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REVIEW ARTICLE

An overview of the sand fly salivary proteins in vaccine development against leishmaniases

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ABSTRACT

Leishmaniases are a group of vector-borne parasitic diseases transmitted through the infected sand flies. *Leishmania* parasites are inoculated into the host skin along with sand fly saliva. The sand fly saliva consists of biologically active molecules with anticoagulant, anti-inflammatory, and immunomodulatory properties. Such properties help the parasite circumvent the host's immune responses. The salivary compounds support the survival and multiplication of the parasite and facilitate the disease progression. It is documented that frequent exposure to uninfected sand fly bites produces neutralizing antibodies against specific salivary proteins and further activates the cellular mechanisms to prevent the establishment of the disease. The immune responses due to sand fly saliva are highly specific and depend on the composition of the salivary molecules. Hence, thorough knowledge of these compounds in different sand fly species and information about their antigenicity are paramount to designing an effective vaccine. Herein, we review the composition of the sand fly saliva, immunomodulatory properties of some of its components, immune responses to its proteins, and potential vaccine candidates against leishmaniases.

Keywords: Sand fly; Salivary proteins; *Leishmania*; Vaccine; Immunity

INTRODUCTION

Leishmaniases are a group of diseases caused by protozoan parasites of *Leishmania (L.)* species. The parasite is transmitted to its mammalian hosts through the bite of infected sand flies of the genera *Phlebotomus (Ph.)* and *Lutzomyia (Lu.)* in the Old World (OW) and New World (NW), respectively. The promastigote forms of the parasite are found in the infected sand flies and are transmitted to the host skin during the blood meals where they are engulfed by the macrophages. Upon entering the macrophages, the promastigotes replicate intracellularly and transform into the amastigote forms which elicit the pathology. Leishmaniases are manifested by diverse clinical presentations, from asymptomatic subclinical infection to cutaneous form (i.e., Cutaneous Leishmaniasis; CL) and even visceral forms (i.e., Visceral Leishmaniasis; VL), associated with significant mortalities (1).

Female sand flies are the vectors of different species of *Leishmania*. The sand fly introduces promastigotes along with saliva into the skin during biting (2). As a result, pharmacologically-active compounds in

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the sand fly saliva counteract the host's hemostatic and immune systems. These compounds also support the survival and multiplication of the parasites and facilitate the disease progression (3-5). On the other hand, specific individual salivary proteins are known to protect from subsequent infections (6, 7). With this notion, the presence of antibodies against the salivary proteins of sand flies in humans and animals was sought after and their identification paved the way for researchers to consider them as markers of exposure as well as vaccine candidates for both humans and animals (8, 9). The present review focuses on the proteins with biological functions and immunogenic properties of the sand flies' saliva, with the potential applications of vaccine candidates for leishmaniases.

The anti-hemostatic and pharmacologically active components of sand fly saliva. Salivary proteins of sand fly prevent processes of hemostasis and inflammation which facilitate successful blood-feeding. The functions and features of such specific proteins are described below.

