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Leishmania-macrophage interactions modulate mitochondrial metabolism: integrating mitochondrial function with parasite proliferation

By

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To my mother Amparo Ospina, my father Albeiro Acevedo, my sister Dafne Acevedo Ospina and my fiancée Marie-Michèle Guay-Vincent.

May they live forever with me in the science memories through my work

A mi madre Amparo Ospina, mi padre Albeiro Acevedo, mi hermana Dafne Acevedo Ospina y mi prometida Marie-Michèle Guay-Vincent.

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ABSTRACT

Leishmania ssp. are protozoan parasites and etiological agents of a complex human vector-borne disease known as leishmaniasis. *Leishmania* presents a digenetic life cycle in which the parasite is transmitted to mammals by phlebotomine sandflies through the inoculation of metacyclic promastigotes that target mononuclear phagocytic cells. To colonize macrophages, *Leishmania* employs a panoply of virulence factors, including the glycolipid lipophosphoglycan (LPG), which impairs different host cell processes and rewires host cell metabolism creating a metabolically-adapted microenvironment required for pathogen replication. At the core of *Leishmania*-macrophage interactions, there is a constant interplay between parasite factors and host responses, which is crucial to define the infection outcome. Whereas the role of the SIRT1-AMPK axis in mediating *Leishmania*-induced changes in host cell bioenergetics profile has been established, our knowledge of the nature of both the host cell receptors and *Leishmania* effectors involved in the macrophage metabolic reprogramming remains fragmentary. In the present study, we investigated the mechanisms by which *L. donovani* alters host cell mitochondrial biology. In particular, we sought to determine the potential role of the parasite cell surface glycolipid lipophosphoglycan (LPG) in this process.

To decipher the possible mechanisms governing the *Leishmania*-mitochondrial interactions, we use as our main model, bone marrow-derived macrophages (BMM) infected with *L. donovani,* a genetically and structurally defined *L. donovani* mutant defective in the synthesis of LPG *(*Δ*lpg1*) and its complement counterpart *(*Δ*lpg1+LPG1*). Furthermore, the mitochondrial ultrastructure during chronic infection was evaluated *in vivo.* TLR4-, TLR2-, endosomal TLRs-, and IFNAR-deficient macrophages were used to validate the connection between host-receptor and mitochondrial reprogramming. Finally, the importance of mitochondrial reprogramming for *Leishmania's* fate was disclosed.

Here, we describe that the induction of oxidative phosphorylation and mitochondrial biogenesis by *Leishmania donovani* requires the virulence glycolipid lipophosphoglycan, which stimulates the expression of key transcriptional regulators and structural genes associated with the electron transport chain. *Leishmania*-induced mitochondriogenesis also requires a lipophosphoglycan-independent pathway involving type I interferon (IFN) receptor signalling. The observation that pharmacological induction of mitochondrial biogenesis enables an avirulent lipophosphoglycan-

defective *L. donovani* mutant to replicate in macrophages supports the notion that mitochondrial biogenesis contributes to the creation of a metabolically-adapted environment propitious for parasite replication. Noteworthy, the enhanced heme biosynthesis and its dependence for *Leishmania* replication support the importance for the parasite to induce mitochondrial biogenesis and of OXPHOS for its development within the mammalian hosts.

In summary, this study provides novel insight into the complex mechanisms leading to mitochondrial biogenesis and metabolic reprogramming in macrophages infected with *L. donovani*. Our results are consistent with the notion of pathogen-specific metabolic rewiring, which results from the intricate interplay between complex sets of pathogen molecules and host cell receptors.

Keywords: Macrophages, mitochondrial metabolism, *Leishmania*, Lipophosphoglycan, IFNAR.

RÉSUMÉ

Leishmania ssp. est un parasite protozoaire et l'agent étiologique de la leishmaniose, une maladie humaine complexe transmise par un insecte. *Leishmania* a un cycle de vie digénétique au cours duquel le parasite, sous sa forme promastigote métacyclique, sera transmis à son hôte par la piqûre d'une mouche phlébotome femelle. Le parasite infecte les mammifères, et ira cibler les cellules phagocytiques mononucléaires. Afin de coloniser les macrophages, *Leishmania* emploi une panoplie de facteurs de virulence, incluant le glycolipide lipophosphoglyane (LPG), qui altère différents processus de la cellule hôte et réorganise son métabolisme, créant un microenvironnement métaboliquement adapté nécessaire à la réplication intracellulaire du parasite. Au cœur de l'interaction *Leishmania*-macrophage, il y a un échange réciproque constant entre les facteurs du parasite et la réponse de l'hôte, qui déterminera l'issue et la sévérité de l'infection. Bien que le rôle de la voie de signalisation SIRT1-AMPK dans la médiation des changements du profil bioénergétiques induits par *Leishmania* a déjà été établis, notre connaissance est plutôt limitée par rapport à quels récepteurs de la cellule hôte et quels effecteurs de *Leishmania* sont impliqués dans la reprogrammation métabolique. Lors de cette étude, le mécanisme par lequel *L. donovani* altère la biologie du macrophage de la cellule hôte a été examiné. Plus précisément, il était question de déterminer le rôle potentiel du glycolipide de surface parasitaire LPG (lipophosphoglycane) dans ce processus.

Afin d'élucider les possibles mécanismes gouvernant l'interaction *Leishmania*-macrophage, des macrophages dérivés de la moelle osseuse (BMM) infectés soit par L. donovani, un mutant L. donovani génétiquement modifié afin qu'il ne synthétise pas LPG (Δ*lpg1*) ou son homologue complémenté (Δ*lpg1+LPG1*). De plus, l'ultrastructure mitochondriale pendant l'infection a été évaluée in vivo. Des macrophages déficients pour TLR4, TLR2, TLRs endosomaux et IFNAR ont aussi été utilisés afin de valider la relation entre les récepteurs de l'hôte et la reprogrammation mitochondriale. Finalement, l'importance de cette reprogrammation pour le sort de *Leishmania* a été élucidée.

Notre étude décrit que l'induction de la phosphorylation oxydative et la biogénèse mitochondriale par *L. donovani* requiert le facteur de virulence LPG, qui stimule l'expression de mécanismes de régulation transcriptionnels et gènes structurels associés à la chaîne de transport d'élections. La mitochondriogénèse induite par *Leishmania* requiert également, d'une

manière LPG indépendante, une voie impliquant la signalisation de l'interféron de type I (IFN). Il a été observé que l'induction pharmacologique de la biogénèse mitochondriale permet au mutant *L. donovani* déficient pour LPG (Δ*lpg1*) de se répliquer normalement dans les macrophages. Ceci supporte l'hypothèse que la biogénèse mitochondriale contribue à la création d'un environnement métaboliquement adapté et propice à la réplication du parasite. À noter que l'augmentation de la biosynthèse de l'hème et sa dépendance pour la réplication efficace de *Leishmania* supporte l'importance pour le parasite d'induire la biosynthèse mitochondriale et l'OXPHOS pour son développement dans la cellule hôte.

En résumé, cette étude démontre de nouvelles connaissances par rapport aux mécanismes complexes menant à la biogénèse mitochondriale et reprogrammation métabolique dans les macrophages infectés par *L. donovani*. Ces résultats sont l'exemple-même de la notion de remodelage métabolique spécifique à une infection, qui résulte de l'intrigante interaction entre les récepteurs cellulaires et les facteurs du pathogène.

Mots-clés: Macrophages, métabolisme mitochondriale, *Leishmania*, Lipophosphoglycane, IFNAR.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS iii

ABSTRACT v

RÉSUMÉ vii

TABLE OF CONTENTS ix

LIST OF FIGURES AND TABLES xiii

LIST OF ABBREVIATIONS xv

CHAPTER 1: LITERATURE REVIEW 1

1 MACROPHAGE AND THEIR ROLE IN IMMUNITY 3

1.1 History and discovery of the macrophage 3

1.2 Macrophage development 8

1.3 Pattern recognition receptors 11

1.3.1 Toll-like receptors (TLRs) 12

1.3.2 C-type lectin receptors (CLRs) 12

1.3.3 Nucleotide-binding oligomerization domain-like receptors (NOD) 14

1.3.4 Retinoic acid-inducible gene I-like receptors (RIG-I) 14

1.3.5 DNA-sensing cyclic GMP–AMP synthase receptor (cGAS) 15

1.3.6 Secreted PRRs 16

1.4 Phagocytosis and phagosome maturation 18

1.5 Macrophage polarization 23

1.6 Type I interferon and its role in immunity 28

2 *LEISHMANIA* BIOLOGY 31

2.1 Epidemiology of leishmaniasis 31

2.2 *Leishmania* life cycle 34

2.3 *Leishmania* virulence factors 38

3 MACROPHAGE-*LEISHMANIA* INTERACTION 43

3.1 Initial interactions and *Leishmania* recognition 43

3.2 Phagosome biogenesis and transition into the parasitophorous vacuole 46

3.3 Modulation of host immune response 50

4 MITOCHONDRIAL BIOLOGY 53

4.1 History and discovery of the mitochondria 53

4.2 Mitochondria at the core of ATP production 58

4.2.1 Glycolysis 59

4.2.2 Anaerobic respiration 62

4.2.3 TCA cycle 62

4.2.4 Electron transport chain 65

4.3 Mitochondria life cycle 68

4.3.1 Mitochondrial biogenesis 69

4.3.2 Mitochondrial dynamics 70

4.3.1 Mitophagy 74

4.4 Metabolic reprogramming in macrophage polarization 78

4.5 Host Immune-metabolic interactions with *Leishmania* 83

CHAPTER 2: HYPOTHESES AND OBJECTIVES 87

CHAPTER 3: PRIMARY ARTICLE 91

CHAPTER 4: DISCUSSION OF THE PRIMARY ARTICLE 125

5 DISCUSSION AND CONCLUSIONS 127

5.1 Mitochondria at the core of immunometabolism 127

5.2 Energetic metabolisms at the interface between *Leishmania* and macrophages 129

5.3 Host mitochondrial biogenesis: a common process during *Leishmania* pathogenesis 132

5.4 Signalling axis involved in mitochondrial biogenesis 138

5.5 Importance of type I IFN and mitochondrial biogenesis for *Leishmania* 142

5.6 Can mitochondrial metabolism be targeted to prevent leishmaniasis? 144

5.7 General conclusion and final remarks 146

CHAPTER 5: RÉSUMÉ COMPLET EN FRANÇAIS 149

6 RÉSUMÉ 151

6.1 Introduction et objectifs de recherche 151

6.2 Méthodologie 153

6.3 Résultats 153

6.4 Discussion 159

6.5 Conclusion ... 163

APPENDIX 1: Complementary publications ... 165

7 Publication in related fields ... 167

7.1 VAMP3 and VAMP8 Regulate the Development and Functionality of Parasitophorous Vacuoles Housing *Leishmania amazonensis* ... 167

7.2 Cell-intrinsic Wnt4 ligand regulates mitochondrial oxidative phosphorylation in macrophages ... 185

7.3 Understanding TGEV–ETEC Coinfection through the Lens of Proteomics: A Tale of Porcine Diarrhea ... 201

References ... 205

LIST OF FIGURES AND TABLES

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Figures

Figure 1.1. Elie Metchnikoff's original draws and legends. 4

Figure 1.2. Pathogen clearing by the phagolysosome. 6

Figure 1.3. Mononuclear phagocyte system. 7

Figure 1.4. Embryonic hematopoietic programs. 8

Figure 1.5. Development of macrophage populations. 10

Figure 1.6. TLR receptors and their respective adaptors 13

Figure 1.7. dsDNA recognition by cGAS receptor. 16

Figure 1.8. Phagosome maturation. 22

Figure 1.9. Macrophages subsets. 25

Figure 2.1. Leishmaniasis endemicity. 32

Figure 2.2. *Leishmania* morphology. 35

Figure 2.3. *Leishmania* life cycle. 37

Figure 2.4. *Leishmania* promastigote virulence factors. 38

Figure 2.5. *Leishmania* virulence factors. 39

Figure 2.6. LPG structure. 41

Figure 3.1. *Leishmania* entry receptors in phagocytes. 45

Figure 3.2. *Leishmania* subverts macrophage biology. 49

Figure 3.3. Phagosomal assembly during *L. donovani* infection. 49

Figure 4.1. Mitochondrial ultrastructure. 56

Figure 4.2. ATP molecular structure. 59

Figure 4.3. Glycolysis pathway. 61

Figure 4.4. Anaerobic homolactic fermentation 62

Figure 4.5. Cory cycle. 63

Figure 4.6. TCA cycle. 64

Figure 4.7. Electron transport chain. 66

Figure 4.8. Mitochondrial network. 69

Figure 4.9. Mitochondrial biogenesis. 71

Figure 4.10. Mitochondria fusion. 72

Figure 4.11. Mitochondrial fission. 75

Figure 4.12. Mitophagy. 77

Figure 4.13. M1 macrophages. 81

Figure 4.14. M2 macrophages. 82

Figure 5.1. Metabolic print of *L. donovani* infected macrophage. 131

Figure 5.2. Mitochondria-*Leishmania* contact site 136

Figure 5.3. Parkin/Tom20 puncta accumulation 137

Figure 5.4. Type I IFN signalling axis 139

Figure 5.5. Graphical respresentation sumarizing the present findings. 147

Tables

Table 1. C-type lectin receptors. 14

Table 2. NLR and RLR. 17

Table 3. Opsonic and non-opsonic receptors. 20

Table 4. Molecular markers in polarized macrophages. 27

Table 5. IFNs family. 29

Table 6. Leishmaniasis and its associated etiological species. 33

Table 7. Mitochondria landmark discoveries 57

LIST OF ABBREVIATIONS

AKT	Protein kinase B
ADP	Adenosine diphosphate
AICAR	5-aminoimidazole-4-carboxamide ribonucleoside
AMP	Adenosine monophosphate
AMPK	AMP-activated protein
AP-1	Activator protein-1
APCs	Antigen-presenting cells
Arg	Arginine
ATP	Adenosine tryphosphate
BMM	Bone marrow-derived macrophages
C3b	Complement component 3b
CSF-1	Colony stimulating factor 1
CpG	Cytosine–phosphate–guanine
CPT-1	Carnitine palmitoyltransferase I
DAMPS	Damage-associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
eIF4E	Eukaryotic translation initiation factor 4E
ECAR	Extracellular acidification rate

EMPs	Erythromyeloid progenitors
eNOS	Endothelial nitric-oxide synthase
ER	Endoplasmic reticulum
ETC	Electron transport chain
ERK	Extracellular signal-regulated kinase
FAD	Flavin adenine dinucleotide
Fc	Fragment crystallizable region
FCCP	Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
G-CSF	Granulocyte colony-stimulating factor
GP63	Major surface glycoprotein 63
HIF-1α	Hypoxia-inducible factor 1 alpha
IFN	Interferon
iNOS	Inducible nitric oxide synthase
IRF	IFN-regulatory factor
IRG1	Immunoresponsive gene 1
JAK1	Janus kinase 1
LAMP-1	Lysosomal-associated membrane protein 1
LC3-II	Microtubule-associated protein light chain 3 phosphatidylethanolamine conjugate
LDHA	Lactate dehydrogenase A
LKB1	Liver kinase B1

LPG	Lipophosphoglycan
LPS	Lipopolysaccharide
M-CSF	Monocyte colony-stimulating factor
mTOR	Mammalian target of rapamycin
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NLRP3	NLR family pyrin domain containing 3
NO	Nitric oxide
NOX2	NADPH oxidases
NRF	Nuclear respiratory factor
MHC	Major histocompatibility complex
OCR	Oxygen consumption rate
PDH	Pyruvate dehydrogenase
PDK	Pyruvate dehydrogenase kinase isoform
PER	Proton efflux rate
PFK	Phosphofructokinase
PGC-1α	Proliferator-activated receptor-gamma coactivator-1α
PI3K	Phosphoinositide-3-kinase
PPARɣ	Peroxisome proliferator-activated receptors gamma

PTP-1	Protein-tyrosine phosphate
Rab7	GTPase protein 7
RET	Reverse electron transfer
ROS	Reactive oxygen species
SDH	Succinate dehydrogenase
SNARE	N-ethylmaleimide-sensitive factor attachment protein receptor
STAT	Signal transducer and activator of transcription
SYNT	Syntaxin
TGF-β	Transforming growth factor beta
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
TYK2	Tyrosine kinase 2
v-ATPase	Proton-ATPase
VL	Visceral leishmaniasis
TRAF	TNF Receptor Associated Factor 1
TNF	Tumor necrosis factor
TYRO	Tyrosine-protein kinase receptor
VAMP	Vesicle-associated membrane protein
WT	Wild type
α-KG	α-ketoglutate

CHAPTER 1: LITERATURE REVIEW

1 MACROPHAGE AND THEIR ROLE IN IMMUNITY

1.1 History and discovery of the macrophage

The macrophages are heterogeneous accessory cells present in most tissues across Metazoans that share the same core functions during general homeostasis. Macrophages play an important role in wide-ranging processes including, sensing micro-environmental changes (nutrients or microbial products), phagocyte detrimental or foreign cells (dead cells, abnormal cells and pathogens) and remodelling the micro-environment (Arroyo Portilla *et al.*, 2021; Man *et al.*, 2017; Wynn *et al.*, 2013). The word macrophages mean large eaters from the Greek (*μακρός* (*makrós* = large) and *φαγεῖν* (*phagein* = to eat). They compose one of the first defensive barriers to face invasive pathogens and an important link between the innate immunity and the adaptative immunity due to their capacity to present antigens to naïve T-lymphocytes (Gordon, 1998).

Macrophage discovery occurred over a century ago by the zoologist and embryologist Elie Metchnikoff (1845-1916). In the year 1880, Metchnikoff described that during the starfish larvae development, wandering amoeboid cells (now referred to as macrophages) were accumulating in different tissues surrounding and engulfing different materials composed of dead cells, allowing tissue resorption. Metchnikoff's interest to understand the nature of these ameboid cells led him to further observe how those cells were surrounding and devouring rose thorns introduced into the starfish body, a process that was further established as phagocytosis, raising the hypothesis that macrophages are involved in the detection, engulfment and clearing of foreign bodies. Metchnikoff extends his studies to other organisms to complement these findings, observing that a mix of fungi spores with the water flea *Daphnia* promotes mobile cells surrounding and engulfing the spores. In superior organisms including rabbits, Metchnikoff observed similar reactions after the exposition of anthrax, especially in attenuated forms, which strengthened these ameboid cells' role in immunity and gave rise to the concept of cellular innate immunity (Metchnikoff, 1905) **Figure 1.1**. The landmark discovery of macrophages, the close link between inflammation and phagocytosis of foreign bodies among different species, granted Metchnikoff to become the co-winner of the 1908 Nobel Prize in Physiology along with Paul Ehrlich.

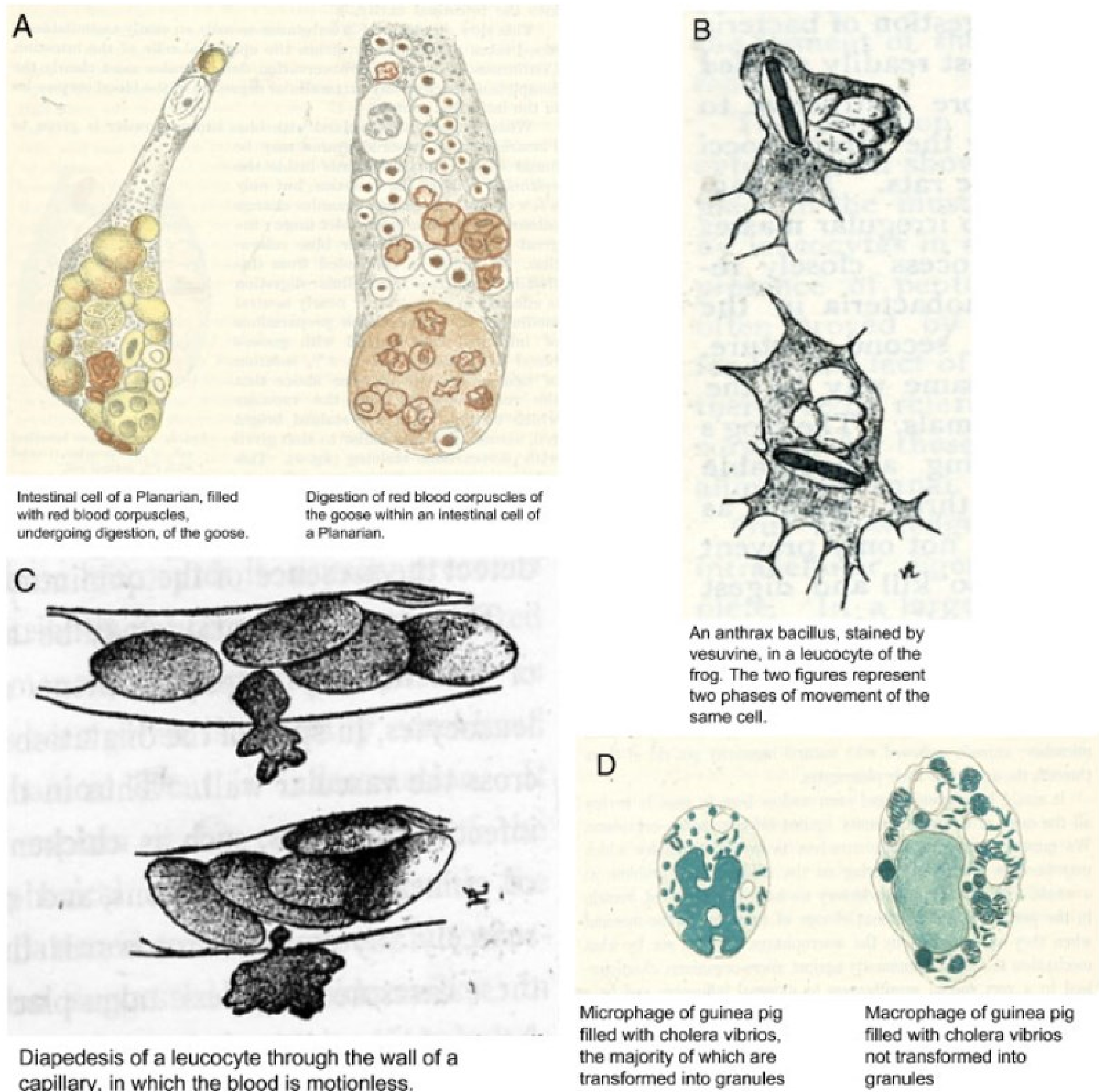


Figure 1.1. Elie Metchnikoff's original draws and legends.
Graphical representations of Metchnikoff's research in the landmark discovery of the macrophage (Gordon, 2008).

