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Muco-cutaneous leishmaniasis in the New World: The ultimate subversion

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nfection by the human protozoan par

asite Leishmania can lead, depending

primarily on the parasite species, to either
cutaneous or mucocutaneous lesions, or
fatal generalized visceral infection. In
the New World, Leishmania (Viannia)
species can cause mucocutaneous leish-
maniasis (MCL). Clinical MCL involves
a strong hyper-inflammatory response
and parasitic dissemination (metastasis)
from a primary lesion to distant sites,
leading to destructive metastatic second-
ary lesions especially in the naso-
pharyngeal areas. Recently, we reported that
metastasizing, but not non-metastatic
strains of Leishmania (Viannia) guya-
nensis, have high burden of a non-seg-
mented dsRNA virus, Leishmania RNA
Virus (LRV). Viral dsRNA is sensed by
the host Toll-like Receptor 3 (TLR3)
thereby inducing a pro-inflammatory
response and exacerbating the disease.

Leishmania parasites exist as free-
living promastigotes in the sand fly vector.
Following differentiation to the infective
metacyclic form, parasites are deposited in
the skin of vertebrate host by the sand fly
bite. There promastigotes encounter sev-
eral host cell types including neutrophils,
dendritic cells and skin macrophages,
ultimately transiting and differentiating
into amastigotes which go on to replica-
cate within the phagolysosome of macro-
phages. Leishmania parasites must change
their metabolism and adapt themselves to
this new environment, and resist the oxi-
dative and other attacks activated by the
innate immune system of the host.

Leishmania species of the L. (Viannia)
subgenus, including mainly L. brazili-
ensis, L. guyanensis and L. panamensis, give
rise to CL but are also responsible for
MCL in up to 5–10% of cases. MCL is
clearly distinguishable from other cuta-
neous leishmaniases by its chronic, latent
and metastatic behavior. It is character-
ized by the dissemination of parasites and
secondary distant lesions development
(metastasis), especially in the oral and
nasopharyngeal areas of the face, and is
accompanied by extensive tissue destruc-
tion concomitant with high immune cell
infiltration, intense activation of inflam-
matory cells and parasite presence (albeit
at low levels).1 MCL can appear con-
comitantly, several years after the initial
infection, or even in patients without
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Additional host factors are thought to
play significant roles in determining the
clinical course of the disease as well.

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comitantly, several years after the initial
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healing and are more resistant to antimony
treatment than the primary lesions, with frequent relapses. The factors responsible for these relapses are not known; both the emergence of antimony resistance as well as differences among the infecting L. (Viannia) species and its virulence have been suggested.  

Reactivation of L. (Viannia) infection can occur following stress or immunosuppression at a site of local inflammation, raising the challenging question of how these factors interact with slow-growing or dormant parasites and the immune system to favor the reemergence of disease pathology. Thus far, little is known about the pathogenesis of MCL, especially factors involved in the immune response of the host, in the parasite dissemination, or in reactivation. It is likely that both L. (Viannia) oxidative stress and antimony resistance as well as genetic background of the host (e.g., particular alleles encoding TNFα, TNFβ, IL-6, CXCR1 and CCL2/MCP1) and particular species and/or isolate specific virulence factors are important parameters in the development of MCL. The definition of such factors and of the immune response of the host could be extremely useful, not only to predict the outcome of the disease and diagnosis tools, but also to understand the metastatic process and the inter-relationships of the parasite with its host. Currently the immunological mechanisms of protection and factors controlling relapse and avoiding reactivation of the infection are not well understood.

In MCL, the immune response to infection differs from that observed in other types of leishmaniasis. After a primary lesion, metastatic lesions can appear at other body sites, accompanied by tissue inflammation. This pathology has been associated with hyperactivity of the specific T cell immune response, with an exuberant, usually progressive, inflammatory response, that is not yet well understood. High levels of pro-inflammatory cytokines such as IFNγ and TNFα, and decreased responses to IL-10 and TGFβ, have been described in references 5 and 6. MCL development is associated with persistent immune responses having elevated pro-inflammatory mediator expression (higher levels of TNFα, CXCL10 and CCL4), with a mixed intra-lesional Th1/Th2 phenotype and elevated cytotoxic T cell activity. However, cells from MCL patients display impaired control of the immune response due to a defect in their ability to respond to IL-10. The production of the different inflammatory cytokines by the host is likely to increase cellular recruitment and contribute to the pathology of the disease. Thus by these and potentially other mechanisms, immunological hyperactivity contributes to MCL pathology. In turn measures diminishing uncontrolled inflammation could be one promising alternative or complement to the conventional drug therapy. Interestingly, treatment with the anti-inflammatory TNFα inhibitor pentoxyphylline in combination with antimony was effective in MCL patients unresponsive to antimonial therapy alone.

