Microreview

The *Leishmania*–macrophage interaction: a metabolic perspective

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Summary
Protozoan parasites belonging to the genus *Leishmania* exhibit a pronounced tropism for macrophages although they have the capacity to infect a variety of other phagocytic and non-phagocytic mammalian cells. Unlike most other intramacrophage pathogens, the major proliferative stage of *Leishmania* resides in the mature phagolysosomes of these host cells. In this review we highlight some of the strategies utilized by the intracellular amastigote stage of *Leishmania* to survive in this compartment. Remarkably, and in contrast to many other intracellular pathogens, *Leishmania* amastigotes have a minimalist surface glycocalyx which may facilitate uptake of essential lipids and promote exposure of phospholipids required for phagocytosis via macrophage apoptotic cell receptors. *Leishmania* amastigotes also differ from many other intracellular pathogens in having complex nutritional requirements which must be scavenged from the host cell. Amino acids and polyamines appear to be important carbon sources and growth-limiting nutrients, respectively, and their availability to intracellular amastigotes may be regulated by the activation state of host macrophages. Metabolic processes in both the parasite and host cell may thus be crucial determinants of disease outcome.

Introduction
*Leishmania* spp. are protozoan parasites that cause a spectrum of diseases in humans, ranging from self-limiting cutaneous infections to disseminating diffuse cutaneous, mucocutaneous and visceral leishmaniasis. Control of this disease, which affects more than 20 million people worldwide, has been hampered by the absence of a vaccine, limitation with frontline drugs and the increased transmission as a result of co-infections with HIV (Croft *et al*., 2006). Infection of the mammalian host is initiated by flagellated promastigotes, which develop within the midgut of the sandfly vector, and are deposited into the skin during a sandfly bloodmeal. Promastigotes are phagocytosed by macrophages, either directly or after infection of neutrophils initially recruited to the sandfly bite (van Zandbergen *et al*., 2004). Promastigotes are targeted to vacuolar compartments in the macrophage that have the characteristics of mature phagolysosomes, where they differentiate to the smaller aflagellated amastigote stage. Amastigotes proliferate by binary cell division and can spread to other macrophages as well as some other phagocytic (i.e. dendritic cells) and no-professional phagocytic (i.e. fibroblasts) cells. The capacity of these pathogens to target and replicate within the mature phagolysosome compartment is remarkable. With the exception of the Gram-negative bacterium *Coxiella burnetti* (Voth and Heinzen, 2007), no other microbial pathogen resides throughout their replicative cycle in this compartment. In this review we highlight aspects of the surface chemistry and metabolism of intracellular amastigote stages that are required for *Leishmania* survival in the macrophage. The influence of macrophage metabolism on nutrient availability and intracellular growth is also discussed.

