Chapter 5

Vaccines for Human Leishmaniasis: Where Do We Stand and What Is Still Missing?

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Additional information is available at the end of the chapter

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Abstract

Responsible for up to 30,000 deaths annually, leishmaniasis is a complex spectrum of diseases endemic in 97 countries around the globe. Disease control relies heavily on the early diagnosis and treatment of the active cases (relevant for anthroponotic disease), although it is widely accepted that a prophylactic vaccine for human leishmaniasis is the way to achieve the successful elimination of human disease (taking in consideration the vast list of non-human reservoirs that enable the perpetuation of parasites all around the globe). The notion that infection leads to strong and long-lasting immunity against leishmaniasis supports vaccination as an achievable goal. However, and in spite of the different candidates tested along the years, till date, we still do not have an approved vaccine for humans. In this chapter, we will explore the last advances made in the field of vaccines against *Leishmania* without forgetting the historical perspective, essential to the understanding of the road already undergone. We will then discuss the correlates of disease and protection, still neither consensual nor definitive, as well as the issue of pre-clinical to clinical translation. The complete understanding of these issues will be essential for the approval of a successful vaccine for human leishmaniasis.

Keywords: leishmaniasis, human vaccines, correlates of protection, cellular immunity, cross-protection

1. Introduction

Vaccination is undoubtedly one of the greatest achievements of modern medicine, responsible, together with the use of antimicrobials and access to clean water and sanitation, for the global human demographics transformation in the past two centuries [1–3]. The apparently

insignificant proportion of the world population, whose lives are spared annually, thanks to vaccines (0.04 or 0.1%, if we include deaths avoided by smallpox eradication), is equivalent to up to 3 (or 8) million lives spared per year and a cumulative of more than half a billion deaths avoided just in the twentieth century [4, 5]. Nevertheless, and notwithstanding the significant and successful global efforts toward the goal of universal health protection/promotion, the picture could be much better. On the one hand, just by improving global vaccination coverage, an additional 1.5 million deaths could be avoided yearly [5]. On the other hand, there are still many deadly infectious diseases, whose prevention through vaccination is theoretically possible but for which there are no vaccines approved [6–8]. There are different compatible explanations/hypothesis that together justify it. The first one has to do with legal and ethical reasons: to test/approve/administer a pharmaceutical product nowadays is harder than it was 100 years ago [9]. Also in a chronologic point of view, it is not surprising that there are still no vaccines available for emerging diseases (e.g., Zika or MERS-CoV) [10, 11]. Other reasons have to do directly with the convergence of the nature of the pathogens with the evolution of vaccine technologies [12]: (i) almost all vaccines available till date are humoral based, which is not the best option against intracellular pathogens (e.g., *Leishmania* spp., *Trypanosoma cruzi*) [13, 14] and (ii) there are pathogens with immune-evasion strategies dependent on high antigenic variability that poses a challenge in vaccine development [6, 15]. Lastly, but not least important, there are diseases more relevant from an economic standpoint than others: many diseases for which there are still no vaccines available affect almost exclusively the poorest of the poor (neglected tropical diseases—NTDs) [8, 16, 17].

Fortunately, with the arrival of the new millennium, WHO/UN initiatives such as the Millennium Development Goals (Goal 6, Target 3) and more recently the Sustainable Development Goals (Goal 3, Target 3.3) contributed to an increase in the awareness on the NTDs and consequently the investment on strategies to control them [18, 19]. The best example of concrete measures undertaken to "end the neglect" is given by the London Declaration on NTDs, signed in 2012 by 20 parties (including governmental organizations, non-profits and pharmaceutical companies) and endorsed thereafter by many others, that proposes to meet the goals set by the WHO Roadmap to overcome the global burden of NTDs (2012–2020), that include the elimination of five diseases and the control of five others. One of the potential short-term controllable NTDs is the fatal form of leishmaniasis [20].

Endemic in 97 countries around the globe, leishmaniasis is a complex spectrum of diseases [21, 22]. The first layer of complexity is given by its vector-borne nature, which introduces an extra variable (the phlebotomine vector) to the binomium host pathogen. The second one is given by the 20 *Leishmania* species known to cause human diseases (usually in a species/disease-manifestation-specific fashion), which is mostly but not exclusively of zoonotic origin (there are no animal reservoirs recognized for *L. donovani*) [23]. The third one relates to the infectious process, which frequently does not lead to an overt disease but instead to a chronic and "benign" asymptomatic state [24]. These are some of the main challenges to consider within the topic of disease control, which relies heavily on the early diagnosis and treatment of the active cases (whose influence in the diminishment of disease incidence should be relevant in anthroponotic *versus* zoonotic leishmaniasis) [25, 26]. Although till date there is no vaccine available against human leishmaniasis, not only is it widely accepted that the development of an effective vaccine is possible but also it is recognized that vaccination is the only viable option to achieve zoonotic disease elimination [25].

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With this chapter, we propose to explore the broad anti-*Leishmania* vaccines field, with humans as the focus population. Starting from a historical perspective, we will clarify where we stand today by discussing the different candidates and approaches followed along the years, situating them in the vaccine development pipeline. Additionally, we will debate what is missing (focusing mainly, but not only, on the correlates of protection and the disease models) as a way to substantiate why currently there are no vaccines against leishmaniasis approved for humans.

2. Vaccines for human leishmaniasis: where do we stand?

2.1. Leishmanization as the proof of principle of vaccines against leishmaniasis

The close relation of the human host and *Leishmania* parasites is quite ancient: there is evidence of parasite genetic material (identified retrospectively) in mummies from the year 2000 B.C. [23, 27]. However, the major breakthroughs in the leishmaniasis field were only achieved starting from the beginning of the twentieth century, with the identification of the causative agent(s), the incrimination of the vector(s), and consequently the understanding of parasite(s) life cycle and the distinct physiopathologic mechanisms that characterize each of the leishmaniasis forms [23, 28]. The definitive allocation of leishmaniasis within the infectious (or communicable) diseases, in convergence with the "success of variolation" and the birth of vaccination [29], boosted the investigation of the anti-*Leishmania* immune response envisioning the development of an effective prophylactic approach. The first reports date from early 1900 and are based on either contemporary common "medical practices" from Old World Cutaneous Leishmaniasis (CL) endemic countries or directly on evidence produced in human clinical trial-like studies [30, 31]. The general conclusions of these pioneer "vaccine studies" that used as inoculum either material from CL patient's ulcers or live parasites collected from *in vitro* cultures (*L. tropica*) were (i) only the individuals that developed a lesion and then self-healed were resistant to reinfection and (ii) reinoculation of immune individuals led to what is nowadays known as Type I delayed type hypersensitivity (DTH) reaction [30, 31]. Such studies established the dogma accepted today by the scientific community—"previous infection leads to robust immunity against *Leishmania*"—and were the proof of principle of the only prophylactic approach clinically used against leishmaniasis known as leishmanization.

Leishmanization was no more than the controlled induction of the cutaneous disease to prevent the consequences of natural infection, such as the scarification of exposed body parts (particularly the face) and the consequent life-long psychosocial impact and simultaneously to decrease the disease incidence in hyperendemic areas [32, 33]. In the 1970s and 1980s, several trials were performed using live virulent *L. major* parasites with promising results (up to 80% efficacy, **Table 1**) [32, 34–36]. This vaccine approach was accepted in countries such as the former Soviet Union, Iran, Israel, and Uzbekistan [32, 36, 37]. However, it was generally abandoned (with the exception of Uzbekistan, where it is still a licensed approach according to the most recent reviews on the field [34, 36, 38]) due to a number of concerns such as: (i) some individuals (1–2/10,000 inoculations) developed non-healing lesions, hard to resolve with chemotherapy [32, 39]; (ii) live vaccines (even the attenuated) are contraindicated to immuno-suppressed individuals [40] (whose worldwide prevalence has increased in the modern days, due not only to the HIV pandemic but also, for instance, to the increase of organ transplantation

procedures [41]); (iii) batch-to-batch variability issues of such complex immunogens raise reproducibility concerns [36, 42]; and (iv) complex logistics are usually associated with live vaccines [42].

