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Leishmania RNA Virus: Mere Endosymbiont or Major Player in *Leishmania*-Associated Disease?

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SUMMARY Leishmaniasis is a vector-borne disease caused by various species of *Leishmania* parasites. They are transmitted by the bites of infected sand flies and go on to infect host macrophages. Approximately 12 million are currently infected with the disease worldwide, and there may be over 1 million new cases every year. Leishmaniasis has several clinical manifestations including cutaneous, mucocutaneous, and visceral disease. Current treatments involve chemotherapy and medications that directly act against the parasite, but are associated with adverse effects, disease relapse, and drug resistance. Without effective treatments for the disease, individuals can be left with debilitating skin and facial deformities, and can even lead to death if left untreated. Some parasite strains have been found to be infected with virus particles called *Leishmania* RNA virus (LRV). LRV is a small parasitic virus containing a dsRNA genome that encodes a capsid protein and RNA-dependent RNA polymerase, and resides exclusively in the cytoplasm of the parasite. Remarkably, LRV has been implicated with increased rates of disease relapse, drug treatment failure, and exacerbated disease symptoms. Investigating the role LRV plays in *Leishmania*-associated disease may open up novel alternative therapeutic avenues that can better treat Leishmaniasis and prevent future infections. This article provides an overview of the biological relationship between LRV and *Leishmania* parasites, what effects LRV has on *Leishmania*-associated disease, whether the virus can be specifically targeted as a therapeutic strategy and which components of the virus can be potential targets. Understanding LRV's role and contribution to disease pathogenesis will not only increase our understanding of virally-infected parasites, but will also pave the way for considering LRV as a potential treatment target.

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INTRODUCTION

Leishmaniasis is a neglected-tropical disease caused by the protozoan parasites that belong to the genus *Leishmania* [1]. The disease is prevalent in 90 countries across regions in Africa, Asia, Southern Europe, and South America, most of which are developing countries. In addition, there are approximately 700,000 to 1.2 million new cases of

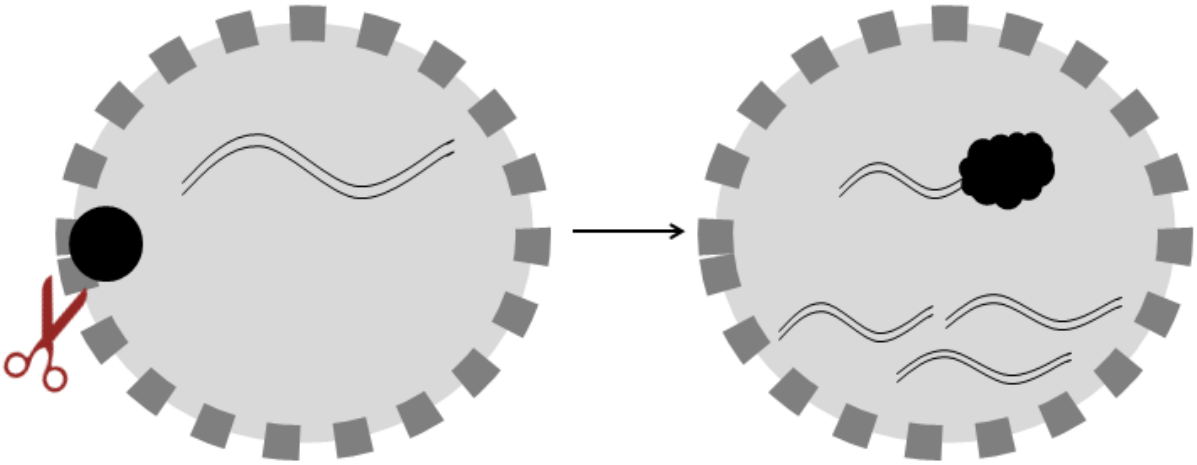

FIG. 1 LRV capsid-RNA-dependent RNA polymerase fusion protein cleavage. A *Leishmania* cysteine protease is responsible for cleaving the fusion protein in order for the RNA-dependent RNA polymerase to become active and replicate the genome.

