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Immunometabolism of *Leishmania* granulomas

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Abstract

Leishmania are parasitic protists that cause a spectrum of diseases in humans characterized by the formation of granulomatous lesions in the skin or other tissues such as liver and spleen. The extent to which *Leishmania* granulomas constrain or promote parasite growth is critically dependent on the host Th1/Th2 immune response and the localised functional polarization of infected and non-infected macrophages towards a classically (M1) or alternatively (M2) activated phenotype. Recent studies have shown that metabolic reprograming of M1 and M2 macrophages underpins the capacity of these cells to act as permissive or non-permissive host reservoirs. In this review, we highlight the metabolic requirements of *Leishmania* amastigotes and the evidence that these parasites induce and/or exploit metabolic reprogramming of macrophage metabolism. We also focus on recent studies highlighting the role of key metabolic signalling pathways, such as mechanistic target of rapamycin (mTOR), adenosine monophosphate-activated protein kinase (AMPK) and peroxisome proliferator receptor gamma (PPARγ) in regulating the pathological progression of *Leishmania* granulomas. These studies highlight the intimate connectivity between *Leishmania* and host cell metabolism, the need to investigate these interactions *in vivo*, and the potential to exploit host cell metabolic signalling pathways in developing new host directed therapies.

Introduction

The parasitic protist *Leishmania* spp. are the causative agents of human leishmaniasis, a spectrum of diseases that includes both localized and disseminating cutaneous and mucocutaneous infections, as well as infections of the liver/spleen/intestine (visceral leishmaniasis, VL) which is fatal in >95% of untreated cases [1]. Despite some success in reducing the incidence of VL, human leishmaniasis remains a significant global health burden with a minimum global case load of 12 million, 0.7-1 million new cases per annum

and 30,000 deaths/yr [2, 3]. A further 120 million people are thought to be asymptomatically infected [4], contributing to transmission and the reactivation of disease in immunocompromised individuals. A safe vaccine has yet to be developed and current drug treatments are suboptimal due to toxicity, expense, low efficacy in generating sterile cure, and emerging resistance [1].

Leishmania develop as flagellated promastigotes in the midgut and mouthparts of the sandfly vector, before being injected into the skin of vertebrate hosts during the sandfly bloodmeal. Non-dividing metacyclic promastigotes are initially phagocytosed by neutrophils, which rapidly infiltrate the site of tissue damage [5]. Infected apoptotic neutrophils and/or released parasites are subsequently taken up by tissue resident macrophages (TRM, dermal macrophages in the skin, Kupffler cells in liver)) and dendritic cells (DC), which triggers the differentiation of internalized parasites into obligate intracellular amastigotes within the parasitophorous vacuole (PV) of these host cells [5]. Further recruitment of monocytes and other immune cells leads to the development of granulomatous lesions in the skin composed of infected and uninfected macrophages, monocytes and DCs, as well as a significant number of neutrophils (that can also be infected) and eosinophils [6-8] (Figure 1a). Expansion of the granuloma occurs through the IFNγ-mediated recruitment of inflammatory monocytes that rapidly outnumber tissue macrophages and actively phagocytose dying infected host cells, promoting parasite transmission between monocytes/macrophages in the lesions [9-11] (Figure 1a). These primary lesions are major sites of parasite expansion or control, as well as a source of parasites that seed secondary granulomas in other dermal sites or internal organs in susceptible hosts. Significantly, parasites can persist long after natural or drug-mediated resolution of primary lesions, providing a parasite reservoir that can lead to subsequent reactivation of disease in immunocompromised individuals, many years after the initial infection.

The extent to which *Leishmania* granulomas promote or control parasite development is dependent on several factors, including the species of *Leishmania* involved, host genetics as well as early innate and subsequent adaptive immune responses. In genetically resistant murine models, the development of self-resolving lesions is associated with a strong $CD4^+$ T cell helper (Th1) immune response and production of proinflammatory cytokines, interferon

gamma (IFNγ) and IL-12, that polarize infected and uninfected macrophages in lesions towards a classically activated M1 state [5]. M1 macrophages secrete inflammatory mediators (e.g. IL-12, TNFα) and express high levels of iNOS and NADPH oxidase (NOX2), leading to production of nitrous oxide (NO) and reactive oxygen species (ROS), respectively, with concomitant containment or clearance of intracellular parasites (Figure 1a). Conversely, acute, non-resolving infections are associated with a Th2 immune response and production of anti-inflammatory cytokines (e.g. IL-4, IL-13 and/or IL-10), and polarization of infected macrophages towards an range of alternatively activated M2 states that promote tissue repair and constitute a permissive host cell reservoir [12]. While this paradigm is supported by many studies, recent work has shown that *Leishmania* granulomas can contain subpopulations of macrophages in both M1 and M2-polarized states despite induction of a strong host protective Th1 response [13] (Figure 1a). M2 macrophages can be maintained under these circumstances by local feedback loops involving IL-4 producing eosinophils and/or excessive local production of NO which suppresses macrophage inflammatory responses [8, 14]. These feedback loops, together with gradients in nutrients, oxygen and other factors. may lead to substantial heterogeneity in the types of host cell that are infected, as well as their replicative potential and state of polarization.

