Chapter

Wnt5A Signaling Antagonizes *Leishmania donovani* Infection

Arijit Chakraborty, Shreyasi Maity and Malini Sen

Abstract

Infection by the parasite *Leishmania donovani* causes Visceral Leishmaniasis, a deadly disease if not properly treated. *L. donovani* captures the immune defense potential of host macrophages. It disrupts immune homeostasis at least partly by inducing expression of anti-inflammatory cytokines and altering the cellular cytoskeletal framework, thereby creating a niche for its own survival. While inhibition of macrophage Wnt5A signaling promotes infection by the parasite, activation of Wnt5A signaling significantly dampens infection. Experimental evidence suggests that the antagonistic effect of Wnt5A signaling on parasite infection in macrophages may be on account of its influence on the actin cytoskeleton. Importantly, the inhibitory effect of Wnt5A on infection is sustained independent of drug sensitivity and resistance. Keeping in mind that drugs used to treat Visceral Leishmaniasis are quite toxic and the parasite develops drug resistance, revamping host Wnt5A signaling may be useful for eliminating infection independent of drug sensitivity or resistance.

Keywords: Wnt5A, *L. donovani*, macrophage, cytoskeleton, Rac1, actin

1. Introduction

Leishmaniasis is a group of neglected tropical zoonotic diseases caused by intracellular obligate parasites belonging to the genus *Leishmania*. The disease is endemic to about 60 countries and may turn out life threatening if not properly treated [1]. Clinical manifestations of the disease range from cutaneous lesions (as in Cutaneous Leishmaniasis) to visceral pathologies (as in Visceral Leishmaniasis) [2]. In this review we will focus on Visceral Leishmaniasis (VL), its causative organism *Leishmania donovani* and host immune response to *L. donovani* infection.

Like many other intracellular parasites, *L. donovani* has evolved a unique ability to sustain a parasitic mode of life. In VL, *L. donovani* resides and then proliferates mainly in the spleen, the liver and the bone marrow. This can cause splenomegaly, hepatomegaly, fever and gray discoloration of the skin—hence the name "kala azar" or "black disease". Associated hematological changes include a hemoglobin level of 7–10 g/dl, leukopenia, thrombocytopenia, pancytopenia, increased ESR and erythroid hyperplasia in the bone marrow and varying degrees of erythrophagocytosis, leukophagocytosis and granulomatous reactions. In some cases clotting abnormalities and deranged platelet function are also observed [3]. Few studies, furthermore, indicate inflammatory changes in the liver, which include hypertrophy and hyperplasia of Kupffer cells, diffused intralobular fibrosis, fibrosis of the wall of the central vein, and pericellular fibrosis [4].

L. donovani invades mostly host macrophages and also dendritic cells and neutrophils, hijacking the cellular machinery for its survival through adopting various strategies to evade immune response [5]. One of the strategies employed by the parasite is to inhibit phagolysosomal maturation by altering host cell lysosomal integrity [6]. *L. donovani* also alters host cytoskeletal dynamics [7, 8]. While various host and parasite specific factors are indicated in the regulation of host phagolysosomal maturation and cytoskeletal dynamics, how such regulation occurs during host pathogen interactions is unclear and a subject of intense study.

The first and the only line of treatment for Visceral Leishmaniasis is the use of chemotherapeutic agents like the pentavalent antimonials, amphotericin and paromomycin [9–11]. Although these drugs are effective for the treatment of *L. donovani* infection, drug administration is associated with serious side effects and other issues. First, these drugs are cytotoxic in nature thus causing serious damage to the hepatic health of the ailing patients [11–13]. Secondly the *L. donovani* parasites evolve drug resistant phenotypes decreasing drug efficacy. Lastly the drugs come at a high cost thereby increasing the economic burden of the already economically challenged individuals. To counteract these challenges researchers all over the world have tried to bring out effective vaccine candidates to counteract the onset of infection and progression of the disease. Both live attenuated parasites and recombinant antigens have been used as target candidates for vaccination. Some of the vaccine candidates are still in clinical trials and their efficacy waits to be tested [14].