2.2. An overview of the vaccine candidates against human leishmaniasis explored since leishmanization until the present day

The knowledge produced by leishmanization trials and campaigns conducted at the end of the last century is the most important evidence that the development of a vaccine against leishmaniasis is quite far from being impossible. The quest for such an essential pharmaceutical, indispensable for the achievement of global disease control, has been continuous (in a scale proportional to the funding for NTD research) and fruitful if we consider the number of candidates and different approaches tested. Here we will separate them into five major groups: live vaccines ("leishmanization like"), first-, second-, and third-generation vaccines, and vector-derived vaccines. **Table 1** compiles the information to be discussed in the next subheadings, presenting not only the different candidates/approaches tested along the years but also the disease form they were destined to prevent, their placement in the vaccine development pipeline, and the main findings reported.

2.2.1. Live vaccine candidates

The success of leishmanization is still used to support the investigation of vaccine approaches based on live parasites (called by some as leishmanization revisitation [38]), that according to the authors have the advantage of at least partially reproducing the normal infectious process (and consequently induce a "close-to-natural" anti-*Leishmania* memory) [43]. This includes for some candidates the long-term parasite persistence in the site of inoculation that will continuously boost the immune system and prevent the loss of immunity to reinfection [44–46]. The first two approaches explored, relying on parasite persistence as the key to effectiveness, are readaptations of leishmanization directed toward the prevention of visceral disease and proposed the controlled infection with either virulent *L. major* parasites or with a virulent but dermotropic *L. donovani* strain to promote heterologous or homologous protection against visceral disease caused by viscerotropic *L. infantum* or *L. donovani* strains, respectively [38, 47–49]. Still, both approaches, although shown effective in the pre-clinical context, will unlikely proceed in the vaccine development pipeline, mainly due to the safety concerns always raised by the use of virulent pathogens. As a way to partially overcome this barrier, different live vaccine approaches proposed the use of attenuated parasites, that would still mimic the natural infection (although in a sub-clinical form) and induce anti-*Leishmania* memory but in most of the cases would then be completely eliminated. In the pre-genomic era (but not only) chemically and physically attenuated parasites were shown to be effective, in pre-clinical trials, against CL, muco-cutaneous leishmaniasis (MCL), and visceral leishmaniasis (VL) [50–52]. These attenuation approaches that did not assure a homogeneous parasite population (with an unpredictable potential of reversion to the virulent form) were almost completely replaced by the genetically modified parasites in the post-genomic era. Two main groups of genetically modified *Leishmania* parasites were used in the pre-clinical context: loss-of-function mutants

Type	Approach/vaccine candidate(s)	Disease form	Vaccine development pipeline	Efficacy/outcome	Reference
	Inoculation of live, virulent *L. major* parasites: Leishmanization	CL	Effective clinical use in the former Soviet Union, Israel and Middle East* (discontinued)	About 80% efficacy	[31, 34, 36]
	Heterologous protection mediated by inoculation of live, virulent *L. major*	VL	Pre-clinical studies in mice	No effect in BALB/c mice; protection in C57Bl/6 mice	[46–48]
Live vaccines	Inoculation of a dermotropic *L. donovani*	VL	Pre-clinical studies in mice	Protection against challenge with viscerotropic *L. donovani* in BALB/c mice	[37]
	Physically attenuated parasites	CL/ MCL/ VL	Pre-clinical studies in mice and hamsters	Homologous protection for *L. major*, *L. tropica*, *L. amazonensis*, *L. donovani* and *L. braziliensis*; no effect for *L. Infantum*	[49–51]
Non-defined composition; live, attenuated and/ or drug-sensitive parasites (through culture, chemical, radiation or genetic manipulation)	Chemically attenuated parasites (N-nitrosamines/ antibiotic pressure)	CL/ MCL/ VL	Pre-clinical studies in mice and dogs	Homologous protection for *L. major* and *L. mexicana* in BALB/c mice; promising results for *L. infantum* in dogs	[51]
	Genetically attenuated parasites (**Lmaj**dhfr-ts, **Lmex**CystProt, **Lmaj**LPG2, **Lmaj**PPM, **Ld**Cen1, **Li**HSP70-II, **Ld**p27, **Ld**ALO and **Ld**BT1 null mutants; **Li**SIR2 sKO)	CL/ MCL/ VL	Pre-clinical studies in mice/hamsters/dogs/ macaques	Homologous protection for *L. major* in mice, but not monkeys; *L. mexicana* in mice and hamsters; *L. infantum* in mice and *L. donovani* in mice, hamsters and dogs; heterologous protection for *L. major* in mice, *L. braziliensis* in mice and hamsters and *L. infantum* in dogs (mediated by *L. donovani* KO parasites)	[51–55]
	Genetically modified parasites (gain of function)—suicide mutants: *L. major* tk-cd+/+ (susceptible to Ganciclovir and 5-flurocytosine), *L. amazonensis* alad-pbgd+/+ (used in the context of photodynamic vaccination)	CL/VL	Pre-clinical studies in mice and hamsters	Homologous long-term protection (lesion free) in mice for *L. major*; heterologous protection against *L. donovani* mediated by *L. amazonensis*: 99% reduction in parasite loads and suppression of disease	[50, 56, 57]

Type	Approach/vaccine candidate(s)	Disease form	Vaccine development pipeline	Efficacy/outcome	Reference
	Immunization with non-pathogenic *L. tarentolae* (wild type or genetically modified strains producing LPG3, **Ld**A2 or **Ld**A2/CPA/CPB)	VL	Pre-clinical studies in mice and dogs	Promising results in mice and dogs	[50, 60, 61]
First generation vaccines	ALM adjuvanted with BCG	CL/VL	Pre-clinical and human clinical studies	Protection in macaques against *L. donovani* challenge; poor efficacy in humans. Protection in mice against *L. major* infection; clinical studies with disappointing results	[54, 65, 67, 68]
	Alum-ALM adjuvanted with BCG	CL/VL	Pre-clinical and human clinical studies	Immunogenic and safe in humans; protective (single dose) in macaques challenged with *L. donovani*; moderate efficacy against canine visceral leishmaniasis; protection in BALB/c mice against challenge with *L. major*	[54, 56, 69]
	Autoclaved *L. donovani*	VL	Pre-clinical studies in mice	Significant levels of homologous protection	[70]
	Phenol or Heat inactivated *L. guyanensis*, *L. braziliensis* and *L. amazonensis* adjuvanted with BCG	CL/ MCL	Human clinical studies	52% Efficacy in endemic area (phenol inactivation); no protection against *L. amazonensis* infection (heat inactivation)	[65, 67]
	Merthiolate-killed *L. amazonensis* (with/without BCG)	CL	Pre-clinical and Human clinical studies	Protection in mice not reproduced in humans	[65, 73, 74]
Non-defined composition; whole killed parasites or parasite fractions	Sonicated *L. donovani* (whole cell or soluble antigens) adjuvanted (MPL-A, BCG, liposomes)	VL	Pre-clinical studies in mice hamsters and monkeys	Good homologous protection in all species; liposomal formulation elicits the best protection in mice	[36, 54, 71, 72]
	Liposomal *L. major* soluble antigen adjuvanted with CpG	CL	Pre-clinical studies in mice	Significant levels of homologous protection	[36]