Leishmaniasis world-wide every year [2]. The parasites are transmitted through the bites of female phlebotomine sand flies and although there are about 500 known species, only 20 are implicated in *Leishmania* transmission. When sucking blood from an infected human or animal, the sand fly becomes infected with *Leishmania* which multiplies over a period of 4-25 days. When this fly bites another person or animal, the parasites are inoculated into the new host and go on to infect their macrophages, thus completing the transmission cycle [3].

There are three major clinical manifestations of the disease: cutaneous Leishmaniasis (CL), mucocutaneous Leishmaniasis (MCL), and visceral Leishmaniasis (VL) [1, 4]. The specific form of the disease developed by an infected individual depends on complex interactions between the virulence of the specific *Leishmania* species, the immune response of the host, and environmental factors [1, 4]. CL is characterized by skin ulcerations that last months to years and result in severe scarring [4]. Untreated CL can lead to MCL which results in total destruction of mucous membranes of the nose and mouth [5]. Finally, VL results in enlargement of the spleen, and liver, causing death if left untreated [4, 6].

In 1988, some parasites were found to contain virus particles called *Leishmania* RNA virus (LRV) [7]. Residing and replicating in the cytoplasm of the parasite, LRV is composed of a non-enveloped icosahedral capsid approximately 40 nm in diameter. It belongs to the *Totiviridae* family that infect protozoa, yeast, fungi, and arthropods. In addition, it contains a non-segmented dsRNA genome approximately 5.3 kb in length encoding a major capsid protein and a capsid-RNA-dependent RNA polymerase (RDRP) fusion protein that needs to be cleaved in order to be functional (Fig. 1) [8]. Although LRV have been found in several *Leishmania* species such as *L. braziliensis* [9], *L. guyanensis* [7], and *L. major* [10], its role in virulence and metastasis of *Leishmania*-associated disease has only recently been explored, suggesting that LRV may actually be playing a role in disease severity and therapeutic outcomes [11].

RESEARCH QUESTIONS

Leishmaniasis is thought to have the second highest mortality and fourth highest morbidity rates across all tropical diseases despite there being established medications available to treat the disease [12]. Currently, there are no vaccines or medications that can prevent *Leishmania* infection and current treatments have high relapse rates, side effects, and drug resistance [13, 14]. In addition, there has been some debate about whether LRV can actually exacerbate symptoms of *Leishmania*-associated disease [15]. Therefore, this paper will clarify the current knowledge of LRV and its implications on disease severity. First, the biological relationship between LRV and *Leishmania* parasites will be elucidated. Second, the effect LRV has on *Leishmania*-associated disease will be reviewed. Finally, the potential

of LRV to be a therapeutic target will be explored. By answering these questions, this paper will shed light on how a virus, parasite, and host can be involved in a complex disease relationship.

PROJECT NARRATIVE

What is the biological relationship between LRVs and *Leishmania* parasites?

Understanding the virus-parasite relationship between LRV and *Leishmania* is very important in order to explore the effects LRV has on disease. Insight into how LRV emerged as a parasitic virus and the current mechanisms used by the parasite to control viral load can provide clues for managing a virally-infected parasite infection.

There are currently two known strains of LRV: those that are found within the *Vianna* subgenus (LRV1) and those found outside the subgenus (LRV2) [8]. While LRV1 has been identified in several parasite isolates, LRV2 has only been found in a single, exceptional isolate of *L. major* and has not been studied as extensively as LRV1[16]. Phylogenetic analyses of the different strains of LRV showed that there is a greater degree of divergence between LRV1 and LRV2 than any other strains, suggesting a long-term association and co-evolution of LRV that pre-dates the divergence of *Leishmania* into its different lineages [17]. Furthermore, LRV may provide survival advantages to parasites by reducing sensitivity to oxidative stress and increasing latency and chronicity to improve host-to-host parasite transmission [8].