In spite of the use of different treatment regimens for attenuating *L. donovani* infection, understanding of one's natural host immune response to *L. donovani* infection is important. Cells of the innate arm of the immune system, for example macrophages, neutrophils and dendritic cells play an integral part in clearance of the parasites. Neutrophils have been suggested to be recruited early during the course of infection [15]. The ability of the neutrophils to produce NETs (Nuclear Extracellular Traps) and oxidative burst often determines the progression of the disease. It has been reported that neutrophils from VL patients show dysregulated NET and ROS (Reactive Oxygen Species) production. Since neutrophils are short lived the next line of defense is taken up by the macrophages. Within the macrophages the parasite tries to subvert immune defense to create a favorable niche for itself. Dendritic cells have a unique role in sustaining immune response against *L. donovani* infection. During infection with the parasite dendritic cells produce IL-12p70, a key cytokine involved in priming and maintenance of microbicidal Th1 responses [16–19]. Several reports also indicate the importance of the complement system in immune defense. The complement system involves a large number of distinct thermolabile proteins, which react with one another to opsonize pathogens and trigger a series of inflammatory responses to combat infection [20]. It has been reported that host cell complement receptors like CR3, CR1, mannose receptors (MR) are responsible for internalization of *Leishmania* [21]. In 1912, W.S. Patton first observed fresh serum mediated lysis of *L. donovani*, suggesting the role of complement system in immune defense against *L. donovani*. Later studies suggest that *L. donovani* can activate complement via the classical and alternative pathways [22]. Complement receptors have also been shown to be involved during maturation of *L. donovani* containing phagosomes [23]. In light of the genetic studies carried out on mice, gene loci *Lsh* and *H2* may be linked to resistance toward *L. donovani* infection. Gene products like Nramp1 from the *Lsh* locus of chromosome 1 in mice influence natural resistance to *L. donovani*. The H2 locus which codes for the MHC (Major Histocompatibility Complex) molecules in mice also determines the fate of the disease in mice [24]. Other gene products involved in macrophage mediated immune defense, for example *Ifn*, *Tnf* and *Nos* play a very important role in clearance of the parasite. Upregulation of these gene products however, does not limit

the disease, suggesting that many other factors may be involved in the interplay of events that bolster immunity to infection [25–27]. Wnt signaling, which is known to regulate different aspects of cell polarity and coordination in tissue patterning during development is an important theme to study in this context [28–30], especially in view of the fact that different levels of cell polarity and coordination are intrinsic to the progression of innate immune responses [31].

Wnts comprise a family of about nineteen glycoprotein ligands that signal upon binding to the Frizzed (about twelve in number) and ROR1/ROR2 transmembrane cell surface receptors (**Figure 1**). While the Frizzled receptors bear resemblance to heterotrimeric G protein coupled receptors, ROR1/ROR2 bear tyrosine kinase like motifs [32]. Wnt signaling in general is divided into two broad categories, -canonical or β-catenin dependent and non-canonical or β-catenin independent. The transcriptional co-activator β-catenin promotes gene expression by LEF/TCF family transcription factors in response to canonical Wnt signaling and transcriptional activators such as NFκB, NFAT and AP1 are associated with non-canonical Wnt signaling. Although the ligands Wnt3A and Wnt5A are mostly considered as representatives of the canonical and noncanonical modes of Wnt signaling respectively, the mode of signaling is in reality governed by the receptor(s) receiving the Wnt signal and the associated adaptor molecule(s) transmitting it [33, 34]. Thus some