Macrophage polarisation is now known to be tightly associated with changes in metabolic signalling pathways and cellular metabolism [15] which underpin many key macrophage functions including motility, phagocytosis, and mediator production (NO, ROS, cytokines, chemokines). Changes in intracellular metabolite levels can also directly modulate signalling transduction pathways (such as HIF-1α) and induce long term epigenetic and transcriptional programs that drive M1 and M2 polarization. Finally, changes in host cell metabolism directly impact on the availability of carbon sources and nutrients needed for growth of intracellular amastigotes. In this review, we summarize how changes in macrophage polarization and metabolism, induced by innate and adaptive immune responses as well as intracellular parasite stages, impact on the growth and survival of *Leishmania*. We also review the role of key macrophage metabolic signalling pathway in determining the outcome of infection.

Living in the phagolysosome; metabolism of intracellular parasite stages

Leishmania proliferate within parasitophorous vacuoles (PV) that have the hallmark of mature acidified phagolysosomes (pH 5.5), while also containing markers for the ER/Golgi, suggesting a hybrid compartment that is continuously fusing with membrane vesicles from both the endo-lysosomal and secretory pathways [16] (Figure 1b). Nutrient levels in the PV lumen are likely to be regulated by the rate of fusion of endocytic/phagocytic vesicles, autophagy, and direct exchange of small molecules across the PV membrane (PVM) (Figure 1b). Recent metabolomic analysis of macrophage lysosomes indicate that these compartments contain high levels of proteogenic amino acids, nucleotides/bases as well as sugars and lipids, consistent with their central role in degrading endogenous and exogenous macromolecules [17]. These analyses also indicate that nutrient exchange across the PVM is highly dynamic and bidirectional, consistent with early studies showing that small molecules can be transported from the host cytoplasm to the lumen of the PV [18], and the finding that supplementation of the medium of *Leishmania*-infected macrophages with specific amino acids (arginine, ornithine) promotes intracellular amastigote growth [19]. Interestingly, bidirectional transport of metabolites across the PVM may not be restricted to polar metabolites. Specifically, *Leishmania* amastigotes form distinct tight junctions with the PVM that may allow two-way transport of lipids between the host cell and amastigotes. These junctions have been shown to contain parasite amastin proteins, as well as the macrophage scavenger receptor, CD36, suggestive of a role in transporting fatty acids or other metabolites across the PVM [20, 21].

Despite having access to a wide range of carbon sources within the macrophage lysosome, recent studies have shown that intracellular amastigotes switch to a slow growth state and activate a stringent metabolic response [22-24]. The latter is associated with reduced expression of glucose and some amino acid transporters, greater dependence on glycolysis coupled with redox balancing succinate metabolism for ATP synthesis, and a truncated TCA cycle which is primarily used to synthesize amino acids, such as glutamine. This metabolic switch appears to be at least partly hardwired into amastigote differentiation, and not a direct response to nutrient limitation, as similar changes also occur in *in vitro* differentiated amastigotes cultivated in rich medium, [22]. Activation of the stringent response may confer protection against host derived NO and ROS, that target mitochondrial enzymes containing iron-sulphur clusters [25]. Consistent with this hypothesis, parasite lines with reduced glucose

uptake, and increased dependency on amino acids are highly attenuated *in vivo* [24]. Thus despite having access to multiple carbon sources, amastigotes are highly dependent on sugars (glucose, glucosamine) as their major carbon source [24].

Leishmania amastigotes lack a glyoxylate cycle and are unable to use fatty acids as a primary carbon source. However, fatty acid uptake and β-oxidation is upregulated in these stages and a recent study has shown that β-oxidation of polyunsaturated fatty acids (PUFA) is essential for virulence [26]. Fatty acid β-oxidation may provide an additional source of carbon for synthesis of glutamate [22, 27]. Alternatively, it may be required to prevent accumulation of toxic levels of PUFA [26]. A similar function has been proposed for the *Leishmania* iron transporter protein, Iron Regulator I (LIR1), which is thought to play a critical role in preventing iron toxicity within the PV [28].

Impact of macrophages metabolism on intracellular *Leishmania* growth

While Leishmania appear to reside within a relatively nutrient-replete niche, some nutrients may become limiting and/or reach toxic levels depending on the macrophage activation state. In particular, changes in host cell amino acid and central carbon metabolism have been shown to impact significantly on amastigote growth and survival.

Host arginine metabolism

Leishmania are arginine auxotrophs and are completely dependent on host arginine for protein synthesis, as well as *de novo* synthesis of other essential metabolites, such as polyamines, trypanothione and the non-proteinogenic amino acid hypusine [29]. Arginine levels within the PV are limiting for parasite growth as modulation of macrophage arginine levels, either by supplementation of the medium with exogenous arginine or inhibition of macrophage arginine uptake, enhances or limits amastigote growth, respectively [30]. Intracellular amastigotes also up-regulate the expression of their arginine transporter, AAG3, and endogenous arginase suggesting that amastigotes need to compete against macrophage PVM arginine transporters [31]. Significantly, *Leishmania* mutants lacking arginase, are still able to cause lesions, although their growth *in vivo* is attenuated suggesting that amastigotes can also scavenge essential polyamines, such as ornithine, from the PV [32-35]. However, *L. donovani* mutants lacking enzymes down-stream of ornithine (i.e. ornithine decarboxylase, spermidine synthase and S-adenosylmethione decarboxylase) are

severely attenuated in virulence, suggesting that PV levels of spermidine or spermine are below levels needed for parasite survival [36, 37].