Maxadilan is one of the most investigated salivary proteins, unique to NW sand flies. This protein is a vasodilatory active molecule, identified only in the NW sand fly *Lu. longipalpis.* Although maxadilan resembles a human calcitonin gene-related peptide (CGRP), its potency and duration of vasodilation effect are much higher (10). Apyrase is a potent antiplatelet aggregation enzyme that is found in both the NW and the OW sand flies' saliva. It hydrolyzes ATP and ADP to AMP plus inorganic phosphate and is dependent on calcium cations for its activity (11). Endonuclease is shared by OW and NW sand flies. The salivary endonuclease from *Lu. longipalpis* (Lundep) prevents blood clotting by inhibiting the intrinsic pathway of coagulation; acts as an anti-inflammatory and destroys the neutrophil extracellular traps (NETs) (12). 5′-nucleotidase is another antiplatelet protein that also has vasodilatory properties and complements the activity of maxadilan and apyrase (2). The protein is unique to NW. RGD motif-containing proteins that contain Arginine-Glycine-Aspartate (RGD) amino-acid sequences are specific to the NW sand fly saliva. Antiplatelet activity has been expected for these proteins based on their RGD motif. Ayadualin is an RGD-containing protein of *Lu. ayacuchensis* which has antiplatelet aggregation properties and inhibits coagulation cascades (13). Odorant Binding proteins (OBP) are represented with D7-related and PpSP15-like proteins in both the OW and the NW sand flies (14, 15). The long-form D7-related proteins bind eicosanoids (cysteinyl leukotrienes and thromboxanes) and perform anti-inflammatory and anti-platelet functions (14). Two SP15-like Proteins - PdSP15a and PdSP15b- isolated from *Ph. duboscqi* have a high affinity for polyphosphates and heparin. Upon binding, they inhibit the autoactivation of coagulation factor XII, factor XI, and prekallikrein, and thereby prevent coagulation (14, 16). Antigen-5 family of proteins has antioxidant and antiplatelet aggregation properties, presents in both the NW and the OW sand flies' saliva. However, these functions are yet to be studied in sand flies (17). Adenosine and its precursor 5′-AMP present in the OW sand fly *Ph. papatasi* -although are not proteins -have potent vasodilatory as well as antiplatelet-aggregation properties (18). C-type lectins, unique to NW sand flies, contain a C-type lectin domain that bind to some of the coagulation factors and inhibit the blood coagulation cascade (19). Lufaxin, shared by OW and NW sand flies, is an inhibitor of factor Xa and has antithrombotic and anti-inflammatory activities (20). Hyaluronidase family of proteins presents in both the NW and the OW sand flies' saliva, catalyze the degradation of hyaluronic acid and create interstitial gaps between the cells in the host, thereby can promote the diffusion of other salivary molecules and dispersal of the parasites (21). Yellow-related proteins are a major protein family in the saliva of OW and NW sand flies. This family has a biogenic amine-binding function. LJM11 and LJM17 from *Lu. longipalpis* are members of this family and prevent inflammation and hemostasis (22, 23). A recombinant yellow-related protein of *Ph. argentipes*, known as pagSP04, has been shown to have a high binding ability toward serotonin and a low affinity to histamine (24).

The immunomodulatory components. Salivary substances from sand flies have immunomodulatory properties; which enhance the ability of pathogens to establish themselves in the host. Maxadilan displays several immunomodulatory properties such as downregulation of LPS-induced TNF-α and nitric oxide (NO) release by macrophages, enhancement of IL-6 production which stimulates Th2 response, and inhibition of T-cell proliferation and DTH reaction (25). It has been shown that an apyrase (rSP01) has a strong inhibitory effect on NO production (26). SALO (Salivary Anticomplement of *Lu. longipalpis*; formerly known as LJM19) is an 11-kDa protein, unique to the NW sand fly saliva and is a specific inhibitor of the classical complement pathway (27). This protein inhibits C4b deposition and cleavage of C4. Yellow-related proteins (PduM10 and PduM35) exhibit neutrophil chemoattractant activity (28). The other proteins of this family (rSP03 and rSP03B) show anti-inflammatory effects by inhibiting TNF-α, and NO, and inducing IL-10 production (26). Adenosine deaminase has been shown to prevent T-cell apoptosis, bind to CD26 on T cells, and exert a co-stimulatory function. Therefore, it putatively interferes with lymphocyte function (29).

Sand fly salivary immunogenic proteins. Sand fly salivary proteins are recognized by immune systems of humans and animal reservoirs. Some of these proteins have been shown to work as biomarkers of vector exposure and/or vaccine.

Antibody response to sand fly saliva. Repeated exposure to saliva from the sand flies bites is immunogenic in nature and increases the antibody titers in the host (30). Some of these proteins are recognized by sera of many individuals which can be used as markers of exposure and for epidemiological risk-assessment studies.