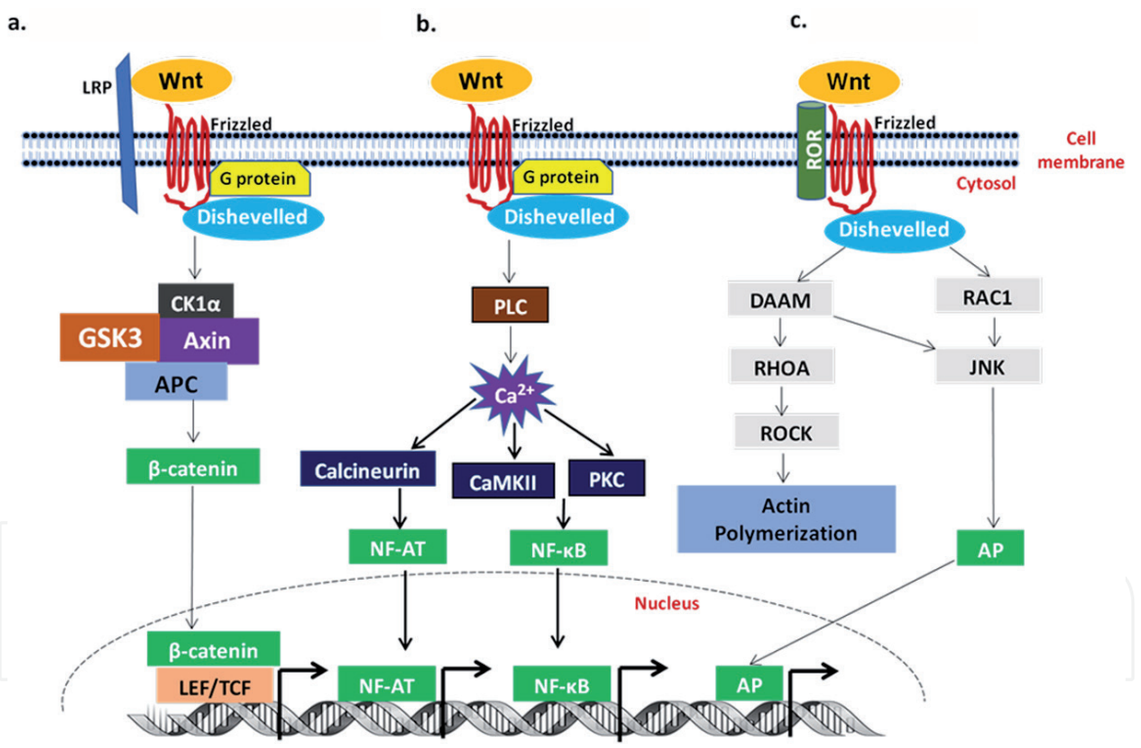

Figure 1.
Overview of Wnt signaling pathways. (a) β-Catenin dependent (the canonical pathway), (b) Wnt-Ca^{2+}-dependent pathway and (c) planar cell polarity pathway (PCP) (noncanonical pathways). (a) Wnt ligand binds to the Frizzled (Fz) family transmembrane receptors and coreceptor LRP leading to the activation of the signaling pathway through Disheveled (Dvl), Casein Kinase 1α (CK1α) and/or G proteins. This leads to inhibition of the destructive complex formation by GSK3, Axin and APC causing the stabilization, accumulation and subsequent nuclear translocation of β-catenin. β-Catenin forms an active complex with nuclear LEF and TCF promoting expression of LEF/TCF responsive genes. In the absence of Wnt ligands, APC complex can degrade β-catenin. (b) In noncanonical Wnt-Ca^{2+}-dependent pathway, Wnt-Fz interaction activates PLC via Disheveled and/or G-protein, which triggers the release of calcium ion. Increased level of calcium ion in turn activates calcineurin, CaMKII and PKC that help in nuclear translocation of NF-AT and NF-κB. (c) In noncanonical PCP pathway, Wnt may bind with ROR and Fz, which then activates Dvl. Activated Dvl can modulate actin cytoskeleton through DAAM, RHOA and ROCK signaling. Dvl also activates Rac1, which triggers JNK activity leading to nuclear translocation of AP and gene expression. LRP, low density lipoprotein receptor-related protein; GSK3 , glycogen synthase kinase 3; APC, adenomatous polyposis coli; LEF, lymphoid enhancer binding factor; TCF, T cell factor.