In parallel, while Metchnikoff was conducting experiments to strengthen his theory, Paul Ehrlich (1845-1915) was working on another revolutionary theory known as the side chain, which later became a principal topic of immunological research. This theory postulates that certain cells present a set of side chains on their surface (further named receptors) responsible for specific toxin recognition upon microbe infections. After an initial encounter with the toxin, the cell

produces an antitoxin (further named antibodies) to bind and neutralize them, allowing for the organism to survive future encounters against the same microbe. Importantly, a small amount of these toxins led to a large production of antitoxins, hence preventing the adverse effects of the toxin. Thus, Ehrlich proposed that the soluble side chains assist in the elimination of microbes as part of the host's defences. This fascinating theory can be linked with the fact that pathogens neutralized by the side chains or dead cell products of host-pathogen interaction, can be cleared by the ameboid cells described by Metchnikoff. Noteworthy, both theories were integrated some years after Metchnikoff and Ehrlich's finding.

In 1924, the description of phagocytes became chaotic and the term "macrophage" was associated with around 30 different names depending of the observed tissue (Gall, 1958). This was the case until Karl Albert Ludwig Aschoff (1866-1942) coined the term reticuloendothelial system (RES) to describe the group of circulating blood cells with the capacity to incorporate vital dyes from circulation. Those cells nowadays are known as monocytes, neutrophils, eosinophils, and basophils (Aschoff, 1924; Ehrlich, 2013; Florey & Florey, 1958). The word reticulo refers to the ability of these phagocytic cells to create a network (reticulum) by cytoplasm extensions, and the word endothelial refers to the phagocyte's proximity to the vascular endothelium (Wintrobe, 1980). Thus, the major role of RES is to detect, phagocyte and clear foreign matter from the blood in superior organisms (Aschoff, 1924; Silverstein, 2011; Tauber, 2003; Yona & Gordon, 2015). Further studies performed by different immunologists described that blood culture over time differentiates into macrophages that actively phagocyte debris from other blood cells (Awrorow & Timofejewskij, 1914; Carrel & Ebeling, 1926; Lewis & Lewis, 1925). Importantly, the fact that blood monocytes were migrating from circulating blood to the site of injury (diapedesis) in a pathological context led to a better understanding of macrophage ontogeny and how blood monocytes are differentiated into macrophages (Ebert & Florey, 1939; Sabin, 1925; Volkman & Gowans, 1965). Subsequently, with the development of electronic microscopy, it was possible to distinguish the early phase of monocyte migration in a mild inflammatory model concluding that monocytes become effector cells that concentrated at the injury site (Gall, 1958; Marchesi & Florey, 1960).

During the '60s, Zanvil Alexander Cohn (1926-1993) emerged as the founder of modern macrophage biology, which better defined the role of macrophages (Steinman & Moberg, 1994). During his research, he described macrophages as large cells that can surround and digest foreign matter, including protozoa and bacteria. His team isolated granules from phagocytic cells

to analyze whether those cells contain preformed bacterial substances to kill pathogens (Armstrong & Hart, 1971). Indeed, by using phase contrast and electron microscopy they found structures further known as lysosomes. Additional studies showed how bacteria were engulfed and internalized by the cells forming a vacuole, followed by a fusion with lysosomes to digest and clear those bacteria (**Figure 1.2**). This finding is considered a major immunological landmark, which established a central field for cell biology (Endocytosis). Subsequent studies established that macrophages could release a multitude of biologically active products derived from the lysosomes (Armstrong & Hart, 1975) and activated macrophages actively release oxygen intermediates such as superoxide and hydrogen peroxide to clear out bacteria and tumor cells (Clifford & Repine, 1982; Wang *et al.*, 1996). Cohn's research went beyond the role of macrophages in the infective process. He also described that macrophages secrete a plethora of molecules into their surrounding environment (known as cytokines, prostaglandins and leukotrienes), affecting other cellular activities, which may lead to either wound healing or tissue disruption (Steinman & Moberg, 1994).

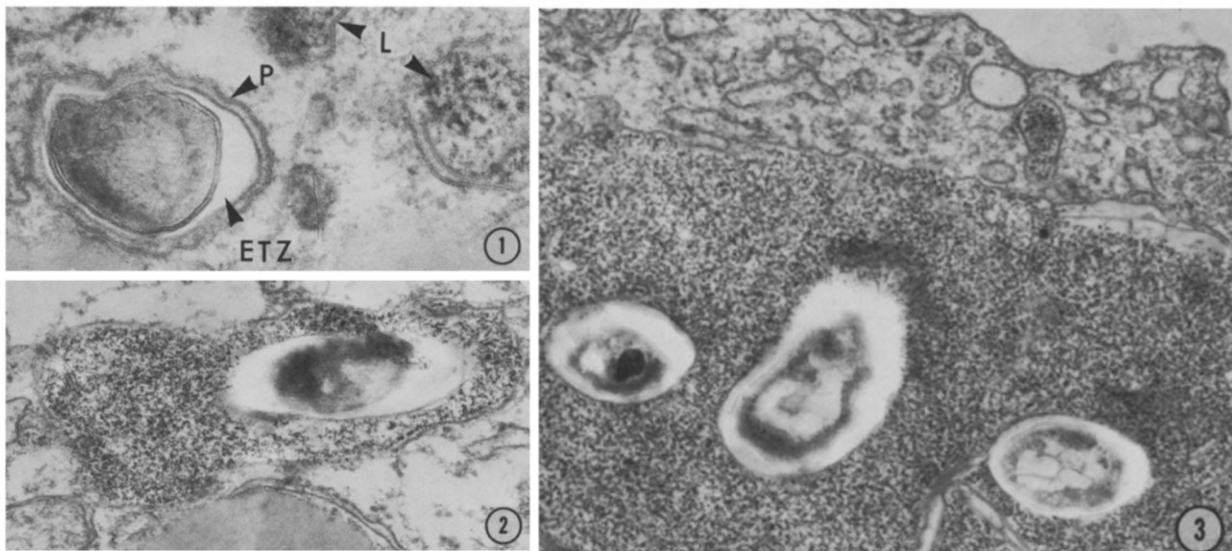


Figure 1.2. Pathogen clearing by the phagolysosome.
Panel 1 shows a pathogen inside a phagosome and two lysosomes. Panel 2 shows the phagosome fusion with a lysosome. Panel 3 shows the phagolysosome in which the pathogen is further destroyed. Plasma membrane (P), Lysosome (L), electro-transparent zone (ETZ) (Armstrong & Hart, 1975).

Even if during the years from 1920 to 1960–a lot of evidence supported that the macrophage originates from circulating blood monocytes, some authors had shreds of evidence that resident

macrophages were independent of circulating monocytes or their antecedents, proposing that resident macrophages may arise from distinct lineages (Maksimov, 1907; Sawyer *et al.*, 1982; van Furth & Cohn, 1968; Volkman *et al.*, 1983). During the scientific meeting in Leiden in 1969, due to the accumulated knowledge since the establishment of the term REM and the missing pieces in the resident macrophage ontology, a group of prominent pathologists and immunologists proposed to relabel the term REM to mononuclear phagocyte system (MPS) for being a more accurate term to describe monocytes and macrophages derived from the bone marrow-derived monocytes **(Figure 1.3)** (Van Furth *et al.*, 1972). This model continued evolving in concept and thanks to complementary analyses that included parabiosis, thymidine incorporation and bone marrow chimera experiments suggesting that a proportion of myeloid cells in the brain, lung, liver, peritoneum, and spleen were dependent on local proliferation rather than monocyte recruitment of bone marrow-derived monocytes (Kennedy & Abkowitz, 1997; Parwaresch & Wacker, 1984). In the late '90s, the landmark discovery that microglia (brain macrophages) are derived cells from the yolk sac (YS) and their progeny support and replicate in number during development, open a new chapter in the macrophages ontology and further straightened for several authors that revealed a fetal origin for most postnatal tissue macrophages, including microglia. (Alliot *et al.*, 1999; Hashimoto *et al.*, 2013; Perdiguero *et al.*, 2015; Schulz *et al.*, 2012; Yona *et al.*, 2013)

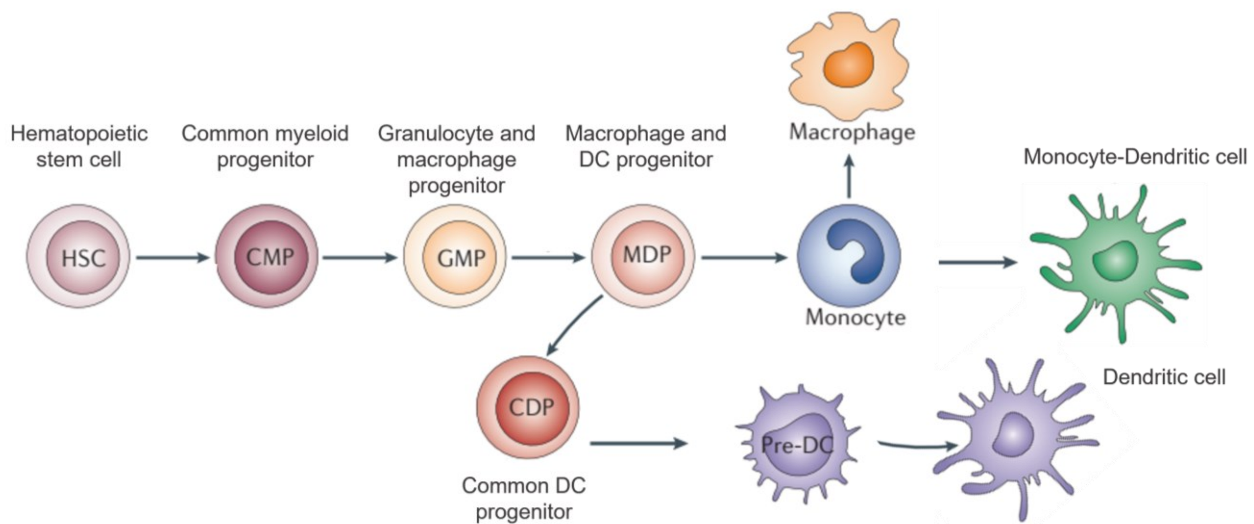


Figure 1.3. Mononuclear phagocyte system.
Hematopoietic-stem cells undergo a differentiation process that generates a common precursor for different cell lineages including, Monocytes, Macrophages and Dendritic cells (Chow *et al.*, 2011)**.**

1.2 Macrophage development

The macrophages are evolutionarily conserved phagocytes that evolved over 500 million years ago and that are found in all vertebrates, invertebrates and most primitive groups of organisms including protozoans, sponges, and cnidarians (Buchmann, 2014; Cooper & Alder, 2006; Gaudet *et al.*, 2017; Wittamer *et al.*, 2011). In mice, the origin of the macrophages occurs in the beginning of the embryonic development in three well-defined waves, that give rise to diverse macrophage populations in the adult state. The first wave starts with macrophages derived from the yolk sac that are independent of blood monocytes and give origin to microglia cells. The second wave involves monocytes from the blood and produces the fetal liver, which in turn produces tissue-resident macrophages. Finally, the third wave produces monocytes derived from the bone marrow hematopoietic stem cells (HSCs) giving origin to monocyte-derived macrophages **(Figure 1.4)** (Hoeffel & Ginhoux, 2018).

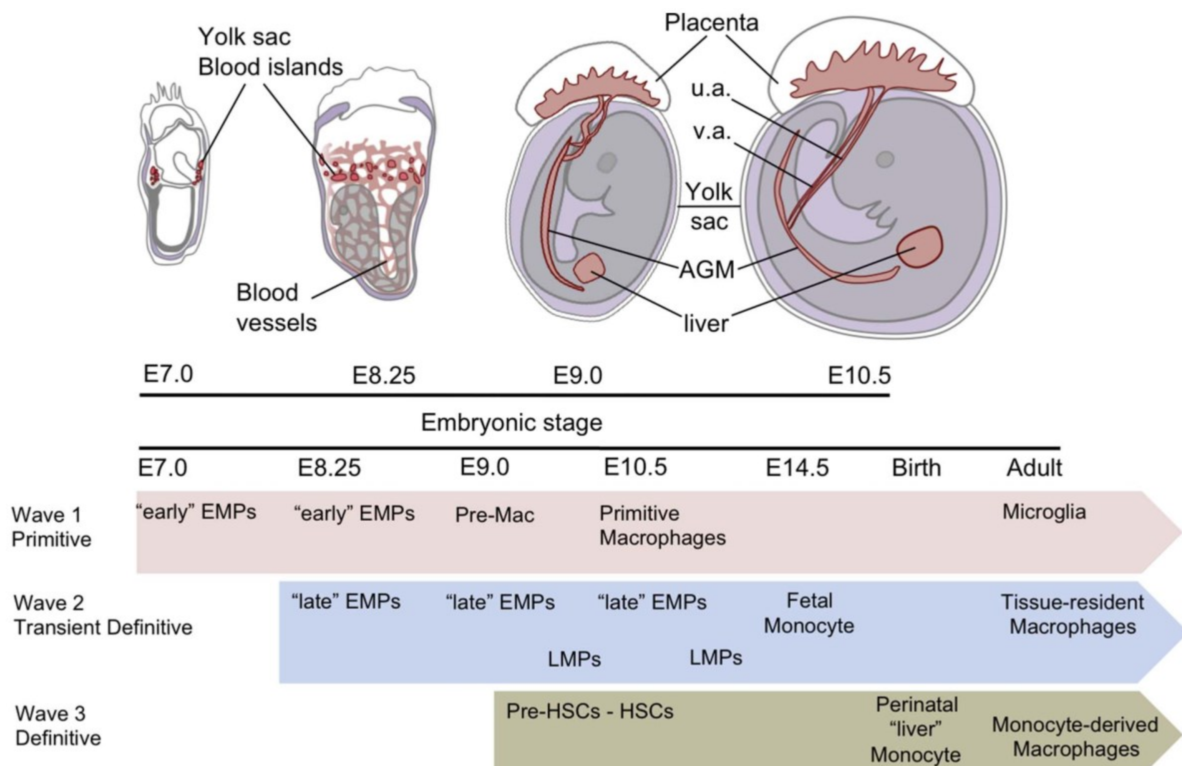


Figure 1.4. Embryonic hematopoietic programs.
Hematopoiesis during mouse embryonic development begins with the first wave during week 7 in which early erythro-myeloid progenitors (EMP) from the yolk sac give rise to primitive macrophages. The second wave begins during week 8.25 in which EMPs differentiate into fetal monocytes and further give rise to Tissue-resident macrophages. The third wave begins during week 10.5 with the emergence of Hematopoietic Stem Cells (HSC) from aorta-gonads-mesonephros (AGM) that give rise to the perinatal liver and further give rise to the mononuclear phagocyte system (Hoeffel & Ginhoux, 2018).

During the first wave (a primitive program), the embryo produces lifelong undifferentiated HSCs from the yolk sac at the embryonic age 7.0 (E7.0) where a unipotent myeloid progenitor restricted to the macrophage lineage emerges among other cells (Mac-CFC). The denomination “primitive” refers to the Yolk Sac macrophages due to their independent development of the transcription factor c-Myb before the beginning of fetal liver hematopoiesis. The primitive macrophages continue proliferating and eventually migrate to the developing brain and different tissues between E10 to E12.5. In contrast, during the next waves, these primitive macrophages are replaced by new emerging macrophages, except for the microglia, which is the unique tissue that preserves the primitive macrophage population (Ginhoux *et al.*, 2010; Hoeffel *et al.*, 2015; Hoeffel *et al.*, 2012; Perdiguero *et al.*, 2015). The primitive macrophages can be traced by using the molecular markers $CD45^+$, $F4/80^+$ and, CX_3CR1^- (Bertrand *et al.*, 2005). During the second wave, (a transient definitive program) alongside the emergence of different cell populations in the yolk sac, a multipotent erythroid-myeloid progenitor (EMP) rises in a c-Myb dependent manner between E7.0 to E11.0 generating among others fetal monocytes, which compose the main progenitor cell populations for almost all tissue-resident macrophages with an intermediate monocytic subset (Ginhoux & Guilliams, 2016; Hoeffel *et al.*, 2015; Palis *et al.*, 1999). Further, between E12 to E14, the fetal monocytes reach the blood circulation and colonize every tissue excluding the brain (Hoeffel *et al.*, 2015; Hoeffel *et al.*, 2012).

The EMP can be traced by using the molecular markers $CD45^-$, $c\text{-}Kit^+$, and CX_3CR1^+ (Bertrand *et al.*, 2005). During the third wave (definitive program) there is an emergence of HSC in the intra-embryonic region, in vitelline and umbilical arteries at E9.5 (Clements & Traver, 2013; Lin *et al.*, 2014; Mikkola *et al.*, 2005; Tavian & Peault, 2003) that further migrate to the fetal liver to massively expand and mature. On D16.5 these HSCs from the fetal liver migrate and colonize the developing fetal bone marrow, where they remain throughout adulthood and generate all blood cell lineages (myeloid linage) (Christensen *et al.*, 2004). After the third wave and following the embryo development, the myeloid system is established as a layered system supported by the coexistence of resident tissue macrophages, HSCs and other cell lineages, that in turn support the self-renewal of the different macrophage populations (Shepard & Zon, 2000).

The mononuclear phagocyte system includes a myeloid hematopoietic cell linage that gives rise to circulating blood monocytes, that can enter different tissues, thus becoming tissue-resident macrophages (Yona & Jung, 2010). During the macrophage differentiation process, several markers have been identified to belong to different subpopulations, which present a panoply of

functions. The common monocyte-macrophage marker F4/80 recognizes the extracellular domain of Epidermal Growth Factor (EGF)-like calcium-binding domains and is present in the majority of the MPS system (Hume, 2006; Waddell *et al.*, 2018). Circulating Blood monocytes can be distinguished in at least two principal populations: classical monocytes (inflammatory) expressing the markers Ly6C^{hi}, CD43^{+}, CCR2^{+}, CD62L^{+}, CX$_{3}$CR1^{Low} and non-classical (resident) monocytes expressing the markers Ly6C^{low}, CD43^{++}, CCR2^{-}, CD62L^{-}, CX$_{3}$CR1^{hi} **(Figure 1.5)** (Hoeffel *et al.*, 2015; Mosser & Edwards, 2008; Yona & Jung, 2010; Ziegler-Heitbrock *et al.*, 2010). The circulating monocytes have a half-life time estimated at around one day (Liu *et al.*, 2007) and under steady conditions, Ly6C^{lo} monocytes contribute to the replenishment of resident macrophages in different tissue compartments, which has an important role in the anti-inflammatory response, tissue remodelling and angiogenesis. In contrast, Ly6C^{hi} monocytes can, under inflammatory conditions, give rise to macrophages with high microbicidal capacity (Serbina *et al.*, 2008; Yang *et al.*, 2014)

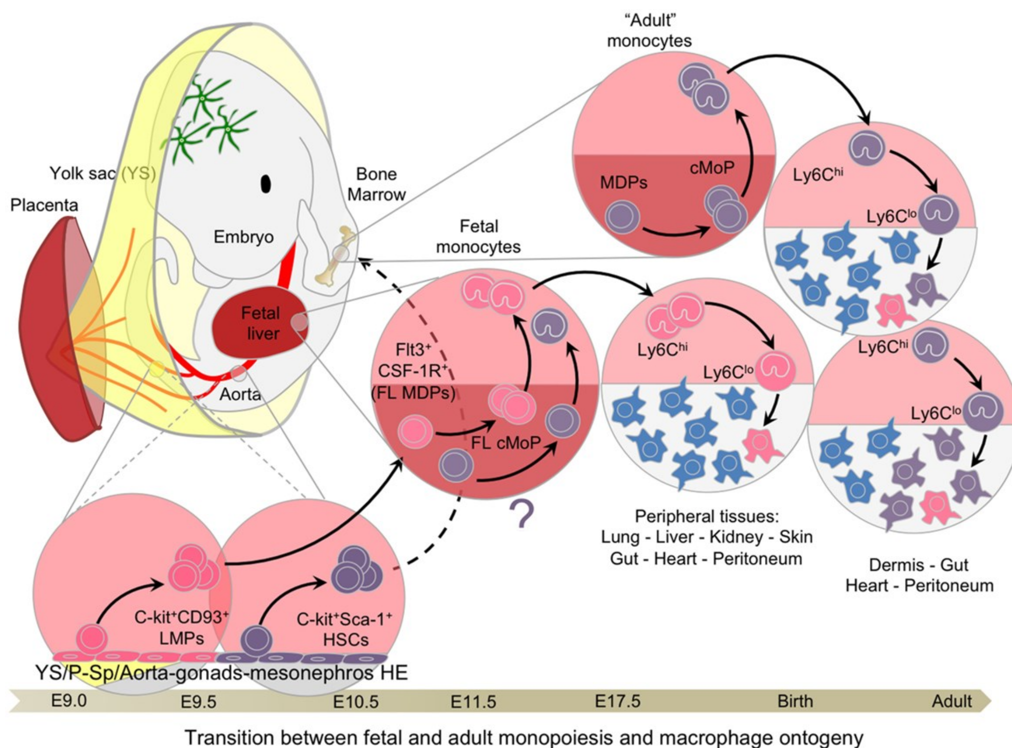


Figure 1.5. Development of macrophage populations.
Primitive macrophages replicate and maintain their population in the brain (Microglia) during adult life. During the embryo development, fetal monocytes establish the tissue-resident macrophage network including adipose tissue, blood, bone, gastrointestinal tract, liver, lung serosal tissues, skin, and spleen, after the macrophage replenishment is principally supported by bone marrow during adult life. Circulating monocytes from the bone marrow rise all the tissues (excluding the brain) and act as immune sentinels for pathogens and tissue remodelling (Hoeffel & Ginhoux, 2015).

