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Review Article

DNA Vaccines against Protozoan Parasites: Advances and Challenges

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Over the past 15 years, DNA vaccines have gone from a scientific curiosity to one of the most dynamic research field and may offer new alternatives for the control of parasitic diseases such as leishmaniasis and Chagas disease. We review here some of the advances and challenges for the development of DNA vaccines against these diseases. Many studies have validated the concept of using DNA vaccines for both protection and therapy against these protozoan parasites in a variety of mouse models. The challenge now is to translate what has been achieved in these models into veterinary or human vaccines of comparable efficacy. Also, genome-mining and new antigen discovery strategies may provide new tools for a more rational search of novel vaccine candidates.

1. INTRODUCTION

In spite of the success of vaccines in public health, there are still numerous pathogens, and in particular protozoan parasites such as *Plasmodium falciparum*, *Trypanosoma* sp., or *Leishmania* sp. against which there are still no effective vaccine. However, the discovery that the direct injection of plasmid DNA encoding foreign proteins could lead to endogenous protein biosynthesis and a specific immune response against it opened new perspectives in vaccine development. Over 15 years later, DNA vaccines have gone from a scientific curiosity to one of the most dynamic fields of research and may offer new alternatives for the control of infectious diseases [1]. Indeed, the first two DNA vaccines have been licenced, in recent years, to protect horses from west nile virus and salmons from infectious hematopoietic necrosis virus, confirming the usefulness of this biotechnology. We review here some of the advances and challenges for the development of DNA vaccines against two well-studied protozoan parasites, *Leishmania* sp. and *Trypanosoma cruzi*. Both belong to the trypanosomatidae family and are ranked among the three major protozoan parasites affecting humans. Leishmaniasis is a complex disease caused by at least 18 species of parasites from the *Leishmania* genus and transmitted to humans by hematophagous sandflies. With an estimated 12 million cases, it has a major public health impact in several regions, and in particular in India, Sudan, and Brazil [2]. Clinical manifestations range from self-healing cutaneous lesion to fatal visceral form, and this variety can be attributed in part to the respective parasite species, and each presents specific relationships with the host and diverse mechanisms of pathogenesis [3, 4], which represents an additional difficulty for the development of treatments and vaccines. On the other hand, *T. cruzi* is the agent of Chagas disease, which is present from southern Argentina to the southern USA. An estimated 16–18 million persons are infected in the Americas and close to 100 million people are at risk of infection. After a short benign acute phase (a few weeks) and a very long (several years) asymtomatic phase, about 30–40% of infected patients develop chronic chagasic cardiomyopathy and eventualy die of heart failure. Current chemotherapy relies on nitrofurans (Nifurtimox), or nitroimidazoles (Benznidazole). However, the usefulness of these drugs is limited by their reduced efficacy (mostly during the early stages of the infection), serious side effects, and the emergence of drug-resistant strains of parasites, and new treatments are slow to develop [5].

2. WHY DNA VACCINES?

DNA vaccines induce a complete immune response against the encoded antigen. The exact mechanisms involved in this proccess are still poorly understood, and particularly the type of $CD4^+$ and $CD8^+$ effector and memory cells activated, and

some of these aspects have been reviewed in detail elsewhere [1]. Apart from their immunogenicity and efficacy that will be discussed below, there are several features of DNA vaccines that make them very advantageous against tropical diseases. First, they are extremely safe as they do not contain any pathogenic organism that may revert in virulence. The major concern of genomic integration of the plasmid DNA has also extensively been studied in safety studies and found to be rather unlikely [6]. Additional safety issues such as anti-DNA antibodies or autoimmunity have also been addressed in a growing number of preclinical and clinical studies [7], which confirmed the high safety of these vaccines. With respect to manufacturing, storage, and distribution, they also present major benefits in that the production process is the same for any DNA vaccine, which is not the case for other types of biologicals and vaccines, for which a specific protocol has to be developed for each. This makes production easy and costs will likely go down as this type of vaccines become mainstream and future technological improvements are implemented. Also, plasmid DNA is a very stable molecule, specially compared to recombinant or live attenuated vaccines, which would greatly facilitate storage and distribution of DNA vaccine in tropical settings with limited health infrastructure as the huge costs associated with the cold chain may be offset. Administration is also easy as simple IM or ID injections can be sufficient, and multiple plasmids can be combined for the elaboration of multivalent vaccines [1]. Overall, DNA vaccines may thus represent an ideally affordable alternative for disease control, which explains in part the growing interest in their development for the control of tropical parasitic diseases such as malaria, leishmaniasis, or Chagas disease.