level of overlap between the two modes of signaling is quite frequent. Interestingly, the intracellular adaptor molecule Disheveled acts as a mediator of both β-catenin-dependent and β-catenin independent Wnt signaling. Heterotrimeric G proteins, which have been reported to couple with Frizzled receptors, add to the complexity of Wnt signaling [35, 36]. Whether heterotrimeric G proteins cooperate with Disheveled during canonical and non-canonical Wnt signaling is unclear. Despite some evidence of the involvement of lipid molecules such as cholesterol in switching Disheveled between the canonical and noncanonical modes of Wnt signaling [36], the molecular details of such presumed conformational switches remain undocumented.

In this chapter we will focus on the role of Wnt5A signaling in host immune response and its influence on *L. donovani* infection. Our present knowledge in the field of host parasite interaction limits us to the use of chemotherapeutic intervention to limit *L. donovani* infection. Therefore it is necessary to delve deep into understanding the cell biology of infection in the context of immune modulators like Wnt5A. Quite justifiably this kind of study will not only help us to understand host pathogen interaction in a better way but also aid in the formulation of novel therapeutic strategies through regulation of immune response.

2. Role of Wnt5A signaling in immune response

Primary studies on the association of Wnt5A signaling, a prototype for the non-canonical mode of Wnt signaling with immune response were focused on synovial fibroblasts from RA patients, where a correlation between Wnt5A signaling and proinflammatory cytokine expression was established [37]. Subsequent experimental evidence suggested that Wnt5A signaling may regulate IL-12 and IFN-γ expression by macrophages in the context of mycobacterial infection [38]. Similar studies carried out in bone marrow stromal cells and synovial fibroblasts also suggested that Wnt5A activates secretion of cytokines (IL-6, IL-1β) and chemokines (CCL2, CCL5, CXCL1 and CXCL5) upon interaction with Frizzled-5 and ROR, putative cell surface receptors to Wnt5A through NFκB activation [39].

It is now known that Wnt5A signaling plays an important role in stimulating microbial phagocytosis and sustenance of immune homeostasis through alteration in actin assembly and maintenance of an appropriate cytokine milieu [40–43]. The role of WNT5A signaling in stimulating microbial phagocytosis has been shown to be dependent on Rac GTPase and Rho GTPase activity in macrophages [41]. These GTPases are known to regulate cytoskeletal changes. Immune cells like macrophages, dendritic cells and other antigen presenting cells depend heavily on the cytoskeletal modulators to phagocytose and process various antigens so that they can be effectively presented to the T cells [44]. Alteration of cytoskeletal dynamics by Wnt5A signaling therein may be correlated with better assembly of NADPH oxidase subunits on phagosomal membranes and efficient production of microbicidal ROS [45, 46]. The increase in phagocytic activity and microbial killing by Wnt5A signaling may also be dependent upon the change in lipid raft organization of the macrophages in association with actin assembly [41–43]. Suppression of Wnt5A production by IWP2 (Inhibitor of Wnt production-2) accordingly abrogates both phagocytic activity and microbial killing in macrophages [40, 41, 47].

Several lines of evidence indicate that Wnt5A signaling is important for macrophage differentiation and survival. When stimulated with granulocyte monocyte-colony stimulator factor (GM-CSF), bone marrow from Wnt5A conditional knock-out mice show less potency to differentiate into $F4/80^+$ and $CD11b^+$ macrophages compared to bone marrow from control mice [48]. Furthermore,

Wnt5A-depleted BMDMs (Bone Marrow Derived Macrophages) show reduced expression of the anti-apoptotic molecules Bcl2 and Bcl-xl, and increased expression of the pro-apoptotic molecule Bax, leading to decreased survival of macrophages [40].

