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PI3K signaling in *Leishmania* infections

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Abstract

PI3K signaling plays a role in the host response to *Leishmania* infections. At the cellular level PI3K signaling is engaged by the parasite to control several cellular processes, which ensures parasite persistence. At the systemic level, there is evidence that recruitment of regulatory cells into lesions is impaired in the absence of robust PI3K signaling. In this mini-review the more recent studies that investigated the roles of PI3K signaling in *Leishmania* infections are discussed.

1. Overview of Phosphatidyl-Inositol 3-Kinase (PI3K) signaling

Amongst other head groups of phospholipids in membranes, the inositol head group plays an important role in signal transduction. Phosphorylation at positions 3 to 5 of the inositol ring transforms it into substrates to which lipid binding proteins are recruited and phosphorylated. These phosphorylated proteins in turn phosphorylate downstream molecules thus initiating a signal transduction cascade. Three classes of enzymes called phosphatidyl-inositol (PtdIns) 3 kinases phosphorylate the 3 position of the inositol ring (summarized in Table 1). These enzymes differ in their structure, their preferred substrate and the stimuli that activate them. The substrate of the Class III enzymes is an unphosphorylated inositol ring, which upon phosphorylation at the 3 position becomes PtdIns -3-phosphate (also known as PI3P). The substrates for the prototypic Class III enzyme, vps34, are primarily found on internal membranes that delimit organelles, which explains why they play important roles in vesicular trafficking (1). The Class II enzymes phosphorylate PtdIns - 4-phosphate (PtdIns(4)P) converting it to PtdIns 3,4 phosphate (PtdIns(3,4)P₂) (2). These enzymes are the least well understood of the 3 classes. The substrates for Class I enzymes are PtdIns 4,5-phosphate (also known as PIP₂) that are found predominantly on the plasma membrane; PIP₂ are phosphorylated to PtdIns 3,4,5 phosphate (PIP₃) (3). The Class I enzymes are further divided into 2 subclasses that differ in their overall structure and in the stimuli that activate them. The Class Ia enzymes function as heterodimers that include a catalytic subunit and a regulatory subunit. The catalytic subunit of 110kDa is encoded by 3 genes termed pik3ca (gene product is PI3K α), pik3cb (gene product is PI3K β), and pik3cd (gene product is PI3K δ). The regulatory or adaptor subunit of 85kDa is encoded by 4 genes

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Pik3r1, Pik3r2, Pik3r3 (gene products p85 α , p85 β and p85 γ ; alternative transcription initiation from Pik3r gives rise p55 α , p50 α and p55 γ). The Class Ia kinases are recruited via the Src homology domains on the regulatory subunit to receptor tyrosine kinases. These tyrosine kinases may be associated with growth hormone receptors or antigen receptors. The Class Ib kinases are composed of a single member, PI3K γ , which is homologous to the p110 catalytic subunit of the Class Ia enzymes. Unlike the Class Ia enzymes, Class Ib enzymes associate with adaptors different from the p85 subunit and are activated primarily by G-protein coupled receptors. Only the contributions of the Class I PI3kinases have been evaluated in *Leishmania* infections.

Phosphorylation of the 3 position of the inositol ring is also dependent on the action of phosphatases. The phosphatase and tensin homologue deleted on chromosome 10 (PTEN) dephosphorylates PtdIns at the 3 position by converting PI(3,4,5)P3 to PI(4,5)P2 (4). Removal of the 3-phosphate on the inositol ring down modulates PI3K mediated signaling. Another relevant phosphatase is the SH2 domain-containing inositol 5-phosphatase type 2 (SHIP2) that converts PI(3,4,5)P3 to PI(3,4,)P2 (5). The role of PTEN has been evaluated in *Leishmania* infections as well.

All three PtdIns substrates that are phosphorylated at position 3 of the inositol ring PI3P, PI(3,4,)P2 and PI(3,4,5)P3 recruit proteins with distinct lipid binding domains. Molecules with a pleckstrin homology domain such as the Ser/Thr kinase protein kinase B (PKB)/Akt and Tyr protein kinases BTK, bind to PI(3,4,5)P3 and PI(3,4,)P2 (3), while molecules with a PX homology domain or FYVE domain such as p40-phox or the early endosomal antigen 1 (EEA1), respectively bind to PI3P (Figure 1). Differential recruitment of these lipid binding proteins determines the signal transduction scheme and consequently the outcome of the activation of PI3K signaling.