The susceptibility of the golden hamster to infection with species of the L. (Viannia) subgenus has provided a useful experimental model of mucocutaneous leishmaniasis. Hamsters infected with L. (Viannia) guyanensis iso- lated from human MCL lesions reproduce the metastatic phenotype with primary and metastatic lesion development. Different species and individual strains often differ in their propensity to cause hyperinflammatory cutaneous secondary metastatic lesions. Diversity was even seen within a single strain, as infective clones from the isolate of L. (Viannia) guyanensis (L.) (WHI/BR/78/M5313) were either highly metastatic, moderately metastatic or non-metastatic in the hamster model. Non-metastatic (M) clones formed lesions only at the site of inoculation and did not disseminate, whereas metastatic (M') clones gave rise to metastases in 60% to 80% of hamsters. The metastatic phenotype was stable over several passages and exacerbated by non-specific or immunologically induced inflammatory responses.

Molecular approaches have provided some insights into factors potentially playing a role in MCL. One of the most surprising difference between the genomes of L. braziliensis, L. major and L. infantum is the maintenance in L. braziliensis of genes encoding the RNA-mediated interference (RNAi) machinery, telomere-associated transposable elements and splice leader-associated SLACS. The RNAi machinery was recently shown to be functional in L. braziliensis and in L. guyanensis. A second remarkable feature the presence of Leishmania RNA viruses in many isolates of the L. (Viannia) species. These Leishmaniaviruses have been classified as Totiviridae, which includes RNA viruses detected in other protozoa such as Trichomonas vaginalis and Giardia lamblia and a variety of fungi including Saccharomyces cerevisiae. Totiviruses have a small unsegmented dsRNA genome between 5–7 kb in length, which encodes a capsid protein and a capsid-RNA dependent RNA polymerase (RDRP) fusion protein essential for replication.

The existence of cytosolic dsRNA viruses within Leishmania was first shown in two L. guyanensis strains: MHOM/SR81/CUMC1A and MHOM/BR/75/M4147. Currently Leishmania viruses are given arbitrary identifiers at the time of discovery, namely LRV1-1 and LRV1-4 for the viruses of the L. guyanensis CUMC-1 and L. guyanensis M4147 strain respectively. These two viruses share an overall 76% nucleotide sequence identity. LRVIs have since been identified in many isolates of New World Leishmania (L. braziliensis and L. guyanensis), but in just one isolate of Old World species L. major, which was showed sufficient nucleotide sequence divergence to be termed LRV2-1 (compare taxonomy browser at www.ncbi.nlm.nih.gov). LRV1 are present not only in laboratory strains of L. guyanensis and L. braziliensis but importantly also in biopsies and parasite cultures isolated from clinical cases of leishmaniasis. LRV-positive strains of Leishmania originated from both active and healing lesions or scars of patients living in Brazil, Peru, Guyana and Colombia. It was also shown that LRV1 can be occasionally be lost, thus far in just one line of L. guyanensis. Such isogenic lines are invaluable tools in evaluating the impact of LRVs specifically on the parasite and on the immune response.

The study of M and M' line is one approach that may shed light on what parameters underlie the metastatic phenotype and the hyperinflammatory response observed in MCL patients. To investigate whether the immune response of the host cell could serve as a readout assay we
performed preliminary experiments on the response of host macrophage infected by M' and M lines, keeping in mind that *L. (Viannia) guyanensis* could be infected by a dsRNA virus. Using a 15k cDNA microarray, we concluded that infection of bone marrow derived macrophages (BMMφ) with M' parasites induced 294 annotated differentially expressed genes when compared with BMMφ infected with non metastatic (M-) parasites that had at least a 1.5-fold change (p < 0.05). Given the importance of the immune response in *M. tuberculosis* infection, we were potentially harbor genetic differences when compared with BMMφ infected with *L. guyanensis* parasite dsRNA and specifically the LRV1 RNA.

showed only trace levels of LRV1 RNA other than the presence of LRV, we were able to show definitively that LRV1 was responsible for the cytokine responses by comparing isogenic *L. guyanensis* bearing or lacking LRV1–4. As before, BMMφ infected with *L. guyanensis* M4147 (LRV1high) produce significantly higher cytokine and chemokine than the isogenic virus-free *L. guyanensis* M4147 (LRV1low) in a TLR3-dependent manner. To analyze whether TLR3 and LRV1 play a role in leishmaniassiasis development in vivo, TLR3-/-, TLR7-/- and C57BL/6 wild-type (WT) mice were infected in the footpad. A significant decrease in footpad swelling peak and diminished parasite load could be observed in mice lacking TLR3 infected with *L. guyanensis* M4147 (LRV1low) parasites compared WT mice. No distinguishable difference in disease phenotype was observed in mice infected with *L. guyanensis* M4147 or *L. guyanensis* M4147 (LRV1high) parasites to compared WT mice. No distinguishable difference in disease phenotype was observed in mice infected with *L. guyanensis* or between WT and TLR7-/- infected mice with any parasite isolates.