Depletion of steady state Wnt5A signaling reduces IFN-β and IFN-γ production by macrophages through inhibition of IκB kinase β (IKK2) activity. The reduction in IKK2 activity, which causes reduced IκB degradation and p65 (NFκB) nuclear translocation relates to decreased expression of immune regulators such as CD14 that are key components of immune responses during microbial infection [40]. Other reports suggest that depletion of Wnt5A signaling correlates with increase in microbial infections in mice [40, 43]. Thus Wnt5A signaling may be a crucial player in the sustenance of immune functions.

3. Intracellular life of *L. donovani*: the role of Wnt5A signaling therein

Intracellular parasitism is a strategy by which parasites build a niche to sustain within the host. Parasites such as *L. donovani* have developed sophisticated strategies to counteract host defense machinery. One such strategy to adapt to a parasitic mode of life is the dimorphic life cycle in *L. donovani*. *L. donovani* resides in the gut of its vector (Phlebotomine sand flies) in a flagellated infective form (promastigote). During a blood meal of the sand fly on its mammalian host these promastigotes are transferred to the blood, where they are phagocytosed by neutrophils and macrophages. While residing in macrophages, the parasites lose their flagella and transform to amastigotes. Amastigotes divide and thrive within the host, causing disease. The parasite life cycle is repeated with blood meal of sandflies from parasite-infected patients. The mechanism of entry of *L. donovani* into macrophages has been debated for long. It has been shown that host cell receptors (for example Complement receptors and Fcγ) influence *L. donovani* internalization and this interaction is partially dependent on the presence of promastigote flagella [49]. It is also documented that host cell membrane microdomains influence internalization of the parasite [5, 7, 50] . In order to hijack the cellular defense machinery *L. donovani* interacts with components of endoplasmic reticulum and the trans-Golgi network (TGN) [7, 51]. *L. donovani* containing vacuoles take up necessary nutrients like glucose, amino acid and essential ions like Fe^{2+} from the trans-Golgi network (TGN). These parasite harboring vacuoles/parasitophorous vacuoles (PV) while acquiring nutrients also disrupt the transport of different proteins to their designated vacuoles (endosomes/lysosomes) from the TGN and endoplasmic reticulum (ER), thus compromising their function [51]. The internalized parasite also delays the fusion of PV with the lysosomes through the action of lipophosphoglycan (LPG), a parasite derived molecule. Parasitophorous vacuoles accordingly become encapsulated with host F-actin, myosin and F-actin nucleating factors, thus producing a halo of F-actin surrounding the vacuole and inhibiting its lysosomal fusion [6]. The parasitophorous vacuole also expresses the early endosomal marker EEA1, and the small GTPases Rab5 and Rab 7 [52] preventing lysosomal degradation. The altered acidification of parasitophorous vacuoles is instrumental in promastigote to amastigote transformation and sustenance of infection [53]. Such remodeling of PV may lead to alteration in host lipid microdomains and alter assembly of the NADPH oxidase complex, which holds a key to microbial elimination through generation of microbiocidal Reactive Oxygen Species (ROS). The influence of Wnt5A signaling on actin cytoskeletal dynamics, organization of lipid raft microdomains and organelle polarity and assembly [30, 40, 41] suggests that host macrophages can potentially counteract the establishment and progression of *L. donovani* infection through Wnt5A signaling.

L. donovani infection is accompanied by increase in anti-inflammatory cytokine expression, which may help the intracellular amastigotes to build a safe niche within the macrophage. Increase in anti-inflammatory cytokines is often associated with decrease in production of ROS or Nitric oxide, which is unfavorable for amastigote growth [54–56]. Host macrophage Wnt5A signaling may be instrumental in attenuating the effect of anti-inflammatory cytokines by maintaining a proinflammatory cytokine signature [37, 40, 41].