It is largely unknown how maintenance and transmittance of the virus occurs. Transmission has been hypothesized to take place during genetic exchange, but this form of mating is highly infrequent in *Leishmania* parasites. Instead, random segregation of the virus during parasite replication is more likely [17]. Although there have been attempts to infect virus-free *Leishmania* parasites with LRV, it was only transiently maintained, indicating that LRV is unlikely to be transmitted extracellularly [18]. RNA interference (RNAi) in the parasite may be the reason why stable infection with LRV is so difficult to achieve. RNAi is a mechanism where dsRNA, such as foreign viral genomes, becomes degraded and prevents viral replication [19]. Most *Leishmania* species do not possess RNAi, but LRV is remarkably only found in those that do [20]. Since LRV doesn't have any anti-RNAi activity to protect itself, their retention is thought to be due to a balance between RNAi and LRV replication. RNAi may be playing a regulatory role in terms of viral titre, where too much RNAi leads to viral elimination and too little RNAi leads to an overload of virus, thereby killing the parasite. In fact, this characteristic allowed researchers to enhance the RNAi pathway in a *L. braziliensis* strain containing LRV and tilt the balance towards elimination in vitro [21]. This method was not only able to specifically target LRV, but also highlights how the virus-parasite relationship is one more of balance and symbiosis.

What effect does LRV have on *Leishmania*-associated disease?

Although *Leishmania* parasite infection with LRV was observed several decades ago, the interest in its functional and clinical role remained unexplored until recently. Understanding how LRV may contribute to disease progression is imperative in order to consider LRV as a potential therapeutic target. It may also shed light on why certain infected individuals experience disease relapse while others infected with the same parasitic strain do not. An interest in LRV-related virulence re-emerged when it was observed that mice infected with LRV1-bearing strains exhibited greater footpad swelling and higher parasite numbers compared to LRV-deficient strains [22]. They concluded that LRV1 in metastasizing parasites subverted the host immune response and promoted parasite persistence, increasing the severity MCL [22]. In humans, presence of LRV1 in clinical isolates of *L. braziliensis* and *L. guyanensis* correlated with significantly increased drug treatment failure and higher relapse rates compared to patients without LRV-infected parasites [23, 24, 25].

One reason for this increased disease severity is that when parasites containing LRV eventually die, viral dsRNA is released and recognized by toll-like receptor 3 (TLR-3) in host macrophages (Fig. 2). This induces pro-inflammatory cytokines and chemokines, specifically IL-6 and TNF-a, which are hallmarks of MCL and further exacerbate the disease [25, 26]. In addition, TLR-3 recognition of LRV1 induces miRNA expression. Out of 1179 miRNAs analyzed, only miRNA 155 (miR-155) was upregulated. MiR-155 plays a role in activating