1.3 Pattern recognition receptors

Macrophages are a main component of the innate immune system, involved in different processes including the distinguishing of self-tissues from a microbial invader, effector response to clear or control microbe infections and tolerating potential collateral damage induced by the effector responses. The process by which macrophages establish different responses relies on the detection of different molecular structures (ligands) by the germline-encoded host receptors known as pattern-recognition receptors (PRRs) expressed on the cell surface, in intracellular compartments, secreted into the bloodstream and present in interstitial fluids raising different tissues (Areschoug & Gordon, 2008; Medzhitov & Janeway, 1997; Medzhitov & Janeway Jr, 1997; Medzhitov *et al.*, 1997). The PRRs can recognize two different classes of ligands. Depending on the ligand nature and the PRR's location, the macrophage may trigger different responses including pathogen clearance, initiation of the antigen-specific adaptive immune response, cytokine release and antitumoral effects among others. The first group of ligands are the Pathogen-associated molecular patterns (PAMPs), which are associated with microorganism recognition such as bacteria, protozoans, and fungi. PAMPs include small conserved molecular motif such as LPS, Glycans, lipoteichoic acid, and lipoproteins among others. In general, PAMPs recognition triggers an activation response in macrophage along with cytokine and interferon production (Bianchi, 2007; Gordon, 2016; Takeuchi & Akira, 2010; Wilson *et al.*, 1989). The second group of ligands are Damage-associated molecular patterns (DAMPs), which are associated with the recognition of the host's cell components released during homeostasis, from dead or damaged cells and trauma due to a microorganism infection or physical damage. DAMPs include molecules such as Uric acid, heat-shock proteins, ATP, and nucleic acids among others. In general, recognition of DAMPs triggers a non-infectious inflammatory response and initiates a healing process (Bianchi, 2007; Gong *et al.*, 2020; Kroemer *et al.*, 2022).

Based on the PRR's localization, they may be divided into membrane-bound PRRs (Toll-like receptors and C-type lectin receptors) and cytoplasmic PRRs (NOD-like receptors, RIG-1-like receptors and cGas receptors) (Creagh & O'Neill, 2006; Gordon, 2002; Xia *et al.*, 2016).

1.3.1 Toll-like receptors (TLRs)

Toll-like receptors (TLR) were initially discovered in *Drosophila* and their function was associated to trigger host cell defences, such as cytokine production, and play an important link between the innate and adaptive immune responses. TLR homologues have been described in a wide range of species and to date, there are twelve identified functional TLRs in mice (TLRs 1–13, where TLR10 is a non-functional pseudogene) (Janssens & Beyaert, 2003; Lien & Ingalls, 2002). TLRs are synthesized in the ER, which are then translocated to the Golgi complex and delivered to intracellular compartments or the cell surface (Vijay, 2018). They are composed of an ectodomain with conserved extracellular Leucine-rich repeat (LRR) motifs that mediate ligand recognition. They also have a transmembrane domain (TMD) that mediates membrane attachment and a cytoplasmic Toll/IL-1 receptor (TIR) domain that activates downstream signalling (Luo *et al.*, 2019). Depending on the TLR, they may interact with the ligand as a homo or heterodimer in conjunction with accessory molecules and co-receptors (Botos *et al.*, 2011). After ligand recognition, TLRs recruit the TIR domain along with adaptor proteins including myeloid differentiation primary response 88 (MYD88), TIR-domain-containing adapter-inducing interferon-β (TRIF) or both, and TRIF-related adaptor molecule (TRAM), which in turn, activate downstream signalling pathways that lead to the activation of factors such as NF-κB, IRFs, or MAP kinases that regulate cytokine and chemokine expression, inflammation induction and type I IFN production among others **(Figure 1.6)** (Baumann *et al.*, 2010; Kawai & Akira, 2010; Zanoni *et al.*, 2011).

1.3.2 C-type lectin receptors (CLRs)

C-type lectin receptors (CLRs) are soluble and membrane-bound receptors involved in recognizing a myriad of pathogens. They are the principal receptors for fungi recognition and detecting self-ligands that regulate immune homeostasis (García-Vallejo & van Kooyk, 2009). The CLRs recognize proteins with at least one C-type lectin domain (CTLD) and its function is mediated dependently or independently of $Ca2^{+}$. The CLRs are divided into two groups: Type I CLRs (mannose receptor family) with a transmembrane protein containing several carbohydrate-recognition domains (CRDs or CRD-like) such as selectins, DEC-205 and the macrophage mannose receptor (MMR); and Type II transmembrane CLRs (asialoglycoprotein-receptor family) containing a single CRD domain, which encompass a multifamily group of

receptors such as hepatic asialoglycoprotein receptors (ASGPRs), Dectin-1, Dectin-2, macrophage-inducible C-type lectin (Mincle), the dendritic cell-specific ICAM3-grabbing nonintegrin (DC-SIGN), and DC NK lectin group receptor-1 (DNGR-1). To date, based on their structure, function and phylogeny, they are categorized into 17 sub-groups. (Drickamer, 1999; Zelensky & Gready, 2005). After the ligand recognition, CLRs activate downstream signalling pathways that lead to the activation of NF-κβ and mitogen-activated protein kinases (MAPK), triggering different cellular responses, such as phagocytosis, inflammasome activation, respiratory burst, and soluble mediator production. These receptors can also drive the development of adaptative Th1 and Th2 immunity **(Table 1)** (Drummond *et al.*, 2011; Kerrigan & Brown, 2011; Sancho & Reis e Sousa, 2012; Strasser *et al.*, 2012).

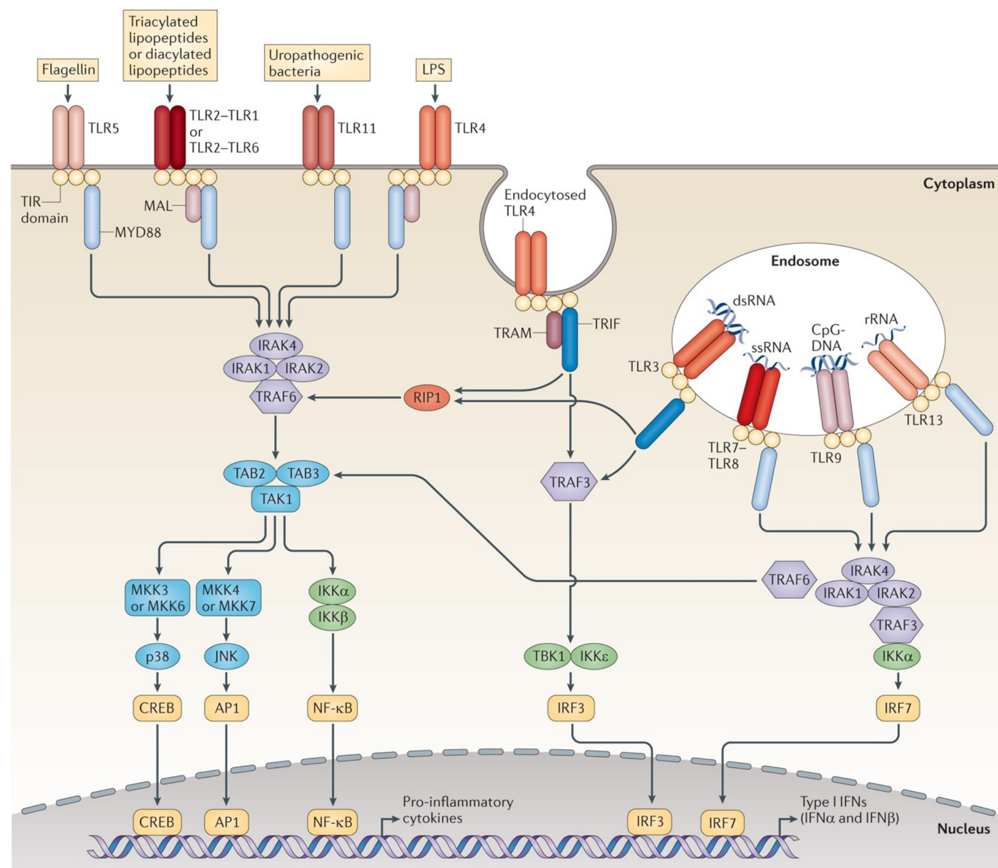


Figure 1.6. TLR receptors and their respective adaptors
Extracellular and intracellular TLRs bind to their respective ligands inducing it dimerization in conjunction with accessory molecules and co-receptors such as MYD88, TRIF and TRAM. Engagement of the signalling adaptors stimulates downstream signalling pathways that active transcription factors such as IRFs, CREB, AP1 and NF-κB, which in turn induces their respective response (O'Neill *et al.*, 2013).

Table 1. C-type lectin receptors.
C-type lectin receptors with their respective ligands and their cellular location (Zhang & Mosser, 2008).

Receptors and their ligands		Locations
C-type lectin receptors		
MR*	Ligands bearing mannose, fucose, or *N*-acetyl glucosamine	Cell surface
DC-SIGN	ICAM2/3; HIVgp120; *M. tuberculosis* ManLAM	Cell surface
Dectin-1	Fungus β-glucans; zymosan	Cell surface

1.3.3 Nucleotide-binding oligomerization domain-like receptors (NOD)

Nucleotide-binding oligomerization domain-like receptors (NLRs) are highly conserved intracellular receptors found in several species, which principally recognize bacterial peptidoglycans and intracellular molecules released during cellular stress or damage (Dolasia *et al.*, 2018; Ting *et al.*, 2008). NLRs are composed of three domains. The central NACHT (NOD or NBD – nucleotide-binding domain) domain, which is common to all NLRs and important for nucleic acid binding and oligomerization. The C-terminal leucine-rich repeat (LRR) domain is present in the majority of NLRs and is involved in ligand recognition. The variant N-terminal effector domain is involved in protein-protein interactions (Martinon & Tschopp, 2005). Depending on the N-terminal domain type, NLRs are divided into five subfamilies: the NLRA subfamily (A for the acidic transactivating domain), the NLRB subfamily (B for the baculovirus inhibitor of apoptosis protein repeats), the NLRC subfamily (C for the caspase activation and recruitment domain), the NLRP subfamily (P for the pyrin domain) and the NLRX subfamily containing other NLR effectors, which do not have significant homology to an N-terminal domain (Kim *et al.*, 2016). In general, once the ligand is recognized by the LRR, the NACHT domain is activated and the N-terminal effector domain recruits adaptor molecules to initiate the corresponding transduction signal, which activates downstream signalling pathways that lead to inflammasome activation, apoptotic and proinflammatory responses **(Table 2)** (Kanneganti, 2010).

1.3.4 Retinoic acid-inducible gene I-like receptors (RIG-I)

Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) are cytosolic receptors involved in the recognition of viral RNA and mediation of antiviral host responses. RLRs are members of the DEAD-box helicase family and include three members: retinoic-acid inducible gene or DDX58

(RIG-I), melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) (Barral *et al.*, 2009; Kawai & Akira, 2009). RLRs receptors share a common domain containing a catalytic helicase core made up of two RecA-like domains that coordinate ATP and RNA binding. Also, a C-terminal domain (CTD) binds viral RNA. Two N-terminal CARDs (caspase active recruitment domains) are essential for the initiation of downstream signalling in RIG-I and MDA5. In contrast, LGP2 is implicated as a positive and negative regulator of RIG-I and MDA5. Once the RNA is recognized by the RLR, the CARD domains oligomerize and interact with the mitochondrial antiviral signalling protein (MAVS) inducing TRAF3 and IKKε/TBK1 recruitment that lead to type I and III interferon production **(Table 2)** (Ng *et al.*, 2012; Rehwinkel & Gack, 2020).

1.3.5 DNA-sensing cyclic GMP–AMP synthase receptor (cGAS)

The DNA-sensing cyclic GMP–AMP synthase receptor (cGAS) is involved in the recognition of host cytosolic dsDNA derived from nuclear DNA or mitochondrial DNA (DAMPS), which is length-dependent (>45 bp for a proper immune response). Also, cGAS is involved in the recognition of dsDNA derived from pathogens, such as bacteria or viruses (Xia *et al.*, 2016). Strikingly, the distinction of host DNA from pathogen DNA is critical to establish a specific immune response. Contrary, improper DNA distinction may lead to autoimmune diseases (Ablasser *et al.*, 2013; Ahn & Barber, 2014; Li *et al.*, 2013). Once cGAS recognizes dsDNA, it undergoes a conformational change that allows the ATP and GTP entry to the catalytical pocket, which synthetize the cyclic guanosine monophosphate–adenosine monophosphate cGAMP, that in turn act as a strong activator of interferon genes (STING) (Civril *et al.*, 2013). The adaptor STING is mostly localized on the ER; however, it also can be localized on the ER-mitochondria associated membranes (Smith, 2020). cGAS-STING interaction promotes STING migration from the ER to the Golgi, where it induces TBK1 activation, that in turn induces IRF3 phosphorylation and subsequent type I interferon production. Also, cGAS-STING promotes IKK-NF-κβ activation an its respective immune response **(Figure 1.7)** (Takeuchi & Akira, 2010).

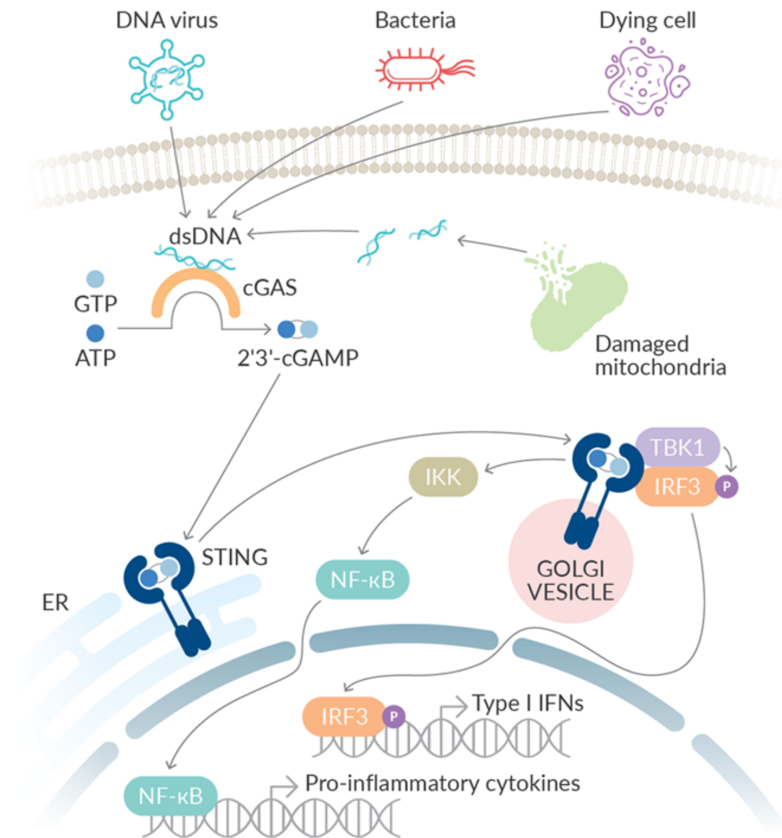


Figure 1.7. dsDNA recognition by cGAS receptor.

dsDNA is recognized by cGAS and promotes STING recruitment. Downstream signaling leads to IKK-NF-κβ and TBK1-IRF3 activation that lead to pro-inflammatory cytokines and type I IFNs production respectively (Taken from https://www.caymanchem.com/news/targeting-cgas-activity-in-the-sting-pathway).

1.3.6 Secreted PRRs

In addition to the PRRs mentioned above, there are also secreted PRRs present in different intracellular compartments, circulating in the interstitial fluid and bloodstream. Secreted PRRs are involved in the recognition of PAMPs and DAMPs, which in general lead to inflammatory regulation and pathogen clearance (Gao *et al.*, 2008; Iwasaki & Medzhitov, 2010). Collectin and ficolin are receptors that recognize oligosaccharides, lipids, and carbohydrates, involved in pathogen recognition and clearance (Holmskov *et al.*, 2003). Pentraxins are receptors that recognize a wide range of self and non-self ligands including growth factors, extracellular matrix components, and some pathogens giving them a multisided role in host protection (Mantovani *et*

al., 2008). Peptidoglycan recognition proteins (PGRPs) are highly conserved receptors that mainly recognize the bacteria cell wall peptidoglycan and are involved in pathogen clearance (Dziarski, 2004). In addition to this, other receptors, including complement receptors, apoptotic receptors, scavenger receptors, mannose receptors and β-glucan receptors that can recognize several ligands and induce among others, a phagocytic response. Nevertheless, their specific function will be mentioned in the next section.

Table 2. NLR and RLR.
NLR and RLR family receptors with some examples of their respective ligand and the associated pathogens (Zhang & Mosser, 2008)

Receptors and major ligands	Live infectious bacteria and virus
NLR family	
CARD subfamily	
NOD1 GM-tripeptide; *meso*-lanthionine; *meso*-DAP; γ-D-Glu-DAP; FK156 (D-lactyl-L-ala-γ-Glu-meso-DAP-Gly); FK565 (Heptanoly-γ-Glu-meso-DAP-D-ala)	*Chlamydia* spp.; enteroinvasive *E. coli*; *Helicobacter pylori; Listeria monocytogenes*; *Pseudomonas* spp.; and *Shigella flexneri*
NOD2 M-TRILys (MurNAc-L-Ala-γ-D-Glu-L-Lys); MDP	*L. monocytogenes; Salmonella flexneri*; *S. typhimurium*; and *Streptococcus pneumoniae*
IPAF Bacterial flagellin from *Coxiella, Legionella* and *Salmonella*	*S. typhimurium*
Pyrin subfamily (14 NALPs)	
NALP1 LeTx (anthrax lethal toxin); MDP	*Staphylococcus aureus* and *L. monocytogenes*
NALP3 ATP; bacterial mRNA; maitotoxin; nigericin; uric acid crystal; R848	
BIR subfamily	
NAIP Flagellin from *Bacillus, Legionella* and *Salmonella*	*Legionella pneumophila*
RNA helicases	
RIG-1 Uncapped 5′-triphosphate-RNA	Newcastle disease virus, Sendai virus, influenza virus, vesicular stomatitis virus, Japanese encephalitis virus
MDA5 Encephalomyocarditis virus dsRNA; poly (I:C)	Encephalomyocarditis virus; picornaviruses; Thyler's virus and Mengo virus

1.4 Phagocytosis and phagosome maturation

Macrophages play an important role in the homeostasis of higher metazoans, mediating different biological processes including development, removal of apoptotic bodies, tissue remodelling and pathogen clearance (Arroyo Portilla *et al.*, 2021; Gordon, 2016; Wynn *et al.*, 2013). To do this, macrophages generally detect and uptake several targets through a process known as phagocytosis in which particles >0.5 µm are completely engulfed within the plasma membrane and give rise to the phagosome. Then, the phagosome goes through multiple vesicular changes and maturation processes to generate a phagolysosome, which degrades its cargo due to the acidified microenvironment and action of hydrolytic enzymes (Desjardins, 1995; Desjardins *et al.*, 1997; Flannagan *et al.*, 2012; Vieira Otilia *et al.*, 2003). There are two main types of phagocytic bodies: foreign bodies (microorganisms) and self-bodies (apoptotic and necrotic) that contrast the consequence of their uptake into a pro- or anti-inflammatory response respectively, excluding the necrotic cell uptake that promotes inflammation as an exception (Uribe-Querol & Rosales, 2020). Importantly, depending on the cargos' nature, phagocytosis may induce antigenic presentation that would lead to the activation of the adaptative immune response (Jutras & Desjardins, 2005). Thus, it is a challenge for the macrophage to distinguish between the self and non-self ligands, living cells from damaged or dead cells and pathogens from commensal microorganisms, to establish an adequate immune response. The phagocytic process involves four principal phases: detection of a target body, activation of the uptake process, phagosome formation and maturation of the phagosome into a phagolysosome.

The way the macrophage distinguishes different bodies and induces a phagocytic response is mediated by receptors that may include opsonic receptors and non-opsonic receptors **(Table 3)** (Flannagan *et al.*, 2012). Opsonic receptors are involved in the recognition of foreign bodies by soluble immunoglobulins (IgG) and by fractions of the complement system such as iC3b, C3b and C4b that are constantly circulating in the bloodstream and interstitial fluids. These molecules act as phagocytic coadjutants by attaching to the foreign bodies in a process known as complementation. Furthermore, the complemented bodies are recognized by FcγR receptors and complement receptors that trigger the phagocytic process (Anderson *et al.*, 1990; Dustin, 2016; Ross *et al.*, 1992). The FcγR receptors recognize the Fc portion of the immunoglobulin G (IgG-coated targets) and are composed of FcγR I, FcγR II and FcγR III. These receptors contain an intracellular adaptor ITAM (immunoreceptor tyrosine-based activation motif) involved in the transduction signal that activates the Wiskott–Aldrich Syndrome protein (WASp) by the action of

RAC and Cdc42. This process induces lipid rearrangements and cytoskeletal remodelling that extend the plasmatic membrane into protrusions (lamellipodia) forming a phagocytic cup around the bodies, followed by a complete engulfment that creates the phagosome (Park & Cox, 2009; Prehoda Kenneth *et al.*, 2000). The complement receptors recognize bodies coated by iC3b, C3b and C4b and are divided into three family members: the short consensus repeat (CR1 and CR2), the β2 integrin (CR3 and CR4) and the immunoglobulin G (CRIg). The transduction signal induces the tyrosine kinase Syk and Rho GTPases for CR3. Nevertheless, the pathways involved in this process are not well characterized. Unlike FcγR, the complement receptors induce phagocytosis without cytoskeleton extensions. Instead, there is an induction of membrane ruffles that surround the opsonized bodies and a complete engulfment that creates the phagosome (van Lookeren Campagne *et al.*, 2007). The non-opsonic receptors recognize foreign bodies independently of the complement fractions or IgG and may include the recognition of apoptotic bodies. These groups include receptors such as Scavenger receptors, C-type lectin and Dectin-1. The scavenger receptors are a subclass of membrane-bound receptors and recognize different PAMPs and DAMPs. Depending on their structure, scavenger receptors are involved in different processes including phagocytosis of pathogens, antigen presentation and the clearance of apoptotic cells (Canton *et al.*, 2013; Penberthy & Ravichandran, 2016; van der Laan *et al.*, 1999).

During tissue homeostasis, many cells constantly die and need to be replaced by new oncoming cells. The apoptotic cells express specific molecules such as lysophosphatidylcholine and phosphatidylserine (PS) that are only exposed in the external part of the plasma membrane and act as an “eat me” signal. The macrophage presents a panoply of receptors that recognize that signal, inducing a phagocytic process in a non-inflammatory context **(Table 3)** (Nagata *et al.*, 2016; Segawa & Nagata, 2015). Importantly, phagocytic receptors may synergize with some PRRs that do not induce phagocytosis directly, but, which can prime the cell for an efficient recognition of ligands, cause receptors aggregation and initiation of the phagocytic processes (Gordon, 2016). Independent of the receptors involved in the phagocytic process, the mechanism by which the phagosome forms is similar. The ligands are engaged by the receptors and activate a transduction signal that induces changes in the membrane composition and the cytoskeleton, resulting in the formation of membrane protrusions that cover the body and fuse at the distal edge of the membrane creating a new phagosome that pinche out from the plasma membrane. Once the internalization process begins, the phagosome undergoes a series of

changes in its membrane composition and contents, transforming into a phagolysosome in a process known as phagosome maturation in which the cargo can be safely degraded **(Figure 1.8)** (Desjardins, 1995; Desjardins *et al.*, 1994; Levin *et al.*, 2016). The maturation process starts when the early phagosome recruits small Rab GTPases, such as Rab5 and other accessory vesicle fusion molecules, which are acquired from the fusion of early endosomes and the plasma membrane with the phagosome.