3. DNA VACCINES AGAINST *LEISHMANIA*

3.1. Correlates for protection

As mentioned above, leishmaniasis is caused by at least 18 species of parasites with diverse relationships with the host and mechanisms of pathogenesis [3, 4]. Early studies of cross-protection between *Leishmania* species clearly showed that it is a complex problem, with infection by one species protecting or not from subsequent infection by another species, depending on the species and the order of infections. Most vaccine studies have thus been focusing on homologous protection, although a single vaccine able to protect against all pathogenic species would be ideal.

The correlates for protection have been extensively studied in the case of *L. major*, and contributed considerably to the development of the Th1/Th2 paradigm [8]. Thus, there is a general agreement that a Th1-type immune response, characterized by a high IFNγ and low IL-4 and IL-10 production, leads to control of *L. major* infection, while a Th2-type immune response does not [8]. Antibodies may have an exacerbatory role [9], but may also contribute to T cell responses [10, 11]. Both IFNγ producing $CD4^+$and $CD8^+$T cells seem to contribute to protective immunity, and induction of NO production by macrophages is central to parasite elimiation [12, 13]. While it was assumed for a long time that this Th1/Th2 paradigm was applied to all *Leishmania* species, it has become clear in recent years that each species has a distinct relationship with the host, different mechanisms of pathogenesis, and possibly different correlates for protection [3, 4]. Nonetheless, IFNγ production seems to be a general requirement, although not necessarily sufficient, for protection against most if not all *Leishmania* species.

3.2. Single antigen DNA vaccines

The earliest DNA vaccine experiments against *Leishmania* used *L. major* GP63 antigen, which has been extensively used as a recombinant or peptide vaccine. Immunization with a plasmid encoding GP63 was able to induce a Th1-type cytokine profile and a significant reduction of lesion size after challenge of the immunized mice with *L. major* [14–17]. Subsequent studies investigated DNA vaccines encoding a large variety of *Leishmania* proteins (Table 1) and showed that many different DNA vaccines were able to induce a Th1 immune response, and confer variable degrees of protection as assessed by reduction in skin lesion size and/or parasite burdens in mouse models. However, given the large variety in experimental models and designs, it is difficult to compare the effectiveness of the different vaccines to induce a protective immune response. Nonetheless, it is clear from studies comparing different DNA vaccines that the nature of the antigen encoded by the vaccine is a key parameter for efficacy.

Also, a few studies provided interesting comparisons of the same antigens administered as recombinant protein or DNA vaccines and showed that the latter were overall more effective than their recombinant protein counterparts. Indeed, DNA vaccines were able to induce a stronger Th1 bias in the immune response, a longer-lasting immunity, and/or a better protection against disease progression [19, 31, 32, 35, 40, 44]. While most of these studies have used a rather artificial infectious challenge based on the injection via nonnatural routes of high parasite doses, an experimental system criticized by some authors, the superior efficacy of DNA vaccines was also observed using a low-dose intradermal challenge in the ear, which was proposed to more closely mimick natural infection [45]. In these studies, both DNA and protein vaccination were able to induce very similar level of short-term (2 weeks postvaccination) protection against infection with *L. major*, but only DNA vaccine was able to induce long-term (12 weeks postvaccination) protection [45].

These results thus confirmed the strong potential of DNA vaccines against *Leishmania*, but also indicated that in most cases only partial protection was achieved. Prime-boost immunization protocols have been tested with various antigens to increase vaccine potency (Table 1). They are based on priming the immune response with a DNA vaccine and boosting with the corresponding recombinant vaccine based on recombinant virus or protein (Table 1). In some studies, such immunization protocol resulted in increased immunogenicity of the vaccines and better protection levels [26, 27], but in others, DNA only remained the best formulation for optimum efficacy [32]. Nonetheless, a major drawback of

Table 1: DNA vaccines tested against murine leishmaniasis.