Stage-specific modulation and functions of the *Leishmania* surface glycocalyx
The promastigote stages of *Leishmania* are coated by a thick glycocalyx composed of glycosylphosphatidylinositol (GPI)-anchored proteins, the GPI-anchored phosphoglycan, lipophosphoglycan (LPG) and a family of free GPI glycolipids termed glycoinositolphospholipids (GIPLs) (Naderer *et al*., 2004). Analysis of *Leishmania* mutants lacking individual or multiple surface components indicates that only LPG plays a critical role in macrophage infection, while loss of other surface components has
relatively little affect on promastigote virulence in the mammalian host (McConville et al., 2007). Analysis of the LPG-deficient Leishmania major mutant, Δlpg1, indicated that LPG protects promastigotes from a transient rise in reactive oxygen species (ROS) generated during phagocytosis (Spath et al., 2003). LPG may scavenge ROS directly and/or inhibit macrophage signalling pathways and the recruitment of the NADPH oxidase to the phagosome membrane (Lodge et al., 2006). The latter possibility is supported by a recent study that showed that the secreted proteophosphoglycans (PPGs) of L. major promastigotes (that are elaborated with similar phosphoglycans to LPG) do not compensate for loss of LPG (Capul et al., 2007), suggesting that membrane-bound LPG is essential for infectivity of L. major promastigotes. While most ex vivo studies have focused on the role of LPG in promastigote–macrophage interaction, it is likely that LPG has an equal if not more important role in enabling promastigotes to survive in neutrophils, which may be the major host cell in the initial stages of infection (van Zandbergen et al., 2004; Gueirard et al., 2007). Intriguingly, L. mexicana promastigotes do not require LPG for virulence (Ilg, 2000) and LPG expression is downregulated in infective metacyclic promastigote stages (Ralton et al., 2003). L. mexicana promastigotes and amastigotes are more resistant to oxidative stress and reactive nitrosative species than either L. major or L. donovani (Wanasen et al., 2007), possibly explaining these species-specific differences. Finally, a large number of studies have indicated that LPG is the primary ligand for multiple macrophage opsonic and pattern recognition receptors (Naderer et al., 2004). However, the physiological significance of these findings has been questioned by the finding that the initial uptake of L. major promastigotes by neutrophils is promoted by the presence of dead parasites in the inoculum (van Zandbergen et al., 2006). The dead parasites contain high levels of surface exposed phosphatidylycerine which may promote the uptake of both live and dead promastigotes via neutrophil receptors that are normally involved in apoptotic cell clearance (van Zandbergen et al., 2006). Uptake via the host cell apoptotic receptors prevents the activation of microbicidal responses (as well as pro-inflammatory cytokine release) and may be critical for promastigote survival in neutrophils (Gueirard et al., 2007; van Zandbergen et al., 2007).

In contrast to promastigote stages, intracellular amastigotes downregulate the expression of LPG (and other surface macromolecules) and lack a conspicuous surface coat (Naderer et al., 2004), although they retain a glycosylphosphatidylinositol (GPI)-anchored proteophosphoglycan (McConville and Blackwell, 1991; Winter et al., 1994; Naderer et al., 2004). This chimeric glycosylphosphatidylinositol–anchored proteophosphoglycan may shield membrane-embedded nutrient transporters and other polytopic membrane proteins from lysosomal hydrolases, minimizing the release of parasitically encoded peptides that could be presented to the host immune system by MHC class II proteins. Remarkably, expression of mature GPIs is not essential for the virulence of either L. major or L. mexicana amastigotes (Garami and Ilg, 2001a; Zufferey et al., 2003; Naderer et al., 2004), suggesting that their function(s) may be compensated for by the equally abundant host glycosphingolipids. Leishmania amastigotes also synthesize inositol phosphoceramide (IPC), using sphingolipid bases salvaged from the host (Zhang et al., 2005), and this abundant phospholipid may also complement the functions of the GPIs. The plasma membrane of Leishmania amastigotes is unusual in several other respects. It contains relatively high levels of externally exposed phosphatidylycerine that may provide a mechanism for entering host cells via apoptotic cell receptors without activating microbicidal processes or pro-inflammatory responses (Wanderley et al., 2006). Whether the surface exposed phosphatidylycerine is parasite- or host-derived has not been investigated. Lesion-derived amastigotes are also heavily opsonized with host IgG antibodies (Kane and Mosser, 2001). Opsonization with IgG promotes amastigote uptake via the macrophage Fc receptors, and the release of anti-inflammatory cytokines such as IL-10 (Kane and Mosser, 2001). As described below, IL-10 induces changes in macrophage microbicidal responses and metabolism that are favourable for amastigote proliferation. The surface epitope(s) recognized by these opsonic antibodies has not been defined, although the abundant amastin proteins (a polymorphic family of proteins with four predicted transmembrane domains and two exoplasmic loops (Rochette et al., 2005) are possible candidates.