Our results confirm that metastasizing *L. guyanensis* parasites derived from secondary lesions of hamsters or humans activate host BMMφs to secrete TNFα, IL-6, CCL5 and CXCL10, elevated levels of which have been associated with human MCL. These TLR3-dependent responses to infecting *L. guyanensis* parasites resulted in increased disease severity in mice. Our work provides evidence that LRV1 within metastasizing *L. guyanensis* parasites is recognized by the host to promote inflammation, and is involved in susceptibility to infection. One question is how the dsRNA found normally within the viral particle is able to interact with TLR3. We know from previous studies that 5–10% of the infecting promastigotes are killed during the first hours of infection. This killing process takes place in the phagolysosome where endosomal TLRs are present (Fig. 1). As recognition of LRV1 within the metastasizing *L. guyanensis* parasites arises early after infection, we hypothesize that the viral capsid is destroyed in the acidic milieu prevalent in the phagolysosome, leading to the release of LRV1 dsRNA, recognition by TLR3, and activation of the signaling cascade via TRIF, leading to the secretion of IFNβ (which could act in an autocrine loop on its receptor). In the next hours, inflammatory cytokines and chemokines are produced leading to the attraction of dendritic and T cells. Importantly, both the presence and levels of LRV are factors impacting the host immune response, as parasites bearing only low levels of LRV failed to activate TLR3. Finally, other nucleic acid derived motifs predicted to arise for parasite destruction may contribute to the host's response, as shown by the somewhat diminished cytokine and chemokine production in TLR7-/- BMMφ infected with *L. guyanensis* (M') parasites. As these effects were less than seen with TLR3-/- infections, and the TLR7-/- mice did not show any reduction in disease progression or pathology, the TLR3-dependent responses appear to dominate.

Our data show that *L. guyanensis* LRV1 induces a specific immune response via dsRNA binding to TLR3 and production of IFNβ early after infection, sufficient to modulate the initial immune response in a way that impairs rather than promotes killing. This is likely mediated through the production of pro-inflammatory chemokines and cytokines, thereby increasing the host's susceptibility to infection and likely parasite dissemination. Thus, *Leishmania* RNA virus, when present in New World *Leishmania* species, plays an important role in subverting the innate immune response. This newly recognized parasite factor could explain some of the differences observed in the different pathologies induced by Old World and New World species. Although the murine model is likely not fully representative of the pathology in humans, it is instrumental for evaluating the role of LRV in MCL. Of course, a role for LRVs in the pathology of MCL does not exclude the likelihood that other parasite or host factors play strong roles as well.

In the future, it will be necessary to investigate the mechanism whereby LRVs confer increased susceptibility to infection with *L. (Viannia)* parasites, and to analyze the critical role of cytokines and chemokines played in the host immune response. Key questions are how LRV1, and the associated hyper-inflammatory immune response conspire together to yield the metastatic phenotype, and whether anti-inflammatory drugs can prevent the development of chronic and secondary
Figure 1. Model of the signaling cascade in response to the release of dsRNA from LRV particles, production of IFNβ and secretion of proinflammatory cytokines and chemokines. The main pathway involved in this process is highlighted in bold. (1) Phagocytosis of LRV infected promastigotes by phagocytes (macrophages); (2) promastigotes differentiate into amastigotes, which reside in phagolysosomes; (3) death of some parasites (promastigotes and amastigotes), release of LRV and dsRNA, which binds to TLR3; (4) activation of TLR3 via TRIF and signal transmission via the transcription factors IRF3 and NFκB; (5) activation and secretion of IFNβ; (6) binding of IFNβ to its receptor and activation of pro-inflammatory cytokines and chemokines genes (autocrine loop); (7) synthesis and secretion of pro-inflammatory cytokines and chemokines such as TNFa, IL-6, CCL5 and CXCL10 leading to increased parasitemia and pathology.
metastatic lesions. These new results should help defining the role of LRV1 in MCL pathology and ultimately facilitate the introduction of new clinical strategies to fight MCL. Since most human MCL is caused by infections of L. braziliensis, it will be important to determine whether the LRV-dependent immune suppression observed with L. guyanensis isolates is also a key determinant of L. braziliensis MCL.

Our study on L. guyanensis has possible applications on the diagnosis, prognosis, treatment and diagnosis of MCL. As CL can emerge prior to MCL, or can recede followed by reactivation to MCL, the presence of LRV1 prior to MCL, or can recede followed by treatment like antimony. Since most human MCL is observed with pentoxifylline plus antimony. Am J Trop Med Hyg 2001; 65:87-9; PMID:11508396.