4. Host defense against *Leishmania donovani* infection in the context of Wnt5A signaling

Macrophages, the primary sentinels of host immune response carry the potential to confront the challenge imposed by *L. donovani* infection. One important strategy adopted by macrophages to limit the pathogenicity from infection is production of ROS and nitric oxide, which are detrimental to the pathogen [57]. Interestingly the production of ROS and nitric oxide is often triggered by cytokine or chemokine signaling in the macrophages. IFN-γ is considered to be one of the major chemokines which bring about production of nitric oxide and ROS in macrophages [58]. IFN-γ null and IFN-γ receptor null mice carry enhanced infection load [50] substantiating the importance of IFN-γ in restraining infection. Overall, cytoskeletal actin modulations in association with organization of protein-lipid microdomains and transcriptional control of immune regulatory genes act in concert to antagonize the attempts of the parasite to settle into a favored niche within macrophages. These aspects of immune response to parasite infection are akin to the already known attributes of Wnt5A signaling as described previously in this chapter. Thus keeping in mind that *L. donovani* tries to subvert immune response by modulating lipid dynamics as well as cytoskeletal dynamics it is important to study the role of Wnt5A signaling in *L. donovani* infection.

Experimental evidence indicates that Wnt5A signaling and *L. donovani* infection are in mutual opposition. *In-vitro studies* have shown that the protein level of Wnt5A in *L. donovani* infected macrophages is significantly lower than that in the uninfected controls, with no significant change in Wnt5A mRNA level. The observed decrease in Wnt5A protein upon *L. donovani* infection is indicative of infection-induced suppression of Wnt5A signaling in the host macrophage. Protozoan parasites like *L. donovani*, are known to harbor a plethora of proteases (including metalloproteases) to counteract host immune response through cleavage of host proteins. GP63, one such well-studied metalloprotease, is expressed in significant amounts in both the promastigote and amastigote forms of the *L. donovani* parasite. Cleavage and destruction of host proteins such as AP1 by GP63 has already been reported [54]. Since, the reduction in Wnt5A protein upon infection of macrophages with *L. donovani* was inhibited by O-phenanthroline, a small molecule inhibitor of metalloproteases, it is possible that infection induced reduction in Wnt5A protein is brought about by the action of parasite specific metalloproteases like GP63. In view of the fact that Wnt5A signaling is known to boost immune homeostasis [40, 41], reduction in Wnt5A protein level may help the parasite to evade immune response. In contrary to our observation, the mRNA levels of Wnt5A are found to increase during mycobacterial and ehrlichial infection in macrophages [38, 59]. Changes in Wnt5A mRNA and protein levels may depend on the type and load of infection. Thus, further validation of proteomic data from various samples such as sera and spleen aspirates from *L. donovani* infected individuals will be needed to further analyze and understand the experimental findings.

On the basis of the understanding that the steady state level of Wnt5A signaling is significantly reduced during *L. donovani* infection, we hypothesized that revamping Wnt5A signaling in macrophages might have a debilitating effect on parasite load. Indeed we found that upon treating macrophages with recombinant Wnt5A prior to infection there is significant reduction in parasite load. The decrease in the parasite load was seen to be dose and time dependent. Interestingly, a decrease in parasite load was also seen when Wnt5A was exogenously added to infected macrophages, suggesting that there may be a therapeutic role of Wnt5A signaling during *L. donovani* infection [42]. Depletion of Wnt5A signaling through application of IWP-2 or transfection with Frizzled5 (putative receptor to Wnt5A) siRNA resulted in enhancement of infection by *L. donovani*, corroborating the importance of Wnt5A signaling in limiting *L. donovani* infection.