Table 3. Opsonic and non-opsonic receptors.
PRRs, opsonic and apoptotic receptors with their respective ligand (Flannagan *et al.*, 2012).

Receptors	Ligands
Pattern-recognition receptors	
Mannose receptor (CD206)	Mannan
Dectin-1 (CLEC7A)	β1,3-glucan
CD14	Lipopolysaccharide-binding protein
Scavenger receptor A (CD204)	Lipopolysaccharide, lipoteichoic acid
CD36	*Plasmodium falciparum*–infected erythrocytes
MARCO	Bacteria
Opsonic receptors	
FcγRI (CD64)	IgG1 = IgG3 > IgG4
FcγRIIa (CD32a)	IgG3 ≥ IgG1 = IgG2
FcγRIIc (CD32c)	IgG
FcγRIIIa (CD16a)	IgG
FcαRI (CD89)	IgA1, IgA2
FcεRI	IgE
CR1 (CD45)	Mannan-binding lectin, C1q, C4b, C3b
CR3 ($\alpha_M \beta_2$, CD11b/CD18, Mac-1)	iC3b
CR4 ($\alpha_V \beta_2$, CD11c/CD18, gp150/95)	iC3b
$\alpha_5 \beta_1$	Fibronectin, vitronectin
Apoptotic corpse receptors	
TIM-1	Phosphatidylserine
TIM-4	Phosphatidylserine
BAI1	Phosphatidylserine
Stabilin-2	Phosphatidylserine
Mer	Gas6, protein S
$\alpha_V \beta_3$	MFG-E8
$\alpha_V \beta_5$	Apoptotic cells
CD36	Oxidized lipids

Rab5 is critical to phagosomal development, due to its importance in the acquisition of phosphatidylinositol 3-phosphate (PI(3)P) that leads to further recruitment of the early endosomal antigen 1 (EEA1) and NOX2 NADPH oxidase. During this process, the new phagosome combines with several other compartments and removes unnecessary material via recycling endosomes (Barr, 2013; Flannagan *et al.*, 2012). This event includes the loss of Rab5 and the recruitment of Rab7 on the membrane that mediates the fusion of the phagosome with late endosomes along with the recruitment of the vacuolar V-ATPase on the phagosome membrane inducing acidification (pH 5.5–6.0) by proton translocation (H+) into the phagosome lumen. At the final maturation stage, the phagosome fuses with late endosomes and lysosomes. In parallel, there is also recruitment of lysosomal-associated membrane proteins (LAMPs) and luminal proteases (cathepsins and hydrolases) that promotes further acidification (pH 4.5-5.0) and the phagosome becomes a phagolysosome (Fairn & Grinstein, 2012; Vieira Otilia *et al.*, 2003). The proteome composition of phagosomes varies in function of the internalized cargo (Gotthardt *et al.*, 2006; Goyette *et al.*, 2012; Jutras *et al.*, 2008; Trost *et al.*, 2009). For instance, latex bead-containing phagosomes discloses around 140 proteins in which hydrolases, proton pump ATPase subunits, and proteins of the fusion machinery are identified and are well characterized. However, other sets of proteins involved in antigen cross-presentation were identified indicating the phagosome capacity to present antigens (Garin *et al.*, 2001). In this regard, phagosomes harbouring intracellular pathogens such as *mycobacteria*, *E. coli*, *S. typhimurium*, *B abortus* and *Leishmania* have the molecular machinery to stimulate cross-presentation (Houde *et al.*, 2003).

Overall, the phagolysosome is a fundamental microbicide organelle in which the very acidic microenvironment, the NADPH oxidase that produces oxygen reactive species (ROS), the inclusion of hydrolytic enzymes (cathepsins, proteases, lysozymes, and lipases) contributes to the pathogen degradation and may also induce antigenic presentation during pathogen clearance.

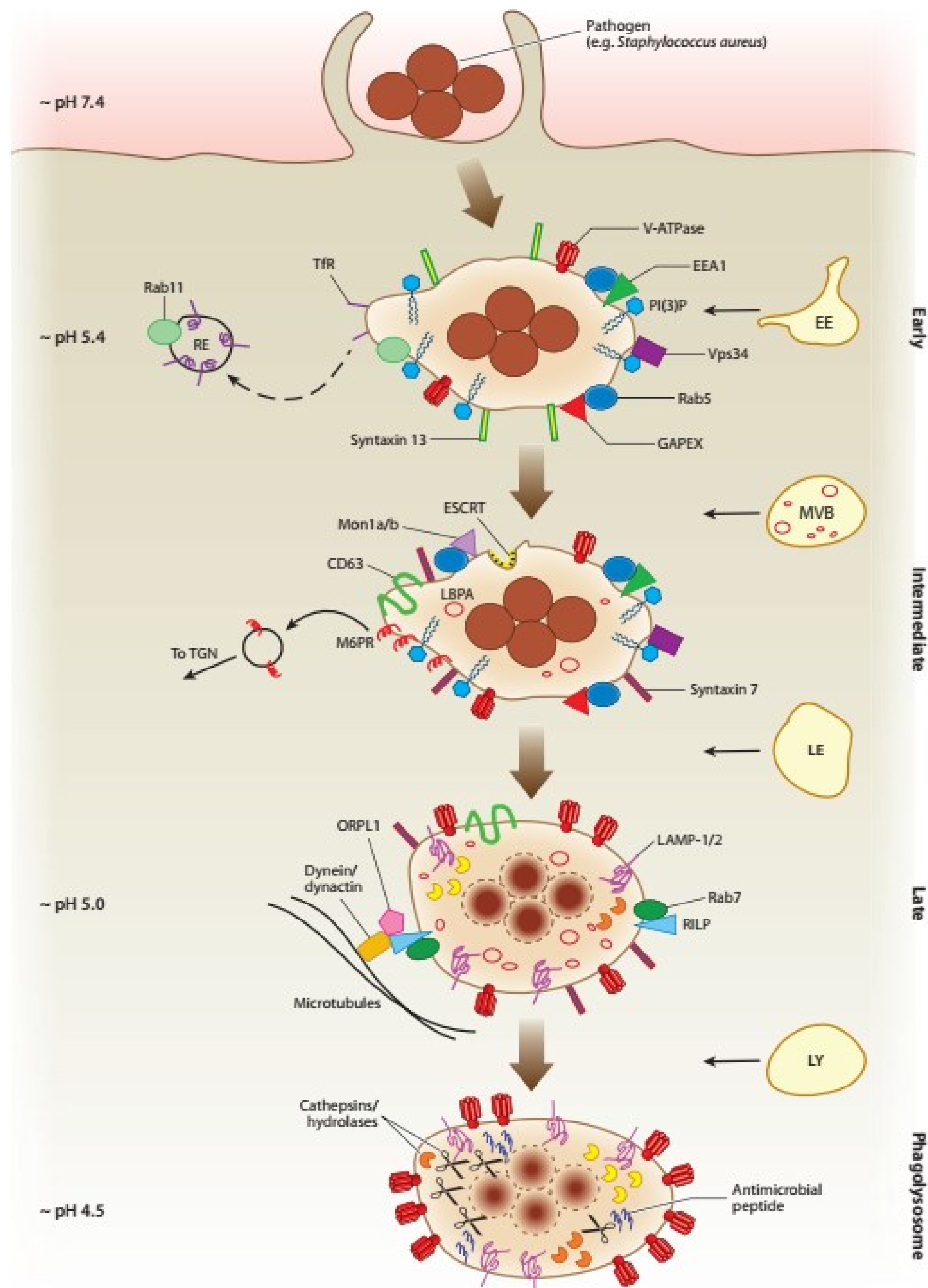


Figure 1.8. Phagosome maturation.
A pathogen is detected and phagocyted. In the early maturation step, a phagosome is formed, then, Rab5 and accessory vesicle fusion molecules are recruited. In the intermediate maturation step, the phagosome combines with endosomes and is acidified throughout the V-ATPs enzyme. In the late maturation step, the phagosome fuses with lysosomes creating a phagolysosome and there is a recruitment of cathepsins and hydrolases. Abbreviations: EEA1, early endosomal antigen 1; ESCRT, endosomal sorting complex for transport; M6PR, mannose 6-phosphate receptor; LAMP, lysosome-associated membrane protein; MVB, multivesicular body; ORPL1, oxysterol-binding protein-related protein 1; PI(3)P, phosphatidylinositol 3-phosphate; RILP, Rab7-interacting lysosomal protein; TfR, transferrin receptor (Flannagan *et al.*, 2012).

1.5 Macrophage polarization

Macrophages are plastic cells that integrate multiple signalling axis and adopt different functional programs depending on the surrounding stimulatory context. The process by which the macrophages are activated at any given point in space and time is known as macrophage polarization. The different classes of activated macrophages have specific roles during tissue homeostasis, pathogen encounters and a critical role in inflammatory modulation (Mantovani *et al.*, 2013; Murray, 2017; Pollard, 2009). Traditionally, the nomenclature to distinguish different macrophage subsets was derived from the type of immune response and the prevalent secreted cytokines after stimulation by using specific molecules *in vitro* (Mills *et al.*, 2000). Thus, M1 macrophages or classically activated macrophages arise in an inflammatory context, triggered by TLRs agonists and interferon responses, which includes a Th1 response *in vivo*. The M1 subset is generally associated with immunity against intracellular pathogens. Contrary to M1, M2 macrophages or alternatively activated macrophages arise in an anti-inflammatory context, thus developing a Th2 response *in vivo* and generally are associated with helminth immunity and tissue repair (Muraille *et al.*, 2014). The macrophage nomenclature M1/M2 was initially introduced around the year 1990, by using specific polarizing molecules that drive the macrophage activation and cytokines produced in each subset. Nevertheless, it is a simplified nomenclature based on *in vitro* studies, which, only represents two extremes of a broad spectrum of differentiation states, which also lacks defined criteria to describe the different phenotypes and doesn't completely fit in the embryonal-macrophage polarization structure, nor in the actual population of macrophages found *in vivo*. Noteworthy, this nomenclature was set in a pre-genomic era, where there was a lack of understanding of information about the transcriptomic profiles and transcriptomic regulation. Nowadays, more complete information on macrophage transcriptomic signatures, epigenetic pathways involved in macrophage regulation and effect of the immunometabolism on cellular fate disclose a broader spectrum of polarized macrophages.

Macrophage polarization is triggered and regulated by extrinsic, intrinsic and tissue environmental signals (Ginhoux *et al.*, 2016; Xue *et al.*, 2014). M1 polarization generally induces a prototypic inflammatory response and is principally driven by the stimulation of three inductors (IFN-γ, LPS and GM-CSF). The main cytokine associated with the M1 polarization is IFN-γ, which may come from other cell sources or the macrophage itself. The macrophage recognizes IFN-γ via the interferon-gamma receptor (IFNGR) and induces a transduction signal that

involves the adaptors JAK1/2 along with STAT1 and the interferon regulatory factors (IRFs). The activation by IFN-γ induces gene expression programs that include the production of cytokine receptors (IL2RA, IL6R, RA and CSF2RB), cell adhesion molecules (ICAM1, MUC1 and ITGAL) and cell activation markers (CD16, CD32, CD36, CD38, CD69, CD80, CD86 and CD97). The interaction with different bacterial components such as LPS, lipoteichoic acid and muramyl dipeptide, drive the M1 polarization through the activation of different TLRs. The ligand interaction with different TLRs induces a transduction signal that involves the adaptors MyD88 and TRIF leading to a strong pro-inflammatory response mediated by type I IFN , IL-6, IL-12, TNF and IL-1α production. Also, it causes the release of chemokines (CCL2, CXCL10 and CXCL11) that recruits different cell population and promotes antigen presentation molecules, which is important for further activation of the humoral response. Stimulation with LPS has a certain degree of overlap with IFN-γ, which shares regulators that include NF-kB, activator protein 1 (AP-1) and early growth response (EGR) factor. The Granulocyte-macrophage-stimulating factor (GM-CSF) is a glycoprotein produced by several cell types including macrophages, T-cells, NK cells and fibroblasts that promote macrophage proliferation and further differentiation into the M1 subset. GM-CSF is recognized by tyrosine kinase receptors (CSF-1R) and, just like IFN-γ and LPS stimulation, induces recruitment of the adaptors JAK2 and STAT5 and a transduction signal that involves extracellular signal-regulated kinase (ERK), V-Akt murine thymoma viral oncogene homolog 1 (AKT) and the nuclear translocation of NF-kB. GM-CSF stimulated macrophages induce cytokine production (IL-1β, IL-6, IL-8, G-CSF, M-CSF and TNF). Other stimuli that share pro-inflammatory properties and drive the polarisation to M1 are induced by TNF, IL-1ß, and IL-6, which complement the heterogeneous group of the M1-like macrophages **(Figure 1.9)**. In general, independent of the pathway involved during the polarization process, the M1 subset drives an enhanced immune response required for pathogen clearance along with a Th1 polarized *in vivo* response. The activated macrophage employs different mechanisms to destroy intracellular pathogen including nutrient and iron restriction (Gruenheid & Gros, 2000), production of reactive oxygen and nitrogen species (RONS) that includes two classes of molecules: reactive oxygen species (ROS) and reactive nitrogen species (RNS). These molecules are free radicals generated by NADPH oxidase, myeloperoxidase, monooxygenases, and nitric oxide synthase (NOS), which reduce arginine into nitric oxide (NO) and cause severe oxidative damage to biomolecules (MacMicking *et al.*, 1997; Rath *et al.*, 2014).

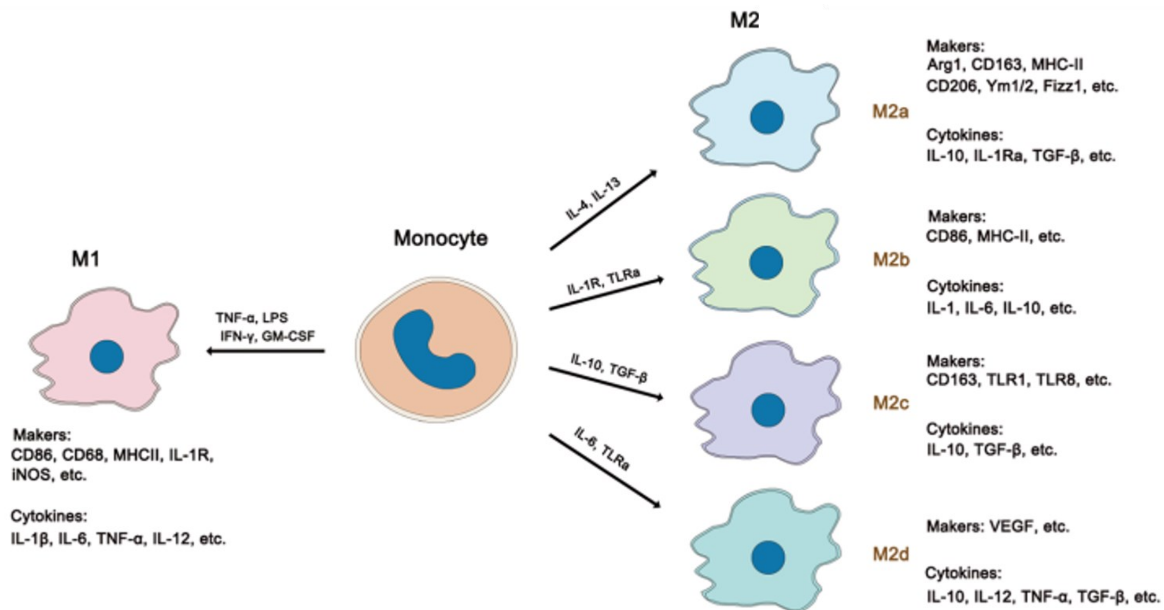


Figure 1.9. Macrophages subsets.
Macrophage polarization presents a broad spectrum of differentiation states. The M1 and M2 subsets represent the two extremes of the polarization states in which M2 can be subdivided into 4 groups. Each macrophage subset presents its respective polarization inductor, the associated markers and the main released cytokines (Wang *et al.*, 2020).

M2 polarization generally induces an antagonistic inflammatory response and, depending on the nature of the stimulatory factor, may be classified as a M2a, M2b, M2c and M2d subset. The main cytokines associated with the M2 polarization are IL-4 and IL-13, which may come from Th2 cells such as eosinophils, basophils, or macrophages themselves. Those cytokines are recognized by the receptors IL-4Ra1, IL13Rα1 and IL13Rα2, which activate JAK3, leading to a transduction signal that involves STAT6 and c-Myc (Chen & Paul, 1997). The M2 subset is characterized by an increase in the receptors DC-SIGN, Scavenger A and B-1, mannose and Dectin-1 (Martinez *et al.*, 2009) and is highly regulated for the transcription factors peroxisome proliferator-activated receptor γ, δ (PPARγ and PPAR δ) and Krueppel-like factor 4 (KLF-4) (Charo, 2007; Sharma *et al.*, 2012). The M2a polarization is driven by IL-4 and IL-13 and is characterized by decreased phagocytosis, enhanced activity of transglutaminase 2 (TGM2), cholesterol hydroxylase CH25H, prostaglandin-endoperoxide synthase (PTGS1), an induction of mannose receptor (MRC1) overexpression, which is important during pathogen interactions. M2a macrophages promote the recruitment and differentiation of Th2 cells through the action of CCL17 and CCL22, reduction of the inflammatory response by the secretion of the receptor

antagonist IL-1RA (Martinez *et al.*, 2009; Pechkovsky *et al.*, 2010) and prevention of the NF-kB activation that in turn prevents M1 polarization (Kapoor *et al.*, 2015). The principal function of M2a macrophages is to contribute to tissue homeostasis and wound healing. The M2b polarization is driven by the interaction with immune-complex, TLRs agonist or IL-1R agonist and expresses high levels of CCL1 and TNF. M2b macrophages are referred to as regulatory macrophages for their capacity to regulate inflammation by decreasing IL-12 production and by releasing the anti-inflammatory cytokine IL-10 along with other anti-inflammatory cytokines such as IL-1 β, IL-6, and TNFα (Ito *et al.*, 2017). The M2c polarization is driven by the exposure to glucocorticoids, IL-10 and TGF-β. M2c macrophages are characterized for down-regulating inflammation by releasing high amounts of IL-10, enhancing tissue remodelling by releasing TGF-β and phagocytosis of apoptotic cells (Mantovani *et al.*, 2004; Rőszer, 2015; Zizzo *et al.*, 2012). The M2d polarization is driven by TLR agonists that activate the adenosine A(2A) receptor. M2d macrophages are characterized for suppressing the expression of TNF-α, IL-1 and IFN-γ. Also, M2d cells produce high levels of IL-10 and vascular endothelial growth factor (VEGF). This phenotype is associated– with promoting angiogenesis and tumour expansion (Ferrante *et al.*, 2013; Pinhal-Enfield *et al.*, 2003). The M2 subset is a heterogeneous group that drives an anti-inflammatory response by downregulating proinflammatory mediators such as IL-1β, TNFα, IL-6, IL-8, IL-12, and GM-CSF through the action of IL-4 and IL-13. Also, M2 is involved in the metazoan parasite clearance by reducing their motility, viability, and fecundity and contributes to the expulsion process. It promotes wound healing principally supported by an enhanced arginase activity that converts ornithine into polyamines **(Figure 1.9)** (Gordon & Martinez, 2010; Nahrendorf & Swirski, 2013)

Macrophage *in vivo* polarization is a highly coordinated and dynamic process that regulates the polarization level, maintains different macrophage subsets at a desirable level and can be reversed in accordance to specific stimulus at given time (Guiducci *et al.*, 2005; Saccani *et al.*, 2006). For instance, during certain pathogen infections, a mixed population of M1 and M2-polarized states is displayed in which M1 macrophages are involved in pathogen clearance and M2 macrophages rise as an important regulatory mechanism to avoid tissue damage in later stages of infection, damping the inflammatory response and promote tissue repair (Biswas & Lopez-Collazo, 2009). Similar scenarios, that involve a mixed M1/M2 population, were observed during co-infection with intracellular pathogens and worms (Salgame *et al.*, 2013). Moreover, in a non-pathogenic context, an imbalance in the M1-M2 polarization can drive non-pathogenic

diseases including, atherosclerosis, osteoporosis, tissue damage, imbalanced tissue remodelling and the development of tumour-associated macrophages in cancer (TAM) (George *et al.*, 2000; Jakubzick *et al.*, 2004; Mantovani *et al.*, 2011; Mantovani *et al.*, 2002; Martinez *et al.*, 2009; Vignery, 2000).

Currently, the M1/M2 classification is a simplified way to distinguish two principal groups of macrophage subsets that occur under the circumstances mentioned above. Thus, the expression of different markers and gene signatures are the most efficient way to distinguish the polarization state of macrophages at a specific time, even though some markers are shared between the different subsets **(Table 4)**.

Table 4. Molecular markers in polarized macrophages.
Gene signatures associated with M1 and M2 macrophages (Murray, 2017)

M1 markers		M2 markers	
IL1α	IκBζ	FIZZ1, RELMα	Ccl8
IL1β	Ccl1	Mgl2	Mela
IL6	Cxcl13	Ccl17	Dectin-1
IL12α	Eotaxin	Eotaxin-2	PD-L2
IL12β	Cxcl2	Irf4	Socs2
IL23α	A20	Chitinase 3	Cadherin 1
IL27	Socs3	Mannose receptor	PPARδ
Tnf	Marco	Arginase-1	PPARγ
G-CSF	iNOS	Ear11	Ccl22
GM-CSF		Ear2	

1.6 Type I interferon and its role in immunity

During the innate and adaptative immune responses, there are different factors regulating the kind of response in which the Interferon signalling axis plays an essential role due to this autocrine and paracrine function. Interferons (IFNs) belong to a large family of cytokines discovered in 1957 and associated with the interference of influenza virus replication in mammalian cells (Isaacs & Lindenmann, 1957). Initially, the role of Interferon was associated with the immune response against the virus. Nowadays, we know that the role of interferon is also involved in immunity against bacteria, parasites, fungi and protozoans, during tumour development and regulation of the inflammatory response.