2. PI3K signaling in *Leishmania* infections

In *Leishmania* infections the contributions of PI3K signaling to the host response has been evaluated by assessing the role of molecules at critical points of the signal transduction scheme. Some studies have evaluated the role of individual PI3K catalytic subunits or the regulatory subunits in *Leishmania* infections. More recent studies that have focused the p110 δ and p110 γ catalytic subunits have shown that impaired function of either of these enzymes results in limited *Leishmania* infections. Studies of the role of molecules at the next level of PI3K signaling have focused primarily on the role of PKB/Akt; activation of this molecule mediates parasite persistence by promoting cell survival, production of leishmanicidal responses such as NO and secretion of anti-inflammatory cytokines. Beyond PKB/Akt signaling some studies have evaluated the role of the target of rapamycin (mTOR) and glycogen synthase kinase. Finally, the role of the phosphatase PTEN has also been evaluated in *Leishmania* infections. In light of the recent review by Lambertz et al (2012) (6), this review will primarily focus on newer studies.

2.1 Role of the catalytic subunits of PI3K enzymes

The laboratories of Uzonna and Satoskar have each independently and together, explored the role of either p110 δ or p110 γ in *Leishmania* infections. They have exploited the availability

of mice deficient in either of these enzymes and also the availability of a specific inhibitor to p110 γ to explore how these molecules control the progress of experimental infections by several *Leishmania* species. The P110 δ catalytic subunit is expressed more abundantly by hematopoietic cells including B cells and T cells. A number of studies from Vanhaesebroeck's laboratory had shown that p110 δ plays essential roles in B cell and T cell development and in their activation via the antigen receptor (7, 8); moreover they showed that in mice in which p110 δ function was compromised by insertion of an inactivating knock-in mutation in the p110 δ (referred to as p110 δ (D910A)) the function of CD4⁺CD25⁺Foxp3⁺ regulatory T cells (T-regs) was altered. In light of a large number of studies that had established an essential role for CD4⁺ T cell subsets in resistance or susceptibility in the *L. major* experimental *Leishmania* model, Uzonu and colleagues (9,10) assessed the progress of *L. major* infections in the p110 δ (D910A) mice. Whether on 'resistance' or 'susceptible' backgrounds, *L. major* infections exhibited reduced lesion size and accelerated parasite clearance. In additional experiments that included adoptive transfer of enriched T-regs from wild-type mice into p110 δ (D910A) mice they showed that increased resistance was due to impaired expansion and effector functions of Tregs. In a follow-up study, Khadem et al (2014) (11) showed that infection of p110 δ (D910A) mice with *L. donovani* parasites, the causative agents of visceral leishmaniasis, also resulted in a limited infection course that presented as reduced splenomegaly and hepatomegaly and limited parasitemia in these organs. They also showed that in *L. donovani* infections as well, Treg expansion was impaired, which was interpreted to mean that loss of Treg function was the major determinant of resistance in the D910A mice infected with *L. donovani*. Another PI3K subunit, the p110 γ , which is the lone Class Ib PI3K catalytic has also been evaluated for its role in the progress of experimental *Leishmania* infections. The studies of Satoskar and colleagues were feasible because of the availability of mice in which the p110 γ gene had been deleted (p110 γ ^{-/-} mouse) and the availability of a specific inhibitor of p110 γ , AS-605240 (12,13). The course of infection with *L. mexicana*, a causative agent of cutaneous leishmaniasis, was found to be suppressed in the p110 γ ^{-/-} mice as compared to C57BL/6 wild type controls. Treatment of infected mice with AS-605240 had a comparable effect to the p110 γ ^{-/-} mice. The authors showed that limited infections in these mice was due in part to limited phagocytic uptake of *Leishmania* parasites by macrophages and neutrophils where loss of PI3K activity impairs phagocytosis. The limited infection course was also due to impaired secretion of T_H2 cytokines that otherwise promote susceptibility to leishmaniasis. Moreover, they showed that blockade of p110 γ ^{-/-} mediated signaling reduced the recruitment of CD4⁺ FoxP3⁺ Tregs to the infection lesion site. Taken together, these studies established an important role for PI3K signaling in *Leishmania* infections. It is plausible to propose that these molecules are great targets for the control of leishmaniasis. In light of the fact that small molecules such as AS-605240 have been and continue to be developed that specifically target the different PI3K subunits, it can be anticipated that studies of inhibitors to PI3K molecules will lead to the control of these infections.