Yellow-related proteins from sand fly saliva of both OW and NW such as *Lu. longipalpis, Ph. orientalis*, and *Ph. perniciosus* are the best biomarkers of vector exposure in dogs and humans (8, 9, 22). From this family of salivary proteins LJM11 and LJM17 as specific markers of *Lu. longipalpis* exposure in humans and dogs (22, 31, 32); PorSP24 as a marker of *Ph. orientalis* exposure in humans, sheep, goats, and dogs (8); rSP03B as a marker of *P. perniciosus* exposure in mice, dog, hare and rabbit (9, 33-35) have been suggested as biomarkers of exposure. Apyrases are one of the most antigenic salivary protein families recognized by sera of repeatedly bitten hosts. Two apyrases namely rSP01B and rSP01 (from the saliva of *Ph. Perniciosus*) were identified as the best candidates for evaluating the exposure of mice and dogs, hares, and rabbits to *Ph. perniciosus* bites and for estimating the risk of canine leishmaniasis (33, 34). A significant correlation between antibody response against SGH of *Ph. orientalis* and recombinant apyrase rPorSP15 in sera of sheep, goats, and dogs was also detected (8). LinB-13 and mAG5 (antigen 5-related proteins) were shown to be the best biomarkers of *Lu. intermedia* and *Ph. orientalis* exposure in humans, respectively (36, 37). PpSP32, a silk-related protein was identified as the best marker of human exposure to the bites of *Ph. papatasi* sand flies (38, 39). The D7-related protein LJL13 was recognized by dogs naturally exposed to *Lu. longipalpis* but not by sera from foxes and humans from an endemic focus of *L. infantum* (22).

Sand fly saliva-based anti-*Leishmania* vaccine candidates. The resistance to *Leishmania* species infection is characterized by the stimulation of the CD4+ Th1 response, characterized mainly by IFN-γ expression; while, susceptibility to the infection is correlated with the development of CD4+ Th2 response and its associated increase in IL-4-producing cells (2). Kamhawi et al. have described that prior exposure of mice to the uninfected bites of sand flies results in powerful protection against *L. major* infection in the form of DTH and increased IFN-γ (2). In addition, Individuals living in endemic areas of leishmaniases are less susceptible to the infection as compared to the newcomers, due to the repeated exposure to the uninfected sand flies (40). Pre-exposure with sand fly salivary proteins triggers antibodies and/or cell-mediated immune responses against the sand fly saliva (41). It is generally accepted that the main protective immunity is mediated by DTH reaction and enhanced IL-12/IFN-γ production. This was further confirmed by the experiments conducted on B lymphocytes–deficient mice which were vaccinated with saliva-derived plasmid and protected against *L. major* (42). However, there is evidence for the involvement of neutralizing antibodies, based on the protein antibody-mediated-inactivation of saliva immunomodulatory components, which may inhibit the establishment of the parasite (19, 43).

When the host is exposed to the parasite via infectious vector bites, the fast cellular recruitment and Th1 polarization against the salivary proteins will result in a less successful *Leishmania* establishment in the host and lead to earlier priming of the immune system toward an anti-*Leishmania* immunity (15).

Oliveira et al. conferred that different SGH proteins of sand fly differed in their immune response against *Leishmania* infection (44). Some of the salivary protein vaccine candidates which are investigated so far against CL or VL are listed in Table 1.

Table 1. Salivary vaccine candidates from *Phlebotomus (Ph.)* and *Lutzamia (Lu.)* species.