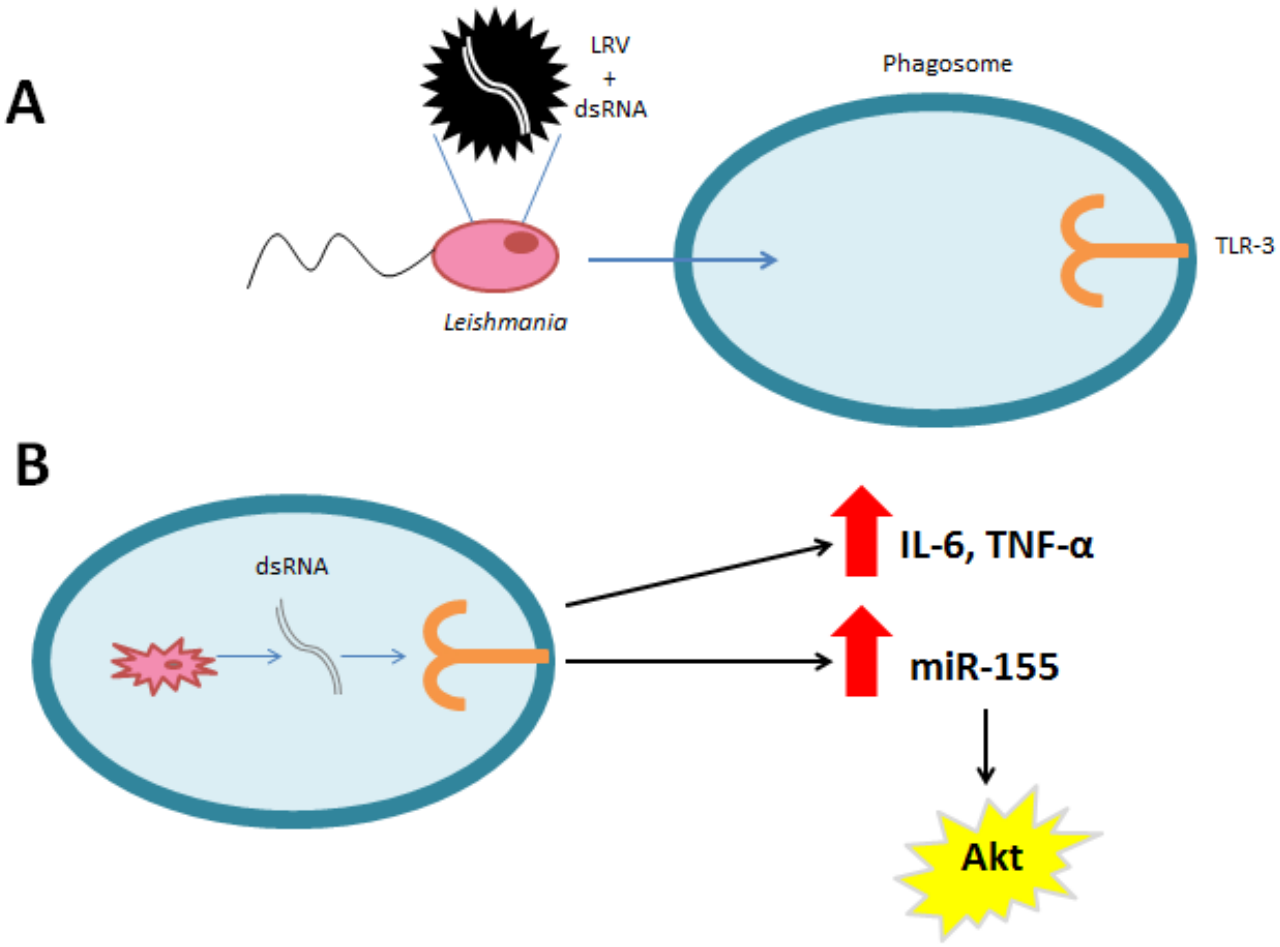

FIG. 2 Overview of how LRV contribute to *Leishmania*-associated disease pathogenesis. A. A *Leishmania* parasite containing LRV enter the macrophage through a phagosome. B. Some parasites eventually dies, LRV replication stops, dsRNA released in phagosome, and dsRNA is recognized by toll-like receptor 3 (TLR-3). This causes an increase in pro-inflammatory cytokines including IL-6 and TNF- α, and upregulation of miR-155 which activates the Akt pathway. This results in increased macrophage, and therefore parasite

the Akt-pathway, a signal transduction pathway that promotes macrophage survival, and therefore, parasite persistence. Moreover, miR-155 knockout mice experienced less footpad swelling and decreased parasite numbers compared to wild-type mice [27]. Interestingly, miR-155 has been implicated in exosomes of human melanoma cells and colorectal cancer [28, 29]. However, there have been no studies investigating which miRNAs are implicated in humans containing LRV-infected parasites.

Despite all these observations, it is important to know that some studies found no exclusive associations between LRV infection and disease severity [15, 30]. This may be due to surveying patients from small geographical areas in Brazil. Since those studies, more recent investigations that were described previously do support LRV exacerbating the pathogenesis of *Leishmania* infections [23, 24, 25].

Could LRVs be a potential target for therapeutics?