2.2. Role of PKB/AKT in leishmaniasis

Amongst the PI3K membrane proximal transducers of signaling, PKB/Akt has received the greatest attention. PKB/Akt contains a pleckstrin homology domain through which it binds to PI(3,4,5)P₃ or PI(3,4) on membranes. There, PKB/Akt is phosphorylated at Thr308 by the

phosphoinositide-dependent kinase 1 (PDK1) and also by several kinases including the mammalian target of rapamycin (mTORC2) at Ser473. PKB/Akt has numerous downstream targets, which upon their phosphorylation, they promote several processes including metabolism, proliferation and cell survival. As stated earlier, Lambertz et al (2012) (6) presented a comprehensive review of studies that demonstrated the critical of PKB/Akt signaling in *Leishmania* infections. More recent studies include a report by Calegari-Silva et al (2015) (14) who evaluated the role of PKB/Akt in the inhibition of nitric oxide release in *L. amazonensis* infections. Specifically, they considered the role of PKB/Akt in the activation of NF κ B, which has been shown to control the transcription of the iNOS gene. Activation of NF κ B can either result in the translocation of p50 and p65 subunits into the nucleus where they positively regulate relevant gene expression or translocation of p50 homodimers instead, which results in suppression of gene expression. They showed that even upon incubation of cells with lipopolysaccharide (LPS) which is a potent activator of NO, the activation of PKB/Akt by *L. amazonensis* infection, resulted in the translocation into the nucleus of the inhibitory p50 subunits that form p50 homodimers in the nucleus. This led to the suppression of iNOS transcription and consequently NO wasn't produced in response to LPS in *L. amazonensis* infection. In another study, Vázquez-López et al (2015) (15) showed the effects of activation of PKB/Akt by *Leishmania* infection wasn't limited only to macrophage infections. They showed that in dendritic cells as well, *L. mexicana* parasites activate PKB/Akt to promote cell survival in response otherwise potent apoptotic inducers, while suppressing the MAP Kinase p38 and JNK.

The role of PKB/Akt in the production of or response to cytokines in *Leishmania* infections is complex. Recently, a study by Mukherjee et al (2013) (16) reported on an intriguing role for PKB/Akt in the resistance of *L. donovani* parasites to antimony. They showed that antimony resistance by *L. donovani* (Sb^RLD) had a two-stage mechanism of resistance. In the first stage, Sb^RLD parasites induce the production of IL-10. Interestingly, production of IL-10 was attributable to the presence of a unique glycan with N-acetylgalactosamine as a terminal sugar expressed exclusively by resistant parasites. Moreover, this production of IL-10 wasn't dependent on PKB/Akt activation. To fully achieve resistance to antimony these parasites induced the increased synthesis of the multi-drug resistance gene (MDR-1), which presumably is necessary for drug efflux from infected cells. They determined that the upregulation of MDR-1 was PI3K/Akt dependent and was largely due to IL-10 that acted in an autocrine fashion. The regulation of IL-10 production by *Leishmania* is complex, which might explain why several studies have arrived at different conclusions. In support for a role of PI3K/PKB/Akt in the regulation of IL-10 production in *Leishmania* infections, Nandan et al (2012) (17) evaluated *L. donovani* infections of primary human monocytes as well human and mouse macrophage cell lines. They showed that infection with *L. donovani* activated PI3K signaling as evidenced by the appearance of phosphorylated PKB/Akt. Activated PKB/Akt resulted in the phosphorylation of glycogen synthase kinase β (GSK β), which relieves its suppression of CREB, an IL-10 transcription factor. IL-10 production in infected cells was inhibited by agents that suppress PI3K/PKB/Akt signaling. This confirmed the observations of Ohtani et al (2008) (18) that LPS induced IL-10 production was suppressed by inhibitors of PI3K signaling that prevent phosphorylation of GSK β , which had a comparable effect as lithium chloride (LiCl) and other direct inhibitors of GSK β function.

Ruhland and Kima, (2009) (19) had also shown that PKB/Akt activation results in suppression of the production of pro-inflammatory cytokines such as IL-12 while promoting the production of anti-inflammatory cytokines such as IL-10.