IFNs are classified into three distinct families: type I (IFN-I), type II (IFN-II), and type III (IFN-III) **(Table 6)** (Silva-Barrios & Stäger, 2017a). IFN-I is a multi-gene-related cytokine family that includes two main subgroups (IFNα and IFNβ) in which IFNα encodes, in humans, 13 and, in mice, 14 partially homologous genes and IFNβ is encoded by a single gene. IFN-I also encompasses other less studied IFNs (IFN-ϵ, IFNδ, IFNκ, IFNτ, IFNω). IFN-I signalling is regulated through the common heterodimeric IFN-I cell surface receptor (IFNAR1 and IFNAR2) (de Weerd & Nguyen, 2012). IFN-II includes a single gene product, IFN-γ, which is mostly produced by T-lymphocytes and natural killer (NK) cells. IFN-II signalling is regulated through the heterodimeric IFN-γ receptor (IFNGR) expressed in a wide range of cells (Schoenborn & Wilson, 2007). IFN-III family, also known as IFN-λ, includes four different subgroups: IFN-λ1, IFNλ2, IFN-λ3, and IFN-λ4. IFN-III signalling is regulated through a unique IFN-λ receptor (IFN-λ) expressed mainly in cells of epithelial ontology (Egli *et al.*, 2014).

IFN-I can be produced by almost all cell types in response to different stimuli. Nevertheless, the majority of IFN-I is secreted by infected cells in response to PAMP recognition mediated by membrane and/or cytosolic PRRs **(Chapter 1.3)**. IFN-I production promotes three major functions: activation of cell-intrinsic antimicrobial programmes, promoting a balance of the innate immune response that enhances antigen presentation and control of the inflammatory and cytokine responses as well as activation of adaptive immune responses by promoting high-affinity antigen-specific T and B cell responses and immunological memory. Also, IFN-I production and regulation depends on the PRRs nature and the cell type, which plays a varying role on the course of the immune response (Ivashkiv & Donlin, 2014; van Boxel-Dezaire *et al.*, 2006).

Table 5. IFNs family.
IFNs classification, receptors involves in their recognition and signalling molecules related to transduction signal (de Weerd & Nguyen, 2012).

IFN type	*Interferons*	*Receptor*	*Signaling molecules*
Type I	α (13 types), β, δ, ε, κ, τ, ω, ζ	IFNAR1, IFNAR2	JAK1, Tyk2 STAT-1, -2, -3, -4, -5 MAPK, PI3K, Akt, NFκB p53, PRMT1
Type II	γ	IFNGR1, IFNGR2	JAK1, JAK2 STAT-1,-2, -3, -5 MAPK , PI3K, Akt, NFκB
Type III	λ	IFNLR1, IL10RB	JAK1, Tyk2 STAT-1, -2, -3, -4, -5 MAPK , PI3K, Akt

The IFN-I signalling axis is the best characterized of the IFNs group. During the canonical induction, IFNα and IFNβ bind IFNAR1 or IFNAR2, which in turn induce a co-recruitment of other subunit and cause a dimerization of the IFN-α/β receptor complex (IFNAR). IFNAR engagement activates the receptor-associated protein tyrosine kinases JAK1 and TYK2, which phosphorylate the cytoplasmic transcription factors STAT1 and STAT2 inducing their dimerization (Levy & Darnell, 2002; Stark & Darnell Jr, 2012). Dimerized STAT1 and STAT2 are further translocated to the nucleus promoting a trimolecular complex assembly with IRF9 known as IFN-stimulated gene factor 3 (ISGF3), which binds to the DNA sequences TTTCNNTTTC. Also, dimerized STAT1 and STAT2 bind to the Gamma interferon activation site (GAS) at the DNA sequences TTCNNNGAA. ISGF3 and GAS promote gene transcription of IFN-stimulated response elements found in the promoter regions. Then, ISG- and GAS-encoded proteins restrain pathogens by viral replication, transcription, and translation inhibition as well as by degrading viral nucleic acids and alteration of cellular lipid metabolism (Decker *et al.*, 1997; MacMicking, 2012; Schoggins *et al.*, 2011). Different immune cells present and express constitutively the same IFN I core pathway (IFNAR, JAK1, TYK2, STAT1, STAT2 and IRF9) to establish type IFN I-dependent responses. Importantly, immune cells can rapidly mount an IFN I response even under low levels of IFNβ and IFNα due to an autocrine loop that maintains the homeostatic condition (Gough *et al.*, 2012).

During viral infection, the role of type I IFN is associated with antiviral protection through the induction of an ISG-based cellular antiviral program and by enhancing immune responses. However, during the last decades, several studies showed that the role of IFN I may be detrimental to the host during viral infections. Hence, during acute viral infections, IFN-I

promotes DC antigen presentation, which favours the T-cell response and establishment of a proper humoral response (Fink *et al.*, 2006; Le Bon *et al.*, 2003; Montoya *et al.*, 2002). In contrast, during chronic viral infections, IFN-I promotes an immunosuppressive role in humoral responses and reduces IFN-γ macrophage responses (Moseman *et al.*, 2016; Rayamajhi *et al.*, 2010; Wilson *et al.*, 2013). Also, the dual role of IFN-I has been evaluated in intracellular bacterial infections such as *C. pneumoniae, L. monocytogenes* and *R. akari,* although less is known about the factors and the mechanisms regulating the fate of these bacteria in the IFN-I context (Auerbuch *et al.*, 2004; Kazar *et al.*, 1971; Rothfuchs *et al.*, 2001). The dual role of IFN-I highlights the fact that depending on the infection stage, the microorganisms and their interaction with their host can drive different host responses that may lead to pathogen replication or clearing.

2 *LEISHMANIA* BIOLOGY

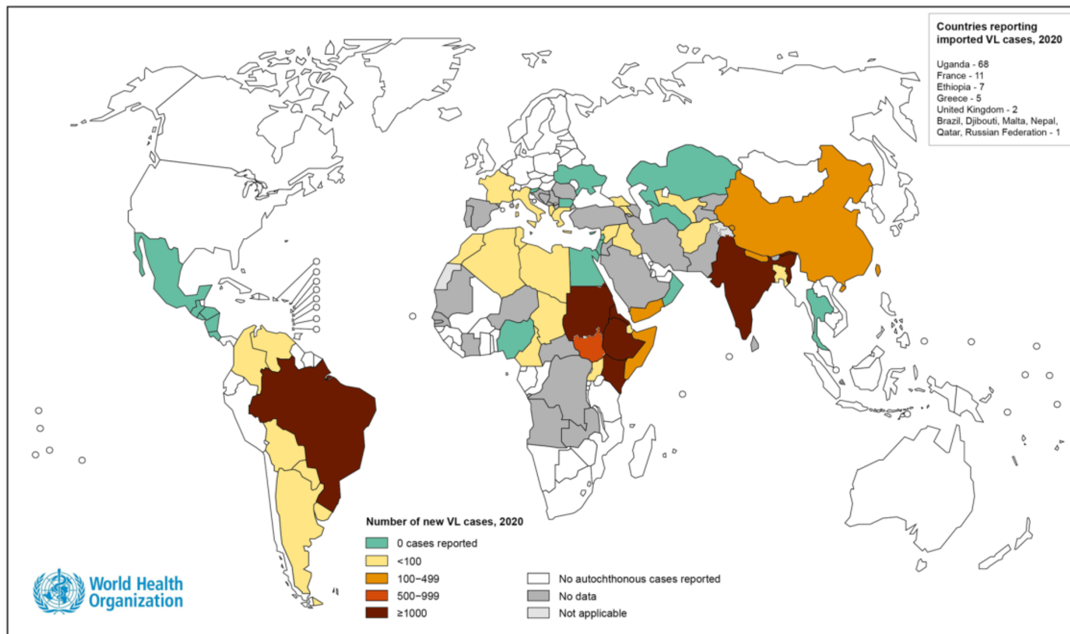
2.1 Epidemiology of leishmaniasis

Leishmaniasis is a complex human vector-borne disease caused by protozoan parasites of the genus *Leishmania*, which is considered one of the world's most neglected diseases (Alvar *et al.*, 2012; Mathers *et al.*, 2007) as well as the second and fourth most common cause of death and disease respectively, among tropical parasitic infections (Bern *et al.*, 2008; Pace, 2014). *Leishmania* is transmitted by arthropod vectors of the genera *Phlebotomus* and *Lutzomyia* from the Old and New Worlds respectively. This disease is widely distributed in tropical and sub-tropical regions across 98 countries, being endemic in most of them **(Figure 2.1)** (Bern *et al.*, 2008). According to the World Health Organization (WHO), annually between 1.5 - 2 million people develop leishmaniasis and 350 million people live in endemic areas enhancing the contagion's risk. Additionally, there is a constant flow of *Leishmania* between humans (anthropozoonotic) and domestic animals (zoonotic) (Gradoni, 2018).

At least, there are 20 *Leishmania* species pathogenic to humans (Akhoundi *et al.*, 2017). For instance, old World species of *Leishmania,* including *L. donovani* and *L. major*, cause pathology in southern Europe, the Middle East, southern Asia and Africa, whereas new World species such as *L. amazonensis*, *L. brasiliensis*, *L. mexicana* and *L. chagasi* are found throughout South and Central America and the southern states of the United States (Berman, 2005). Leishmaniasis progression results in different clinical manifestations, which are species-dependent and variate according to the host's immunological status. The prevalent forms are visceral leishmaniasis or kala-azar (VL), which affects the mononuclear phagocytic system, presents systemic manifestations and is deadly if not treated properly. During a chronic state, visceral leishmaniasis is characterized by prolonged fever, pancytopenia, splenomegaly and hypergammaglobulinemia. Cutaneous leishmaniasis (CL) is the most common form, which can generate warty lesions, nodes, or ulcers at the inoculum site. Another less common acute form includes mucocutaneous leishmaniasis (MCL), which is developed from parasites metastasis of the nasopharyngeal mucosa **(Table 6)**. (David & Craft, 2009; Ready, 2014; Reithinger *et al.*, 2007). Leishmaniasis is a disease linked with poverty, due to deteriorated environmental sanitation, poor nutrition associated with immunosuppression and poor housing conditions that integrate sand fly vectors where human and animal reservoirs are key elements in the

transmission chain (Alvar *et al.*, 2006). Although most of the countries affected by leishmaniasis are developing countries, climate change is also promoting vector migration and spread of the parasite to places where it was not found before (Antinori *et al.*, 2012).

Status of endemicity of visceral leishmaniasis worldwide, 2020



Status of endemicity of cutaneous leishmaniasis worldwide, 2020

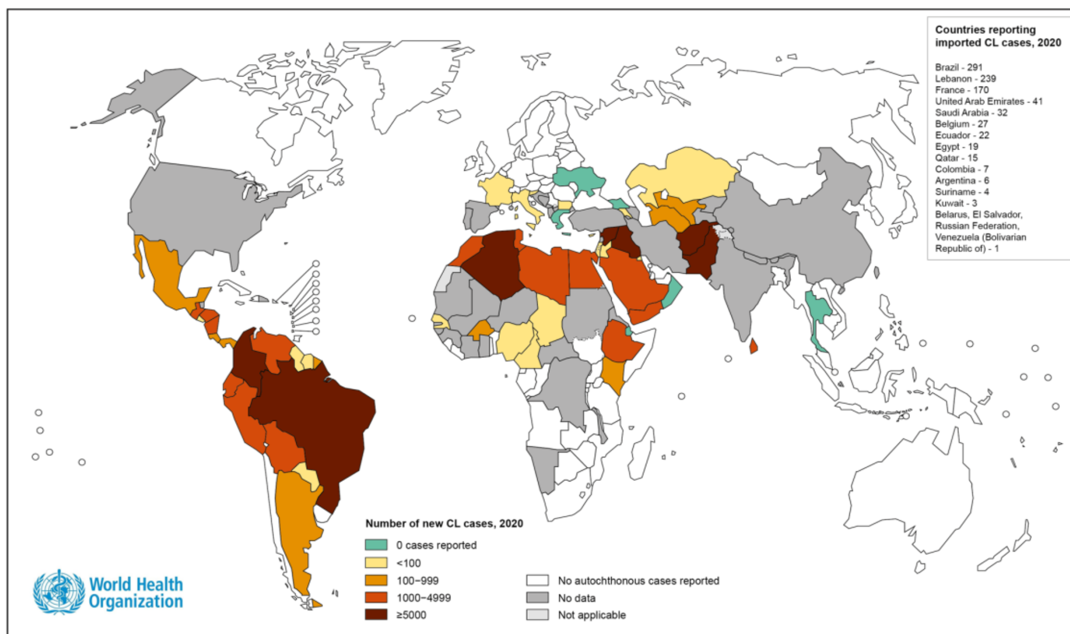


Figure 2.1. Leishmaniasis endemicity.
Maps of visceral and cutaneous leishmaniasis new reported cases per country in 2020. Image obtained from World Health Organization (WHO).

Traditionally, visceral and cutaneous leishmaniasis have been treated with pentavalent antimonials such as meglumine antimoniate (Glucantime), sodium stibogluconate (Pentostam) and paromomycin as a first compound line since their development in 1945 (Tiuman *et al.*, 2011). As a second compound line, pentamidine and amphotericin B are used. Nevertheless, these compounds present difficult parenteral administration, high costs, high toxicity and causes the rise of several *Leishmania*-resistant strains due to the use of those treatments (Bhattacharya *et al.*, 2020). Therefore, proper parasite identification is important to select the appropriate treatment (Arevalo *et al.*, 2007). Currently, there are no available prophylactic or preventive vaccines, thus there is a constant effort to develop a vaccine, and new synthetic and natural base anti-leishmaniasis treatments (Alves *et al.*, 2018).

Table 6. Leishmaniasis and its associated etiological species.
Leishmaniasis progression depending on the *Leishmania* species can result in different clinical manifestations including (VL), (CL) and (MCL). The same specie may progress in different clinical manifestations.

Clinical manifestation	Associated specie
Visceral Leishmaniasis (VL)	*L. infantum, L. donovani, L. amazonensis, L. tropica, L. martiniquensis, L. colombiensis*
Cutaneous Leishmaniasis (CL)	*L. aethiopica, L. braziliensis, L. amazonensis, L. major, L. mexicana, L. tropica, L. venezuelensis, L. guyanensis, L. lainsoni, L. lindenbergi, L. naiffi, L. panamensis, L. peruviana, L. shawi, L. martiniquensis, L. colombiensis, L. infantum*
Mucocutaneous Leishmaniasis (MCL)	*L. braziliensis, L. panamensis, L. aethiopica, L. amazonensis, L. mexicana, L. guyanensis, L. peruviana*

2.2 *Leishmania* life cycle

The existence of the *Leishmania* genus dates from prehistoric times. The most ancient, documented sandfly fossil infected with *Leishmania*-like species is 100 million years old (Poinar Jr & Poinar, 2004). Even if the occurrence of leishmaniasis is reported since ancient times (Steverding, 2017), the discovery of *Leishmania sp* was made by the Scottish pathologist Lieutenant General Sir William Boog Leishman (1865–1926) who discovered ovoid bodies in smears from a spleen of a dead soldier that presented splenomegaly in 1900 (Leishman, 1903; Leishman, 1904). A few weeks later, the Irish doctor Charles Donovan (1863–1951) reported finding similar bodies in splenic samples from native Indians that presented enlarged spleens (Donovan, 1903). Initially, those ovoid bodies were identified as degenerated trypanosomes. Nevertheless, further analyses identified these bodies as a new parasite specie (Steverding, 2017). The genus *Leishmania* was thus established in honour of William Boog Leishman and the specie *L. donovani* in honour of Charles Donovan for their respective discoveries (Ross, 1903).

Leishmania is a protozoan parasite from the Mastigophora subphylum, Kinetoplastida order, Trypanosomatidae family and *Leishmania* genus, which includes more than 53 species with similar morphological characteristics. Therefore, to identify different *Leishmania* species it is necessary to employ genetic or biochemistry characterizations (Graça *et al.*, 2012; Rioux *et al.*, 1990). The genus *Leishmania* is divided into two subgenera depending on where the parasite grows in its vector: the subgenus Viannia groups the species growing in the hindgut of the vector, while the subgenus *Leishmania* groups the species growing in the midgut of the vector (Shaw, 1994). *Leishmania* presents two distinct developmental stages. The promastigote is the extracellular form, which colonizes the arthropod vectors and, which infects different phagocytic cells including neutrophils, dendritic cells, monocytes and macrophages in mammals, the later being their principal target. They present an elongated shape with a length between 10-20 µm, a well-developed and long flagellum, a central nucleus, a terminal kinetoplast within the extension of the mitochondrion, the axoneme arising from the basal body from the flagellar pocket and a complex glycoconjugate set of proteins including LPG that are important during infection. The amastigote is the intracellular form, which is the main target of the macrophage. They present a rounded morphology with a diameter between 1.5 and 5 µm, and structures such as an enlarged nucleus, a small kinetoplast and a thin filament that joins the kinetoplast and basal body (Sunter & Gull, 2017). The kinetoplast is a substructure present in a single large mitochondrion, with

unique DNA and is strictly associated with the flagellar pocket and the basal body of the flagellum **(Figure 2.2)**.

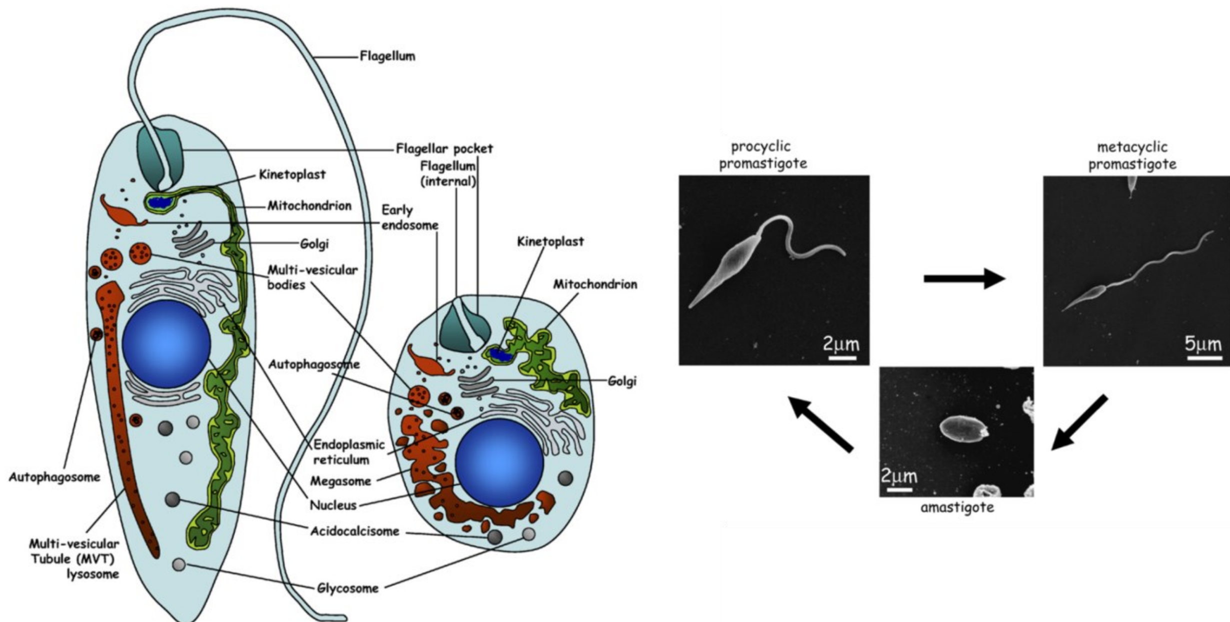


Figure 2.2. *Leishmania* morphology.
Leishmania undergoes a morphological change when it alternates from the sand fly to the mammalian host. On the left, we observe the principal elements of each stage and, on the right, the morphology from electron microscopy images. Adapted from (Besteiro *et al.*, 2007).

Leishmania presents an intercalated di-genetic life cycle in which a female sandfly ingests amastigotes from an infected vertebrate host during a blood meal. Further, the amastigotes travel through the sandfly's digestive tract until they reach the intestine where both the temperature change from 37ºC to 26ºC and the pH increase from 4.5 to 6.9 as well as the rich nutrient microenvironment, induce the differentiation of the amastigote into procyclic promastigote, which has a high replication rate and adherence to the intestine walls (Dostálová & Volf, 2012). Subsequently, the parasites undergo a change from procyclic promastigotes to highly infectious stage metacyclic promastigotes, which present high motility and a longer flagellum that allows them to migrate from the intestine walls to the anterior digestive tract (Rogers *et al.*, 2002). The morphological change from procyclic promastigote to metacyclic promastigote occurs through a process known as metacyclogenesis (Da Silva & Sacks, 1987). Subsequently, during the next sandfly blood meal of another mammalian host, it may inoculate the metacyclic promastigotes into a new host. During the blood meal, the sandfly regurgitates

the metacyclic parasites on the skin, thus releasing them in large numbers (around 1000). Subsequently, those parasites are recognized by phagocytic cells, followed by their internalization process. After, the parasites undergo a morphological change from metacyclic promastigotes to amastigotes, induced among others, by the thermal shock, nutrient depletion in the phagosome and the low phagolysosome pH. These changes induce a panoply of morphological and biochemical changes characterized by the expression of enzymes with optimal activity at low pH, changes in virulence factor composition such as LPG and GP63 (Ríos Yuil & Sousa, 2010) and regulation of gene expression required for differentiation and adaptation in the host. The parasite's survival and the ability to establish a long-term infection rely on the parasite's ability to be recognized and phagocyted by host cells as well as a complex host-pathogen interaction that rewires the macrophage biology to create a safe environment for the parasite differentiation and replication **(Figure 2.3)** (Descoteaux & Turco, 2002).

The promastigote differentiation into amastigote naturally occurs in mammals, however, *in vitro* stimulation recreating the lower pH of the phagolysosome and increased temperature promotes differentiation into amastigote-like, that present similarities to macrophage-differentiated amastigotes (Holzer *et al.*, 2006; Walker *et al.*, 2006). Differentiation of promastigotes into amastigotes may be summarized in four different key processes: (1) From 0 to 4 hours, the signals that induce the differentiation program are activated along with an overexpression of the glycoconjugate A2. (2) From 5 to 9 hours, the parasite reduces its motility and induced a cell cycle arrest in G1. (3) Between 10- and 24 hours, the promastigote changes its shape to an amastigote. (4) Between 25 and 120 hours, the parasite differentiates completely into an amastigote, downregulates LPG expression and upregulates proline transporters. After the parasite differentiates into an amastigote, it replicates within the phagolysosome and, eventually, infected cells rupture thus releasing amastigotes outside, enabling them to infect more surrounding phagocytic cells (Ambit *et al.*, 2011; Teixeira *et al.*, 2002; Wheeler *et al.*, 2011). To survive within the hostile macrophage environment, *Leishmania* developed different strategies to counter the microbicidal cellular response. In part, the rewiring of the macrophage biology is mediated by virulence factors that modulate different signalling programs that control among others, parasite detection and internalization, phagolysosome formation, macrophage fate, and energetic and nutrient- metabolism.