Metabolism and nutrient environment in the macrophage phagolysosome

Most species of Leishmania proliferate within individual, tight-fitting vacuoles, although amastigotes of the L. mexicana complex (L. mexicana, L. amazonensis and L. pifanoi) reside within large communal vacuoles (Antoine et al., 1998) (Fig. 1). These vacuoles have the characteristics of mature phagolysosomes and can fuse with late endocytic vesicles, phagosomes and autophagosomes, suggesting that there is a continuous flux of low molecular weight metabolites and macromolecules into the lumen of this compartment (Antoine et al., 1998; Burchmore and Barrett, 2001). Based on the known and predicted auxotrophies of wild-type Leishmania (McConville et al., 2007; Oppenheim and Coombs, 2007), as well as the in vivo growth phenotype of metabolic mutants, it is likely that the phagolysosome lumen contains a variety of carbon sources and essential nutrients, but is hexose poor (Table 1). L. major and L. mexicana mutants lacking
enzymes required for gluconeogenesis, or the de novo synthesis of inositol and mannose, respectively, are poorly virulent in mice (Garami and Ilg, 2001a; Ilg, 2002; Naderer et al., 2006), suggesting that the morphologically distinct vacuoles occupied by these species are hexose-depleted. Paradoxically, a L. mexicana mutant lacking three glucose transporters is also highly attenuated in ex vivo macrophage infection studies (Burchmore et al., 2003), suggesting that gluconeogenesis is not sufficient to supply all the hexose requirements of amastigotes. This requirement for hexose synthesis/uptake may be linked to the need for the pentose phosphate pathway [required for regeneration of NADPH and precursors for RNA and DNA synthesis (Maugeri et al., 2003)] and/or the production of intracellular mannan (linear oligomers of β1–2mannose), the major short-term energy reserve of these parasites (Sernee et al., 2006). The possibility that the latter pathway of carbohydrate metabolism is required for amastigote growth is supported by the finding that Leishmania mutants with defects in mannose metabolism are avirulent in macrophage and mice (Garami and Ilg, 2001b).

Table 1. Nutrient composition of Leishmania-occupied phagolysosome.

| Metabolite         | Level* | Evidence                                                                 | Reference                  |
|--------------------|--------|--------------------------------------------------------------------------|----------------------------|
| Carbohydrates      |        |                                                                          |                            |
| Hexoses            | Limiting | L. major Δfbp proliferate slowly in macrophages or mice                 | Naderer et al. (2006)      |
| Mannose            | Limiting | L. mexicana Δpmi unable to synthesize mannoglycoconjugates              | Ilg (2002)                 |
| myo-inositol       | Limiting | L. mexicana Δino1 avirulent in macrophages and mice                      |                               |
| Amino acids        |        |                                                                          |                            |
| Arg, His, Ile, Leu, Lys, Phe, Trp, Tyr, Val AldoMet, Met Polyamines | Not limiting | WT parasites auxotrophic for these amino acids                       | McConville et al. (2007)    |
| Ornithine          | Not limiting | L. major Δmthfr virulent in macrophages and mice                        | Vickers et al. (2006)       |
| Essential vitamins |        |                                                                          |                            |
| Folic acid, bipterin, pantothenate, pyridoxine biotin, thiamine, haeme | Not limiting | WT parasites auxotrophic for these vitamins, although a pathway for ascorbic acid predicted | Opperdooes and Coombs (2007) Ouellette et al. (2002) |
| Purines            |        |                                                                          |                            |
| Nucleobase/tides   | Not limiting | WT parasites auxotrophic for all purines                               | Landfear (2001)             |
| Pyrimidines        | Limiting | L. major thymidine auxotroph (Δdhfr-ts) has attenuated virulence in macrophages and mice | Ouellette et al. (2002)     |
| Thymidine          |        |                                                                          |                            |
| Lipids             |        |                                                                          |                            |
| Sphingolipids      |        |                                                                          |                            |
| ethanolamine       | Not limiting | L. major amastigote auxotrophic for sphingosine and/or ethanolamine. L. major Δspt2 and Δspl mutant virulent in mice | Zhang et al. (2007)         |

a. A nutrient is considered non-limiting if it is essential for wild-type (WT) parasites, or if Leishmania mutants auxotrophic for that nutrient grow with similar kinetics to WT parasites in macrophages or susceptible mice. A nutrient is considered limiting if mutant auxotrophs exhibit reduced growth in macrophages and/or murine infections.
b. Genes encoding following enzymes: dhfr-ts, dihydrofolate reductase-thymidylate reductase; fbp, fructose 1,6-bis phosphatase; arg, arginase; pmi, phosphomannose isomerase; ino1, myo-inositol phosphate synthase; mthfr, methylenetetrahydrofolate reductase, spl, sphingosine-1-phosphate lyase; spt2, serine palmitoyltransferase.