Sand fly species	Salivary protein/ adjuvant SP15	Animal Model	Platform/vehicle	Root of administration	Challenge with	Root of inoculation	Protection	Type of Leishmaniasis	Ref.
	-SP15	C57BL/6 mice	DNA/VR1020	i.d.	*L. major*+ SGH (*Ph. papatasi*)	i.d. (needle)	+	CL	(42)
	-SP44	C57BL/6 mice	DNA/VR2001	i.d.	*L. major*+ SGH (*Ph. papatasi*)	i.d. (needle)	+	CL	(44)
	SP15+CPA,CPB						-		
Ph. papatasi		BALB/c mice	DNA/VR1020+ rLive/rL. tarentolae	s.c.	*L. major*+ SGH (*Ph. papatasi*)	s.c. (needle)	+	CL	(66)
		C57BL/6 mice							
	PpSP15/CpG	BALB/c mice	rLive/rL. tarentolae	s.c.	*L. major*+ SGH (*Ph. papatasi*)	s.c. (needle)	+	CL	(67)
	SP15	BALB/c mice	DNA/rLactococcus lactis	s.c.	*L. major*+ SGH (*Ph. papatasi*)	s.c. (needle)	+	CL	(68)
Ph. papatasi	SP15-T2A-SP9/CpG	BALB/c mice	rLive/rL. tarentolae	s.c.	*L. major*+ SGH (*Ph. papatasi*)	s.c. (needle)	+/-	CL	(69)
Ph. sergenti					*L. tropica*+ SGH (*Ph. sergenti*)		+		
Ph. duboscqi	SP15/GLA-SE	Rhesus macaques	DNA/VR2001-rProtein	i.d.	*L. major*	sand fly bites (*Ph. duboscqi*)	+	CL	(45)
Ph. sergenti	SP9	BALB/c mice	DNA/VR1020	i.d.	*L. tropica*+ SGH (*Ph. sergenti*)	i.d. (needle)	+	CL	(47)
Ph. duboscqi	Multi epitopes of	C57BL/6 mice	DNA/VR2001	i.d.	*L. major*	sand fly bites (*Ph. duboscqi*)	-	CL, VL	(70)
Lu. longipalpis	Sp15 + LJL143	BALB/c mice				(*Lu. longipalpis*)	+		
	Maxadilan/Freund	CBA/CaH-T6J mice	Synthetic Protein	s.c.-i.p.	*L. major*+ SGH (*Lu. Longipalpis*)	s.c. (needle)	+	CL	(51)
	-LJM19	Syrian golden hamsters	DNA/VR2001	i.d.	*L. infantum chagasi*+ SGH	i.d. (needle)	+	VL	(7)
	-LJM17				(*Lu. Longipalpis*)		-		
	-LJM11						+		
	-LJL11						-		
	- LJL143	Beagle Dog	DNA/VR2001-rProtein-DNA/r	i.d.-i.m.	*L. infantum chagasi*	sand fly bites (*Lu. longipalpis*)	+	VL	(56)
	- LJM17		canarypoxvirus				+		
	/CpG								
	-LJM11	C57BL/6 mice	DNA/VR2001	i.d.	*L. major*+ SGH (*Lu. Longipalpis*)	i.d. (needle)	+	CL	(23)
	-LJM04						-		
	-LJL143						-		
	LJM19	Golden Syrian Hamsters	DNA/VR2001	i.d.	*L. braziliensis*+ SGH (*Lu. Intermedia*)	i.d. (needle)	+	CL	(50)
Lu. longipalpis	LJM11	C57BL/6	rProtein	i.d.	*L. major*	sand fly bites (*Lu. longipalpis*)	+	CL	(53)
	LJM11	C57BL/6 mice	DNA/r L. monocytogenes	i.v.	*L. major*	sand fly bites (*Lu. longipalpis*)	+	CL	(55)
					L. major+ SGH (*Lu. Longipalpis*) *L.*	i.d. (needle)			
	LJM19 and/or KMP11	Golden Syrian Hamsters	DNA/VR2001 and pcDNA3	i.d.	*chagasi*+ SGH (*Lu. Longipalpis*) *L.*	i.d. (needle)	+	VL	(63)
	LdCen-/- +LJM19	Syrian golden hamsters	Live/attenuated L. donovani-protein	i.d.	*donovani*+ SGH (*Lu. Longipalpis*)	i.d. (needle)	+	VL	(62)
	LJL143+KMP11+LeishF3/	BALB/c mice	rProtein/Virus-Like Particles (VLP)	i.m.	-	-	*	VL	(64)
	GLA-SE	-BALB/c mice	Synthetic Protein and peptides/	s.c.	*L. major*+ Maxadilan	s.c. (needle)	+	CL	(52)
	Maxadilan/CLDC	-C3H-HeN mice	CLDC						
		-C57BL/6 mice							
	LJM11	BALB/c mice	rProtein	i.d.	*L. braziliensis*+ SGH (*Lu. Longipalpis*)	i.d. (needle)	+	CL	(54)
					L. braziliensis+ SGH (*Lu. Intermedia*)		-		
Lu. intermedia	Linb-11	BALB/c mice	DNA/ VR2001	i.d.	*L. braziliensis*+ SGH (*Lu. Intermedia*)	i.d. (needle)	+	CL	(57)

i.d.:intradermal; s.c.: subcutaneous; i.m.: intramuscular; i.v: intravenous; i.p.: intraperitoneal
CLDC: cationic lipid DNA complex
LdCen-/- : *L. donovani* Centrin Knock-Out
*Immunogenicity was positive