Antigen	Dose	Challenge	Immune response	Protection	Reference
GP63	$2 \times 100\,\mu$g IM	*L. major*	Th1	+++	[14–17]
LACK	$2 \times 100\,\mu$g IM	*L. major*	Th1, IFNγ	+++	[18–21]
LACK	$2 \times 30\,\mu$g IN	*L. amazonensis*		+++	[22]
LACK	$2 \times 100\,\mu$g IM	*L. mexicana*		–	[23]
LACK	$2 \times 100\,\mu$g IM or SC	*L. chagasi*	Th1, IFNγ	–	[24]
LACK	$2 \times 100\,\mu$g ID or SC	*L. chagasi*	Th1	–	[25]
LACK	Prime/boost	*L. major*	Th1	+++	[26]
LACK	Prime/boost	*L. major*	Th1	+++	[27]
LACK	Prime/boost	*L. major*	Th1, IFNγ	+++	[28]
LACK	Prime/boost	*L. infantum*	IFNγ	+++	[29]
TRYP	Prime/boost	*L. major*	IFNγ	+++	[30]
LiP0	$2 \times 100\,\mu$g im	*L. major*	Th1	++	[31]
SP1	$2 \times 100\,\mu$g SC or prime/boost	*L. major*	IFNγ	++	[32]
Histones mix: H2A, H2B, H3, H4	$3 \times 200\,\mu$g IM	*L. major*	Th1, IFNγ	+++	[22]
LmSTI1,TSA	$3 \times 100\,\mu$g IM	*L. major*	Th1	+++	[33]
LACK, LmSTI1, TSA	$1 \times 300\,\mu$g SC	*L. major*	Th1	+++	[13]
CPb/CPa	$2 \times 100\,\mu$g IM	*L. major*	Th1	+++	[34]
PSA-2	2×20–$50\,\mu$g IM	*L. major*	Th1	+++	[35]
LACK, PSA2, Gp63, LeIF, p20 Ribosomal like protein	$1 \times 50\,\mu$g IM	*L. major*		– to +++	[36]
Meta 1	$3 \times 100\,\mu$g IM	*L. major*	Th2	–	[37]
P4	$3 \times 100\,\mu$g various sites	*L. amazonensis*	Th1	+++	[38]
CPb, GP63, GP46	$2 \times 100\,\mu$g IM	*L. mexicana*		+ to +++	[23, 39]
NH36	$2 \times 100\,\mu$g IM	*L. domovani*		+++	[40]
		L. mexicana		++	
NH36, GP63	$2 \times 20\,\mu$g IM	*L. mexicana*	IFNγ	+++	[41]
CPa/CPb	Prime/Boost	*L. infantum*	Th1, IFNγ	+++	[42]
ORFF	$3 \times 100\,\mu$g IM	*L. donovani*		+++	[43]

IM: intramuscular; IN: intranasal; SC: subcutaneous; –: no protection; +: little protection; ++: fair protection; +++: very good protection.

such vaccine formulation remains its complexity, which may limit their practical use.

3.3. Multiple antigen DNA vaccines

An alternative way to broaden vaccine immunogenicity and increase its efficacy has been to use combination of plasmids encoding various antigens. For examples, cysteine proteinase (CP) a and b DNA vaccines are not protective when used individually, but immunization with a combination of both plasmids induces long-term protective immunity [34]. Alternatively, gene fusion has also been successively used to achieve expression of an antigenic fusion protein from a single plasmid construct [33]. Overall, expression of several antigens mostly resulted in increased efficacy, but this also depended on the antigen combination [13, 22, 23, 41, 45]. Most authors thus argue that a successful *Leishmania* vaccine is likely to be based on multiple antigens.