Changes in macrophage activation and polarization lead to significant changes in intracellular arginine pools, microbicidal NO and growth promoting polyamines, which directly impact amastigote survival (Figure 2). IFNγ-activated M1 macrophages have elevated levels of arginine uptake, due to increased expression of the CAT2B arginine transporter, as well as increased expression of iNOS [38] (Figure 2). iNOS catalyzes the two step, NADPH-dependent conversion of arginine through N^{ω}-hydroxy-L-arginine (NOHA) to citrulline and NO. Both NOHA and NO have potent anti-microbial activities and play a key role in controlling *Leishmania* growth during both acute and chronic phases of murine infections [39-41]. Increased expression of iNOS and NO production also strongly polarizes macrophages towards a glycolytic M1 phenotype, as NO is a potent inhibitor of mitochondrial respiration, and may prevent repolarization to a M2 phenotype [42]. Interestingly, low levels of IFNγ activation can promote intracellular parasite growth by increasing arginine availability while NO levels are below a growth inhibitory threshold [38]. This is particularly evident for members of the *L. mexicana* complex that induce large communal vacuoles and are more resistant to NO than other species of *Leishmania* that inhabit tight-fitting, individual vacuoles [38]. Similarly, sublethal NO levels in acute skin granulomas was found to restrain but not kill intracellular amastigotes [43], and could potentially drive the parasites into a slow growth and/or metabolically quiescent state that is well adapted to survive in the granuloma tissue niche.

Conversely, polarization of macrophages towards a M2 phenotype by IL-4/IL-13 (via activation of STAT6), or other anti-inflammatory cytokines (e.g. IL-10/STAT3) or hypoxic tissue microenvironments, leads to increased expression of arginase-1 (Arg1), as well as the up-regulation of the CAT2B transporter [38] (Figure 2). Arg1 catalyzes the conversion of arginine to ornithine and urea, thereby promoting *de novo* glutamine, proline and polyamine synthesis which sustain collagen production and the tissue repair functions of M2 macrophages. Arg1 is reciprocally regulated with iNOS at the level of transcription (although $Arg1^{+}$/$iNOS^{+}$ macrophages are detected in lesions) as well as metabolically [9]. Specifically, upregulation of Arg1 diverts arginine from NO production, while upregulation of iNOS

results in the generation of NOHA, a potent inhibitor of arginase. As a result, elevated expression of Arg1 in myeloid cells limits the anti-leishmanial effector functions, as well as the inflammatory effects of iNOS/NO. The upregulation of myeloid Arg1 may also deplete arginine levels in tissues and inhibit proliferation of host protective T cells and other immune cell functions [44]. Many studies have investigated whether enhanced expression of Arg1 in *Leishmania* infected M2 polarized macrophages promotes parasite growth beyond antagonizing NO production. In support of such a role, parasites preferentially populate $Arg1^+$ monocytes and macrophages in lesions, and Arg1 expression correlates with increased lesion development in susceptible BALB/c mice [41, 45]. Furthermore, treatment of *L. major* infected BALB/c mice with the non-selective arginase inhibitor (NOHA), or conditional knock-down of Arg1 in hematopoietic and endothelial cells, showed that the hyper expression of Arg1 may prevent uncontrolled disease progression, visceralization and lethality [19, 46]. In contrast, conditional knock-down of Arg1 expression or a block in Arg1 induction in C57BL mice that form self-curing lesions, had no effect on the progression of disease or parasite persistence after granuloma resolution [9, 44]. Moreover, proliferating *L. major* parasites did not exhibit a tropism for $Arg1^+$ macrophages in latent C57BL infections, indicating that elevated expression of host arginase is not essential for intracellular survival [47]. These differences in the significance of host arginase on *Leishmania* infection outcomes likely reflects variability in Arg1 expression in different mouse strains (normally much higher in BALB/c mice) and in the innate microbicidal capabilities of BALB/c and C57BL mice [44]. Interestingly, Arg1-dependent synthesis of putrescine has recently been shown to sustain cycles of macrophage efferocytosis and internalization of apoptotic or pyroptotic cells [48]. Elevated Arg1 expression in newly recruited monocytes may therefore promote parasite transmission between granuloma phagocytes through efferocytosis.

*Host tryptophan metabolism***.**

Leishmania growth within macrophages may also be constrained by the availability of other amino acids, such as tryptophan. IFNγ-activated M1 macrophages upregulate expression of indoleamine-2,3-dioxygenase (IDO) and the conversion of tryptophan to kynurenine metabolites reducing the availability of tryptophan to amastigotes [49]. IDO1 is highly expressed in cutaneous *Leishmania* lesions although the impact of IDO1 expression on parasite burden remains poorly defined. Macrophages also utilize tryptophan for NAD^+

biosynthesis, with tryptophan 2,3-deoxygenase (TDO) catalysing the first step. Although TDO expression is not induced by IFNγ, TDO levels are highly correlated with parasite load in *L. major* cutaneous lesions and TDO inhibition in *ex vivo* infected macrophages promotes parasite growth [49]. Whether IDO and TDO act synergistically or separately to restrict *Leishmania* growth needs further investigation.

Host central carbon metabolism.