First-line anti-*Leishmania* drugs such as pentavalent antimonials are readily available, but many individuals experience adverse effects and drug resistance is on the rise [13]. Considering alternative therapies that specifically target LRV may reduce the aforementioned exacerbated symptoms and prevent disease relapse, a common problem seen in some LRV-positive populations [24]. In addition to regulating the RNAi machinery of parasites mentioned previously, there are several alternative approaches that can be considered (Fig. 3). While there has been extensive research on developing *Leishmania* vaccines, no candidate has been deemed effective enough for worldwide use [31]. However, an original vaccine that

targeted LRV inside parasites was developed to prevent virus-related complications. Mice were immunized with a recombinant LRV1 viral capsid with a T helper-1 polarizing adjuvant that resulted in significant reduction of lesion size and decreased parasite load [32]. More research needs to be done to see if a vaccine that targets LRV would be effective in humans. The greatest hurdle in achieving such therapies is ensuring that LRV-specific drugs are able to penetrate not only human macrophages, but also the parasites within those macrophages.

Another therapeutic option could target the LRV RDRP, which is essential for viral replication and maintenance. Recently, the molecules 2'-C-methyladenosine (2CMA) and 7-deaza-2'-C-methyladenosine (7d2CMA) were identified as potential inhibitors of the viral RDRP. Treatment resulted in 12-fold reduction if LRV1 capsid levels, and therefore viral number. The only limitation was the high concentration of 100 uM needed for inhibition to occur. Observing the effects of these analogs also determined that transmission of the virus is due to random segregation during parasite replication [33]. Before the RDRP can be targeted, it is important to note that the RDRP only becomes functional when it is cleaved from the capsid-RDRP fusion protein. This cleavage event ensures viral maturation and replication. It was determined that a *Leishmania*-encoded cysteine protease, potentially a cathepsin B or L, is responsible for this cleavage (Fig. 1). The molecules antipain dihydrochloride, E-64, leupeptin, and cystatin can inhibit proteolysis, but their effect on viral number has not been studied [34].

Finally, it is of interest to note that *Leishmania* parasites are known to produce exosomes [35]. They exhibit conserved mechanism for exosomal RNA packaging, mostly including non-coding RNAs such as tRNA, rRNA, and siRNA [36, 37]. As mentioned previously, since LRV reside in the cytoplasm, it would be worth investigating whether miRNAs such as miR-155 and other LRV-derived components can be found in these

