps for the incorporation of the gene of interest into the viral genome. The construction of a recombinant vaccinia virus is based on a helper virus-dependent system [22]. Expression of the gene of interest may occur if the gene, under the control of a vaccinia virus promoter, is cloned into a plasmid (shuttle vector). The plasmid is transfected into a permissive cell highly infected with wild-type vaccinia virus. The gene of interest is incorporated into the wild-type vaccinia virus through homologous recombination between the viral genome and the shuttle vector (see **Figure 3**) [39].

4.2 Vaccinia virus in leishmaniases vaccines development

The development of vaccines against smallpox, which culminated in its eradication in the 1970s, resulted in a number of strains of vaccinia virus [40]. The first generation of vaccines against cancer, HIV/AIDS, and other infectious diseases was based on replication-competent strains of VACV, such as WR (Western Reserve strain), Wyeth, and Copenhagen. However, for safety reasons, most of the vectors currently used in vaccine trials are VACV non-replicative strains, such as MVA and NYVAC. Although highly attenuated vectors are capable of inducing protective immunity against various pathogens, their limitation in replicative capacity reduces their potential as compared to replicative vectors. In order to achieve a balance between safety and replication, several VACV strains with intermediate phenotype have been developed [41].

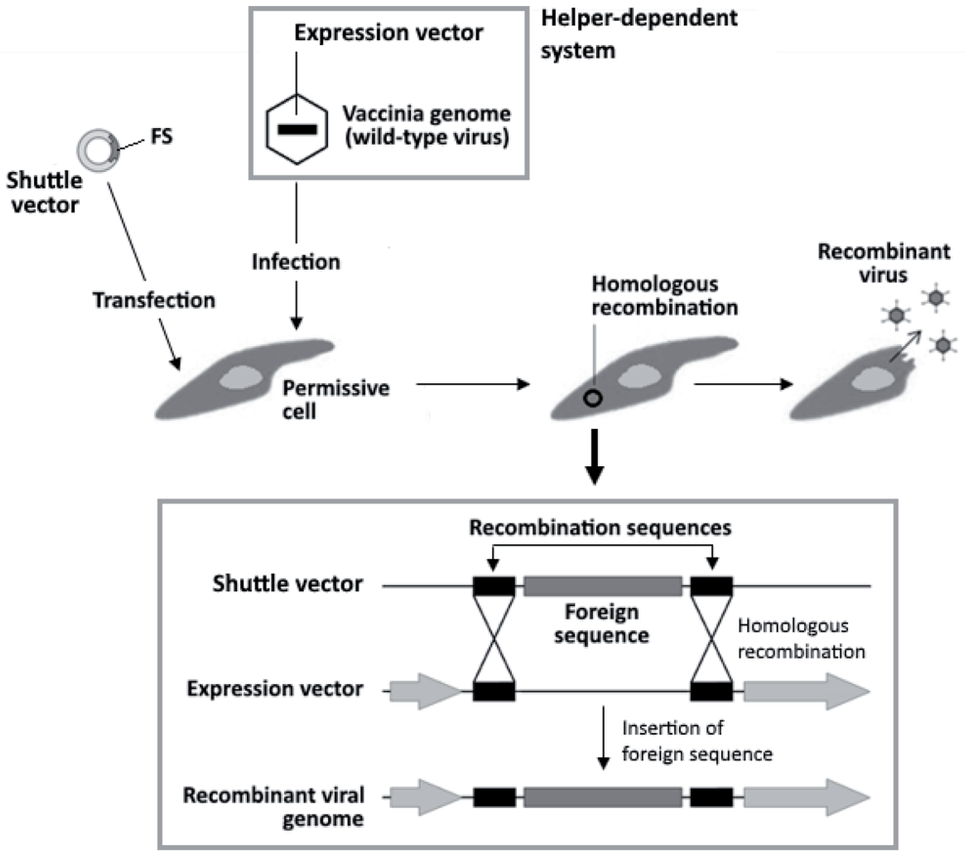

Figure 3.
*The construction of recombinant vaccinia virus occurs by intracellular homologous recombination between the shuttle vector, which contains the foreign sequence (FS), and the viral genome. Generation of recombinant vaccinia virus requires a helper virus-dependent system. *This image has not been previously published.*