Infection of murine macrophages with *L. donovani* increased expression of glucose transporters, leading to an initial defensive switch to enhanced aerobic glycolysis [50]. However, after 24hr, infected macrophages upregulated mitochondrial respiration, consistent with a switch towards an M2 polarized phenotype. Infected macrophages in *L. major* lesions also exhibit elevated oxidative phosphorylation, indicative of M2 polarization *in vivo* [14]. Although the metabolic reprogramming of *Leishmania*-infected macrophages has not been examined in detail, IL-4 mediated polarization of macrophages is associated with increased mitochondrial biogenesis, and the operation of a complete TCA cycle, which is supported by increased glutaminolysis and fatty acid β-oxidation [15, 51]. M2 macrophages also express elevated levels of 3-phosphoglycerate dehydrogenase (3PGDH) that channels glycolytic intermediates into serine, glycine and folate one-carbon metabolism [52]. These metabolic shifts may promote *Leishmania* growth in a number of ways. First, the shift towards more efficient mitochondrial respiration may allow the accumulation of intracellular pools of glucose in the host cell which promotes amastigote growth [25, 50, 53]. However, amastigotes can also utilize sugars and amino sugars generated through the breakdown of extracellular matrix hyaluronan and glycosaminoglycans in the PV and therefore may not be dependent on exchanges of sugars across the PVM [54]. Indeed, uptake of extracellular matrix components is increased in M2 macrophages as part of their tissue repair function and likely contributes to enhanced amastigote growth [55]. Second, *Leishmania* appear to be at least partly dependent on glycine and serine salvage from the PV, although they can synthesize these amino acids *de novo* [56]. Increased levels of glycine/serine, as well as folate metabolites, in M2 macrophages may further promote parasite growth.

On the other hand, IFNγ-activated M1 macrophages efficiently channel internalized glucose

into aerobic (or anerobic) glycolysis and the pentose phosphate pathway for ATP and NADPH production, which in turn is required for production of NOX2 driven ROS [15]. Glycolytically derived pyruvate is still catabolized in the mitochondria, but in an impaired TCA cycle in which flux through isocitrate dehydrogenase (IDH) and succinate dehydrogenase (SDH) is blocked, leading to citrate/isocitrate and succinate accumulation, and increased mitochondrial ROS via reverse electron transport. Citrate accumulation drives the synthesis of fatty acids as well as the antimicrobial metabolite, itoconate, while increased succinate levels stabilize HIF-1α, promoting the transcription of glycolytic enzymes and inflammatory cytokines (i.e IL-1β), that are collectively detrimental to *Leishmania* proliferation.

Lipid body accumulation

Infection of murine and human macrophages with different *Leishmania* species leads to the accumulation of lipid bodies in the host cytoplasm [57, 58] as well as transcriptional changes in many lipid biosynthetic genes [59]. Lipid body accumulation is most pronounced in macrophages from susceptible BALB/c background, and the hyperaccumulation of lipid bodies in HIF1α-negative macrophages strongly promotes intracellular growth of *L. donovani* amastigotes [60]. Macrophage lipid bodies often accumulate around *Leishmania* PVs and, in some cases, are even present in the PV lumen. These lipid bodies may provide amastigotes with a source of polyunsaturated fatty acids (PUFA) that can be used as a carbon source [26] and/or may protect parasites from oxidative stress.

Lipid bodies are also sites for synthesis of omega-3 and omega-6 PUFA derived lipid mediators that have profound anti-inflammatory and inflammatory activities. A number of studies have shown that enzymes involved in the synthesis of anti-inflammatory prostaglandin E2 (PGE2) synthesis, such as cyclooxygenase 2 and phospholipase A2 are upregulated in *L. donovani* infected macrophages [61], while levels of both omega-6 derived prostaglandins and leukotriene (PGF2, LTB4) and omega-3-derived resolving D1 (RvD1) are increased in the serum of human patients with visceral or diffuse cutaneous leishmaniasis [62]. Both PGE2 and the resolvins are potent immune modulators that inhibit Th1 immune response and polarize macrophage towards a M2 phenotype, though activation of a PPAR signalling (see below). Addition of RvD1 to *L. amazonensis* infected macrophages was also

associated with increased expression of heme oxidase -1 which promotes parasite growth by reducing ROS production [63] .

Role of macrophage metabolic signalling pathways in *Leishmania* infection

Macrophage polarization within different tissue niches is regulated by local levels of cytokines, growth factors/chemotactic proteins, TLR ligands, and nutrient levels that, in turn, lead to activation of metabolic signalling pathways and the reprogramming of metabolism geared to different effector cell functions. Key metabolic signalling pathways in macrophage activation include the mTOR, AMPK and HIF1a pathways [64]. There is increasing evidence that these pathways play important roles in regulating *Leishmania* granuloma formation as well as the polarization of macrophages within these tissues to form both permissive and non-permissive niches within these tissues as outlined below.

mTOR signalling and regulation of Leishmania *growth*

Mechanisitic target of rapamycin (mTOR) is a key regulator of central carbon metabolism in most eukaryotes. Macrophages express two mTOR multi-protein complexes, mTORC1 and mTORC2, which have non-redundant signalling functions and differ in regulating adaptor proteins and in their sensitivity to rapamycin. The mTORC1 complex is recruited to the lysosome (and other organellar) membranes together with the central adaptor protein, Raptor, through interactions with the Rag GTPase, which in turn is activated by the availability of amino acids in the cytoplasm and the lysosome lumen [65]. mTORC1 activation occurs via GTP-bound RHAB which is, in turn, inhibited by tuberous sclerosis complex (TSC1/2). Genetic loss of the TSC1/2 proteins therefore leads to constitutive activation of mTORC1 in the presence of adequate nutrient levels [66]. Additional regulation is provided by cross signalling from other metabolic sensors, such as AMP kinase (AMPK), that negatively regulates mTORC1 by phosphorylation of the TSC2 protein.