Salivary vaccine candidates from *Phlebotomus* species. PpSP15 was the first salivary protein to be identified as a potential vaccine. Immunization of mice with PpSP15 plasmid induced a delayed-type hypersensitivity (DTH) response that was correlated to protection (42). Then, the protective effect of PpSP15 against *L. major* in mice by induction of a DTH, IFN-γ, and IL-12 expression was validated (44). Immunization of *Rhesus macaque* with SP15 of *Ph. duboscqi* (PdSP15) has also revealed protectivity against CL, characterized by a significant reduction in the lesion and parasite burden (45). Moreover, sera and PBMC of individuals naturally exposed to Ph. duboscqi bites could recognize PdSP15, suggesting its immunogenicity in humans (45). However, although antibodies against PpSP15 have been detected in the sera from individuals naturally exposed to the bite of *Ph. Papatasi*; there was not any IFN-γ production after stimulation of PBMCs with PpSP15 (46). This indicates that proteins that are immunogenic in other hosts may not be immunogenic in humans. Besides, the immunogenicity of proteins from different species of sand flies may vary in humans. It has been also demonstrated that *Ph. sergenti* SP9, a member of the SP15 family proteins, protects mice against *L. tropica* (47). Immunization with plasmids coding for two yellow-related proteins (PpSP42, and PpSP44) although induce DTH could not protect mice against *L. major* challenge. Indeed, the PpSP44 protein produces a Th2 response that results in disease enhancement after *L. major* infection (44). Therefore, salivary proteins which can induce IFN-γ along with DTH can be considered vaccine candidates for leishmaniasis.

It is believed that immunization with closely-related species of sand flies could result in cross-protection. For instance, immunization with *Ph. papatasi* saliva is documented to induce protection against *L. major* with *Ph. papatasi* or *Ph. duboscqi* saliva (48) while only mice immunized with *Lu. longipalpis* saliva could induce protection against *L. amazonensis* plus *Lu. longipalpis* saliva challenge and not mice immunized with *Phlebotomus* species saliva; indicating species-specificity (49). Tavares et al. have also reported induced protection against *L. braziliensis* and *Lu. intermedia* on immunization with *Lu. longipalpis* saliva, indicating the existence of common antigens across different species (50).

Salivary vaccine candidates from *Lutzomyia* species. Maxadilan is a specific salivary component to *Lu. longipalpis.* Immunization of mice with maxadilan protects animals against challenges with *L. major* plus SGH from *Lu. longipalpis* by developing both cellular immunity and antibodies (51). Wheat et al. have shown that a combination of maxadilan and cationic liposome DNA complexes (MAXCLDC) was effective in preventing exacerbation of the infection by *L. major,* with an increase in IFN-γ and a decrease in IL-4 secreting CD4+ cells in mice (52). These suggest that MAXCLDC vaccine targeting sand fly maxadilan improves the host immunity against maxadilan-mediated immunomodulation.

The potential role of another set of salivary proteins of *Lu. longipalpi*s, namely, LJM19, LJM11, and LJM17 as promising vaccine candidates against *Leishmania* infection, have also been evaluated. LJM11 and LJM19 induce a protective immune response against different species of *Leishmania* transmitted by different species of sand fly (7). Immunization with LJM19 (SALO) protects hamsters against challenges with *L. infantum* plus *L. longipalpis* SGH and *L. braziliensis* plus *L. intermedia* SGH (7, 50). Moreover, immunization of hamsters with LJM19 DNA plasmid or *Lu. longipalpis* saliva resulted in a reduction of the lesion size and the parasite load against challenge with *L. braziliensis* plus *Lu. lntermedia* saliva. DTH response and IFN-γ expression were induced; while, the expression of IL-10 and TGF-β were reduced (50). LJM11 is an abundant salivary protein from *Lu. longipalpis.* The recombinant LJM11 (rLJM11) as well as the plasmid encoding this protein efficiently control CL caused by *L. major* in mice. The protection was correlated with a strong DTH response and high IFN-γ induction following exposure to *L. longipalpis* SGH (23, 53). The rLJM11 was effective in providing ulcer-free protection against *L. major* infection (53). Moreover, LJM11 protects hamsters against VL up to 2 months post-infection with *L. infantum*; however, three months later the parasite load was comparable to that of the control group (7). A significant reduction of parasite load in LJM11-immunized mice when challenged with *L. braziliensis* plus *Lu. longipalpis* SGH has been reported; however, cross-protection in the challenge with *L. braziliensis* plus *Lu. intermedia* SGH has not been indicated (54). Abdallah et al. have also demonstrated a Listeria-based vaccine expressing LJM11 which confers long-term protection against CL in mice 3 months post-vaccination (55). LJM17, the other salivary protein of *Lu. longipalpis*, induced a strong DTH response in dogs previously exposed to uninfected sand flies bites. Moreover, PBMCs from LJM17-immunized dogs produced a high amount of IFN-γ upon *in vitro* stimulation with rLJM17, confirming the Th1 profile of the generated immune response. Additionally, immunized dogs showed a strong predominantly IgG2 humoral immune response to LJM17 (56). It is noteworthy that hamsters immunized with plasmid coding LJM17 exhibited significant DTH in response to SGH from *Lu. longipalpis*; however, they were not protected against challenge with *L. infantum chagasi* plus SGH (7).