The first report of the use of recombinant vaccinia virus in the induction of protection against *Leishmania* infection was made by McMahon-Pratt *et al*. (1993). The *L. amazonensis* GP46/M2 membrane glycoprotein was cloned into a live, highly attenuated strain of vaccinia virus (MuLEISH vaccine). Immunization by MuLEISH was able to induce protection in 45–75% of BALB/c mice challenged by *L. amazonensis*, in addition to generating memory T cells. This study demonstrated that recombinant vaccinia virus has great potential in the development of a safe and effective leishmaniases vaccine [41].

Since then, several *Leishmania* spp. antigenic subunits were cloned into recombinant VACV and used in leishmaniases preclinical and clinical vaccine trials. Over the past 10 years, studies using recombinant VACV in prophylactic immunizations have emphasized three antigenic subunits of *Leishmania* spp.: TRYP, LACK, and KMP-11 (see **Table 1**). Tryparedoxin peroxidase (TRYP, also known as TSA) was isolated from *L. major*, is highly conserved among *Leishmania* species, presents high expression in promastigote and amastigote forms, and plays a protective role against oxidative stress to the parasite [42]. LACK (also known as p36), the *Leishmania* homolog for receptors of activated C kinase, is an intracellular protein expressed in promastigote and amastigote forms, highly conserved among *Leishmania* species and highly immunogenic [43]. Kinetoplastid membrane protein-11 (KMP-11) is a protein present in all kinetoplastid protozoa and considered a potential candidate for leishmaniases vaccine [44].

The recombinant MVA vaccine vector expressing TRYP was used in a phase I clinical trial in dogs, the main VL domestic reservoirs caused by *L. infantum*, and has been shown to be safe and immunogenic. Uninfected, unexposed outbred endemic dogs immunized with TRYP-DNA plasmid prime and MVA-TRYP boost produced a

Antigen	Antigen delivery	Adjuvant	Animal model	Challenge	Outcome of vaccination	Reference
LACK	rVV, MVA and DNA plasmid	---	Dogs	*L. infantum*	**Prime DNA + Boost rVV:** ↓ Ab; ↑ T cell activation; ↑ Th1 cytokines; ↓ clinical symptoms; protection. **Prime DNA + Boost MVA:** ↓ Ab; ↑↑ T cell activation; ↑ Th1 cytokines; ↓↓ clinical symptoms; protection.	[47]
TRYP	MVA and DNA plasmid	---	Dogs Phase I trial	---	**Prime DNA + Boost MVA:** ↑ IFN-γ+ Th1 cells; TRYP-specific memory cells; ↑ IgG2; ↓ IgG1; safety	[45]
TRYP	MVA and DNA plasmid	Pam3CSK4 during DNA priming	BALB/c mice	*L. panamensis*	**Prime DNA + Boost MVA:** ↑ IFN-γ+CD4+ and CD8+ T cells; ↑ CD4+ and CD8+ memory cells; ↓ IL-10; ↓ IL-13; ↓ parasitism; protection	[46]
LACK	MVA and DNA plasmid	---	BALB/c mice	*L. major*	**Prime DNA + Boost MVA:** ↑ LACK-specific CD4+ and CD8+ T cells; ↑ LACK-specific CD4+ and CD8+ T effector memory cells; protection	[49]
LACK	M65, M101 and DNA plasmid	---	BALB/c mice	*L. major*	**Prime DNA + Boost M65:** ↑ LACK-specific CD4+ T memory cells; protecion. **Prime DNA + Boost M101:** ↑ LACK-specific CD8+ T memory cells; protection	[50]
				L. amazonesis	**Prime DNA + Boost M65:** ↑ LACK-specific CD4+ T memory cells; no protecion. **Prime DNA + Boost M101:** ↑ LACK-specific CD8+ T memory cells; no protection	
KMP-11	rVV and DNA plasmid	---	BALB/c mice	*L. donovani* SB-S or SB-R	**Prime DNA + Boost rVV:** ↑ IgG2a; ↑ TNF-α; ↑ IFN-γ; ↑ CD8+ T cells; ↑ cytolytic activity; ↓ (90%) splenic and hepatic parasite load; protection	[53]
LACK	NYVAC-C7L and DNA plasmid	---	BALB/c mice	*L. major*	**Prime DNA + Boost NYVAC-C7L:** ↑ CD4+ and CD8+ primary adaptive and memory T cells; ↑ IFN-γ; ↑ TNF-α; ↑ IL-2; ↓ lesion size; protection	[52]
LACK	MVA, M65 and DNA plasmid	---	Golden hamsters	*L. infantum*	**Prime DNA + Boost MVA or M65:** ↑ LACK-specific IgG; ↓ tissue damage and inflammation; ↓ splenic and hepatic parasite load; protection	[51]