Activation of murine macrophages with anti-inflammatory cytokines, such as IL-4 or IL-13, promotes mTORC1 activation through the PI3PK/Akt pathway leading to increased protein synthesis and a switch to anabolic metabolism which is needed to sustain the energy-demanding functions of cell migration, cytokine production and tissue repair [51, 64]. Infection of murine macrophages with *L. donovani* also leads to the activation of mTOR and

polarization towards a permissive M2 phenotype, while inhibition of mTORC1 (via rapamycin treatment) limits parasite growth in *ex vivo* infected macrophages and granuloma development in mice [67-69]. Other studies have shown that mTORC1 activates a number of key down-stream targets and transcription factors, including HIF1α, PGC1α, SREBP1 and PPARγ that lead to transcriptional upregulation of proteins involved in glucose uptake, glycolysis, mitochondrial biogenesis, oxidative phosphorylation, and fatty acid and cholesterol biosynthesis, that individually or collectively may promote intracellular *Leishmania* growth [51, 64]. Remarkably, constitutive mTORC1 activation in murine macrophages (via TSC2 knockdown) results in spontaneous formation of macrophage-rich granulomas in a murine model for sarcoidosis [66] suggesting that activation of mTORC1 by *L. donovani* may stimulate this innate host immune response.

Activation of mTORC1 by *Leishmania* is likely mediated by PI3PK-Akt signalling which is strongly linked to intracellular growth of *Leishmania* amastigotes [65, 70, 71]. Akt phosphorylates (i.e. inactivates) TSC proteins that negatively regulate mTORC1 [65], as well as activating enzymes such as ATP citrate lyase. Increased acetyl-CoA biosynthesis is required for histone acetylation and the transcription of a subset of M2 genes (e.g. ARG1), while suppressing NFκB signalling, the production of pro-inflammatory cytokines (e.g. IL-12), iNOS and apoptosis [72]. Pharmacological inhibition or genetic knock-down of PI3K/Akt in human macrophages inhibits *L. amazonensis* growth [70]. While activation of PI3K/Akt by phagocytosis of microbial pathogens is often transient, *Leishmania* appear to induce sustained activation of this pathway by recruiting inositol lipids PI(3,5)P2 and PI(3,4,5)P3 to the PVM, leading to aberrant recruitment of Akt to the PV [71]. The accumulation of PIPs on the PVM may reflect increased fusion of PI4P-containing ER/Golgi derived vesicles with the PVM, although this remains to be confirmed [16].

Activation of mTOR also leads to the nuclear translocation of sterol regulatory element binding protein-1c (SREBP-1c), and the transcriptional upregulation of fatty acid synthase (FASN) and acetyl-CoA carboxylase (ACC), which are both involved in *de novo* fatty acid synthesis and the accumulation of lipid droplets in splenic and liver macrophages [69]. Interestingly, the mTORC1 induced increase in fatty acid synthesis in *L. donovani* infected

murine macrophage was exacerbated following genetic loss of HIF1α [69]. HIF1α is activated by mTORC1 and may redirect the metabolic switch of infected macrophages towards a more glycolytic/M1-like phenotype. Consistent with this possibility, the permissiveness of macrophages isolated from different mouse strains to *L. donovani* infection was inversely related to HIF1α expression [69]. Furthermore, macrophages isolated from humans carrying mutations in HIF-1α and consequently expressing lower HIF-1α levels, have increased lipid production and are more permissive for *L. donovani* infection, highlighting a novel genetic susceptibility to leishmaniasis.

Paradoxically, infection of murine BALB/c macrophages with *L. major* was associated with the proteolytic cleavage of the mTOR kinase by the parasite surface protease, gp63 [73]. Inhibition of mTORC signalling in *L. major*-infected macrophages led to activation of the translational repressor, 4E-binding protein-1 (4E-BP1) and a global reduction in host protein synthesis. This is turn was associated with reduced secretion of type-1 interferons, IFN-α/IFN-β [73] which can polarize macrophages towards an M1 phenotype with an impaired TCA cycle that generates succinate, citrate and itoconate. These metabolites activate HIF1α and/or deleterious for *Leishmania* growth [42, 64, 74]. In contrast to the situation in *L. donovani* infected macrophages and animal models, mTORC1 activation in the *L. major*-BALB/c model appears to be host protective as rapamycin treatment resulted in enhanced parasite growth *in vivo*, while mice lacking 4E-BP1 had lower parasite burdens and pathology than wild type mice [73]. How gp63 accesses the host mTOR complex and the universality of this mechanism in other *Leishmania*-macrophage interactions remains to be defined.

AMPK signalling also modulates Leishmania growth

Macrophage polarization is also regulated by AMP kinase (AMPK), a key component in a second metabolic signalling complex that is often reciprocally regulated with mTOR [64]. AMPK is normally activated under nutrient limiting conditions and is involved in sustaining homeostasis and catabolic metabolism in non-growing cells [64]. In mammalian cells, AMPK is activated by changes in the bioenergetic state, particularly the ratio of AMP/ADP relative to ATP and glucose deprivation [64], as well as by up-stream regulators, such as sirtuin-1 (SIRT1) and Liver Kinase B (LKB) [64]. Infection of murine macrophages with *L. infantum* was

associated with AMPK activation and a switch to increased mitochondrial oxidative phosphorylation [50]. Activation of AMPK was dependent on LKB/SIRT1, with SIRT1 being up-stream of LKB [50]. AMPK activation led to increased expression of the glucose transporter, Slc2a4, as well as the co-regulator of PPAR-γ (PGC-1α)[50], which stimulates mitochondrial biogenesis in both *ex vivo* cultivated and granuloma macrophages. The AMPK-dependent metabolic reprogramming of *L. infantum* infected macrophages may promote intracellular parasite growth by increasing the availability of glucose through more efficient mitochondrial metabolism [50, 53]. *L. infantum* lesion development in AMPK, SIRT1- or LBK1-knock-out mice was attenuated, suggesting that the AMPK-LBK1-SIRT1 axis is important for intracellular parasite growth *in vivo* [73]. Activation of AMPK could also account for an observed increase in autophagy in *L. donovani* infected macrophages [68]. Increased autophagy promotes intracellular *Leishmania* growth, presumably by increasing nutrient levels in the PV compartment [75]. This is in contrast to the situation for many intracellular bacterial pathogens where increased autophagy results in their more efficient delivery to and degradation within the lysosome [15, 76].