Type	Approach/vaccine candidate(s)	Disease form	Vaccine development pipeline	Efficacy/outcome	Reference
	Fucose-Manose ligand adjuvanted with saponin	VL	Pre-clinical studies in mice and hamsters; "clinical" studies in dogs	Protection in mice and hamsters challenged with *L. donovani* (homologous); effective heterologous protection (against *L. Infantum*) in dogs; transmission blocking potential; commercialized as a canine anti-*Leishmania* vaccine with the name Leishmune in Brazil (commercialization license suspended in 2014)	[66, 75]
	L. infantum or *L. amazonensis* excreted-secreted antigens adjuvanted with saponin	VL	Pre-clinical/"Clinical" studies in dogs	Significant, long-lasting protection against canine VL in a field trial in an endemic area (**Li**); promising results in terms of heterologous protection against *Leishmania infantum* (**La**); commercialized as a canine anti-*Leishmania* vaccine with the name CaniLeish in Europe (**Li**)	[54, 66, 76, 77]
Second generation vaccines	Membrane proteins: native **Ld**Dp-72, gp63 and PSA-2 and recombinant **Li**LCR1, **Ld**HASPB1, KMP-11 and gp63; adjuvanted (BCG, CpG-ODN, MPL-SE, IL-12, saponin, cationic nanoparticles, liposomes)	CL/VL	Pre-clinical studies in mice, dogs and macaques	Promising results regarding homologous protection in mice; dubious protection in monkeys against *L. major* challenge (gp63)	[50, 54, 71, 83–85]
	"Soluble proteins": recombinant **Ld**A2, **Li**PHB, **Ld**F14, **Ld**p27, **Ld**pSP **Ld**P45, **Ld**PDI, **Ld**TPI, **Ld**TPR, **Li**P0, **Lmaj**STM1, **Li**TDR-1, **Lb**Hyp, EiF5a, eIF-2, NH, CPA and CPB, SMT, PEPCK, Histone H1, Heat shock proteins (HSP), **Li**Ribosomal proteins, **Li**Hypothetical amastigote-specific protein, cysteine proteinases, LACK; adjuvanted (BCG, ALD, *P. acnes*, CpG-ODN, MPL-SE, IL-12, saponin, cationic nanoparticles)	CL/MCL/VL	Pre-clinical studies in mice, hamsters and dogs; *ex vivo* human studies	Promising results in mice and hamsters; a major limitation is that most of the antigens were not tested in superior models; positive response in *ex vivo* human studies for **Ld**eIF-2; partial homologous and heterologous (*L. infantum*) protection in dogs and heterologous protection in mice challenged with *L. infantum* and *L. amazonensis* (**Ld**A2: licensed veterinary product in Brazil—LeishTec)	[36, 50, 54, 67, 84, 85, 87–90]

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Type	Approach/vaccine candidate(s)	Disease form	Vaccine development pipeline	Efficacy/outcome	Reference
Defined antigens: (native) or produced through DNA recombinant technology (more frequent)	Peptides: CPA, GP63, LmSTI1, LiKMP-11, PEPCK; often associated DC-based vaccination or nano-sized vaccine-delivery systems; adjuvanted (MPLA, CpG-ODN)	CL/VL	Pre-clinical studies in mice	Partial protection for *L. infantum*; differential protection for *L. major*	[71, 89, 93, 94]
	Fusion protein/polyprotein: Q protein, Leish-F1 (Leish 110-f), Leish-F2 (Leish 110-f), Leish-F3, Leish-F3+, KSAC, 8E + p21 + SMT, KMP-11 + LJL-143 + Leish-F3 + (in virosomes), rLiHyp1 + rLiHyp6 + rLiHyV+rHRF multiepitope; adjuvanted (BCG, Saponin, CpG-ODN, GLA-SE, MPLA, ALD and MPL-SE)	CL/VL	Pre-clinical studies in mice, hamsters, dogs and macaques; human clinical studies	Promising results in mice (CL and VL) and hamsters; protection conferred to dogs against challenge with *L. infantum* (Q protein, Leish 110-f, Leish-F1, KSAC); protection of macaques challenge by *L. major* (Leish-F1); vaccines safe and immunogenic in humans (Leish-F1, Leish-F2 and Leish-F3); licensed veterinary product in Europe (Q protein—Letifend)	[54, 84, 85, 95–105]
Third generation vaccines	DNA plasmidic vaccines (usually self adjuvanted): LdPDI, tuzin, HbR, A2, Histones+p36, LACK, TSA + LmSTI1, gp63, KMP-11, CPB, ORFF, NH36, TRYP, PSA-2, γGCS, PEPCK, LeIF, GP63 + HSP70, LeIF+/orTSA; MIDGE-Th1 vectors encoding conserved T-cell epitopes from KMP11, TSA, CPA, CPB, and P74	CL/VL	Pre-clinical studies in mice, hamsters, dogs and macaques; *ex vivo* human studies	Generally good protective responses in mice and hamsters correlated with the induction of Th1 immunity; partial (Histones+p36) and good (LACK, cysteine proteinase) protection in dogs; protection in macaques (TSA + LmSTI1); effective in mice in immuno-chemotherapeutic approaches (MIDGE Th1); strong possibility of human immunogenicity (MIDGE Th1)	[36, 50, 54, 84, 85, 89, 110–117]
DNA vaccination and/or modified expression systems	Recombinant viral vectors: recombinant/modified vaccinia virus expressing TRYP, LACK, KMP-11; recombinant Influenza virus expressing LACK; (non-replicative) recombinant adenovirus expressing A2, Leish-F3 or KMP-11-HASPB; recombinant lentivirus expressing KMP11-HASPB	CL/VL/PKDL	Pre-clinical studies in mice, dogs and macaques; human clinical studies	Promising results obtained in all animal models; vaccine safe and immunogenic in humans (replication defective adenovirus coding for KMP-11-HASPB)	[50, 83, 119–123]

Type	Approach/vaccine candidate(s)	Disease form	Vaccine development pipeline	Efficacy/outcome	Reference
	Live recombinant bacterial vectors: *Lactococcus lactis* expressing A2 and LACK+IL-12; recombinant *S. typhimurium* vaccine strains expressing gp63, LinJ08.1190 and LinJ23.0410; recombinant *L. monocytogenes* (attenuated) expressing LACK	CL/VL	Pre-clinical studies in mice	Different results obtained, varying from disease exacerbation (A2 *L. lactis*), to limitation of pathology (LACK *L. monocytogenes*) or protection	[51, 124]
Vector-derived vaccines	Th1 immunity inducing sand fly salivary proteins: recombinant or DNA encoding LJM-19 (SALO), PdSP-15, PpSP15 (also *L. tarentolae* based), PpSP-44, LJM-143, LJM-17, LJM-11 (also *L. monocytogenes* based); alone, or in combination with common anti-*Leishmania* vaccine	CL/ MCL/ VL	Pre-clinical studies in mice, hamsters, dogs and macaques	Evidences or described effect in protection from (natural) infection in all animal models, except with PpSP-44 which leads to exacerbation of cutaneous disease	[128–135]
Recombinant or DNA coding for sand fly derived proteins (including heterologous expression systems)	Insect-based transmission blocking vaccine: anti *P. papatasi* galectin (sand fly midgut protein) antibody	CL	*In vitro* and *in vivo* insect studies (artificial feeding)	86% reduction of sand fly-midgut *L. major* infection; impairment of metacyclogenesis	[136–137]