↑: high level; ↑↑: remarkably high level; ↓: low level; ↓↓: remarkably low level; Ab: antibodies; KMP-11: kinetoplastid membrane protein-11; LACK: *Leishmania* activated C-kinase; M65 and M101: vaccinia virus mutants; MVA: modified vaccinia virus Ankara; NYVAC-C7L: replication-competent NYVAC that expresses C7L (gene C7L that allows the virus to replicate in human cells); Pam3CSK4: TLR1/2 agonist; rVV: recombinant vaccinia virus derived from the wild-type Western Reserve (WR) strain; SB-R: *Leishmania* pentavalent antimony resistant; SB-S: *Leishmania* pentavalent antimony sensitive; TRYP: tryparedoxin peroxidase (or TSA).

Table 1.
Recombinant vaccinia viruses used as experimental leishmaniases vaccines within the last 10 years.

type 1-dominated pro-inflammatory cellular immune response which is necessary for protection against *Leishmania* challenge and an immune memory that persists for at least 4 months postvaccination in the absence of restimulation or infection [45]. Mice also immunized by DNA/MVA prime/boost vaccines expressing TRYP were protected against challenge by *L. panamensis*. This protection was achieved specifically through the expansion of antigen-specific effector $CD8^+$ T cells. However, protection was dependent on modulating the innate immune response using the TLR1/2 agonist Pam3CSK4 during DNA priming. Heterologous prime-boost vaccination using only DNA fails to protect [46].

Ramos *et al*. [47] constructed two poxviral vectors: (I) a vaccinia virus derived from the wild-type WR strain (rVV), replicative and (II) an MVA, both expressing LACK. These vectors were used in a clinical vaccine trial to evaluate efficacy and immune response against CVL. This study showed that dog vaccination priming with DNA-LACK followed by a booster with MVA-LACK or rVV-LACK triggered a Th1 type of immune response, leading to protection against challenge by *L. infantum*. In addition, MVA-LACK in the booster demonstrated an advantage when compared to replication-competent rVV-LACK as a vaccine vector against CVL [47]. DNA-LACK/MVA-LACK prime/boost vaccines were also able to protect mice later challenged by *L. major* [48]. In both cases, protection was mediated by a Th1-like immune response against LACK antigen. However, a deep study of the immune populations involved in protection was still needed. Sánchez-Sampedro *et al*. [49] performed an in-depth analysis of the T cell populations induced in BALB/c mice during the DNA-LACK/MVA-LACK vaccination protocol, as well as after challenge with *L. major* parasites. In the adaptive response, there is a

polyfunctional $CD4^+$ and $CD8^+$ T cell activation against LACK antigen. At the memory phase, the heterologous vaccination induces high-quality LACK-specific long-term $CD4^+$ and $CD8^+$ effector memory cells. After parasite challenge, there is a moderate boosting of LACK-specific $CD4^+$ and $CD8^+$ T cells. The immune parameters induced against LACK and triggered by the combined vaccination DNA/MVA protocol could be relevant in protection against leishmaniases [49].