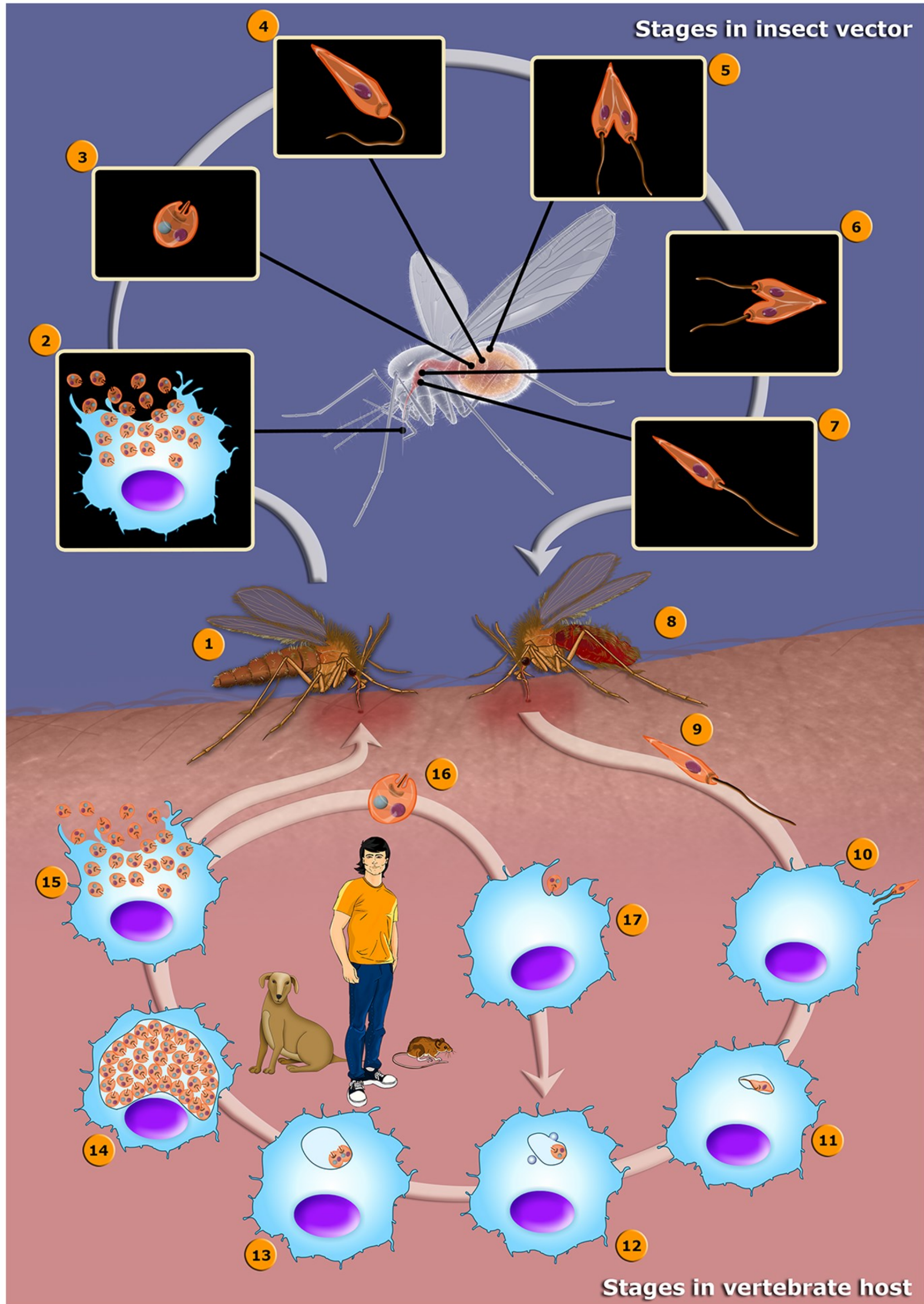


Figure 2.3. ***Leishmania*** **life cycle.**
(1) Sand fly blood meal. (2, 3). Amastigotes are released from macrophages and migrate to the sand fly gut. (4-6) Differentiation to procyclic promastigotes and replication. (7) Metacyclogenesis. (8,9) Sand fly blood meal and promastigotes inoculation. (10,11) Detection and internalization by macrophages. (12-14) Differentiation to amastigotes and replication. (15,16) Amastigotes burst out of the cell. (16-17) Free amastigotes infect new macrophages and reintegrate the cycle in 12. Adapted from (Teixeira *et al.*, 2013).

2.3 *Leishmania* virulence factors

A common feature employed by different pathogenic microorganisms to colonize and establish an infection within the host is the use of factors known as toxins or virulence factors. These factors can be secreted, cytosolic or associated with the membrane. In general, they provoke a panoply of metabolic changes in the host that attenuate the immunological response and facilitate the microorganism's adaptation. To understand how *Leishmania* can survive in the harmful macrophage environment and replicate inside the phagolysosome, it is important to mention the critical role of its virulence factors **(Figure 2.4)**. The *Leishmania* life cycle encompasses two completely different environments in the mammalian and vector hosts. For instance, the strategy to replicate in the vector's gut, rich in digestive enzymes and where nutrients can be easily absorbed, is different from the strategy to replicate within the macrophage phagolysosome with reduced nutrient availability. Thus, in general, the parasite's fate is determined by it's ability to adapt to the sand fly, resist the complement pathway in the bloodstream before internalization and survive in the hostile hydrolytic environment, low pH and accumulation of microbicidal molecules from the oxidative burst in the phagolysosome. During its life cycle, *Leishmania* synthetizes different groups of virulence factors that are required for parasite survival in the different hosts. The most abundant *Leishmania* glycoconjugates belong to the phosphoglycans (PP) family and are characterized by a common structure known as the disaccharide-phosphate Gal(β1,4) Man(α1-PO4→6). This group includes the major surface lipophosphoglycan (LPG), glycoinositolphospholipids (GILP), proteophosphoglycans (PPGs), glycophosphatidylinositols (GPI), and the glycoprotein 63 (GP63) **(Figure 2.5)** (Descoteaux & Turco, 1999; Turco & Descoteaux, 1992).

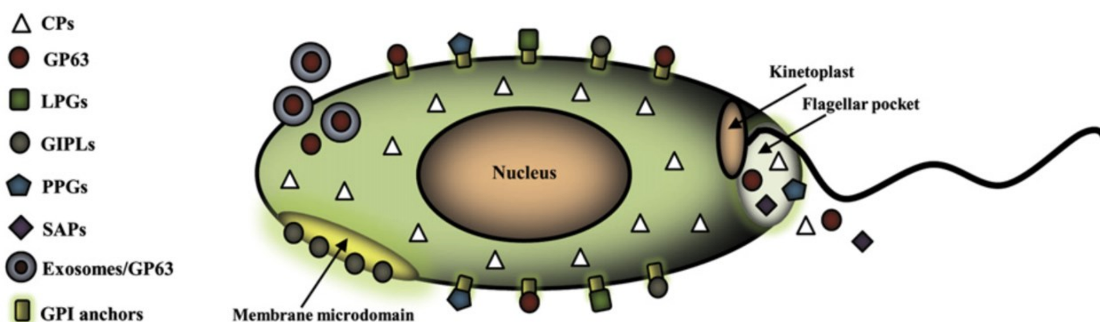


Figure 2.4. *Leishmania* promastigote virulence factors.
Representation showing the GPI-anchored surface molecules GP63, LPGs, PPGs and GIP and not membrane-anchored molecules GP63, PPGs, SAPs and CPs. (Olivier *et al.*, 2012)

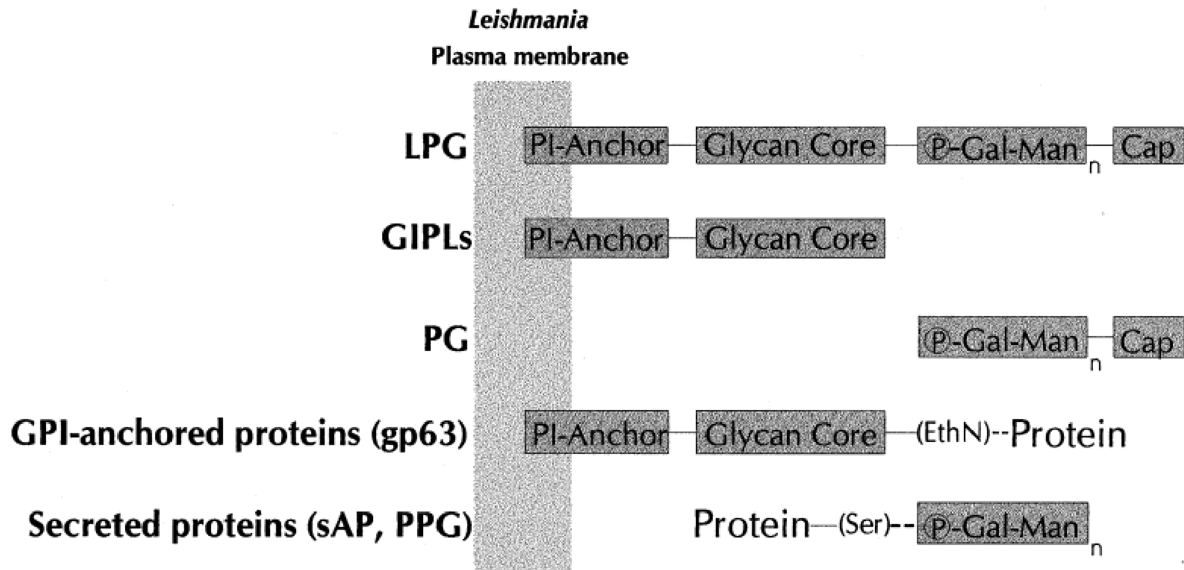


Figure 2.5. *Leishmania* virulence factors.
The glycan cores of LPG, GILPs and GPI-anchored molecules such as GP63 have a conserved Man(α1,4)GlcN(α1,6)-myo-inositol motif. sAP and PPG are secreted factors (Descoteaux & Turco, 1999).

Lipophosphoglycan (LPG) is the most abundant glycoconjugate in all *Leishmania* species and creates a dense glycocalyx surrounding the cell surface and the flagellum. LPG is principally synthesized in the promastigote state. There are more than 5 million copies per cell and it is composed of four domains: PI-anchor (GPI), a glycan core, repeating disaccharide phosphate units and a small oligosaccharide cap **(Figure 2.6).** The lipid anchor, glycan core and Gal-Man-PO4 backbone are conserved across all *Leishmania* species, nevertheless, different *Leishmania* species can have additional oligosaccharide chains branching off the backbone (Descoteaux & Turco, 1999). There are three types of LPG. LPG type 1 do not present sugar changes in the backbone and is the principal LPG component in *L. donovani*. LPG type 2 presents glycosylated galactose in the backbone and LPG type 3 is specific to *L aethipica* with 35% of mannosylated mannose in the backbone (McConville *et al.*, 1995). LPG is very dynamic over *Leishmania* life cycle and undergoes important modifications. For instance, in the sandfly, LPG enables the attachment of the parasites to the midgut and acts as a shield against the digestive enzymes. During *L. donovani* metacyclogenesis, the LPG structure is elongated by an increase from 15 to 30 repeating units approximately, which is important for the parasite release from the sand fly midgut and protection from the complement in the mammalian host (McConville *et al.*, 1992; Sacks *et al.*, 1995). Low expression levels are seen in amastigotes and are typically not required

during their colonization process (Desjardins & Descoteaux, 1997; McConville & Blackwell, 1991). One of the first reports introducing the role of LPG in macrophage modulation by *L. donovani* showed inhibition of protein kinase C activation. Importantly, after stimulation with purified LPG, the macrophages were unresponsive to LPS stimulation (Descoteaux & Matlashewski, 1989). The role of LPG during the infection process will be mentioned in more detail in chapter 3.

Leishmania promastigote and amastigote surfaces are also coated with abundant GILPs, PPGs and PPs. GILPs can be related to or independent of LPG and GPI anchors (Descoteaux & Turco, 1999). Those virulence factors interact with the macrophage surface mannose receptors and are important during the phagolysosome adaptation and amastigote progression stages (Blackwell *et al.*, 1985). Like LPG, GILPs have shown to be efficient in PKC activity inhibition along with reducing the NO and IL-12 production (Assis *et al.*, 2012; Yoneyama *et al.*, 2006). PPGs can be found as secreted, filamentous and membrane-bound forms (Ilg, 2000). This virulence factor is involved in the mid-gut sandfly gel-like networks formation, which promotes sandfly regurgitation and increases the number of bites to facilitate the promastigote transmission to the mammalian host (Rogers & Bates, 2007; Rogers *et al.*, 2004). PPGs are involved in the macrophage recruitment to the inoculation sites, increase in the arginase activity towards polyamines production and downregulation of the pro-inflammatory response in macrophages (Piani *et al.*, 1999; Rogers *et al.*, 2009).

The Glycoprotein 63 (GP63) is a zinc-dependent metalloprotease mostly expressed in promastigotes and down-expressed in amastigotes. This virulence factor is found attached to the membrane through a glycophosphatidylinositol (GPI), in soluble form and can also secreted within exosomes (Silverman *et al.*, 2010a). GP63 presents a conserved motif of (HExxHxxGxxH) and has a wide range of substrates including casein, gelatin, albumin, hemoglobin, and fibrinogen (Yao *et al.*, 2003). GP63 plays an important role in host-response modulation and has been shown to cleave key proteins for different biological processes including protein tyrosine phosphatases, transcription factors, intracellular signalling and vesicular transport. The role of GP63 during the infection will be mentioned in more detail in chapter 3.

Leishmania also synthesizes other virulence factors such as Cysteine protease (CPs), an inhibitor of serine peptidase (ISP), and secreted acid phosphatases (SAPs). The cysteine protease is expressed in promastigotes and amastigotes and is involved in host-immune

response modulation. For instance, *L. mexicana* CPB mutant does not induce lesion growth in BALB/c mice and shifts the predominantly Th2-associated immune response to a Th1-associated response (Alexander *et al.*, 1998). Different studies have described the *L. mexicana* CPB role in the degradation and inhibition of several macrophages substrates involved in immune activation such as the transcription factors STAT1 and AP-1, the subunits of nuclear factor IkB-α, IkB-β and NF-kB, and components of the major histocompatibility complex (MHC) class II to prevent antigen presentation (Cameron *et al.*, 2004; De Souza Leao *et al.*, 1995; Mottram *et al.*, 1996). The principal role of inhibitors of serine peptidase is the inhibition of host enzymes including neutrophil elastase, trypsin and chymotrypsin, which prevent the PKR host activation in *L. major* infected cells (Eschenlauer *et al.*, 2009). The secreted acid phosphatases are conserved in all *Leishmania* species (Shakarian *et al.*, 2003), which are localized on the parasite surface and are constantly secreted. Initial reports have shown that *L. donovani* SAPs prevent oxidative bursts in neutrophils (Remaley *et al.*, 1985) and avirulent strains present significantly reduced acid phosphatase activity (Katakura & Kobayashi, 1988). A characteristic of the SAPs is the optimal catalytic activity at low pH, which is found in the sand fly gut and the phagolysosome (Das *et al.*, 1986)

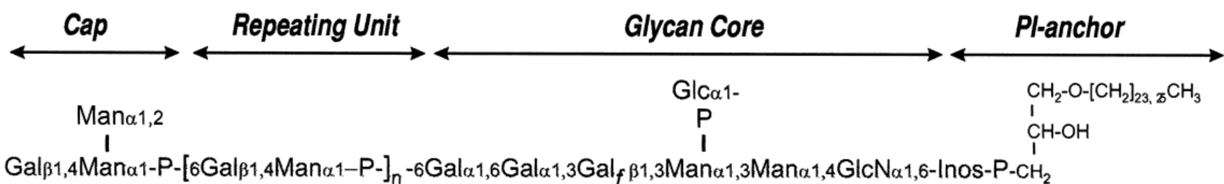


Figure 2.6. LPG structure.
L. donovani LPG presents four domains (1) 1-O-alkyl-2-lyso-phosphatidyl(myo)inositol anchor, (2) a glycan core, (3) repeating disaccharide phosphate units, and (4) a small oligosaccharide cap (Descoteaux & Turco, 1999)

3 MACROPHAGE-*LEISHMANIA* INTERACTION

3.1 Initial interactions and *Leishmania* recognition

The primary metacyclic promastigote interaction with the vertebrate host after the inoculation includes a set of parasite and sandfly factors that play an important role in infection establishment. During the blood meal, the sand fly injects metacyclic promastigotes along with its saliva that contains molecules such as Maxadilan peptide, adenosine, 5′-AMP and prostaglandins among others that promote vasodilatation, inflammation, and recruitment of phagocytic cells to the inoculation site (Rogers & Titus, 2003; Titus, 2000). In addition, the injected metacyclic promastigotes produce large amounts of proteophosphoglycans that stimulate macrophage recruitment (Rogers *et al.*, 2009). Before parasite recognition, there is an important process mediated by GP63 that enhances parasite migration and recognition that activates different cellular mechanism associated with the phagocytic process. Released GP63 in the first instance degrades different components of the extracellular matrix at a subcutaneous level including collagen type IV and fibronectin, which may facilitate parasite migration through the extracellular matrix (McGwire *et al.*, 2003). In parallel, GP63 mediates the complement system inactivation in the serum by degrading the fraction C3b that is converted into the inactive form C3bi, thereby causing a reduction of C5-C9 complex assembly, hence preventing the attack complex formation and enhances parasite resistance to complement-mediated lysis. Importantly, the C3bi fraction acts as an opsonin that coats the parasite surface, which is crucial during parasite recognition and enhances phagocytosis through the macrophage complement receptors CR1 and CR3 (Da Silva *et al.*, 1989). Another important mechanism used by *Leishmania* to survive the complement attack is the enlarged LPG coat on metacyclic promastigotes that prevents the membrane attack complex attachment to the membrane, whereas procyclic promastigotes are very sensitive to complement-mediated lysis (Puentes *et al.*, 1990). In both promastigote stages, there is a deposition of covalently bound C3b on the parasite surface. However, in metacyclic promastigotes, there is a limited C5b-9 fraction able to access the parasite membrane, preventing membrane attack complex assembly (Puentes *et al.*, 1988).

It is important to mention that *Leishmania* inoculation by the sand fly is a mixed population of live and apoptotic-like promastigotes that promote an important response in the host during the initial

interactions. For instance, apoptotic-like promastigotes express on their surface the phosphatidylserine (PS) residue important to activate phagocytosis in a non-inflammatory context along with the secretion of anti-inflammatory molecules including TGF-b, PGE2 and PAF at the inoculation site to prevent an aggressive macrophage response (Fadok *et al.*, 1998; Wanderley *et al.*, 2013). Another beneficial response induced by the apoptotic-like promastigote recognition is the alternative internalization process mediated by LC3 known as LC3-associated phagocytosis (LAP). It reduces T-cell-mediated parasite clearance, provokes anti-inflammatory responses and reduces antigen presentation (Crauwels *et al.*, 2015). On another hand, viable *Leishmania* is detected by different macrophage receptors including complement receptors (CRs), mannose receptors, fibronectin receptors and Fcγ receptors (FcγRs) (Ueno & Wilson, 2012). The phagocytosis associated with each receptor may impact infection outcome. The complement receptors (1 and 3) are beneficial for the parasite, since they inhibit the strong inflammatory response, reduce the oxidative burst and promote the accumulation of lysosomal markers LAMP1 and Cathepsin D, which create more favourable conditions for the parasite within the phagosome (Podinovskaia & Descoteaux, 2015). On the contrary, fibronectin receptors trigger a stronger inflammatory response, whereas Mannose receptors enhance hydrolytic enzyme accumulation in the phagosome and FcγR receptors show higher NADPH oxidase assembly in the phagosome (Polando *et al.*, 2013). Although promastigotes may bind to macrophages through different receptors, the *in vivo* attachment normally occurs through the CR1 and CR3 receptors. This is due to parasite opsonization, with the glycoconjugates LPG and GP63 as principal acceptors of the fragments C3b and C3bi (Mosser & Rosenthal, 1993). The importance of *Leishmania* promoting phagocytosis mediated by complement receptors is associated to induce internalization without triggering the oxidative burst (Wright & Silverstein, 1983). Another way for *Leishmania* to enter the macrophages is mediated by phagocytosis via PMNs of neutrophils harbouring promastigotes. This entry mechanism is advantageous for the parasite because the neutrophile acts as a Trojan horse, due to the non-inflammatory responses associated with this kind of phagocytosis (Peters *et al.*, 2008; Peters & Sacks, 2009). Noteworthy, amastigotes present reduced LPG and GP63 levels on their surface, which leads them to enter by employing a different mechanism compared to promastigotes. Hence, different *Leishmania* amastigotes were found to be coated with IgG1 *in vivo*, which mediates the recognition through FcγR. Also, other family receptors including FnRs and CR3 have revealed that similar to promastigotes, those receptors may mediate amastigote phagocytosis

(Figure 3.1) (Bosetto & Giorgio, 2007; Guy & Belosevic, 1993; Peters *et al.*, 1995; Wozencraft *et al.*, 1986; Wyler *et al.*, 1985).

After *Leishmania* is recognized and bound by complement receptors, the parasite internalization process is induced by Rho kinases (RhoA, Rac1 and Cdc42). Further, there is an accumulation of periphagosomal F-actin (Lodge & Descoteaux, 2005) and cholesterol-rich membrane lipid caveolae that enhance parasite uptake (Rodríguez *et al.*, 2011). The parasite internalization into the macrophage occurs from the anterior part of the *Leishmania* body, which in part is stimulated by the parasite's flagellar motility. Importantly, The flagellar membrane is enriched in lipid raft domains and actin-interacting proteins involved in the phagosome formation through actin polymerization within the flagellar environment (Diniz *et al.*, 2009). This interaction triggers a pseudopod formation initiating at the flagellar tip and extends toward the parasite cell body and, eventually, there is a parasite oscillation that reorientates the flagellum in the host cell periphery, which induces a host cell plasma membrane wounding followed by lysosome recruitment and lysosome exocytosis. This parasite-driven host cell injury is an important event during *Leishmania* host cell infection (Forestier *et al.*, 2011).