3.4. Antigen discovery

Immunization with large number of plasmids is also the basis for expression library immunization, a powerful but labor-intensive strategy for vaccine discovery [46], which has been used with *Leishmania*. Immunization of mice with *L. major* genomic expression library fractions was able to induce significant protection, but these authors did not pursue library fractionation further [47]. In another study, the identification of protective library subsets from an *L. donovani* amastigote cDNA library and their successive fractionation into smaller protective libraries lead to the identification of novel protective antigens [48]. Interestingly, most of the antigens identified would not have been predicted to be good

vaccine candidates. Indeed, they were not surface or secreted proteins, neither stage-specific, but were intracellular and some very conserved such as histones, or ribosomal proteins [48]. Vaccine discovery is also the next logical step following the recent completion of the *L. major* genome sequencing [49]. In one approach, the random screening of 100 genes upregulated in amastigotes tested as DNA vaccine allowed the identification of 14 novel protective and 7 exacerbating antigens [50, 51]. Again, function and cellular localization would have been poor predictors of the protective efficacy of these antigens, as most were not predicted to be localized on the surface, but shared similarity with ribosomal proteins, cytoskeleton, or metabolic enzymes [51]. It is thus becoming increasingly clear that there is little rationale to limit *Leishmania* vaccine discovery searches to surface or secreted antigens. Rather, new criteria need to be considered for the rational identification of vaccine candidates as strategies based on such random screening cannot be applied to large genomes such as that of *Leishmania*, with over 8000 annotated genes.

3.5. Therapeutic vaccines

An additional advantage of DNA vaccines is their potential as therapeutic vaccines, aimed at reinforcing or redirecting the immune response of an infected host to control disease progression [58]. The major advantage of this strategy in addition to its efficacy is that it relies on short treatment regimens, and it is thus an attractive alternative to chemotherapy, particularly in the case of *Leishmania* with so few chemotherapeutic options. Thus, administration of as little as two doses of a DNA vaccine encoding PSA-2 can control an ongoing infection with *L. major* in mice [59]. The therapeutic effect is due to a shift of the immune response towards a Th1 immune response [59]. Similarly, a DNA vaccine encoding *L. donovani* nucleoside hydrolase NH36 has therapeutic activity against murine visceral leishmaniasis caused by *L. chagasi* [60]. The simplicity of such treatment makes them very advantageous compared to chemotherapy. In addition, the fact that the same DNA vaccine can be effective for both the prophylaxis [40] and the therapy of *Leishmania* infection is thus very promising as this would provide a versatile tool for the control of this parasite.

3.6. Cross-protection against multiple Leishmania species

As mentioned above, an added challenge to *Leishmania* vaccine development is the large number of species, as well as the variability within species. Indeed, studies on the polymorphism of leading antigens such as GP63 quickly revealed that it was a very polymorphic [61, 62]. Such polymorphism has important implication for vaccine development as it may limit their efficacy against variant strains of parasites or novel escape mutants, and thus restrict vaccine protection to a single species [63, 64]. Antigen polymorphism between multiple strains and species is thus becoming a major issue in many vaccine development studies [65, 66]. In the case of *Leishmania*, few DNA vaccines have been tested against multiple species. LACK antigen, initially identified in *L. major*, and found to be very conserved between *Leishmania* species, can protect mice against *L. major* [20] and *L. amazonensis* [67], but not against *L. mexicana* [23], *L. donovani* [25], or *L. chagasi* [24]. On the other hand, *L. amazonensis* nuclease protein P4 can protect against both *L. amazonensis* and *L. major*, but cross-protection requires a different formulation (IL-12 or HSP70 as adjuvant, resp.) [38]. In other studies, antigens from one species were used to induce protection against another species [31], but the extent of cross-protection against various species was not investigated. More recently, a single formulation of *L. donovani* NH36 DNA vaccine was found to induce a very good protection against both *L. chagasi* and *L. mexicana*, suggesting that this DNA vaccine may be able to provide broad protection against various *Leishmania* species [40]. Importantly, no DNA vaccine has yet been tested against *L. braziliensis*, in spite of this species being responsible of most cases of cutaneous leishmaniasis in South America.