How AMPK is activated in *Leishmania*-infected macrophages remains unclear. *Leishmania* infection can lead to the efflux of ATP [77], which would alter intracellular pools of AMP/ADP/ATP and potentially activate AMPK in the host cell. The released ATP is converted to adenosine by the surface expressed ecto-nucleotidases, CD39/CD73, and adenosine deaminase (ADA2) leading to the activation of G-protein receptors, $A_{2A}R$ and $A_{2B}R$, which have also been shown to promote M2 polarisation [77]. AMPK can also be activated by cytoplasmic galectins that detect lysosomal damage [78], although whether *Leishmania* amastigotes activate this pathway is unknown. More broadly, it remains unclear whether visceralizing *Leishmania* species simultaneously activate both AMPK and mTORC1 signalling pathways to their advantage. AMPK and mTOR negatively regulate each other by multiple mechanisms, and also drive opposing catabolic and anabolic pathways. However, both signalling networks have been shown to support M2 macrophage polarization and *Leishmania* may have evolved mechanisms for exploiting aspects of both pathways that benefit amastigote survival.

Down-stream pathways - PPAR signalling and lipid metabolism

The Peroxisome Proliferator Activated Receptors (PPAR-α, -γ, -δ) are nuclear receptors and master regulators of lipid metabolism in macrophages. Activation of PPARγ results in increased expression of genes involved in macrophage fatty acid uptake (via CD36), lipid storage, lipolysis and β-oxidation. Activation of PPAR-γ down-stream of mTORC1 signalling also leads to increased mitochondrial biogenesis, oxidative phosphorylation and Arg1 expression characteristic of M2 polarization [53, 79, 80]. A number of studies have shown that activation of macrophage PPARγ in both cutaneous and visceral infections promotes disease progression, while pharmacological inhibition or genetic silencing of PPAR-γ delays lesion development and reduces the size of cutaneous and visceral granulomas [79-81]. In an important study, Beattie and colleagues showed that a transcriptional network centred around retinoid X receptor-α (RXRα), is aberrantly sustained in infected macrophages in *L. donovani* induced liver granulomas, but down-regulated in uninfected macrophages from the same tissue [82]. RXRα forms functional heterodimers with PPAR proteins, suggesting that sustained RXRα/PPAR signalling is important for parasite growth and survival in liver granulomas, and that infected macrophages are less susceptible to being polarized towards an M1 phenotype in this inflammatory tissue environment. Consistent with RXRα/PPAR signalling being parasite supportive, pharmacological inhibition of RXRα results in reduced parasite load in the spleen and liver [82]. The role of other PPAR isoforms in *Leishmania* granulomas has not been extensively studied.

PPARγ-δ are activated by anti-inflammatory cytokines (IL-4, IL-13, IL-10), TLR receptors, as well a range of lipids and other stimuli, including oxidized LDL, PUFA (arachidonic acid, 5-hydroxyicosatetraenoic acid and 5-oxo-eicosatetraenoic acid), oxidized linoleic acid (13-HODE), and prostaglandins (D2, PGE2, PGF2, thromboxane B2) that are variously present in *Leishmania* granulomatous tissues [83]. Interestingly, *Leishmania* derived PUFA polarize murine macrophages towards an M2 phenotype when added exogenously [84]. As noted above, *Leishmania* amastigotes form a tight junction with the PVM, raising the possibility that parasite lipids may be directly transported to the host cell and modulate host cell signalling. Moreover, *Leishmania* have recently been shown to express an aldo-keto reductase that functions as a prostaglandin F2α synthase. This enzyme is exported to the

host cell cytoplasm, possibly via released exosomes and may hijack host responses by inducing the synthesis of anti-inflammatory prostaglandins [85, 86].

Future perspectives

Considerable progress has been made in understanding how changes in host cell metabolism impact on the outcome of granulomatous diseases caused by pathogens such as *M. tuberculosis*, *Salmonella typhimurium* and *Schistosoma* spp [15]. While fewer studies have been undertaken on the immunometabolic aspects of *Leishmania* granulomas, these protozoal diseases represent an excellent model system for studying granuloma dynamics given the extensive literature on host immunity to *Leishmania* (including the first description of Th1/Th2 immune response), the availability of robust animal models for both dermal and liver granuloma induction and the relative simplicity of *leishmania* granuloma histology compared to other infectious granulomas. As in other granulomatous diseases, the extent to which *Leishmania* granulomas function to restrain or allow parasite expansion is dependent in large part on the extent to which macrophage/monocytes are polarized towards a M1 or M2 phenotypes. Importantly, macrophages can exist in different polarization states within the same granuloma, reflecting spatial and temporal heterogeneity in the ontology of different macrophage/monocyte populations (i.e. TRM versus recruited monocytes), and the levels of cytokines, chemokines and host immune factors (e.g. NO), as well as gradients in extracellular nutrients and oxygen levels. Future studies on the metabolic reprograming of host cells in *Leishmania* granulomas therefore need to take into account this phenotypic diversity of host cells in the granuloma microenvironment and the orthoganol metabolic signals that contribute to polarization.