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Leishmania are auxotrophic for many amino acids and intracellular stages must scavenge their essential amino acid needs from the phagolysosome (McConville et al., 2007). In addition to their use in protein and polyamine biosynthesis, amino acids may be a major carbon source for amastigotes. The fact that Leishmania mutants with additional amino acid auxotrophies grow well in macrophages indicates that amastigotes can salvage all of their amino acid requirements from the phagolysosome (Table 1). Free amino acids are taken up by a large family of amino acid permeases, several of which have been shown to be upregulated in amastigote stages (Geraldo et al., 2006). Amastigotes also upregulate the expression of lysosomal cysteine proteases and endocytose host proteins from the lumen or limiting membrane of the phagolysosome, providing an alternative source of amino acids (Besteiro et al., 2007). Leishmania mutants lacking the CpB and CpA classes of lysosomal cysteine proteases are attenuated in their virulence supporting a role for lysosomal degradation in nutrient acquisition (Cameron et al., 2004; Besteiro et al., 2007). A shift toward increased amino acid metabolism and mitochondrial respiration in amastigote stage might lead to elevated levels endogenous ROS production via leakage of superoxide from the electron transport chain. Indeed, Leishmania mutants lacking single alleles of the glycosomal superoxide dismutase or trypanothione reductase are severely attenuated in their virulence (Tovar et al., 1998; Plewes et al., 2003), while increased expression of peroxiredoxins promotes parasite survival in macrophages (Barr and Gedamu, 2003). A slow growth phenotype would reduce respiratory ROS production and may be beneficial in such an environment. While there is very little information on the growth rate of Leishmania amastigotes in vivo, it is notable that some mutant strains selected for rapid growth are poorly virulent in mice (Hoyer et al., 2004).

Leishmania amastigotes may also utilize fatty acids as a carbon source. The Leishmania genomes are predicted to contain mitochondrial and glycosomal-targeted enzymes that can catabolize fatty acids to acetyl-CoA (Ivens et al., 2005) and fatty acid β-oxidation appears to be elevated in lesion-derived amastigotes (Hart and Coombs, 1982; Rosenzweig et al., 2007). Interestingly, there is no evidence that acetyl-CoA feeds into the TCA cycle (Naderer et al., 2006), and in the absence of an obvious glyoxylate shunt, acetyl-CoA cannot be used to synthesize sugars (McConville et al., 2007). These observations raise the possibility that acetyl-CoA is hydrolyzed to acetate via mitochondrial acetyl-CoA synthase or succinyl-CoA ligase (Oppendoes and Coombs, 2007). Although the yield of ATP from this pathway is low, acetyl-CoA hydrolysis bypasses the NADH-generating steps in the TCA cycle and may therefore minimize electron transport chain activity and oxidative stress in amastigote stages. As mentioned previously, complex host lipids can be intercalated into the plasma membrane of amastigotes and also used as precursors for parasite synthesized sphingolipids, such as IPC (McConville and Blackwell, 1991; Winter et al., 1994; Zhang et al., 2005). Unusually, Leishmania promastigotes utilize de novo synthesized and salvaged sphingosine as a source of ethanolamine for phosphatidylethanolamine biosynthesis (Zhang et al., 2007). This pathway, like the de novo sphingosine biosynthesis pathway, is downregulated amastigotes, suggesting that the intracellular stages salvage all of their ethanolamine and/or phosphatidylethanolamine requirements from the host (Zhang et al., 2007) (Table 1).