3.7. Non-Leishmania antigens as vaccines

While all the above DNA vaccines were based on *Leishmania* antigens, an alternative approach has used antigens derived from sand-fly saliva. Indeed, it has been shown that sand-fly saliva can exacerbate *Leishmania* infection [68, 69], and preexposure of mice to saliva components may be sufficient to induce protection against infection [70]. Thus, a number of salivary antigens have been tested as vaccines against *Leishmania*. Maxadilan is a potent vasodilator from sand-fly saliva and was found to be responsible of most of the exacerbatory effects of whole saliva on *Leishmania* infection [71]. Immunization with this antigen (as a recombinant vaccine) protected mice against *L. major* infection [71]. Other salivary components, such as *Phlebotomus papatasi* SP15, have been tested as DNA vaccines and found to protect mice against *L. major* and while the vaccine induced both humoral and DTH responses, protection seemed to be mostly accounted for by the latter, as B-cell deficient mice remain protected [72]. Thus, characterization of sand-fly salivary proteins may lead to the identification of new vaccine candidates [73, 74]. However, as for *Leishmania* antigens, salivary protein polymorphism remains an important issue and may limit the usefulness of such antigens as vaccine candidates [75, 76].

3.8. DNA vaccines against nonmurine leishmaniasis

Based on the success of many of these DNA vaccine studies in mice, a few vaccine candidates have been tested in additional animal models, possibly more relevants for the development of a veterinary or human vaccine (Table 2). PFR-2 and KMP11 antigens were tested as DNA vaccines in hamsters, a highly susceptible animal model. PFR-2 was tested as protein, DNA, or DNA-protein immunization, and protection levels against *L. mexicana* varied greatly depending on vaccine formulation, route of immunization, and sex of the animals [52]. Also, contrary to mouse studies, protein vaccination seemed more protective than DNA only vaccination.

Table 2: Preclinical studies *Leishmania* DNA vaccines in nonmurine models.

Antigen	Dose	Challenge	Host	Immune response	Protection	Reference
PFR-2	Variable	*L. mexicana* *L. panamensis*	Hamster		− to +++	[52]
KMP11	$2 \times 100\,\mu$g IM	*L. donovani*	Hamster	Th1-Th2	+++	[53]
PapLe22	$1 \times 100\,\mu$g IM	*L. infantum*	Hamster		+++	[54]
LACK	$2 \times 100\,\mu$g IM or Prime/boost	*L. infantum*	Dog	Th1/Th2 Th1	+ to +++	[55]
10 antigens mix	$2 \times 500\,\mu$g IM	*L. donovani*	Dog	Th1		[56]
CPa/b	Prime/boost	*L. infantum*	Dog	IFNγ	+++	[57]

−: no protection; +: little protection; ++: fair protection; +++: very good protection.

However, as in mouse studies, heterologous prime-boost vaccination with DNA and protein seemed better than DNA only [52]. Another DNA vaccine encoding PapLe22 was found to be immunogenic in hamsters and decreased parasitemia after infection with *L. infantum*, but further assessment of disease was not performed [54]. Immunization with KPM11 DNA induced a mixed Th1/Th2 response, but was able to protect hamsters against visceral leishmaniasis caused by *L. donovani* [53]. In dogs, while several protein vaccines have been tested and a purified protein vaccine has now been licenced for veterinary use [77], very few DNA vaccine studies have been performed. A heterologous prime-boost strategy using CPa and CPb DNA and protein was reported as immunogenic and protective [57], but the study was of limited power given the reduced number of animals. In another study, dogs were immunized with a mixture of DNA vaccines encoding 10 different antigens previously tested in mouse models, and this immunization induced a very good immune response, with a high production of IFNγ [56]. However, evaluation of protection was limited to an acute *in vitro* assay [56] and further studies will be required to assess the potential of this vaccine in dogs. In spite of their limitations, these studies clearly showed that several DNA vaccines can induce a potent immune response in nonmurine animal models, and it is likely that a good level of protection can be achieved in these as well, provided the correct antigens and vaccine formulation are used.