Macrophage polarization in *Leishmania* granulomas has also been shown to be dependent on key metabolic signalling hubs, such as Akt/mTOR, AMPK, PPAR and HIFα. While activation of some of these pathways is commonly associated with macrophage polarization towards M1 or M2-like phenotypes, there is increasing evidence that cross-talk between these pathways can lead to a spectrum of different metabolic responses *in vivo*. In particular, the observation that activation of both mTOR and AMPK promotes liver granuloma formation induced by visceralizing species of *Leishmania* is of interest as these pathways are normally reciprocally regulated and antagonize each other. Similarly, while

activation of mTOR signalling in macrophages can lead to a M2 phenotype that is permissive for *Leishmania* growth, it can also lead to M1 polarization under conditions that also activate HIF1α signalling and/or increased secretion of inflammatory cytokines that constrain *Leishmania* growth. Therefore, dissection of the role of host cell metabolic signalling on *Leishmania* granuloma development will require a holistic examination of antagonistic/synergistic interactions between different pathways.

Finally, consideration of the impact of macrophage metabolic reprograming on *Leishmania* pathogenesis will require a detailed understanding of the metabolic requirements of intracellular amastigote stages, as well as the properties of the PV. In particular, while amastigotes can clearly access a variety of metabolites from the macrophage cytoplasm, it remains unclear whether this occurs through host PVM transporters and/or through PVM-amastigote tight junctions, and the extent to which amastigotes are directly responsive to changes in macrophage metabolism. Conversely, there is increasing evidence that transport of parasite proteins and metabolites across the PVM modulates host signalling pathways. While it has been speculated that parasite proteins may be transported into the host cytoplasm in membrane bound exosomes, how these proteins are released into the cytoplasm is unknown. Finally, it is worth noting that *Leishmania* amastigotes, unlike nearly all other bacterial, fungal and protist pathogens, survive within a phagolysosome-like compartment of macrophages. As such, it is likely that *Leishmania* will be dependent on different macrophage signalling and metabolic pathways for survival compared to other intracellular microbes. For example, while activation of autophagy controls intracellular growth of *M. tuberculosis*, it is beneficial for *Leishmania*. Overall, these studies highlight the intimate interconnection between host and parasite metabolism which ultimately determine the outcome of infection. Further progress in understanding immunometabolic signalling and programming in *Leishmania* granulomas is likely to open new opportunities for developing adjunct therapies that target host processes.

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Conflict of Interest

The authors have no conflict of interest to declare.

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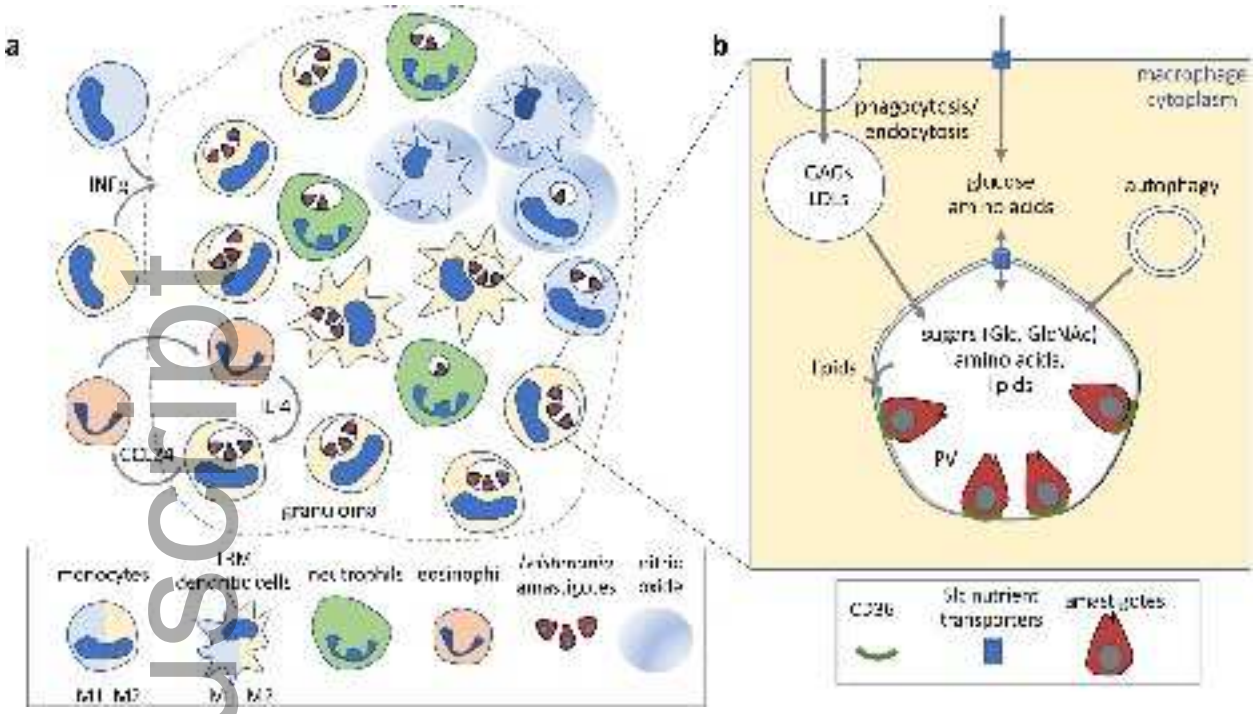
Figure 1. *Leishmania* granulomas. (a) *Leishmania* induced granulomas (dashed lines) comprise aggregates of recruited monocytes, tissues resident macrophages, neutrophils, eosinophils and T-cells (panel A). Granuloma monocytes and macrophages can be classically activated (M1 phenotype) by proinflammatory cytokines (Il-12, IFNγ, TNFα) and by increased diffusible nitric oxide (NO), or they can be alternatively activated (M2 phenotype) by anti-inflammatory cytokines (IL-4, IL-13, IL-10) produced, in part, by granuloma eosinophils. *Leishmania* granulomas contain monocytes/macrophages exhibiting a range of polarization phenotypes from M1 to M2, and may also differ in their capacity to differentiate and self-replicate, directing impacting parasite growth. **(b)** Intracellular amastigotes reside within an acidic phagolysosome-like parasitophorous vacuole (PV) that contains a variety of carbon sources (sugars, lipids, amino acids) and essential nutrients (amino acids, purines, vitamins, heme), needed for parasite growth. These are derived from the breakdown of macromolecules (including glycosaminoglycans (GAGs), low density lipoproteins (LDL)) delivered to the PV via endocytic/lysosomal traffic, autophagy, efferocytosis and/or direct import from the macrophage cytosol by Slc transporters in the PV membrane (PVM). Glc, glucose; GlcN, glucosamine/N-Acetylglucosamine.