PBMC from LJL143 (Lufaxin)-immunized dogs produced a high amount of IFN-γ upon *in vitro* stimulation with recombinant lufaxin and showed a strong IgG2-biased humoral response. Interestingly, the immune response at the site of the bite differed depending on the number of flies used in the challenge. Following exposure to bites of 20 sand flies, TGF-b was the dominant cytokine that was induced with a low expression of IFN- γ, IL-12 and IL-4. In contrast, a high level of IFN- γ and a low level of IL-4 were induced when these dogs were exposed to bites from 5 sand flies (56).

Plasmid coding for Linb-11 protein from *Lu. intermedia* was tested for immunogenicity in BALB/c mice. The results indicated protection against *L. braziliensis* infection which was correlated with decreased parasite load and increased IFN-γ (57).

Immunization with hyaluronidase (LuloHya) and the endonuclease LJL138 (Lundep) from *Lu. logipalpis* led to decreased pathology and parasite burden in mice infected with *L. major* along with sand fly saliva; notably, this protection was dependent on antibody responses because it was not observed in B-cell-deficient mice (58).

Combination of salivary proteins and *Leishmania* antigens as a vaccine candidate. To augment the host immunity, vaccination with sand fly salivary protein combined with *Leishmania* antigens has been proposed and several *in-silico* and experimental studies have been performed in this direction. Fusion Protein Composed of Apyrase-LihyV and LeIF-SP15 have been evaluated immunoinformatically. The results have revealed highly antigenic promiscuous epitopes against leishmaniasis in both studies (59, 60). Ojha et al. have also designed a multi-epitope vaccine targeting 4 salivary proteins of *Ph. argentipes* and 6 parasite-derived antigens to enhance humoral as well as cell-mediated immunity. The immunoinformatic analysis showed that the designed vaccine candidate was antigenic and would be able to initiate the potential immune responses (61).

In another study by Fiuza et al. immunization of hamsters with *L. donovani* Centrin knock-out (Ld-Cen-/-) in combination with LJM19 as an adjuvant, induced a durable protective immune response against VL (62). Silva et al. demonstrated the protective efficacy of KMP11 and LJM19 DNA plasmids combination against a challenge by *L. chagasi* and *Lu. longipalpis* SGH. Strong DTH and IFN-γ productions were correlated with protection. In this case, however, the results were similar to KMP11 and LJM19 vaccines when administered alone and the addition of antigens from sand fly saliva did not enhance the immune responses (63). The immunogenicity and protective efficacy of a multivalent vaccine consisting of LJL143, KMP11, and LeishF3+ (as parasite-derived antigens) in virosomes as antigen-delivery vehicles have also been evaluated. The results indicated improved immune responses against the parasite antigens, significantly lower parasite burdens accompanied by protection against *L. infantum* infection (64, 65).

CONCLUSION

The immune responses mediated by sand fly salivary proteins are highly specific and are dependent on the composition of the salivary molecules. Moreover, salivary components play an important role in disease transmission and the progression of the parasite. Therefore, acquiring a thorough knowledge of the composition of different species and their antigenicity is paramount for designing effective vaccines against leishmaniases or their applications as novel biomarkers of exposure. The formulation of a vaccine that is effective both in combating the infection and is capable of enhancing the host immunity against the parasite is highly suggested.

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