4. DNA VACCINES AGAINST *TRYPANOSOMA CRUZI*

4.1. Correlates for protection

Vaccine development against Chagas disease has been dramatically limited because of extensive debate on the mechanisms involved in this pathology [78, 79]. Indeed, some studies suggested that tissue damage was associated with the presence and replication of intracellular amastigotes, while others proposed that autoimmunity induced by parasite antigens mimicking host proteins was responsible for it. It was thus unclear if the immune response needed to be inhibited, to reduce autoimmunity, or stimulated, to eliminate the parasite. It is now accepted that the presence of parasites in cardiac tissue is necessary to initiate and maintain the inflammatory response, and that therapeutic treatments or vaccines aimed at eliminating *T. cruzi* would limit or prevent the progression towards chronic chagasic cardiomyopathy [80, 81]. There is a growing consensus that protection against *T. cruzi* relies on a Th1 immune response and the activation of cytotoxic $CD8^+$T cells [82–85].

4.2. Single antigen DNA vaccines

The first DNA vaccines to be tested against *T. cruzi* encoded an antigen from the well characterized trans-sialidase family of proteins. There are over 1400 members in this family, making it one of the largest protein families of the parasite, and they are very abundant surface proteins. Several studies have used different members of this family, such as TS or TSA-1 (Table 3) [84, 86–88]. Immunization with TS was found to induce significant antibody titers able to inhibit trans-sialidase enzyme activity, a strong DTH, and lymphoproliferative response [86]. This immune response was protective as determined by an increase in survival and a decrease in parasitemia. Immunization with TSA-1 DNA was found to induce a specific CTL response which also lead to a lower parasitemia and increased survival in both BALB/c and C57BL/6 mice [88].

As in *Leishmania* vaccine studies, a few authors addressed the question of comparing protein and DNA vaccines encoding the same antigen [90, 98]. In A/Sn mice, immunization with recombinant TS induced a higher antibody titer than TS DNA, but a comparable decrease in parasitemia. However, the DNA vaccine was unable to increase survival, which the author attributed to the strain of the mice used, since this DNA vaccine was protective in BALB/c mice [90]. On the other hand, immunization with recombinant CRP or CRP DNA induced a comparable Th1 immune response, but only the DNA vaccine was protective against infection [98].

A number of other studies showed that DNA vaccines encoding various antigens could induce significant protection against *T. cruzi* infection, as evidenced by decreased parasitemia and improved survival of vaccinated mice (Table 3). In addition, a few studies also presented evidence of a reduction in cardiac tissue damage and inflammation at the histopathologic level [87, 97]. Furthermore, T cell analysis confirmed that protection relied on $CD8^+$T cells [84, 91] and recent studies showed that these cells were very rapidly activated following infection of mice immunized with DNA vaccines [101]. DNA vaccines based on defined T cell epitopes from TS antigen have also been tested and it was found that

Table 3: DNA vaccines tested against *Trypanosoma cruzi*.

Antigen	Type of antigen	Dose	Mouse strain	Immune response	Protection	Reference
TS 154	TS family		BALB/c	Th1, CTL	+++	[84, 89, 90]
			A/Sn		−	
TSA-1	TS family	$2 \times 100\,\mu$g IM	C57BL/6 BALB/c	CTL	+++	[87, 91]
ASP-1	TS family	$2 \times 100\,\mu$g IM	C57BL/6	CTL	+++	[87]
ASP-2	TS family	$2 \times 100\,\mu$g IM	C57BL/6	CTL	+++	[87]
Tc13	TS family	$5 \times 50\,\mu$g IM	BALB/c		−	[92]
ASP-clone9	TS family	$4 \times 100\,\mu$g IM	BALB/c	IFNγ	+++	[93]
TSSA	TS family	2 to $4 \times 100\,\mu$g IM	BALB/c, C57BL/6 C3H/Hej	CTL	− to +++	[94, 95]
TS (7 members mix)	TS family	$2 \times 25\,\mu$g IM	C57BL/6		+++	[96]
ASP-clone9, TS	TS family	$4 \times 200\,\mu$g IM	BALB/c	IFNγ	+++	[97]
CRP		$2 \times 100\,\mu$g IM	BALB/c		+++	[98]
cruzipain			BALB/c	CTL		[99]
DHOD		2 to $4 \times 100\,\mu$g IM	BALB/c, C57BL/6 C3H/Hej		−	[94]
LYT1		$2 \times 25\,\mu$g	C57BL/6	IFNγ, CTL	+++	[96]
FCaBP/Tc24		$2 \times 25\,\mu$g	C57BL/6	IFNγ, CTL	−	[96]
Tcβ3		$2 \times 25\,\mu$g	C57BL/6	IFNγ, CTL	++	[96]
Mucin (6 members)		$2 \times 25\,\mu$g	C57BL/6		−	[96]
KMP11		4 doses IM	BALB/c	CTL	− to ++	[100]