A2, amastigote specific protein 2; ALM, autoclaved *L. major*; ALO, arabino-1,4-lactone oxidase; Cen, centrine; BCG, Bacillus Calmette–Guérin; CL, cutaneous Leishmaniasis; CPA/B, cysteine peptidase A/B; CystProt, cysteine proteinase; dhfr-ts, dihydrofolate reductase-thymidylate synthase; elF, elongation factor; GCS, glutamylcysteine synthetase; GLA, glucopyranosyl lipid A; gp, glycoprotein; HASP, hydrophilic acylated surface protein; HbR, hemoglobin receptor; HSP, heat shock protein; IL, interleukin; KMP, kinetoplastid membrane protein; LACK, *Leishmania* homolog of receptors for activated c-kinase; Ld, *L. donovani*; Li, *L. infantum*; LJL, *Lutzomyia longipalpis* Jacobina large; LJM, *Lutzomyia longipalpis* Jacobina medium; Lmaj, *L. major*; Lmex, *L. mexicana*; LPG, lipophosphoglycan; MIDGE, minimalistic immunogenically defined gene expression; MCL, mucocutaneous leishmaniasis; MPL, monophosphoryl lipid A; NH, nucleoside hydrolase; ODN, oligodeoxynucleotides; ORFF, open reading frame fragment; P0, acidic ribosomal protein P0; PDI, protein disulphide-isomerase; PdSP, *Phlebotomus duboscqi* salivary protein; PEPCK, phosphoenolpyruvate carboxykinase; PHB, prohibitin; PKDL, post kala-azar dermal Leishmaniasis; PPM, phosphomannomutase; PpSP, *Phlebotomus papatasi* salivary protein; PSA, promastigote surface antigen; SE, stable emulsion; SIR, silent information regulator; SMT, sterol 24-c-methyltransferase; TDR, thiol-dependent reductase; TPI, triose phosphate isomerase; TPR, trypanothione reductase; TRYP, tryparedoxin peroxidase; TSA, thiol-specific antioxidant; VL, visceral leishmaniasis.*Supposedly still used in some extent in Uzbekistan [56].

Table 1. Different anti-*Leishmania* vaccine candidates explored in the last century.

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(knock-out) and gain-of-function mutants (knock-in). In respect of the first group, nine null mutants [*L. major* dihydrofolate reductase-thymidylate synthase (dhfr-ts$^{-/-}$), *L. mexicana* Cysteine proteases (CPA/CPB$^{-/-}$), *L. major* lipophosphoglycan 2 (LPG2$^{-/-}$), *L. major* phosphomannomutase (PPM$^{-/-}$), *L. donovani* Centrin (Cen$^{-/-}$), *L. infantum* heat shock protein 70 type II (HSP70-II$^{-/-}$), *L. donovani* amastigote specific protein p27 (p27$^{-/-}$), *L. donovani* arabino-1,4-lactone oxidase (ALO$^{-/-}$) and *L. donovani* biopterin transporter 1 (BT1$^{-/-}$)], and one single knock-out [*L. infantum* silent information regulatory protein 2 (SIR2$^{+/-}$)] were proven, in most cases, as effective vaccine candidates (CL, MCL, and VL) [52–56]. Concerning the second group, two gain-of-function mutants were shown effective as vaccines for CL and VL. Both trials relied on the generation of "suicidal mutants" that would be completely eliminated from the immunized host either by the action of chemotherapeutics [L. major thymidine kinase (herpes simplex virus), cytosine deaminase (Saccharomyces cerevisiae) knock-in: tk-cd$^{+/+}$], or by photodynamic therapy (*L. amazonensis* δ-aminolevulinate dehydratase, porphobilinogen deaminase knock-in: alad-pbgd$^{+/+}$) [51, 57, 58]. Yet, although safer in theory than both live virulent and pre-genomic attenuated vaccine candidates, post-genomic live attenuated vaccines still raise safety concerns, both due to the potential for reversion to virulence (higher for gain-of-function parasites but not negligible for knock-out parasites as was reported [59]) and due to the potential risk to the immunosuppressed (that was not explored in most of the trials). The last tested live vaccine approaches we will discuss here propose the use of closely related non-pathogenic parasites as a way to overcome all the live vaccine safety-related red flags. *Leishmania tarentolae* parasites infect reptiles but are unable to generate a sustained infection in humans (although able to enter into human phagocytic cells, there is no evidence of efficient intracellular replication) [60]. Importantly, they share >90% of the gene content with the other *Leishmania* species [60] which makes these parasite species an innocuous source of native *Leishmania* antigens (although some of the important virulence factors of pathogenic parasites that may be essential to the induction of a protective prophylactic response are missing). Using this premise, both wild-type and genetically modified (LPG3, amastigote-specific protein (A2), or A2/CPA/CPB knock-in) non-pathogenic parasites were reported, in the pre-clinical context, as promising vaccine candidates for VL [52, 61, 62].

2.2.2. First-generation vaccine candidates

Together with live attenuated vaccines, killed whole pathogens or fractions of them (inactivated and fraction vaccines) comprise a large proportion of the approved vaccines for humans today [63]. In line with what happened chronologically in modern vaccinology, killed/fractionated vaccines against leishmaniasis were developed both contemporarily and posteriorly to the "leishmanization era," to answer to the safety concerns associated with live virulent/attenuated vaccines. The main advantage of first-generation vaccines in relation to the live vaccine counterparts is consequently their innocuity: the pool of antigens in its native form will still be "delivered" and elicit a specific memory response (diversity in antigenic *repertoire* given by live parasites will be at least partially maintained), while no pathology is expected, even in immunocompromised individuals (no infection = no disease) [64]. This, however, may as well be a disadvantage: while regarding live vaccines, the antigen delivery will be sustained; that will not be true for killed vaccines that may require more than one administration (prime homologous boosts immunization schemes) and/or the co-administration of an immune response enhancer or adjuvant (usually not required in live vaccine approaches) [65], which may or may not be enough to generate long-lasting protection. Additionally, all of the manufacturing and logistics

issues discussed earlier for leishmanization (and live vaccines in general) are also applicable to killed/fractionated vaccines. Notwithstanding, first generation vaccines for leishmaniasis are the better studied ones in the clinical context (the only leishmaniasis vaccine candidates which have undergone phase 3 clinical trials) [66], by itself very relevant for the anti-Leishmania vaccine development field, and are available today in the market as approved vaccines for canine VL (Leishmune® in Brazil and CaniLeish® in Europe) [67]. The better recognized vaccine candidate within this sub-topic is the autoclaved *L. major* (ALM) adjuvanted with BCG, tested in the pre-clinical and clinical contexts, with promising results in the first that were not confirmed in the second [55, 66, 68, 69]. This candidate was then optimized by adsorption of the antigenic fraction to alum (alum-ALM + BCG) and retested once again in both pre-clinical and clinical contexts (CL and VL), with reported different degrees of efficacy in animal models and good immunogenic and safety profiles in humans [55, 66, 70]. A similar parasite-killing approach was used with *L. donovani* parasites, tested in a vaccine pre-clinical trial for VL (mice) that revealed significant homologous protective potential [71]. In parallel, a different inactivation strategy (sonication) was used also with *L. donovani*, and the obtained total or soluble antigens were used together with MPL-A, BCG, or liposomes as vaccine candidates for VL in pre-clinical trials with promising results in all models tested (mice hamsters and monkeys) [37, 55, 72, 73]. Only two other candidates were tested in the clinical context, this time in the New World as CL and/or MCL vaccines. One of them was a trivalent formulation of phenol or heat-inactivated *L. guyanensis*, *L. braziliensis*, and *L. amazonensis* adjuvanted with BCG [66, 68], while the other consisted of merthiolate-killed *L. amazonensis* (with/without BCG) [66, 74, 75]. Curiously, in line with what was verified in the Old World with ALM-BCG, although effective in the pre-clinical context, both candidates generally failed as human vaccines [66, 75]. Apart from crude extracts, parasite fractions have been tested. Liposomal *L. major* soluble antigens adjuvanted with CpG were tested as a vaccine candidate for CL with significant levels of homologous protection observed in mice [37]. A glycoproteic fraction of *L. donovani* parasites (fucose-mannose ligand—FML) adjuvanted with saponin [67, 76] and *L. infantum* (or *L. amazonensis*) excreted-secreted proteins (ESP) also adjuvanted with saponin [55, 67, 77, 78] were tested as vaccine candidates for VL in canines, whose determined efficacy, and safety profiles, was sufficient to warrant their registration as veterinary vaccines (*L. donovani* FML as Leishmune®—out of the market nowadays—and *L. infantum* ESP as CaniLeish®). Nevertheless, they were never tested in the human clinical context, which may be due to different reasons, all connected to the notion that the requirements needed for the approval of a human pharmaceutical are much more strict than the ones required in the veterinary context: (i) the heterogeneous antigen formulation, harder to standardize, may have been considered an obstacle or (ii) the data obtained in the pre-clinical context may not have been sufficient (vaccines conferred only partial protection [67]).