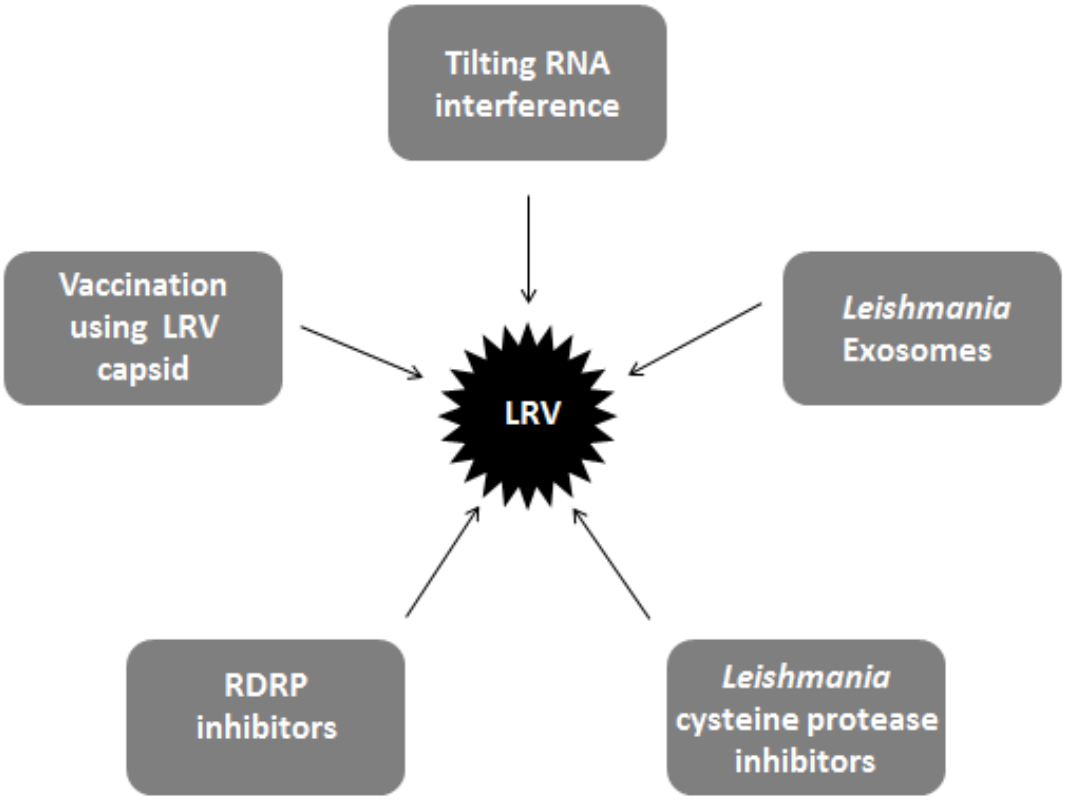

FIG. 3 Summary chart of possible targets against LRV that can be used for developing novel therapeutics. *Leishmania*'s RNA interference pathway can be manipulated to eliminate LRV, vaccination against the LRV capsid may prevent virus-related complications, RNA-dependent RNA polymerase can be inhibited using nucleoside analogs, *Leishmania*'s cysteine protease can be inhibited to prevent LRV pro-protein cleavage, and *Leishmania* exosomes can be examined for containing LRV components.

exosomes. In fact, unknown 'novel' transcripts that could not be attributed to being derived from either human host or parasite were identified and appeared to be specifically enriched in exosomes compared to total cell RNA [37]. Perhaps LRV-derived transcripts may be those unknown 'novel' transcripts that failed to be identified previously. Additionally, there are siRNA coding regions found in *L. braziliensis* exosomes, but not in *L. donovani* which is a strain not known to be infected by LRV [37]. Further research is still needed in order to fully elucidate any relationship between LRV and *Leishmania* exosomes.

SUMMARY AND CONCLUSION

Leishmaniasis is considered to be a disease of poverty, affecting over 12 million people worldwide [2]. *Leishmania* parasites are transmitted through the bites of infected sand flies, and being infected once does not necessarily confer protection against re-infection in the future [3]. The three main forms of Leishmaniasis include CL, MCL, and VL and if left untreated, can lead to severe stigma-forming deformities and death [4]. Some species of *Leishmania* are known to be infected with LRV, a non-enveloped virus containing a dsRNA genome that resides in the cytoplasm of the parasite that may play a role in disease exacerbation [8, 11]. By investigating the biological relationship between LRV and *Leishmania* parasites, there is evidence suggesting that RNAi pathways play a role in maintaining the virus in the cytoplasm [20]. It has also been hypothesized that presence of the virus can benefit the parasite through protection from oxidative stress and increasing parasite virulence [8]. Next, the effect LRV-positive parasites have on *Leishmania*-associated disease was investigated. Although some previous studies found no relationship between LRV and disease severity, recent investigations have established LRV as a contributor to Leishmaniasis exacerbation in humans [23, 24, 25]. Finally, amplification of *Leishmania* infections from LRV provides a unique target for diagnosis and clinical intervention, including LRV vaccination [32], direct-acting antivirals [33], cysteine protease inhibitors [34], and possibly *Leishmania* exosomes [37].

Due to a lack of properly controlled clinical trials, there is currently no consensus about which therapeutic options are best for treating Leishmaniasis [38]. Current treatments that were developed decades ago have been shown to be ineffective, causing drug resistance and adverse outcomes. In addition, with rising global temperatures and increase in international travel, it can be expected that rates of Leishmaniasis in North America and other developed countries will increase [39]. Since most therapies and management options for Leishmaniasis focus primarily on the parasite, it may be of interest to include LRV in the current disease model. Increasing our understanding of LRV infection can lead to the development of novel therapeutics that, when used in combination with established therapies, can reduce the severity of *Leishmania*-associated disease. Studying LRV is not only important for treating Leishmaniasis, but will also elucidate our understanding of other virus-parasite relationships that affect humans such as *Trichomonas vaginalis* in sexually-transmitted disease [40], *Giardia lamblia* in giardiasis [41], and *Babesia bovis* in red blood cell infections[42].

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