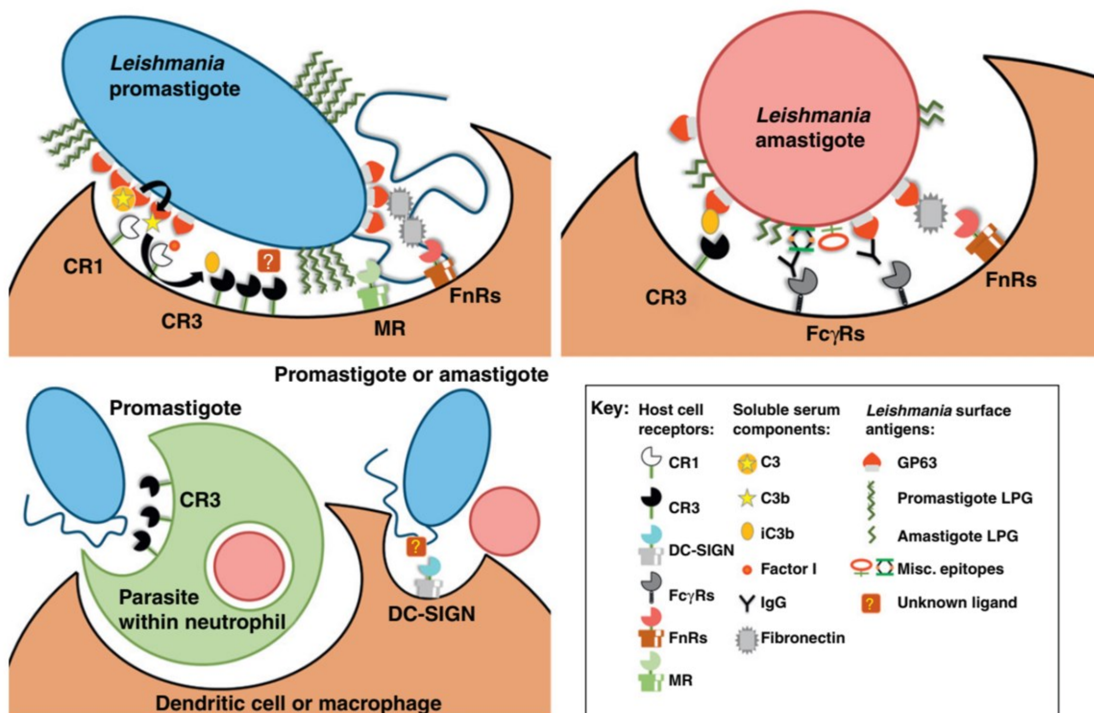


Figure 3.1. *Leishmania* entry receptors in phagocytes.
Leishmania promastigotes and amastigotes are recognized by several host receptors from macrophages, neutrophils and dendritic cells, inducing the parasite entry mediated by phagocytosis (Ueno & Wilson, 2012).

3.2 Phagosome biogenesis and transition into the parasitophorous vacuole

As it was mentioned before, the macrophage detects and internalizes different microorganisms that are cleared in part due to the phagolysosome action. The phagolysosome presents an extremely low pH environment in which the microorganism's fate should lead to death. However, *Leishmania* has evolved to adapt inside the macrophage in a structure known as parasitophorous vacuole (PV). Indeed, the promastigotes are susceptible to being destroyed in the phagolysosome. Nevertheless, the parasite employs several mechanisms that modulate the phagolysosome biogenesis and shape the environment required for its differentiation into amastigotes. The principal virulence factor involved in the phagolysosome modulation is LPG and its dependence on parasite survival was initially described when *L. donovani* and *L. major* LPG-deficient promastigotes induced phagosomes that fuse extensively with endocytic organelles, which ultimately resulted in phagolysosome induced death of the parasites, whereas wild type or LPG-rescued promastigotes that do not promote fusogenic phagosomes managed to survive and differentiated into amastigotes (Desjardins & Descoteaux, 1997; McNeely & Turco, 1990). Nowadays, the LPG's role in sabotaging phagosome biogenesis is well described (Dermine *et al.*, 2005; Dermine *et al.*, 2000; Garin *et al.*, 2001; Scianimanico *et al.*, 1999).

During the initial steps of *Leishmania* phagocytosis, there is an accumulation of F-actin around the phagocytic cup. In a regular phagocytic process, the phagocytic cup disappears, followed by phagosome maturation. However, in *L. donovani*-infected macrophages, even if the F-actin phagocytic cup disappears, F-actin accumulates in the forming phagosome along with microfilament-associated proteins including Arp2/3, Cdc42, WASP, Nck and Myosin II (Holm *et al.*, 2001; Lodge & Descoteaux, 2005). The F-actin sterically prevents the recruitment of signal transducer and phagosome accessory proteins that mediate the phagosome maturation including Rab 7, which promotes the fusion of phagosomes with late endosomes. Importantly, phagosomes harbouring LPG mutant parasites present enhanced transformation into phagolysosomes, which is the result of losing the early endosome markers EEA1 and transferrin receptor, and recruiting the late endocytic and lysosomal markers Rab 7 and LAMP1 (Dermine *et al.*, 2000; Desjardins & Descoteaux, 1997; Scianimanico *et al.*, 1999). In addition, LPG inhibits PKC-α activation, which also promotes F-actin accumulation and delays LAMP1 recruitment to the phagosome hence delaying the maturation process (Dermine *et al.*, 2005; Moradin & Descoteaux, 2012; Vinet *et al.*, 2009). LPG modulates phagosome maturation by transferring LPG from the parasite to the surface of lipid microdomains present in the phagosome

membrane. The LPG accumulation induces microdomain changes into inverted hexagonal structures resulting in reduced fusogenicity by preventing the formation of new lipid microdomains (Dermine *et al.*, 2005; Dermine *et al.*, 2000; Miao *et al.*, 1995). The disruption of lipid microdomains and the periphagosomal F-actin accumulation promotes a truncated phagosome maturation and presents significant functional consequences, thus, allowing the promastigotes to survive the initial interactions inside the macrophage and are crucial for the parasite fate.

During phagosome maturation, there are two key events involved in the microorganism clearance: the action of reactive oxygen species (ROS) and the phagosome acidification by the v-ATPases. ROS production is mediated in part by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex in which the cytosolic $p47^{phox}$ is phosphorylated, inducing a p40/p67phox heterodimers recruitment to form the $p47/p67/p40^{phox}$ heterotrimers complex (NOX2) (Bokoch & Diebold, 2002). *L. donovani* promastigotes, in an LPG-dependent fashion block the $p47^{phox}$ and $p67^{phox}$ recruitment to the phagosome membrane, thus inhibiting the NOX complex assembly and ROS production in the phagosome. Importantly, ROS production in *Leishmania* promastigote infected-macrophages is impaired only in the phagosome (Lodge & Descoteaux, 2006). The intraphagosomal acidification is mediated by the enzyme V-ATPase in which ATP hydrolysis transport protons across the phagosome membrane. V-ATPase recruitment is mediated by Syt V (Vinet *et al.*, 2009). The lipid raft disruption in the phagosome caused by LPG excludes Syt V, that in turn prevents the V-ATPase and cathepsin D recruitment, ultimately preventing phagosome acidification (Vinet *et al.*, 2009). It is important to mention that not all *Leishmania* species modulate phagosome biogenesis in the same fashion. For instance, *L. amazonensis* and *L. mexicana* LPG does not play a role in intracellular survival or phagosome maturation (Courret *et al.*, 2002; Ilg, 2000) indicating that host cell rewiring is specie specific and relies on each parasite's need. The PV biogenesis is also mediated by the recruitment of several proteins and membranes from ER, Golgi and recycled endosomes including calnexin, Sec22b, Stx5, Stx18, VAMP3, VAMP8, SNAP23 and Vti1A among others and may change depending on the *Leishmania* species **(Figure 3.2)** (Arango Duque *et al.*, 2019b; Canton *et al.*, 2012; Garin *et al.*, 2001; Séguin *et al.*, 2022; Séguin *et al.*, 2020). Independently of the parasite's capacity to interfere with ROS production, *Leishmania* displays various antioxidant mechanisms that attenuate the oxidative burst in which the most important are trypanothione reductase (TryR)

and trypanothione synthase (TryS) (Tovar *et al.*, 1998). Also, the heat shock proteins (HSPs) are crucial during the thermal and oxidative shock adaptation (Hübel *et al.*, 1995).

Inside the PVs, the promastigotes are differentiated into amastigotes. The signals that trigger the differentiation process are not clear but are believed to be induced by the increasing temperature, decreasing pH and a nutrient-deficient microenvironment. Interestingly, it has been reported that *L. amazonensis* promotes its differentiation by iron uptake and hydrogen peroxide generation (Mittra *et al.*, 2013). In *Leishmania*, the iron uptake is mediated by the ferrous iron transporter LIT1, and the parasite survival and differentiation are highly impaired in LIT1 mutants. Interestingly, iron deprivation media promotes LIT1 up-regulation that is similar to the reduced iron levels in the PV environment (Flannery *et al.*, 2011; Mittra *et al.*, 2013). Once the promastigotes are completely differentiated into amastigotes, the parasite presents a slow replication ratio. Whereas amastigotes from most *Leishmania* species proliferate in a tight individual PV harbouring each parasite, amastigotes of *L. mexicana* complex reside in large communal PV harbouring several parasites in the same vacuole (Real & Mortara, 2012; Real *et al.*, 2008).

It is important to mention that PV modulations during promastigote infection have an important role in the parasite's fate. However, the amastigotes reside and replicate inside acidic, hydrolase-rich phagolysosomes (Alexander & Russell, 1992; Alexander & Vickerman, 1975; Antoine *et al.*, 1990). Thus, the amastigote's entry mechanism and the PV modulation are different from that of the promastigotes. *In vivo*, amastigotes are completely opsonized and detected by the CR3 receptor. The amastigote entry is mediated via Rac1 and Arf6, which lead to a PV formation with all the endosomes and lysosome marker recruitment characteristic during phagosome maturation (Chang & Dwyer, 1978). Indeed, PVs harbouring amastigotes are enriched with late endosomal/lysosomal proteins, such as Rab7, LAMP-1 and LAMP-2 and acidified through V-ATPases (Antoine *et al.*, 1990; Antoine *et al.*, 1998). Equally to promastigotes, amastigotes subvert the ROS generation in the PV through heme degradation and prevention of the NADPH oxidase complex assembly **(Figure 3.3)** (Lodge & Descoteaux, 2006; Pham *et al.*, 2005)

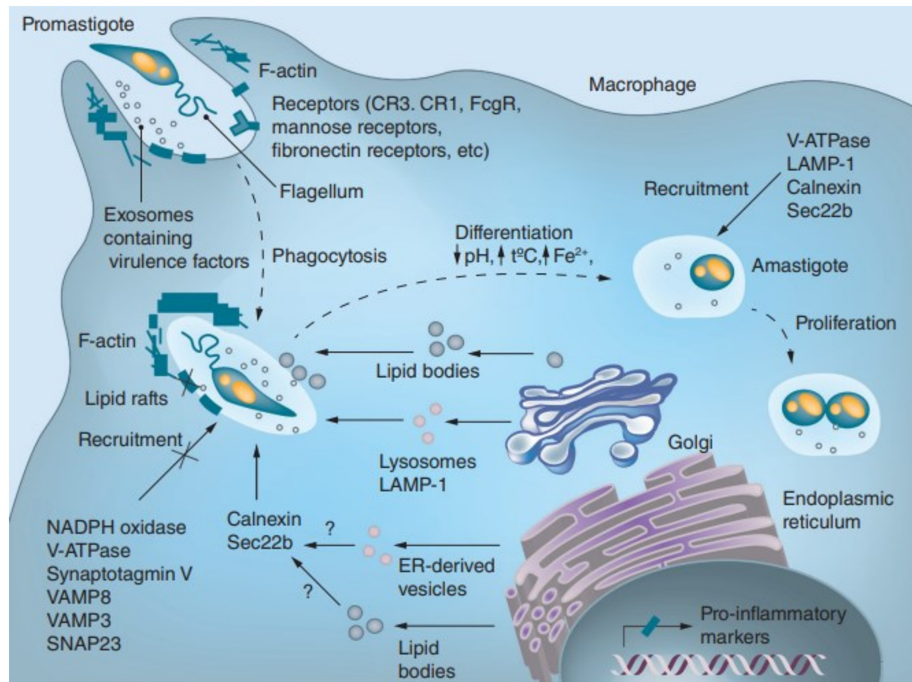


Figure 3.2. ***Leishmania*** **subverts macrophage biology.**
During the colonization process, *Leishmania* interacts with several macrophage receptors inducing its internalization. The phagocytic process and PV formation is subverted by F-actin accumulation and impairment of SNAREs recruitment to the PV, which delays phagosomal maturation. The PV formation involved the recruitment of ER markers. There is a differentiation process from promastigote to amastigote mediated by pH, temperature, and iron uptake among others. PV: parasitophorous vacuole (Podinovskaia & Descoteaux, 2015).

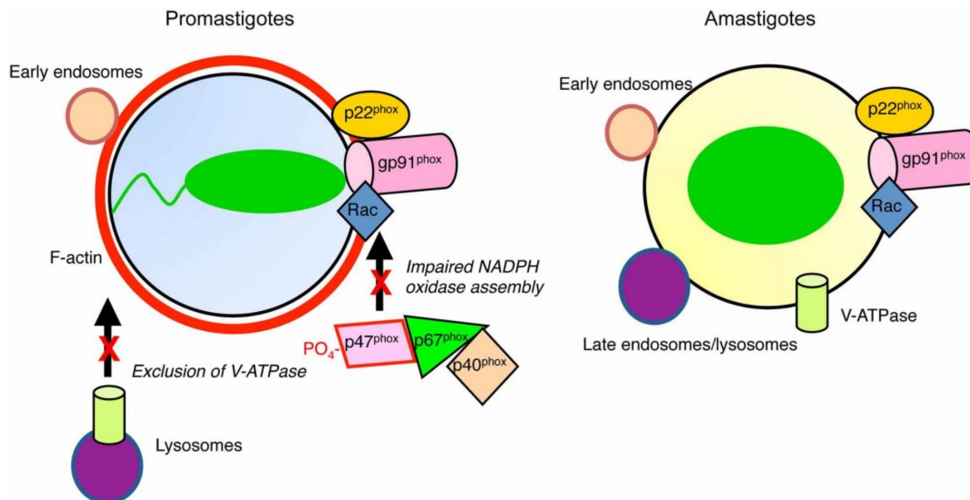


Figure 3.3. Phagosomal assembly during ***L. donovani*** **infection.**
In promastigote-containing PV, the F-actin (red) is accumulated in the PV accumulates, there is an interaction with early endosomes (tan) and the V-ATPase is excluded from the PV. Also, there is poor interaction with late endosomes/lysosomes (purple). In contrast, amastigote-containing PV interacts with late endosomes/lysosomes (purple) and are acidic. Assembly of the NADPH oxidase is impaired in both types of promastigotes and amastigotes PVs (Moradin & Descoteaux, 2012).

3.3 Modulation of host immune response

During the colonization process, *Leishmania* interferes with different signalling axis involved in the immune response and macrophage activation, allowing the parasite to replicate in a long-term infection. In the previous chapters, the role of LPG and GP63 was described during parasite recognition, internalization, and phagosome biogenesis modulation. Additionally, *Leishmania* modulates other macrophage properties that control TLR functions, antigenic presentation, soluble mediator production and other intracellular signalling pathways involved in the macrophage's function. The modulation of these modules may involve different strategies employed by *Leishmania* including the sabotage caused by virulence factors such as LPG or GP63. For instance, in *L. donovani*-infected macrophages, there is a downregulation of TLR4-stimulated IL-12p40 and TLR2 by preventing the MAPK phosphorylation. Moreover, activation of the (ERK)1/2 kinase via phosphorylation, which leads to an increased IL-10 production favouring the Th2 response, is another modulation in *L. donovani* infected macrophages (Chandra & Naik, 2008). Also, in macrophages and NK cells, *Leishmania* LPG is detected through TLR2 resulting in reduced macrophage activity that is important for parasite survival (Becker *et al.*, 2003; Flandin *et al.*, 2006). The LPG's role during TLR2 modulation was disclosed by using two *L. major* strains presenting high and low LPG levels, which were used in *in vitro* infections and were found to induce different TLR9, TGF-β and IL-10 expressions (Srivastava *et al.*, 2013). Another study showed that in humans, *L. Mexicana*-infected macrophages promote in an LPG-dependent manner ERK and p38 MAP kinase phosphorylation through TLR2 and TLR4 recognition, inducing the production of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-12p40, IL-12p70 and IL-10 (Rojas-Bernabé *et al.*, 2014).

In a pathological context, the immune system may combine an innate immune response with an adaptative immune response, which is partly mediated through antigenic presentation to T-lymphocytes. In general, phagocytic cells detect and internalize microorganisms that are degraded in the phagolysosome, which produce specific peptides. Then, these peptides are presented to $CD8^+$ and $CD4^+$ T-cells through the MHC class I and class II molecules respectively to establish an adaptative immune response (Neefjes *et al.*, 2011). The macrophage is a professional antigen-presenting cell and has previously been shown capable of presenting *Leishmania* antigens by cross-presentation (Overath & Aebischer, 1999). However, *L. donovani* and *L. major* promastigotes evade the host immunity by cleaving and excluding VAMP8 and VAMP3 from the phagosome in macrophages in a GP63-dependent manner. This results in the

inhibition of MHC class I presentation and T-cell activation (Matheoud *et al.*, 2013). MHC class II presentation is also reduced in *L. donovani*-infected macrophages independent of VAMP8 recruitment (Meier *et al.*, 2003). Importantly, isolated *L. donovani* exosomes promote a decrease in IL-12p70, TNF-α, and IL-10 cytokine production in human monocyte-derived dendritic cells, causing a failure in the differentiation of naive CD4 T- cells into IFN-γ–producing Th1 cells (Silverman *et al.*, 2010b). *L. amazonensis* also showed to have the capability of modulating antigen presentation by disrupting the CD40–CD40 Ligand Interactions causing an important reduction in protective immune responses (Hodgkinson *et al.*, 1996). Interestingly, antigen cross-presentation is also inhibited via the disruption of membrane lipid microdomains associated with an increase in the phagosome membrane fluidity (Chakraborty *et al.*, 2005). In T-cells, *L. donovani* and *L. major* cleave TLR4 in a GP63-dependent manner, thus reducing the T-cell response to antigen-presenting cells (Hey *et al.*, 1994).

Another functional consequence of macrophage sabotage is the change in soluble mediator production (cytokines and chemokines). *Leishmania* infection in general prevents an effective immune response by attenuating the inflammatory response and promoting the recruitment of different immune cells required for infection establishment. During the establishment of an immunological response, T-lymphocytes play an important role in IFNγ production, which is crucial to promote M1 macrophages and contain the infection with IL-12 as an important regulator in this cellular response (Ivashkiv, 2018; Ma *et al.*, 2015). In macrophages, *L. donovani*, *L major* and *L. mexicana* promastigotes inhibit IL-12 production inducing a prolonged interval of parasite intracellular survival and replication (Belkaid *et al.*, 1998; Carrera *et al.*, 1996). Additionally, *L donovani* induces downregulation of CD80, ICAM-1, which lead to a failure in T-cell activation (Saha *et al.*, 1995), contrary to *L. major* that increases CD40, which in turn increases the T- regulatory cell (T-reg) development, IL-12 production and induces a stronger Th2-response (Campbell *et al.*, 1996). Another important modulator of the immune response is IL-10, which acts as a suppressor of Th1 cells and, which can be used as an indicator of parasite persistence in *Leishmania* infections (Nylén *et al.*, 2007). For instance, BALB/c and C57BL/6 knock-out for IL-10 are highly resistant to *L. donovani* infection that is accompanied by increased production of IFN-γ and nitric oxide in BALB/c (Murphy *et al.*, 2001). Additionally, In *L. donovani*-infected macrophages there is an enhancement of IL-10 production that in turn impairs PKC-mediated signalling, hence reducing the inflammatory response and facilitating intracellular survival (Bhattacharyya *et al.*, 2001). IL-10 production is also enhanced by different *Leishmania*

species (Buxbaum, 2013; Padigel & Farrell, 2005). Similar to IL-10, IL-6 promotes a Th2 response, interferes with the IFNγ production and prevents M1 macrophage activation in *L. major* infections (Chakour *et al.*, 2009; Himmelrich *et al.*, 2000). *In vivo* infections using *L. major* promastigotes revealed induction of IL-6 and TNF in a GP63-dependent manner through the synaptotagmin XI cleaving (Duque *et al.*, 2014). During the colonization process, *Leishmania* has shown to promote a set of chemokines that mediate the recruitment of immune cells that may become new hosts or be involved in the infectious process. For instance, different *Leishmania* species promote the upregulation of chemokines after the inoculation including RANTES/CCL5, MIP-1α/CCL3, IP-10/CXCL10, MCP-1/CCL2, MIP-1β/CCL4, MIP-2/CXCL1, and IL-8/CXCL8, which recruit macrophages, neutrophils, dendritic cells and lymphocytes (Antoniazi *et al.*, 2004; Kobets *et al.*, 2012; Lazarski *et al.*, 2013; Navas *et al.*, 2014; Teixeira *et al.*, 2006).

There is a panoply of different GP63 substrates that have been identified to be cleaved or degraded such as the substrate-related cytosolic proteins for PKC rich in myristoylated alanine residues (MARCKS) (Corradin *et al.*, 1999). Protein tyrosine phosphatases (PTPs) include p130cas, PTP-PEST, SHP-1, and PTP1B (Gomez *et al.*, 2009; Hallé *et al.*, 2009). Transcription factors include p65relA, C-JUN, and C-FOS (Contreras *et al.*, 2010; Gregory *et al.*, 2008). Intracellular signalling such as mTOR, NLRP3, Cortactin and Caspase-3 (Hallé *et al.*, 2009; Jaramillo *et al.*, 2011; Shio *et al.*, 2015). Vesicular transport includes synaptotagmin XI, Syntaxin 5, VAMP3 and VAMP8 (da Silva Vieira *et al.*, 2019; Duque *et al.*, 2014; Matheoud *et al.*, 2013; Matte *et al.*, 2016). The impact of GP63 interaction with those subtracts leads to the degradation or cleaving that, in general, reduces the macrophage inflammatory immune response and activation. Nevertheless, a recent publication revealed artifactual targets including PTP-PEST, mTOR, p65relA, C-Jun, VAMP3, and NLRP3 due to a non-complete GP63 inactivation during the preparation of infected cell lysates. This publication highlights the need to re-evaluate other GP63 substrates that have not been tested in order to properly disclose their role during infection establishment (Guay-Vincent *et al.*, 2022).