IM: intramuscular; CTL: cytolytic activity; −: no protection; +: little protection; ++: fair protection; +++: very good protection.

both $CD4^+$and $CD8^+$T cell epitopes were necessary and sufficient to induce a protective immune response [102].

Taken together, these data clearly demonstrated that vaccination did not result in increased pathology, as initially feared, but allowed at least partial control of disease progression, thus confirming the central role of parasite persistence for Chagas disease pathogenesis and opening the way to further assessment of DNA vaccines against *T. cruzi*. However, it has to be noted that many of the antigens tested belonged to the trans-sialidase family of protein, so that there is still little diversity in terms of the antigens tested as vaccines against *T. cruzi* (Table 3).

4.3. Strategies for potentiating T. cruzi DNA vaccines

Because protection induced by single antigen DNA vaccine remained partial, a number of studies have evaluated strategies to increase vaccine efficacy. These include the use of cytokine/chemokine encoding plasmids to potentiate the immune response induced by the vaccine, and two of the most studied molecules have been IL-12 and GM-CSF, which both were generally able to potentiate protection (Table 2). Alternatively, mixtures of plasmids encoding distinct antigens were used for immunization, and as mentioned above for *Leishmania* vaccines. For example, immunization of mice with plasmids encoding TS and ASP-2 proteins resulted in a specific immune response against both antigens and an increased protection against infection [97]. On the other hand, an immunization with a mixture of DNA vaccines encoding up to 6 proteins from the mucin family resulted poorly protective, while a mixture of up to 7 proteins from the TS family was protective, but not as much as a single antigen vaccine encoding the TS-like antigen ASP-2 [96]. Similarly, a mixture of DNA vaccines encoding ASP-1, ASP-2, and TSA-1 had a similar protective activity as TSA-1 alone [87]. The lack of efficacy of these multivalent vaccines may be attributed to the presence of shared or immunodominant epitopes since they have significant sequence similarity that may not have resulted in a broader immune reponse.

Heterologous prime-boost approach has also been evaluated and immunization with some combinations of DNA and recombinant TS was found to enhance Th1 immune response, but protection was not significantly different from that obtained with DNA alone [103]. Taken together, these studies suggest that additional strategies need to be investigated to potentiate DNA vaccine efficacy against *T. cruzi*.

4.4. Therapeutic DNA vaccines

Therapeutic administration of DNA vaccines to control an ongoing infection with *T. cruzi* may represent an additional

alternative for Chagas disease control. The concept was demonstrated in mice acutely or chronically infected, and in both cases the administration of only two doses of DNA vaccine encoding TSA-1 or Tc24 antigens was sufficient to limit disease progression, as treated mice presented increased survival and reduced cardiac tissue damage, as assessed by histopathologic analysis [104]. A comparative study of different DNA vaccines identified Tc52 antigen as another therapeutic vaccine candidate, while DNA vaccines encoding antigens from the TS family previously found to be protective had no signifiacnt therapeutic effect [105]. It was found that therapeutic vaccination rapidly induced spleen cell proliferation, including IFNγ-producing $CD4^+$ and $CD8^+$T cells, while the effects on cardiac tissue inflammation and parasite burden take longer to be detectable [106]. Importantly, in all these studies, therapeutic vaccination of *T. cruzi* infected mice did not result in an increased inflammatory reaction in the heart, confirming that it is safe to stimulate the immune response of *T. cruzi* infected mice and that attacking the parasite can lead to a reduction of pathology. These studies thus open very attractive perspectives for the control of *T. cruzi* infection, and further studies on the efficacy of DNA vaccines encoding other antigens and on the immune mechanisms underlying their therapeutic effect should provide clues for the optimization of this strategy.