In 2013, Sánchez-Sampedro *et al*. constructed two vaccinia virus mutants, M65 and M101. These replication-competent mutants were generated after 65 and 101 serial passages of persistently infected Friend erythroleukemia (FEL) cells. Mice immunized in a DNA prime/M65 or M101 boost regimen with viral vectors expressing the LACK showed protection or a delay in the onset of CL. In immunized mice, DNA-LACK/M65-LACK protocol preferentially induced $CD4^+$ T cell, whereas DNA-LACK/M101-LACK preferentially induced $CD8^+$ T cell responses. Although both mutants were able to induce protection in mice challenged by *L. major*, they did not induce protection against *L. amazonensis* infection. Protection was similar to that triggered by MVA-LACK [50]. Nevertheless, the protocol of DNA-LACK prime/MVA-LACK or M65-LACK virus boost vaccination significantly reduced the parasite load in the liver and bone marrow of hamsters challenged by *L. infantum*, with no differences recorded between the use of MVA or M65 virus vector options [51].

In addition to MVA, NYVAC is one of the most studied attenuated strains of vaccinia virus. NYVAC was derived from a plaque-cloned isolate of Copenhagen smallpox vaccine strain by selective deletion of 18 open reading frames (ORF) involved in virulence, pathogenicity, and host range regulation. Sánchez-Sampedro *et al*. [52] constructed a NYVAC capable of expressing LACK with insertion of the viral host range gene C7L that allows the virus to replicate in human cells. DNA-LACK-prime/NYVAC-LACK-C7L boost protocols were able to induce preferentially LACK-specific $CD8^+$ T cell responses, with a reduced $CD4^+$ T cell response and reduction in lesion size in mice immunized and challenged by *L. major*. The type and potency of the immune response induced by NYVAC-LACK were improved by C7L insertion [52].

Finally, a heterologous prime-boost immunization strategy using KMP-11-DNA priming followed by boosting recombinant vaccinia virus (rVV) expressing the same antigen was able to induce protective immunity in both hamsters and in mice against VL caused by both antimony resistant (Sb-R) and sensitive (Sb-S) *L. donovani*. Parasite load is kept significantly low in the vaccinated groups even after 60 days postinfection in hamsters, which are extremely susceptible to VL. Protection in mice is correlated with strong cellular and humoral immune responses. Generation of polyfunctional $CD8^+$ T cell was observed in vaccinated groups, which is one of the most important prerequisites for successful vaccination against VL [53].

5. Conclusion

The declaration of smallpox eradication by the World Health Organization, in 1980, and the discovery that genes encoding heterologous antigens could be inserted into the genome of attenuated vaccinia virus, in 1982, resulted in a burst of scientific publications highlighting the potential clinical benefits of the recombinant poxvirus vectors as vaccines against various pathogens. Among the most attractive and efficient viral vectors in inducing a cellular immune response, vaccinia virus has been the most used in leishmaniases vaccine trials, especially in combination with DNA vaccines (heterologous prime/boost protocols). However, studies showed that greatly enhanced immune responses could be obtained when two different viral vectors expressing the common antigen were

used following the prime-boost immunization protocol, which may be experienced in future leishmaniases vaccine efficacy studies. Although highly attenuated vectors, especially MVA and NYVAC, are safe and capable of inducing protective immunity against infection by several *Leishmania* species, their limitation in replicative capacity reduces their potential when compared to replicative vectors. For a safety and replication balance, VACV strains with intermediate phenotypes are desirable. Accordingly, in the last 5 years, two replicating competent mutants were developed, M65 and M101, derived from WR strain, capable of inducing a protective immune response against murine infection by *L. major* (mice, M65 and M101) and *L. infantum* (hamsters, M65), as well as recombinant strain NYVAC-C7L, a highly attenuated vector but competent to replicate in human cells that was also able to potentiate the protective immune response against murine infection by *L. major*. Furthermore, TLR1/2 modulation may be useful in vaccines where $CD8^+$ T cell responses are critical. In conclusion, the potential of poxviral vectors as promising tools for vaccine development against leishmaniases can be explored by the development of new-generation vectors with refined specificity and improved efficacy through the use of co-stimulatory molecules, deletion of viral immunomodulatory genes still present in the poxvirus genome, enhancing both virus promoter strength and vector replication capacity, optimizing expression of foreign heterologous sequences, and the combined use of adjuvants. An optimized poxvirus vector triggering long-lasting immunity with a high protective efficacy against leishmaniases should be sought and can be feasible.

Conflicts of interest

The authors declare that there are no conflict of interests regarding the publication of this paper.

Authors' contributions

All authors have contributed equally to this work.

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