There is evidence that Wnt5A signaling mediated killing of *L. donovani* within the macrophages is brought about by change in Wnt5A induced cytoskeletal and membrane dynamics. Revamping host Wnt5A signaling by exogenous Wnt5A leads to reduction in parasite survival probably because the lay out for a self-sustaining parasite niche in the form of parasitophorous vacuole (PV) is not compatible with the cytoskeletal alterations and associated endosomal/lysosomal vesicle movements induced by Wnt5A. Enhanced endolysosomal fusion in infected macrophages occurred through Wnt5A signaling in infected cells as judged by live cell microscopy

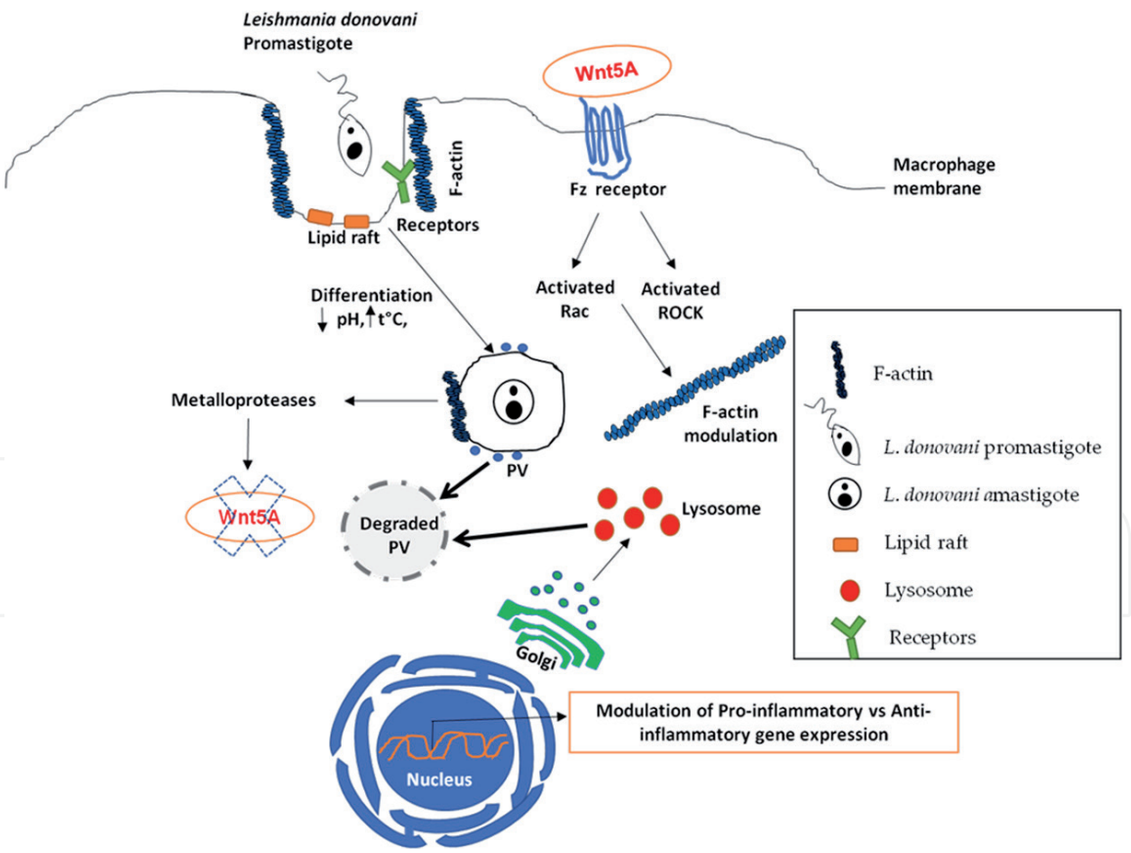

Figure 2.
Schematic representation of Wnt5A signaling-mediated inhibition of Leishmania donovani *infection.* Leishmania *promastigote interacts with the macrophage through different macrophage receptors. This interaction leads to the formation of parasite containing early endosome where several factors like low pH and increased temperature help in differentiation of the parasite into amastigote form. It secretes metalloproteases, which can degrade Wnt5A. Activated Wnt5A signaling modulates actin cytoskeleton and promotes phagolysosomal fusion leading to degradation of the parasite. Wnt5A signaling may also alter the expression of pro-inflammatory and anti-inflammatory cytokine genes thus promoting parasite clearance. PV, parasitophorous vacuole; ROCK , rho-associated coiled coil kinase; Rac, Ras-related C3 botulinum toxin substrate.*