4.5. Antigen discovery

As for any vaccine, the nature of the antigen used remains a key factor for vaccine efficacy, and there is still little variety in terms of antigens evaluated as DNA vaccine candidates against *T. cruzi*. Thus, a number of studies have aimed at identifying novel antigens through various strategies. The most classical approach has been the screening of cDNA libraries using antibodies and screening an amastigote library allowed the identification of a novel antigen Tcβ3, and two previously characterized ones, LYT1 and FcaBP/Tc24 [96]. DNA vaccines encoding these antigens induced variable levels of protection, the best one being LYT [96]. Alternatively, expression-library immunization, described above for *Leishmania*, was also tested with *T. cruzi*, and found to be immunogenic, but there was no attempt at fractionating the library or identifying protective antigens [107]. A likely reason is that such strategy may be too labor-intensive for large genomes/libraries, and its usefulness may be limited to pathogens with small genomes. The availability of *T. cruzi* genome sequence also opens new possibilities for antigen discovery. In one of the first studies using such resource, a combination of bioinformatics analysis were used to identify GPI-anchored or secreted proteins, and most of the identified clones were immunogenic as DNA vaccines [108]. Further studies may confirm the usefulness of these new vaccines to protect against *T. cruzi* infection. Nonetheless, as discussed above for *Leishmania*, the rationale for limiting antigen searches to surface proteins may not be totally relevant, and additional strategies should also be used to include unbiaised genome-wide surveys for antigen discovery.

5. FUTURE DIRECTIONS

As detailed in this review, there have been considerable advances in DNA vaccines against *Leishmania* and *T. cruzi* in recent years. Taken together, these studies clearly validated the concept of using DNA vaccines for both protection and therapy against these protozoan parasites in a variety of mouse models. While sterile immunity seems to be an irrealistic goal for either *Leishmania* or *T. cruzi*, a reduction in disease severity and in the development of the pathology seems clearly within the reach of DNA vaccines. Nonetheless, the relevance of such mouse models for the development of veterinary or human vaccines against these parasites has been challenged by some authors. The few DNA vaccine studies in nonmurine models of leishmaniasis suggest that some extrapolation may be feasible, but certainly not completely. Additional advanced preclinical studies of DNA vaccine candidates in nonmurine animal models such as rats, hamsters, dogs, or monkeys are thus warranted in the next few years, to further explore the immunology and efficacy of DNA vaccines against these parasites. As already observed in such studies for other pathogens, this will lead to the challenge of achieving in these species an immunogenicity of comparable level and protective efficacy as that obtained in murine models. However, advances in adjuvants, DNA vaccine formulation, and delivery systems are likely to contribute to such results [1, 109].

Another major issue is that of antigen discovery, and while a number of DNA vacines tested so far against *Leishmania* or *T. cruzi* have shown promise, we are still unsure if these are the best possible antigens, particularly since these parasites have relatively large genomes, and only a limited variety of antigens have been tested. The availability of the genome sequences of these parasites will without doubt be a key resource for genome-wide screenings for new protective antigens. A key lesson from the initial studies reviewed here [48, 51, 108], together with other similar antigen discovery studies, seems to be that cellular localization and protein function are poor predictors of the antigenicity and protective efficacy of a protein. Alternative criteria should thus be used so that potent vaccine candidates are not missed, and the important development of genome-mining and bioinformatic tools is providing new tools for a more rational search of vaccine candidates [110].

To conclude, those DNA vaccines represent a promising approach for the control of *Leishmania* sp. and *T. cruzi*, and such vaccines would have a major impact in developing endemic countries. Thus the question does not seem to be if DNA vaccines can control these parasites, since many studies have clearly showed that this is the case, but how to translate what has been achieved in mouse models into veterinary or human vaccines of comparable efficacy.

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