Although it is a topic we do not explore in this chapter, it is important to stress that killed vaccines, different from what was observed in the prophylactic context, have shown great promise in a therapeutic context (revised in [79]).

2.2.3. Second-generation vaccine candidates

The birth and evolution of the molecular biology field contributed immensely to the rhythm of science in general. Today, the production of a single antigen is usually easily achievable,

as it is the possibility of scaling-up the process to an industrial level. Second-generation vaccines are a consequence of this scientific evolution (although some are native proteins, most of them are recombinant antigens) and consist of defined antigens, generally together with an immune response enhancer. They are usually accepted by the scientific community, as well as by the regulatory entities that so far have approved three vaccines for human use (including the hepatitis B recombinant vaccine that replaced the traditional plasma-derived one [80]). The main advantage of these vaccines in relation to the ones earlier discussed is the defined composition that allows an easier standardization. Another advantage we can think of is the elimination of immuno-dominance events that invariably occur if a complex antigen mixture is used as a vaccine and may hinder the potential of good vaccine candidates [81]. As disadvantages, the following should be considered: (i) the limited duration of antigen availability might impact the memory pool and limit the "protection window" [82] (more complex immunization schemes have to be used) and (ii) recombinant proteins, usually expressed in heterologous systems, may be slightly different from native proteins (particularly concerning post-translational modifications [83]) which might impact their immunogenic potential (more relevant for humoral responses, considering conformational epitopes).

Second-generation vaccines against leishmaniasis are the group with higher representativeness. Here, for the sake of clarity, we separate them into four different groups: membrane and soluble proteins (full single recombinants), peptides, and polyproteins (multivalent), whose main candidates are enumerated in **Table 1**. The studies from fractionated parasites postulated that parasite membrane proteins had a good vaccine potential. Because of that, and also due to their relative abundance, relevant in terms of antigen presentation, many membrane proteins were explored as vaccine candidates in the pre-clinical context for both CL and VL with promising results [51, 55, 72, 84–86]. Among these is the well-known, and extensively studied in the context of anti-*Leishmania* vaccination, kinetoplastid membrane protein-11 (KMP-11) [87]. Importantly, most of these proteins were identified by classical immuno-proteomic approaches considering always the amastigote parasite form as the most relevant in the human infectious process and are known virulence factors. This fact is also true for most of the non-membrane proteins (we name here "soluble proteins") also tested in the last decades as vaccines against leishmaniasis, although most of them only in rodent models of CL, MCL, and VL (translatability to humans is not assured) [37, 51, 55, 68, 85, 86, 88–91]. Ribosomal proteins (e.g., P0), metabolic enzymes (e.g., TPI), stress-related proteins (e.g., HSP), antioxidant-machinery components (e.g., TPR), and even hypothetical proteins (**Table 1**) are found among them. One of these candidates, *L. donovani* A2, is today a licensed veterinary vaccine against leishmaniasis in Brazil—LeishTec® (that needs however to be optimized, according to a recent efficacy field trial performed in an endemic area with high transmission rates [92]). In the past few years, the development of vaccine candidates against leishmaniasis became more refined and rationale based, following the trends of twenty-first-century vaccinology [93]. New studies are now usually based in an initial *in-silico* prediction of immunogenicity, validated later ideally through *ex vivo* studies using samples from exposed human individuals, all performed before the design of any clinical trial. Furthermore, the antigens/antigen portions should be "broad spectrum"—conserved in all the pathogenic *Leishmania* spp.—and very different from human "self-antigens." From the application of such approaches and selection criteria, promising new candidates were proposed. Among them, peptide vaccines, chosen from immunogenic portions of known vaccine candidates such as KMP-11, were tested pre-clinically, often associated with DC-based vaccination

strategies or nanosized vaccine-delivery systems [72, 94, 95]. Interestingly, a recently published work proposes a peptide vaccine candidate (from *Leishmania* phosphoenolpyruvate carboxykinase—PEPCK) that may be effective for both VL and CL should the results obtained in the pre-clinical context translate into the clinical one [90]. However some argue that to use a single antigen, or a peptide as vaccine, may be less than optimal, considering that there will be a limitation in terms of epitope diversity. To answer to this, some propose the use of defined polyantigen vaccines (fusion proteins or mixed recombinants), also rationale based, as a way to generate "first-generation-like" second-generation vaccines, increasing epitope diversity and consequently in theory enhancing recognition by human T cells (**Table 1**) [55, 85, 86, 96–101]. Some of these candidates are among the second-generation vaccines that went further in the vaccine pipeline. Q protein (a fusion protein containing portions of *L. infantum* p2a, p2b, and P0 ribosomal proteins and histone H2A) that was demonstrated effective in a pre-clinical trial in dogs infected with *L. infantum* is today the newest approved vaccine for veterinary use—Letifend® [102, 103]. Leish-F1 [fusion protein containing epitopes from *Leishmania* elongation initiation factor (LeIF), thiol-specific antioxidant (TSA), and *Leishmania major* stress-inducible protein 1 (LmSTI1)], Leish-F2 (same immunogenic portions as Leish-F1 but his tagged), and Leish-F3 (fusion protein containing portions of *Leishmania* nucleoside hydrolase and sterol 24-c-methyltransferase), which revealed promising and safe candidates in the pre-clinical context (for both CL and VL), were tested in phase I/II clinical trials that confirmed the translatability of results obtained with animal models to humans [97, 104–106]. Nevertheless, the researchers involved in these clinical trials think that there is still space for improvements and recently presented an improved version of Leish-F3 (with cysteine protease B as an extra fused antigen—Leish-F3^{+}) that is going through the pre-clinical phase of the vaccine development pipeline, with promising results, either alone [99] or in combination with KMP-11 and the vector-derived antigen LJL-143, within a virosomal formulation [96].

We cannot end this sub-section without stressing that generally these second-generation vaccine candidates require the co-administration of adjuvants to warrant their efficacies as vaccines for leishmaniasis. **Table 2** resumes the relevant information on the topic, extensively covered by two recently published reviews [107, 108].