using live cell dyes [42]. Electron microscopic (EM) images showed Wnt5A induced increased membranous vesicle fusions with PV in the infected cells. The EM images also revealed a low abundance of the PV upon Wnt5A treatment. The apparent membranous wrappings in degraded PV, as suggested by EM may be due to the formation of autophagosomes through fusion of PV with membranous aggregates during cytoskeletal movements [42]. One of the strategies that a host may adopt through Wnt5A signaling is laminate the parasitophorous vacuole so that the solutes cannot easily reach the parasite thereby slowly starving the parasite to death. While laminating the parasitophorous vacuole it may also ensure that the NADPH oxidase is well assembled so as to generate adequate amounts of ROS, which could lead to killing of parasites. The membrane lamination on the parasitophorous vacuoles through enhanced cytoskeletal dynamics could also lead to increased PV-lysosomal fusion thereby promoting rapid degradation of the parasite. Our study demonstrates that Wnt5A signaling mediated killing of *L. donovani* in macrophages is abrogated when inhibitors of cytoskeletal proteins like Rac1 GTPase and Rho kinase are used, thus implying that the effects of Wnt5A signaling on infection are at least partly mediated through the small molecular weight actin associated GTPases. The possible mechanism of Wnt5A signaling mediated parasite clearance is depicted in **Figure 2**.

It will be important to validate the effect of Wnt5A signaling on *L. donovani* infection *in vivo* and also check the load of *L. donovani* infection in Wnt5A heterozygous mice (Wnt5A null are lethal). Analysis of the cytokine milieu *in vivo* upon activation of Wnt5A signaling at the onset of infection will also provide useful information about the mechanism of Wnt5A induced containment of infection.

5. Conclusion

Wnt5A signaling maintains immune homeostasis [40]. If Wnt5A signaling is not sufficient a disturbed immune homeostasis could lead to adverse effects during *L. donovani* infection. Recently, it has been suggested that *L. donovani* infection associated with a skewed hematopoiesis program promotes the visceral disease [60]. Since Wnt5A signaling is involved in hematopoiesis [61], it is important to have a clear understanding of the role of Wnt5A directed hematopoiesis during *L. donovani* infection.

L. donovani through its evolution has undergone various changes to accommodate itself efficiently in its ever-changing environment. Often drugs have been rendered useless by the emergence of drug resistant strains. Therefore, it would be an efficient strategy to identify host cell factors, which act against these infections and revamp them. Our results indicate that host Wnt5A signaling restricts infection by both antimony drug sensitive and resistant *L. donovani* strains at least partly by prohibiting parasite niche formation within host macrophages. Interestingly, in a follow up study we found a similar kind of result with *Pseudomonas aeruginosa* or *Streptococcus pneumoniae*. These pathogenic bacteria degrade Wnt5A from the system and when Wnt5A is added exogenously the macrophages efficiently lower the bacterial load. The clearance of bacteria was found to happen through cytoskeletal reorganization and efficient formation of LC3B containing phagosome [43]. Recently high serum levels of the Wnt antagonist Dkk1 has been correlated with a predominantly Th2 phenotype during the onset of experimental cutaneous leishmaniasis [62]. Since Wnt5A supports a pro-inflammatory cytokine signature its potential for protection against parasite infection may also involve prevention of the predominantly Th2 signature that sustains infection. Thus Wnt5A signaling plays an important role in maintaining an innate immune readiness within macrophages for pathogenic onslaught.

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Conflict of interest

The authors declare that there is no conflict of interest.

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