Leishmania must scavenge other essential nutrients (purines, haeme, vitamins) and cations (iron, magnesium) from the phagolysosome (Burchmore and Barrett, 2001; McConville et al., 2007). Most of the metabolite transporters are proton-symporters that utilize the low pH of the phagolysosome to drive high affinity uptake (Burchmore and Barrett, 2001). Gene deletion studies have shown that some of these nutrient uptake systems are highly redundant, allowing the exploitation of multiple forms of the same metabolite. For example, the purine requirements of amastigotes can be satisfied through the uptake of either nucleotides or nucleobases, which can both be catabolized by intracellular scavenger enzyme systems (Ortiz et al., 2007). The uptake of cations represents a particular challenge because macrophages express a range of chelators and transporters that effectively remove or sequester free cations, severely restricting intracellular growth of many microbial pathogens. Expression of the membrane Fe²⁺ transporter SLC11a1 (formally known as NRAMP1) on the lysosomes of macrophages and tertiary granules of neutrophils restricts the growth of Leishmania, Salmonella and Mycobacterium spp., while mutations in this transporter are associated with susceptibility to infection (Blackwell et al., 2003). Leishmania amastigotes express a high affinity ferrous (Fe²⁺) transporter, LIT1, that scavenges iron from the phagolysosome in competition to the host transporters (Huynh et al., 2006). The LIT1 gene is constitutively transcribed, but the protein is only expressed at high levels in the amastigote stages and in response to recruitment of functionally active host SLC11a1 to the phagosome membrane (Huynh et al., 2006). Promastigotes of the L. amazonensis Δlit1 mutant can differentiate to amastigotes in macrophages, but cannot proliferate in the phagolysosome. This mutant can persist in susceptible mice indicating that amastigotes have alternative mechanisms for scavenging iron in vivo.

In summary, the nutrient requirements of Leishmania amastigotes tend to be more complex than those for intracellular prokaryote and fungal pathogens, with the notable
exception of Coxiella burnetti, one of the few other pathogens to inhabit the macrophage phagolysosome (McConville et al., 2007; Voth and Heinzen, 2007). The phagolysosome compartment of macrophages appears to be hexose poor, but to contain relatively high levels of most nutrients and carbon sources (either in polymeric or monomeric form), and consequently may represent one of the few intracellular niches capable of supporting growth of these pathogens. In contrast, the phagolysosomes of neutrophils are thought to contain low levels of essential amino acids (Rubin-Bejerano et al., 2003), possibly accounting for the inability of Leishmania promastigotes to differentiate or grow in these cells. The metabolic requirements of Leishmania may thus underlie the propensity for these parasites to target host macrophages.

Interplay between parasite and host cell metabolism

There is increasing evidence that the metabolic state of macrophages has a profound effect on intracellular amastigote growth. For example, activation of macrophages with interferon-γ (IFN-γ) and pro-inflammatory cytokines (IL-12, IL-18) results in the activation of metabolic pathways needed for production of ROS and reactive nitrosative species via the phagosome NADPH oxidase and inducible nitric oxide synthase (NOS2), respectively. The NADPH oxidase requires ATP which is generated by increased glycolytic flux, while nitric oxide production is facilitated by increased arginine uptake, the recycling of citrulline, and the inhibition of host enzymes that channel arginine into ornithine and polyamine biosynthesis (Gordon, 2003) (Fig. 2). Leishmania are dependent on exogenous sources of arginine for protein synthesis and as a precursor for polyamine and trypanothione biosynthesis (Roberts et al., 2004) and depletion of host arginine/ornithine levels may inhibit amastigote growth and also increase their vulnerability to oxidative stress. Other essential amino acids, such as tryptophan, may also be depleted in classically activated macrophages owing to upregulation of enzymes such as indoleamine 2,3-dioxygenase (Murray et al., 1989; Schaible and Kaufmann, 2005).