2.2.4. Third-generation vaccine candidates

The notion that intradermal or intramuscular injection of a plasmid into an animal model would be enough to generate antigen-specific immune responses was responsible for the creation of a new arm in the vaccine research field. Although Initially DNA vaccines were not as well accepted as first- and second-generation vaccines, not only due to potential ethical implications (injection of foreign genetic material into humans that could, for instance, integrate within the human genome) but also due to safety concerns such as the possible generation of autoimmune pathologies initiated by the generation of anti-DNA immune responses [109]. However, these potential issues of DNA vaccines were, with time, shown to be irrelevant, both through extensive pre-clinical research and through several clinical trials performed that confirmed DNA vaccines as safe and immunogenic in humans (although for some candidates, the immunogenicity data was not as promising as expected) [109, 110]. Yet, contrary to the other vaccine approaches discussed earlier, we still have no data from phase IV studies of DNA vaccination, since till date there is no third-generation vaccine approved for human use (although there are already four

Adjuvant	Class	Mechanism of action	Type of immune response	Licensed for use in human vaccines
Aluminum mineral salts	Particulate formulation; antigen depot	NALP3, ITAM, antigen delivery, IL-1 secretion, necrosis, inflammasome	Antibody, Th2	✓ (adjuvant of different commercially available vaccines)
Simple or emulsified Lipid A analogues (e.g., GLA, MPL)		TLR-4 agonists	Antibody, Th1	✓ (in combination with Alum in HBV and HPV vaccines)
Imidazoquinolines (e.g., Imiquimod, R848)		TLR-7, TLR-8 agonists	Antibody, Th1	X (clinically tested in cancer immuno-therapy)
CpG-ODN	Immuno-modulatory molecule	TLR-9 agonists	Antibody, Th1, Th2, CD8+ T cells	X (clinically tested in HBV, malaria, influenza and anthrax vaccines and in cancer immuno-therapy)
Saponins (e.g., QuilA, QS21)		Unknown	/	X (clinically tested in combination with cholesterol in HCV, influenza and HPV vaccines and in cancer immuno-therapy)
Nanoparticles (e.g., Virosomes*, Liposomes)	Particulate formulation	Antigen delivery; cross-presentation enhancer*	Antibody, Th1, Th2	✓ (HAV and *Influenza* vaccines)

Adapted from [106, 107].

* The asterisk means that only virosomes are cross presentation enhancers (asterisk in both)

Table 2. Main adjuvants used in anti-*Leishmania* vaccines development.

approaches approved for veterinary use) [109, 110]. However, considering that third-generation vaccines are the most recent approaches (studies started in the 1990s), it is likely a matter of time until the approval of the first DNA vaccines considering some advantages attributed to them: (i) they are easy to design, produce, and scale up (potentially more cost-effective); (ii) they are quite stable, which minimize distribution and logistics-related complications; and (iii) they can induce both humoral and cellular immune responses (including $CD8^+$-mediated cytotoxicity) [110]. Here, we categorize third-generation vaccines in three clusters: DNA vaccines, viral heterologous expression systems, and live bacterial expression systems. DNA vaccines are the more expressive in respect of the number of candidates explored, containing the simplest vaccine candidates: consist of usually non-adjuvanted plasmids (the "real DNA vaccines"). Similar to what was described for second-generation vaccines, both membrane (e.g., KMP-11 and gp63) and non-membrane antigens (e.g., NH, CPB, HSP70, and A2) were explored in the context of plasmid vaccine candidates (**Table 1**), pre-clinically, using animal models for both CL and VL [37, 51, 55, 85, 86, 90, 111–118]. Interestingly, many of the candidates tested as second-generation vaccines (and particularly those that have shown some degree of promise) were retested as DNA vaccines, either individually or in "multi-antigen" approaches (e.g., KMP-11, A2, LACK, and TSA+LmSTI1), showing the adoption of a rationale-based vaccine development [114]. The general reproduction of the results obtained with second-generation vaccines, after immunization with their DNA counterparts (CL and VL models, including mice, hamsters, dogs, and macaques), validated these approaches as potentially effective agents in

the context of anti-*Leishmania* vaccine prophylaxis [51, 55, 85, 86, 90, 114]. In this sub-group, we would like to highlight the LEISHDNAVAX approach, recently proposed for VL. Completely based in a modern vaccine development approach (rationale based), this vaccine candidate, shown to protect mice from an intravenous challenge with *L. donovani*, is composed of five individual plasmid (MIDGE-Th1 vectors) coding for five *Leishmania* antigenic determinants, chosen based on inter-species conservation, "pan-immunogenic" potential (in different human populations), and content of T-cell-restricted epitopes (KMP11, TSA, CPA, CPB, and P74) [112]. This approach, which, according to the authors, is a candidate for clinical trials, has as the main advantage the modular nature: the vaccine is multivalent, but the antigens are not fused together, allowing the rapid modification and adaptation of the vaccine (exchange, addition, or elimination of antigens) [112]. Still within third-generation vaccines, more complex candidates were explored as well, in the form of heterologous expression systems. Among them are viral vectors, referred to as an improvement of classical DNA vaccines, once in one way allow *in situ* antigen expression, and also have an intrinsic adjuvant activity (mediated by pathogen-associated molecular patterns (PAMPs) immune recognition) [119]. One important prerequisite of such vectors is their relative innocuity, being in most cases either human-approved vaccine strains (which have the same counter-indications for immuno-compromised individuals) or replicative-deficient strains. Till date, more than 5 viral-recombinant vaccines (using as viral platforms modified vaccinia virus, influenza virus, non-replicative adenovirus and lentivirus) coding for *de facto* effective antigens such as KMP-11, LACK, Leish-F3 and HASPB, were tested in the pre-clinical context for both CL and VL, with promising results obtained in all animal models used (mice, dogs and macaques) [51, 84, 120–123]. Remarkably, one of them was the first third-generation anti-*Leishmania* vaccine candidate to undergo human clinical trials. The adenoviral-based vaccine (non-replicative strain) expressing a self-cleaving polyprotein (L. donovani KMP-11+HASPB) was shown safe and immunogenic in humans, inducing particularly specific $CD8^+$ T cell responses, and importantly is being proposed as, more than a prophylactic vaccine, a therapeutic vaccine destined to aid in the control of post-kala-azar dermal leishmaniasis—PKDL ("the neglected form of leishmaniasis" in respect of the anti-*Leishmania* vaccine studies) [124]. Last but not the least, some bacterial-based heterologous systems were proposed as anti-*Leishmania* vaccines although with disappointing results in some cases [52]. Also, to these ones, because they are live organisms, the disadvantages discussed earlier for live attenuated vaccines apply (such as counter-indication to immuno-suppressed) with the exception of the use of non-pathogenic organisms, such as *Lactococcus lactis*. From these candidates, we highlight the recombinant *Salmonella typhimurium* vaccine strains and the attenuated *Listeria monocytogenes* expressing different *Leishmania* antigens (e.g., gp63 and LACK), the ones that have shown the most promising results, although only in rodent models of CL/VL [52, 125].