Figure 2. Metabolic reprogramming of macrophages in *Leishmania* granulomas. *Leishmania* granulomas can contain macrophages in different states of polarization that are either restrictive (M1) or permissive (M2) to *amastigote* growth. INFγ-polarized M1 macrophages exhibit (i) elevated glycolytic flux (with corresponding low levels of free glucose), (ii) a discontinuous TCA cycle (resulting in production of succinate, citrate and increased reactive oxygen species (O_2^{2-}) due to reverse electron transport in the respiratory chain) and (iii) increased production of nitrous oxide (NO) from exogenous arginine or arginine produced via the arginosuccinate (AS) and mitochondrial aspartate/fumarate shunt. Conversely, IL-4/13-polarized M2 macrophages are predicted to have (i) reduced glycolytic flux (ii) an intact TCA cycle and mitochondrial respiration, (iii) increased lipid body synthesis, fatty acid β-oxidation (FAO) and synthesis of anti-inflammatory prostaglandins (PGE), (iv) increased arginase-1 (Arg1)-mediated synthesis of ornithine and other polyamines and (v) increased uptake of glycosaminoglycans (HA) and lipoproteins (LDL) that are delivered to the PV. M1-M2 metabolic reprogramming is regulated by key signalling

hubs that include mTORC1, AMPK, HIF1α, PPAR, NF-κB. Pathways and metabolites that are increased in M1 or M2 macrophages are coloured green, while those that are decreased are coloured red.



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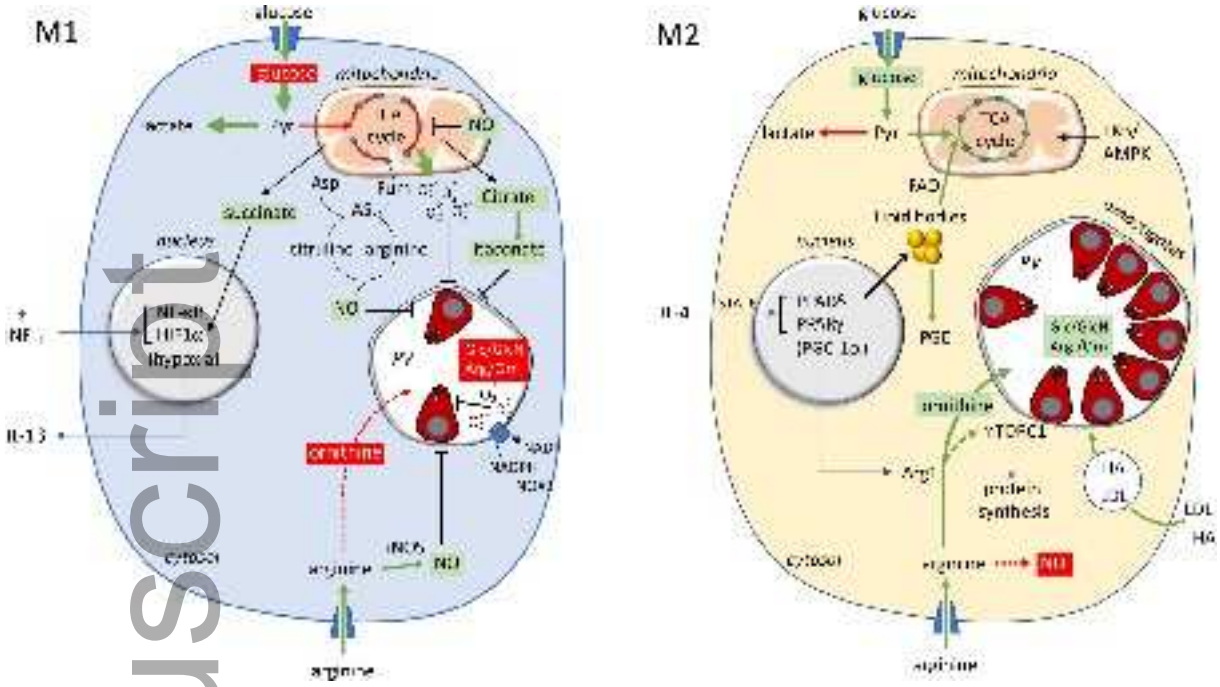

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