2.3. Mammalian target of rapamycin (mTOR) in *Leishmania* infections

mTOR is composed of two complexes that exhibit important differences. There is consensus that activation of the mTORC1-mediated signaling pathway is PKB/Akt dependent. Once activated, this complex in turn phosphorylates ribosomal protein S6 kinase (pS6k), eukaryotic initiation factor 4E (eIF4E) and eukaryotic initiation factor binding protein 1(4EBP1), which promote protein translation and cell growth (20). The activation scheme of mTORC2 is less certain; however, this complex apparently plays a role in the full activation of PKB/Akt in light of evidence that it phosphorylates PKB/Akt at Ser473, which promotes cell survival and other functions ascribed to PKB/Akt. Interestingly, mTORC2 is insensitive to rapamycin. Consistent with the fact that mTORC1 can be placed downstream in the PI3K/PKB/Akt signaling pathway, a few reports have provided evidence of mTORC1's role in cytokine production during *Leishmania* infections (18, 21). These studies showed that *Leishmania* infection mediates suppression of IL-12 production in large part through activation of mTORC1, which also activates IL-10 production. Evidence for a more intriguing effect role of mTORC1 in *Leishmania* infections was presented by Jaramillo et al (2011) (22). They showed that *Leishmania* inhibit host cell protein synthesis by directly targeting mTORC1. Specifically, parasite expressed GP63 cleaves mTORC1, which results in its inactivation and limited phosphorylation of S6k, eIF4E and 4EBP1. This conclusion was arrived at using mutant parasites in which the GP63 gene had been deleted as well as mice in which the 4EBP1/2 genes were deleted. This later observation suggests that *Leishmania* control host cell processes not only by modulating the activation of signaling components but also by targeting key intermediates directly for degradation.

3. Outstanding unresolved observations

1. Although it is known that p110 δ are activated by receptor tyrosine kinases, while p110 γ are activated by GPCRs, it is not known which parasite molecules on promastigotes or amastigotes forms initiate these responses.
2. The preferential sensitivity of Tregs in *Leishmania* infections to loss of PI3K signaling is not explained
3. Most of the studies that have evaluated PI3K signaling in *Leishmania* infections have shown that activation of this pathway is sustained well after (days) parasite entry. There isn't presently a mechanistic explanation for how this is achieved during a *Leishmania* infection. PKB/AKT activation is known to occur at the plasma membrane where PI3K enzymes catalyze the production of PI(3,4)P and PI(3,4,5)P following the activation of a receptor tyrosine kinase or a G-protein coupled receptor as discussed above. Sustained PKB/AKT activation in *Leishmania* infections suggests that there is an intracellular membrane that may have components

of the plasma membrane or complimentary components that are conducive to PKB/AKT activation.

4. In their study Calegari-Silva et al (2015) (14) observed a reduction in infection in cells in which PKB/AKT levels were suppressed by siRNA treatment. This suggests that *Leishmania* survival in macrophages, even in the absence of any additional activation stimulus is dependent on activation of PKB/AKT. This suggests that modulation of PKB/AKT levels could be a productive strategy to control these infections.

Resolution of these questions will enhance our understanding of the role of this critical signaling pathway

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Highlights

- Disruption of PI3K catalytic subunits results in suppressed *Leishmania* infections.
- PKB/Akt activation by *Leishmania* leads inhibition of NO production.
- PKB/Akt activation is sustained in *Leishmania* infections, which implies a non-conventional activation scheme.

Figure 1.

Phosphoinositide metabolism and PI3K signal transduction scheme.

After phagocytic receptor engagement phosphoinositides are phosphorylated by several enzymes. Of interest in this discussion are the enzymes that phosphorylate the 3 position of the inositol ring. The class I and class II kinases promote the production of PtdIns(3,4)P and PtdIns(3,4,5). PKB/Akt is recruited to and binds to PtdIns(3,4)P and PtdIns(3,4,5)P at which point it is phosphorylated by PDK1 and mTORC2. Phosphorylated PKB/Akt has numerous direct targets, some of which are shown. Other downstream targets that PKB/Akt are shown including mTORC1.

Table 1**PI3Kinases**

Enzyme	Gene product	recruitment	Substrate-product
Class I			
Class Ia	110kDa	via src homology domain on regulatory subunit to hormone or antigen receptors	PtdIns(4,5)P – PtdIns(3,4,5)P
Catalytic subunits			
Pik3ca			
Pik3cb			
Pik3cd			
Regulatory subunits	85kDa		
Pik3r1	P85α		
Pik3r2	P85β		
Pik3r (Alternative Transcription Initiation)	P85γ		
	P55α		
	P50α		
	P55γ		
Class Ib			
Catalytic subunit Pi3kcg (PI3kγ)	P110	Activated by G-protein coupled receptors	PtdIns(4,5)P – PtgIns (3,4,5)P
Adaptor subunit			
Pik3R5	P101		
Pik3R6	P84		
Class II			
Pi3kC2α	190Kda		
Pi3kC2β	190kDa	?	PtdIns and PtdIns(4)P – Ptdins(3,4)P
Pi3kC2γ	170kDa		
Class III			
Pik3R4	100kDa		
Hvps34 (catalytic subunit)	150kDa	Recruited to endocytic pathway proteins	PtdIns – PtdIns(3)P –

References: **Fry, M. J.** (2001), *Hawkins et al.* (2006)