2.2.5. Vector-derived vaccine candidates

It has become clear that to consider the sand fly vector only from the perspective of vector-control strategies would be not only reductive but also contribute to a major delay in the achievement of the disease elimination objective. The anti-*Leishmania* vaccine field became more complex from the moment Kamhawi, Belkaid, and colleagues showed that a previous exposure to uninfected sand fly bites (or to sand fly saliva) would be enough to confer protection against CL [126, 127]. Curiously, the anti-saliva-generated DTH responses were shown to be sufficient to negatively impact *Leishmania* parasites (indirectly). And importantly, such responses are apparently not

influenced by constant saliva exposure that could induce tolerization [128]. Such pieces of evidence supported the exploitation of defined sand fly salivary proteins as anti-*Leishmania* vaccine candidates (either as single recombinant proteins or DNA vaccines—both plasmids and heterologous systems—alone or in multivalent approaches together with *Leishmania*-derived antigens) [129–136]. Several antigens, derived from different sand fly species from both New [129–131, 136] and Old [133–135] Worlds, were explored in the pre-clinical context in models of CL, MCL, and VL, most of them with promising results. The most relevant candidate is PdSP15, which was shown to be protective against cutaneous disease in different models, including in non-human primates (DNA protein prime-boost approach) [133–135]. Another candidate that deserves to be highlighted is LJM-19 (or SALO), which was demonstrated simultaneously as a good candidate against visceral ("homologous protection") and mucocutaneous ("heterologous protection") disease [131, 136]. Still within vector-based anti-*Leishmania* vaccine approaches, and although it is an option which is exploited very little, we believe that transmission blocking vaccines deserve to be mentioned. Such vaccines will act by impacting parasites' development within the vector, impeding, therefore, their transmission to a new host [137]. For their engineering, however, the insect midgut proteins that allow parasite attachment during development (assuming such a process is dependent on specific interactions) have to be identified, which was described only for *Phlebotomus papatasi* (galectin—PpGalec) [138]. Interestingly, this study that shows that flies pre-fed with PpGalec murine pre-immune serum and posteriorly infected with *L. major* parasites were reproducibly less infected than the controls (an 86% decrease in the number of parasites retained in the midgut after blood meal excretion which led to at least a 5-fold reduction in the frequencies of mature infection development) is a proof of principle of *Leishmania* transmission blocking vaccines that may be used, for instance (but not only), in animal reservoirs and still impact human disease incidence [137, 138].

2.3. Questions that deserve to be answered

As a connecting point between the current and subsequent sections, we raise some questions for which we still do not have a clear answer today. The first one is if the development of a pan-*Leishmania* vaccine *sensum latum* (both prophylactic and therapeutic; for endemic and non-endemic individuals; against all disease forms) is something over-ambitious. And such a question makes sense, not only because of the time and investment that are expected to be involved—for the case of leishmaniasis, the non-existence of prophylactic agents implies the "faster is better" motto. For instance, in our recent work, we show that the pre-administration of a salivary antigen, followed by a boost with the same salivary antigen together with two other parasite-derived proteins, has a direct impact in the immunogenicity of the latest [96], which may suggest that vaccines for endemic individuals may not work equally in non-endemic ones and *vice versa*. This point is particularly relevant if we use vector- and parasite-derived components in the vaccines against leishmaniasis, which is related with the second question we pose: should vaccines for leishmaniasis always contemplate both parasite- and vector-derived components? Studies that show the improvement of parasite-derived vaccine candidates when co-administered with vector-derived antigens support this hypothesis [54, 64]. However, there are still some issues that have to be addressed, such as the possibility of tolerance induction, that is known to be dependent on the amount of antigen [139] (expected to be higher in a defined antigen-based vaccination approach, compared with a sand fly bite). Furthermore, another question relates to clinical research. How can we test the effectiveness of safe and immunogenic vaccine candidates? The last phase III

clinical trials were performed more than half a century ago and against the cutaneous disease. But, contrary to other deathly parasitic diseases, such as malaria [140], to perform controlled infections with *L. infantum*, *L. donovani* or even *L. braziliensis* or *L. guyanensis* would be unethical, to say the least. Therefore, such trials would have either to evaluate cross-protection to cutaneous disease (controlled infection with *L. major* that still raises ethical issues) and extrapolate results to the mucocutaneous/visceral forms or be designed and conducted directly as phase IV clinical trials (although to use a placebo in this context would probably also not be admissible).

3. Vaccines for human leishmaniasis: what is still missing?

So far, and consciously, we described the different vaccine candidates explored till the present days as vaccines against leishmaniasis, highlighting only their effectiveness in a qualitative way (effective/non-effective, promising or not) and not discussing the immune mechanisms linked to those results: first, because **Table 1** contemplates vaccine candidates developed for the different leishmaniasis forms, whose pathogenic mechanisms are distinct (and not completely understood) [141] and additionally, because the correlates of protection (that may also be distinct, depending on the disease form) are still far from being well established (they are neither consensual nor definitive). Such facts may have different justifications, as (i) we are still missing key insights concerning vector-parasite-host interactions (both in disease and in health states); (ii) the translation value of the animal models used is limited; or (iii) the models used are not adequate.

3.1. From "mice to man": the issue of animal models, correlates of protection, and translation

Being *Leishmania* parasites obligatory intracellular pathogens (in the mammalian host), it is not surprising that humoral-based responses will be less important than cellular-based ones. Indeed, in animal models of VL, the absence of B-cells contributed to decreased susceptibility to infection [142, 143]. Additionally, it has been shown that antibody-opsonized parasites are more efficiently taken by phagocytes that will become "permissive hosts" due to the high IL-10 and low IL-12 secretion phenotype induced by antibody Fc-receptor (FcγR) interactions [144–147]. Importantly, one of the hallmarks of human disease, is hypergammaglobulinemia (that correlates with disease severity), resultant from a polyclonal B-cell activation, being consequently most of the circulating antibodies non-parasite specific [148–150]. Still, and because the development and role of humoral responses in leishmaniasis is controversial and not completely understood, they may be important [151, 152]. For instance, the type and functionality of the antibodies may be relevant from the standpoint of a vaccine approach, considering lytic functions [e.g., antibody-dependent cell-mediated cytotoxicity (ADCC)] or even "Th1-inducing" FcγR ligation [153]. Yet, even for the proper mounting of effective antigen-specific humoral responses, cell-mediated immunity is of paramount importance [154].

What is known today regarding cellular immune responses to *Leishmania* infection was built on top of the Th1/Th2 paradigm defined on the basis of susceptibility *versus* resistance to *L. major* infection (one of the known CL etiologic agents) [155]. Indeed, the IL-12-mediated IFN-γ production by *Leishmania*-specific $CD4^+$ T cells is essential to promote the switch on of the oxidative cell-parasiticidal machinery, important for infection control both in animal models and in human