Conversely, activation of infected macrophages by IL-4 or IL-13, cytokines produced by a T_{H}2-type response, induces a distinct metabolic programme in macrophages that are likely to promote amastigote growth (Sacks and Anderson, 2004). Alternatively activated macrophages are more dependent on mitochondrial respiration for energy and display a marked increase in levels of expression of arginase-1 (Gordon, 2003). Arginase cleaves arginine into ornithine and urea, the precursors for polyamine biosynthesis and competitive inhibitors of NOS2 (Gordon, 2003). Leishmania induce a transient T_{H}2 IL-4 response in genetically resistant mice (prior to establishment of a robust T_{H}1 response), and a persistent T_{H}2 IL-4/IL-10 response in susceptible mice strains (Sacks and Anderson, 2004). Alternative activation of infected macrophages by IL-4/IL-13 and IL-10 clearly promotes amastigote growth, at least in part, by increasing availability of essential nutrients. First, increases in parasite load in both healing and non-healing strains of mice are associated with induction of arginase-1, while resolution of infection in healing mice is associated with reduced host arginase activity (Iniesta et al., 2005; Kropf et al., 2005). Arginase-1 activity also increases when infected macrophages are stimulated with cytokines such as IL-4 and IL-10, that are associated with non-healing phenotype and uncontrolled parasite growth (Sacks and Anderson, 2004; Kropf et al., 2005). Second, inhibitors of arginase-1 reduce the parasite load in Leishmania-infected BALB/c mice (Iniesta et al., 2001; Kropf et al., 2005), while provision of exogenous L-ornithine reverses the effects of these inhibitors and prevents parasite killing in IFN-γ and LPS-activated macrophages (Iniesta et al., 2001). Finally, L. major strains containing elevated levels of arginine induce a
more robust pathology in IL-4-deficient BALB/c mice, suggesting that metabolites downstream of arginine are growth limiting for amastigote growth (Kropf et al., 2003). An implication of these observations is that arginine, polyamines and possibly other small metabolites are imported into the phagolysosome from the host cytosol. Little is known about metabolite transporters in the phagolysosome membrane, although a polyanion transporter is thought to occur in Leishmania-occupied phagolysosomes (Schaible et al., 1999).

To further add to the complexity of the Leishmania–macrophage interaction, there are indications that different species of Leishmania modulate macrophage signalling pathways and metabolism to different extents. For example, activation of L. major-infected macrophages with low levels of IFN-γ is sufficient to clear intracellular amastigotes. In contrast, treatment of L. amazonensis-infected macrophages with IFN-γ promotes intracellular amastigote growth (Qi et al., 2004). IFN-γ treatment stimulated arginine uptake, but did not stimulate NOS2 activity or decrease arginase activity in the infected macrophages (Wanasen et al., 2007). Leishmania amazonensis amastigotes therefore appear to induce a unique activation state in the host cell in which cytosolic levels of arginine are elevated but not further catabolized. Modulation of host cell responses to L. major amastigote infection has also been observed in human THP-1 macrophages (Dogra et al., 2007). Interestingly, apoptotic cells can induce a similar activation state in macrophages in which NOS2 activity is not downregulated, but arginase II activity is elevated and the flux into the polyamine pathway increased (Johann et al., 2007). Leishmania amastigotes appear to mimic apoptotic cells and could direct subsequent host cell responses by engagement of specific receptors and associated signalling pathways. Alternatively, there is increasing evidence that some amastigote proteins can be exported to the host cytoplasm and directly modulate host cell signalling pathways, although the mechanisms underlying this process are poorly defined (Kima, 2007).

Conclusions

In this review, we have highlighted metabolic pathways that are required for Leishmania virulence in the mammalian host. LPG is the only well-defined surface virulence factor in promastigotes stages. Leishmania amastigotes lack LPG, but retain a chimeric surface glycolyx of parasite- and host-acquired (glyco)lipids. While the parasite and host glycolipids appear to have partially redundant functions, parasite phospholipids (such as phosphatidylserine and inositolphosphoceramide), and opsonic host proteins (IgG) may be important in protecting intracellular stages from lysosomal hydrolases and/or mediating amastigote invasion of macrophages, respectively. Metabolic pathways involved in nutrient salvage and carbohydrate metabolism have been shown to be important for intracellular survival. While most aspects of amastigote metabolism are still inferred, there is increasing evidence that the complex nutrient requirements of Leishmania has been a key factor in restricting these parasites to the relatively rich nutrient environment of the macrophage phagolysosome. Finally, Leishmania amastigotes and/or host immune responses can have a profound effect on host cell metabolism which can, in turn, determine the outcome of infection. There is clearly a need to directly measure metabolic processes in both the parasite and host cell in order to delineate this dynamic interaction.

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