disease [156–158] (although in mucocutaneous forms, inflammation is also the cause of pathology [159]). However, while in cutaneous disease a general correlation between Th1 *versus* Th2 responses and immunity *versus* susceptibility is observed, in VL, where the major source of the regulatory cytokine IL-10 is *Leishmania*-specific Th1 cells (Tr1), that is not observed. This mechanism of self-regulation (to prevent inflammation-mediated tissue damage) contributes to parasite persistence [160]. Yet, most of the anti-*Leishmania* vaccine studies rely on the quantification of the levels of IL-10 and IFN-γ-secreted *ex vivo* in response to either the vaccine antigen or to parasite total proteins and use the Th1/Th2 paradigm as a justification for the candidate potential. Others use multi-parametric flow cytometry (or ELISPOT) that allows the characterization of individual cell populations and the disclosure of which cytokines they are producing (most of the times after an *ex vivo* stimulation step): often IFN-γ *versus* IL-10 (individually) or more recently multi-functional T cells [161]. Still, one may claim that results based on such approaches may have a limited validity due to the artificiality of the system: (i) the type and amount of antigen used in the recall and (ii) exclusion of parasite immuno-modulatory potential. To measure directly cytokine expression in the target organ (in an efficacy pre-clinical trial), as is sometimes done in CL models, is a way to bypass the potential limitations of *ex vivo* stimulation approaches. Importantly, the correlates of protection proposed and used should always correlate well with parasite burdens. Another issue that deserves to be emphasized is the cell type(s) we need to look at. Although $CD4^+$ T cells are important in anti-*Leishmania* immunity, so are $CD8^+$ T cells (important from both therapeutic and prophylactic standpoints) that are however many times almost not accounted for in vaccine studies [162]. These cells are nowadays known to be important for resistance to *Leishmania* infection and cure, either by production of IFN-γ (that will activate the microbicidal machinery) or by secretion of cytotoxic mediators that will directly kill infected cells [70, 163, 164]. And because of this, usually, the secretion/expression of IFN-γ or granzyme-B by *ex vivo*-recalled $CD8^+$ cells is used to qualify their responsiveness and considered as potential correlates of protection. Having in consideration what was referred above for $CD4^+$ T cells, an additional problem of translation must be considered. It is known that human $CD8^+$ T cells (and other cytotoxic subsets) produce an antimicrobial peptide (granulysin) with direct parasite-cytotoxic effect, while murine cells do not. Curiously, the infection of a humanized mouse model (granulysin knock-in) with *T. gondi* and *T. cruzi* was less severe than in WT animals, as probably will be reproduced with *Leishmania* spp. [165]. Another important factor to be considered in vaccine effectiveness evaluation is the relevance of the local *versus* systemic responses. Although in CL models, most of the times "specific-systemic responses" are investigated (e.g., recall experiments using splenocytes), it was recently shown that *Leishmania*-specific skin-resident $CD4^+$ T cells are able to confer protection to cutaneous diseases, independently of the central/effector memory pool [166]. However, the immune response in the skin is often not accounted for. Although natural infection (independently of the disease form) always begins by the deposition of parasites in the host dermis (excluding vertical transmission and accidental "human-made" infections resulting from, e.g., blood transfusions), most of the animal models used today in vaccine studies, particularly if we consider VL, completely bypass the skin (controlled infections are performed most of the times either intravenously or intraperitoneally). Therefore, in one way, we may be losing information on the contribution of skin immunity to the protective potential of a given vaccine candidate but on the other hand we may be "overloading the system" and induce responses quite different than the normal ones (too many parasites = excessive inflammation or immuno-modulation)

[167]. Additionally, most of the times in experimental infections, and in this case not only in VL models, the vector is completely disregarded. Importantly, vector saliva was shown to exacerbate infection in different disease models [126, 168, 169]. Also, we have to consider the vector microbiome as a potential infection modulator, as it has been hypothesized [170]. Additionally, parasite-excreted-secreted virulence factors (e.g., promastigote secretory gel and extracellular vesicles/exosomes), or death parasites, all expected to be part of the natural infectious inoculum, were also shown to promote infection [171–173]. Probably, one or the combination of all of these components was the factor responsible for the data published by Peters et al. [174] that have shown the loss of efficacy of the ALM vaccine candidate when tested in the sand fly *versus* needle challenge contexts ("reproducing" the results obtained in the clinical trials of a similar vaccine candidate—ALM + BCG). All of the above discussed point to the use of pre-clinical models to test vaccines that should be as close to what is observed in nature (bearing in mind that even a laboratory-based sand fly transmission model will not be indistinguishable from the natural one, considering the expected differences in the microbiomes [175] and the heterogeneity in vectorial capacities [176]. To improve the chances of translatability (even if the correlates of protection were concrete, the use of an inadequate model would "invalidate" the results), the minimum requirements of vaccine development pre-clinical infection model should be the co-inoculation of parasites together with vector saliva (particularly if the vaccine candidate consists [partially] of vector-derived antigens) in the host dermis, naturally or artificially, by needle injection. No model is perfect and pre-clinical investigation shall ever replace clinical research. However, the system simplification, which is generally used in scientific research as a way to eliminate noise, can also be the reason of loss of translatability. Most of the models used in vaccine development studies have a defined and identical genetic background—they are inbred [177]. Interestingly, vaccine candidates show contradictory results concerning efficacy, depending on the inbred murine model used [178]. We need to have in mind that humans, the target population of the vaccines, are quite a heterogeneous population, with more than 7000 HLA alleles identified so far to which we have to add heterozygosity favored by natural selection [179]. To address this issue, we can start by the vaccine engineering phase that should be more and more rational (using reverse vaccinology approaches [180]) and predict the immunogenicity in different human populations, as a proof of principle that is expected to be validated first pre-clinically and then clinically.

In respect of these three subjects (correlates of protection, animal models, and translatability of pre-clinical studies to humans) that have major overlaps and cannot be separated, there are still too many shades of gray to account for. As a way to eliminate the fogginess, it will be important to identify the divergent and common points of many anti-*Leishmania* vaccine pre-clinical and/or translational studies performed so far. The field would gain a lot from the elaboration and publication of bibliographic statistic studies such as meta-analysis or systematic reviews. Additionally, as suggested by Gannavaram and colleagues, the leishmaniasis research field needs to turn to more complex approaches, such as systems vaccinology, to be able to answer the questions that the community posed a while ago but still remain *quasi*-unanswered [181].

3.2. From "man to mice": the insufficiency of prospective studies

Leishmaniasis animal models have been undoubtedly an extremely useful tool to understand better the host–parasite interactions that influence either resistance to infection or disease

development [157, 182]. This is true for both cutaneous and visceral diseases, although much more relevant in the latest. It would be both unethical and dangerous to biopsy diseased individuals spleen, liver, or bone marrow (target organs of the viscerotropic *L. infantum* and *L. donovani*) just to better understand the infectious process. However, an animal model, even when it combines both conceptual and facial validities, is still just a model; in other words, translation to human health and disease may not always be achievable. In other diseases, prospective studies in human populations have produced valuable information not only from epidemiological and pathological standpoints but also applicable to the vaccine development field [183, 184]. On the other way, till the present days, most of the prospective studies performed in leishmaniasis had an epidemiological character (as invaluable in what respects the common goal of the community, which is disease control) [24, 44]. The development of such studies, focusing on systemic immune responses (particularly cellular based), would be of paramount importance to better understand both disease and resistance in leishmaniasis. For that, there are two target populations that deserve to be studied longitudinally: cured individuals and asymptomatics. While the following of the first population would help to answer the questions related to long-lasting immunity, the following of the second would help to define the potential host factors that determine susceptibility *versus* resistance. Yet, we have to consider as a possible limitation of studies with asymptomatics the less-than-clear and consensual definition of these individuals [24]. Nevertheless, the information generated by such studies would be then possibly "translated back" to animal models, used to better define the correlates of protection to improve vaccine design.

4. Conclusion

Today we still do not have a vaccine approved for human leishmaniasis (regardless of the disease form). Many candidates were tested in the last century, and up to nowadays only vector-derived vaccines were not tested in the clinical context; for all the other parasite-derived candidates, regardless of the vaccine generation they are part of, we have proof of principle of at least immunogenicity and safety (in human healthy individuals) and therefore a precedent is open. Yet, the efficacy clinical trials performed so far (the last more than 50 years ago), excluding leishmanization, were overall disappointing. Such information is however as valuable as any positive result and should be used from a perspective of "learning from our mistakes." There are still many questions to be answered in the anti-*Leishmania* vaccine development field, such as which parameters should be used as correlates of protection and how we should test our vaccine candidates in a way that warrants translation to the clinical context. Additionally, to define the vaccine effectiveness in the clinical context in a controlled way is essential. To address all of these issues, the vaccine development should be more and more rationale based, taking advantage of the modern and of the ancient. Observational studies of target human populations associated with systems biology may for instance help once and for all to disclose the health *versus* disease determinants and contribute to the final establishment of flawless correlates of protection. Additionally, immuno-informatic tools may help to design or refine (through a reverse vaccinology approach) the future vaccine(s) for human leishmaniasis.

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