4 MITOCHONDRIAL BIOLOGY

4.1 History and discovery of the mitochondria

The mitochondria are a double-membrane-bound organelle (mitochondrion in singular) present in most eukaryotic organisms. Mitochondria are traditionally known as the powerhouses of the cell for their capacity to carry out ATP production, which is critical to support the cell's energetic requirements. In eukaryotic cells, the energetic metabolism involves a coordinated action of cytosolic and mitochondrial enzymes that reduce organic molecules to produce ATP in an anaerobic or aerobic manner depending of the cellular needs. One of the principal mitochondrial features is the capacity to reduce organic molecules into high amounts of ATP by using a set of specialized enzymatic complexes known as the electron transport chain in which oxygen plays a critical role as a terminal electron acceptor. Indeed, the mitochondria are also involved in a wide spectrum of cellular functions including maintenance of general homeostasis, cell cycle regulation, control of the anabolism and catabolism, regulation of the immune response and, during the last decade, they have become the central hub of immunometabolism, which dictates cellular fate. To understand the importance of mitochondria in eukaryotic cells and understand why they became the poster child of metabolism, it is important to go back in time around 2 billion years ago; a time in which the ancient fossils confirm the existence of aerobic eukaryotes with ancient mitochondria (Knoll, 1992).

The mitochondrion’s origin is dated to approximately 2 billion years ago (Kurland & Andersson, 2000), a time when the Earth’s oxygen composition increased rapidly from 1% to more than 15% of present levels (Holland, 1994) thanks the action of the oxygenic cyanobacteria metabolism (Johnston *et al.*, 2009). The rise in oxygen levels was probably the stimulus that favored the oxidative system development in some ancient microorganisms. Importantly, the increase in the oxygen levels matches with the ancient origins of the terminal oxidases in archaea, bacteria and eukaryotes, which are characteristic of the modern day mitochondria (Castresana *et al.*, 1994; Cavalier-Smith, 1987; Schäfer *et al.*, 1999). Currently, the introduction of aerobic respiration and the oxidative system in eukaryotic cells is widely accepted to be a product of endosymbiosis with an ancient member of the α-proteobacterium family (Andersson *et al.*, 1998; Gray *et al.*, 1989). Interestingly, phylogenetic reconstructions and distance measurements using the cytochrome c oxidase, the cytochrome b, the heat-shock protein 60 and rRNA sequences showed a

mitochondrion divergence from bacteria between 1.5 and 2.0 billion years ago (Sicheritz-Pontén *et al.*, 1998; Viale & Arakaki, 1994). Indeed, the closest exiting relatives of the mitochondrion's ancestor seem to be the Rickettsia. Hence, the functional genes in *Rickettsia prowazekii* present similarities to those of the current mitochondrial genes. There are no genes related to anaerobic glycolysis in both mitochondria and *Rickettsia* genomes. In opposition, the mitochondria and *Rickettsia* encoded genes that compose the tricarboxylic acid cycle and the respiratory-chain complex are the same, which share a conserved genetic core in the ATP production (Andersson *et al.*, 1998).

Two hypotheses propose how the mitochondria were integrated into the ancient eukaryotic cell: the Ox-Tox model and the hydrogen hypothesis. The Ox-Tox model suggests that the ancient mitochondria were adopted to serve as protectors for the anaerobic host against the toxic effects of oxygen that was raising back in time. As a benefit, the ancient mitochondria acquired an ideal environment abundant with numerous nutrients such as pyruvate. The mutual benefit of those ancient organisms led to a coexistence that eventually would lead to the integration of the ancient mitochondria with the host. As a result, that association evolved into the actual eukaryotic cell containing the modern mitochondria (John & Whatley, 1975; Kurland & Andersson, 2000). Otherwise, the hydrogen hypothesis proposes that the ancient mitochondria were converting organic compounds into hydrogen and carbon dioxide, which in turn were transformed into methane by the methanogenic prokaryotic hosts that cohabitated with ancient mitochondria. The complementary metabolic pathway ultimately resulted in the engulfment and association of the two cell types that became the actual eukaryotic cell containing the modern mitochondria (Martin & Müller, 1998). Both hypotheses remain in discussion. Nevertheless, independently of the mechanisms that led to the mitochondria integration into the host, it is clear that this evolution process led to all eukaryotic life as we know it today.

Mitochondria discovery was not an isolated case. Instead, it happened between 1840 and 1890, when different Cytologist, Biochemist and Physiologist were discovering and describing the different cellular components and their role in different biological systems (Aubert, 1853; Flemming & und Zellteilung, 1882; Henle, 1841; Retzius, 1890). During these years, some reports showed unidentified intracellular structures that probably represent the mitochondria today. However, the first report showing clear evidence of mitochondria was in 1890, when a German histologist, Richard Altmann, described a ubiquitous occurrence of structures named "bioblasts" and concluded that bioblasts were elementary organisms living inside the cells that

carried out vital survival functions (Altmann, 1890). Further in 1898, the term mitochondria was coined by Carl Benda when he described the appearance of these structures during spermatogenesis that tended to form long chains (Benda, 1898). The word mitochondrion comes from the greek mitos" (thread) and "chondros" (granule). Further studies started to elucidate the relationship between the mitochondria and the organic substrate oxidation. For instance, in 1912, the American anatomist Benjamin Freeman Kingsbury proposed that the cytosolic reducing environment is correlated with mitochondria presence. Thus, this discovery shifted the focus on the mitochondria as the source of cellular oxidative respiration (Kingsbury, 1912). Nevertheless, evidence for that hypothesis was only supported in 1925 with the landmark discovery of cytochromes (Keilin, 1925). During the 1930s, Krebs and Kalckar elucidated the energetic pathways of aerobic metabolism by describing what we call nowadays the Krebs cycle and aerobic phosphorylation, which placed mitochondria at the center stage for oxidative metabolism (Kalckar, 1937; Krebs & Johnson, 1937). Further in 1941, Lipmann linked aerobic respiration with the concept of phosphate-bond that carry on energy (ATP) in cellular metabolism (Lipmann, 1941). Indeed, the 1940s was a decade that allowed for a significant advance in mitochondrial biology. For instance, the isolation by ultracentrifugation of mitochondria probed the cytochrome's location within the mitochondria, thus establishing that oxidative phosphorylation and Kreb's cycle take place in the mitochondrion (Claude, 1949; Kennedy & Lehninger, 1948; Kennedy & Lehninger, 1949). During the 1950s, with the advances in fixation and preservation techniques, the optimization of cellular fragmentation methods and the introduction of the electronic microscope, George E. Palade was the first researcher to publish a high-resolution picture of mitochondria **(Figure 4.1)** in which the they were found to be surrounded by a double membrane structure in which the interior membrane is folded and forms ridges (mitochondrial cristae) (Palade, 1953). The methodology to isolate and preserve mitochondria led to the beginning of a new era in mitochondrial ultrastructure studies. Some years later, in 1957, Chance observed that isolated mitochondria catalyze the endogenous NAD^+ reduction by succinate, showing the first evidence of a reversal electron transport (Chance & Hollunger, 1957). Further studies led to the establishment of the general concept that electron transport involves 3 coupling sites of the respiratory chain and it can be reversed in a dynamic equilibrium between respiratory and phosphorylation systems (Ernster & Schatz, 1981; Schollmeyer & Klingenberg, 1961; Williamson *et al.*, 1967). One of the principal breakthroughs in mitochondria studies was made in 1961 by Peter D. Mitchell when his team described how oxidation was coupled to ATP synthesis. He proposed a transition between the chemical

electron coupling that comes from substrate oxidation and transfers of hydrogen and phosphate across the mitochondrial membrane (Mitchell, 1961). These observations further established the basis of the chemiosmotic theory in which ATP synthesis is directly coupled to an electrochemical gradient across the inner mitochondrial membrane and was supported by the proton efflux of the electron transport system (ETS) (Mitchell & Moyle, 1969).

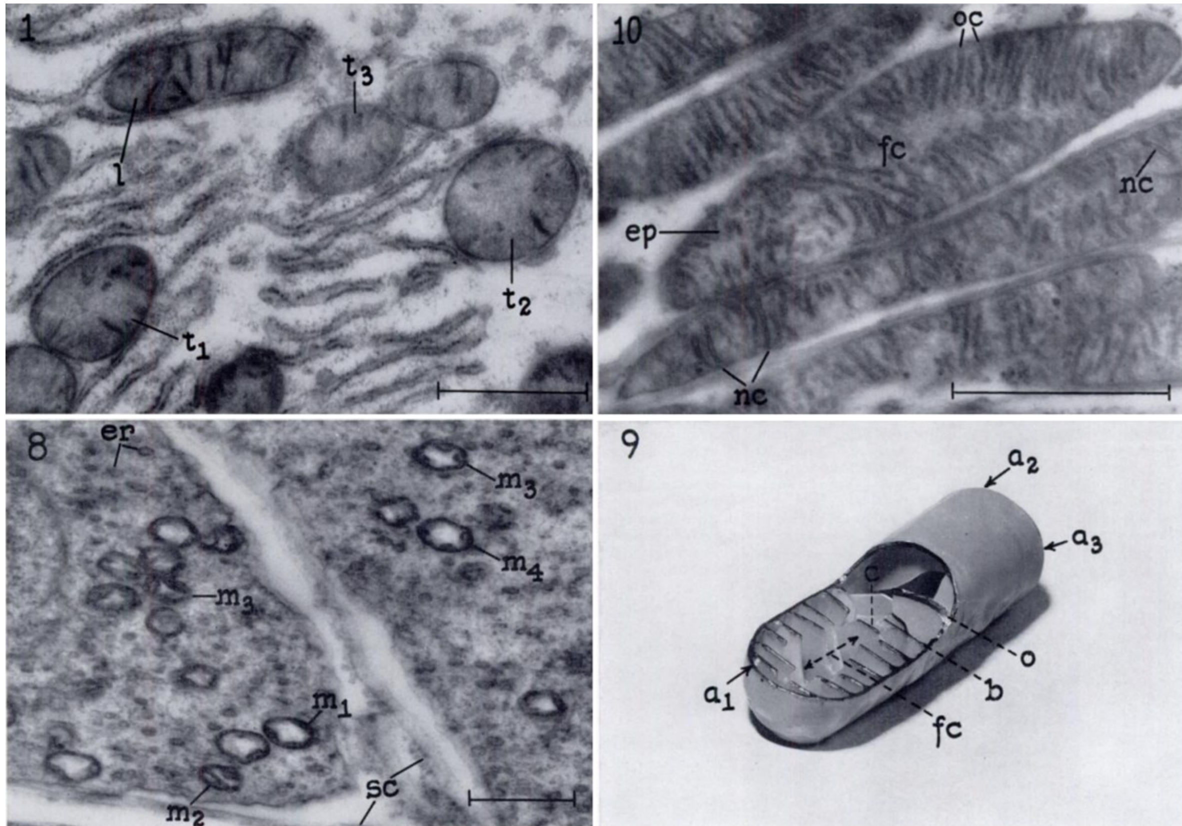


Figure 4.1. Mitochondrial ultrastructure.
The cytoplasm of a parenchymatous liver cell (1), spermatocytes in a seminal tubule (8) and epithelium of the proximal convoluted tubule of the kidney (10), which contains several mitochondrial shapes. Samples were derived from rats. L: longitudinal section, T: traverse section, SC: fine strips of membranes and cytoplasm, M: mitochondrial profiles, ER: endoplasmic reticulum, FC: free channel, NC: cristae, OC: other, EP: the ellipsoidal profile, B: branching cristae and a: sections (Palade, 1953).

It is evident that during almost 200 years of research in mitochondria biology, there are many outstanding discoveries that were made and ,which deserve a place in history; however, there is simply too much to describe in one thesis and thus a few important key events that shaped mitochondria research are summarized in **(Table 7)**. Collectively, these breakthroughs gave us the foundation of our modern understanding of mitochondrial biology. Importantly, the

mitochondria emerge as a central core of ATP production in which the electron transport system plays a critical role in ATP production. Now, one may reasonably ask: how are the organic substrates reduced to transfer energy; how are the different molecules ushered into the mitochondrial energetic machinery; how does the cell dictate whether organic substrates are reduced in the aerobic or anaerobic manner? All these amusing questions will be disclosed and answered in **Chapter 4.2**.

Table 7. Mitochondria landmark discoveries.
Key events that leads to the current understanding of mitochondria biology.

Year	Key event	Reference
1840-1882	First observations of cellular structures that were likely to be mitochondria	(Aubert, 1853; Flemming & und Zellteilung, 1882; Henle, 1841)
1886	The four-band absorption spectrum of cytochromes is observed and named myohaematin	(Munn, 1886)
1890	Cytoplasmic Bioblasts are observed in the cells and are proposed to be elementary organisms	(Altmann, 1890)
1898	The term mitochondria is coined	(Benda, 1898)
1912	The oxidative respiration is proposed to take place in the mitochondria	(Kingsbury, 1912)
1918	Mitochondria hypothesized to be symbiotic bacteria	(Sapp, 1991)
1920	Lactate is an alternative end product in the oxygen absence	(Meyerhof, 1920)
1925	The cytochromes are associated with cellular structures	(Keilin, 1925)
1937	Hans Krebs publishes a paper describing the Krebs cycle	(Krebs & Johnson, 1937)
1946	Cellular fractionation leads to isolate mitochondria/cytochrome and succinoxidase activity is detected in the mitochondria	(Claude, 1946)
1948-1955	Oxidative phosphorylation, Kreb's cycle and fatty acid oxidation take place in the mitochondria	(Green, 1951; Kennedy & Lehninger, 1948; Kennedy & Lehninger, 1949; Lehninger & Sice, 1955)
1953	Publication of the first high-resolution mitochondria	(Palade, 1953)
1955	Oxidative phosphorylation is a multistate process defined by the ADP/O ratio	(Chance *et al.*, 1955)
1958	ATP production is coupled to the Mitochondrial electron transport	(Pullman *et al.*, 1958)
1961	The mitochondrial proton motive force theory is proposed	(Mitchell, 1961)
1963	Discovery of the mitochondrial genome	(Nass & Nass, 1963)

1967	Endosymbiotic theory of the mitochondrial	(Sagan, 1967)
1978	Mitochondrial and bacteria protein sequences present high homology	(Schwartz & Dayhoff, 1978)
1981	Sequencing the mitochondrial genome	(Anderson *et al.*, 1981)
1999	Discovery of the mitosomes	(Tovar *et al.*, 1999)
2006	Energetic state hypothesis of mitochondrial oxygen sensing	(Hardie *et al.*, 2006)
2008	Publication of the mitochondria proteomic catalogue	(Pagliarini *et al.*, 2008)

4.2 Mitochondria at the core of ATP production

Life as we know it today, from the simplest and primitive unicellular organisms to higher eukaryotes, is possible thanks to an energetic flow that sustains all biological processes. Energy transfer involves the action of complex pathways that revolve around the addition of inorganic phosphate (phosphorylation) to adenosine diphosphate (ADP) to synthesize adenosine triphosphate (ATP). ATP is the central molecule storing energy in the phosphate bond, and it can be used when ATP releases the phosphate groups and becomes ADP or AMP **(Figure 4.2).** The simplest organisms carry out ATP production through a basic metabolic pathway that involves glucose degradation in a process known as Glycolysis, which produces low ATP yields. In contrast, in the majority of eukaryotic cells, mitochondria play a critical role in ATP production through a specialized set of enzymes that compose the tricarboxylic acid cycle (TCA) (known also as Krebs cycle or citric acid cycle) and the oxidative phosphorylation system. Indeed, ATP synthesis also occurs at the cytoplasmatic level in which the organic source is degraded independently of the mitochondria. Importantly, ATP production integrates multiple metabolic pathways that break down organic molecules from carbohydrates, lipids or proteins, into simpler energetic intermediates depending on the cellular needs. These intermediates may then feed the anaerobic pathway in the cytosol or aerobic pathway in mitochondria. One of the principal molecules used in energetic metabolism, for its capacity to be reduced into simpler carbon molecules and produce significant levels of ATP, is glucose. Interestingly, glucose anabolism, aside generating different intermediate molecules involved in ATP production, can also link itself with catabolic pathways that promote and amino acid synthesis. In this section, I will explain the pathways involved in ATP production associated with glucose metabolism, which may be divided into 3 principal pathways: glycolysis, lactic acid cycle and complete glucose oxidation through mitochondrial metabolism (TCA cycle and oxidative phosphorylation).

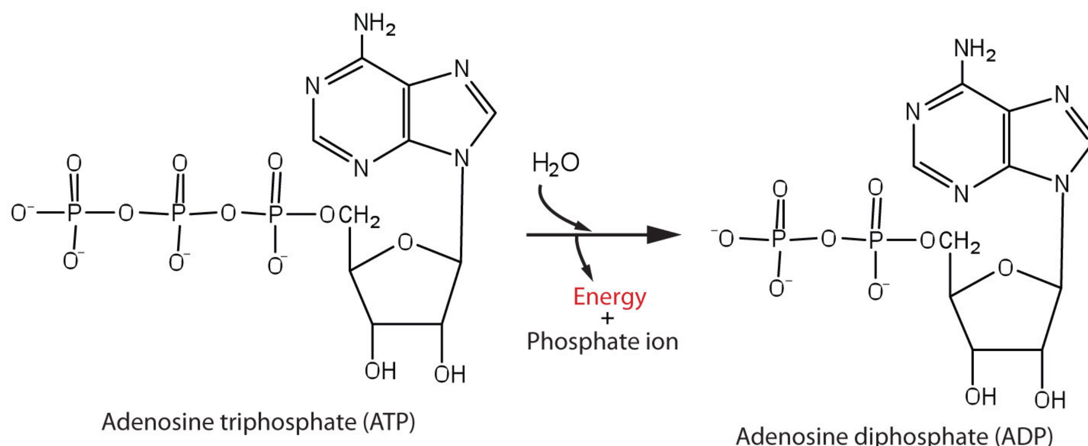


Figure 4.2. ATP molecular structure.
ATP contains three phosphate groups, one ribose sugar and one nitrogenous base. After the ATP breaks down, there is energy release, phosphate ion liberation and ADP formation. (Taken from https://saylordotorg.github.io/text_the-basics-of-general-organic-and-biological-chemistry/s23-01-atp-the-universal-energy-curre.html)

4.2.1 Glycolysis

The process by which glucose is broken down into simpler molecules (pyruvate) is known as glycolysis and it is a common process that occurs in living cells. Glycolysis comes from the Greek *Glukus* = sweet and *Lusis* = rupture. Glycolysis, or as it can also be referred to as Embden-Meyerhof-Parnas (EMP) pathway, was discovered and described by the collective work of Gustav Embden, Otto Meyerhof, and Jakub Karol Parnas (Akram, 2013). The whole pathway involved a set of 10 primary steps, which are driven by 14 different enzymes and take place in the cytoplasm under anaerobic conditions **(Figure 4.3)**. The overall glycolysis process is Glucose + 2 NAD^+ + 2 ADP + 2 Pi → 2 pyruvates + 2 NADH + 2 H^+ + 2 ATP and it can be divided into three phases. The first phase is the energy investment or priming phase in which the glucose is primarily phosphorylated, thus generating glucose 6-phosphate by the action of a hexokinase. During this step, the hexokinase consumes 1 ATP molecule to transfer the phosphate group to the glucose molecule. The second step involves the isomerization of glucose 6-phosphate into fructose 6-phosphate by the action of the glucose phosphate isomerase. During the third step, fructose 6-phosphate is phosphorylated and generates fructose 1, 6-biphosphate via the action of phosphofructokinase. During this step, the phosphofructokinase consumes 1 ATP molecule to transfer a phosphate group to fructose 6-phosphate. The second phase is the splitting phase in which, during the fourth step, fructose

1,6-diphosphate is divided into two three-carbon compounds, known as glyceraldehyde-3-phosphate and dihydroxyacetone phosphate by the action of the fructose-biphosphate aldolase. Dihydroxyacetone phosphate is rapidly converted into glyceraldehyde-3-phosphate by the action of the triosephosphate isomerase during the fifth step. Therefore, the product of this step is the generation of two glyceraldehyde 3-phosphate molecules from each glucose molecule that enters this pathway. The third phase is the pay-off or energy-gaining phase in which, during the sixth step, each glyceraldehyde 3-phosphate is oxidised into the high-energy molecule 1,3-bisphosphoglycerate by the action of the glyceraldehyde phosphate dehydrogenase. During this step, hydrogen is used to reduce the carrier NAD+ into NADH, which is another important energetic intermediate molecule that will generate 3 ATP molecules in aerobic conditions. However, how exactly NADH leads to ATP production will be explained further down. The seventh step involves the phosphate group transfer from the 1,3-bisphosphoglycerate to ADP by the action of the phosphoglycerate kinase. It is during this step that the ATP and the 3-phosphoglycerates synthesis occurs. Indeed, the end result is the production of two ATP and one 3-phosphoglycerate due to the initial breakdown of glucose into two glyceraldehyde 3-phosphate molecules. The eighth step involves the isomerization of 3-phosphoglycerate into 2-phosphoglycerate by the action of phosphoglycerate mutase. Then, the ninth step promotes the conversion of 2-phosphoglycerate into phosphoenolpyruvate by the action of the enolase. During the tenth step, the pyruvate kinase transfers the phosphate group from the phosphoenolpyruvate to ADP, thus generating a new ATP molecule and a pyruvate molecule. At this point, glycolysis is complete, concluding in the generation of 4 ATP, 2 NADH and 2 pyruvate molecules. It is important to note that during the initial glycolysis steps, 2 ATP molecules are used, resulting in the net gain 2 ATP molecules. The two NADH and pyruvate molecules can follow other metabolic pathways to produce additional ATP, which will be explained later in the TCA cycle and the electron transport chain sections.

The glycolytic pathway produces different intermediate molecules that support other metabolic pathways rather than going through glycolysis for ATP production. For instance, glucose 6-phosphate, in cases when the ATP production is higher than what the cell requires, can be transformed into glucose-storing molecules such as glycogen. Glycogen is a multi-branched glucose polysaccharide that can be quickly transformed back into glucose 6-phosphate to replenish cellular energetic demands under glycolysis. Additionally, glucose 6-phosphate can be used to generate NADPH, precursors for nucleotide synthesis (5-carbon sugars) and aromatic

amino acid synthesis (4-carbon sugars) in the pentose phosphate pathway. Dihydroxyacetone phosphate can be transformed into glycerol-3-phosphate, which can be further used for the synthesis of triglycerides and phospholipids. Importantly, these molecules can be broken down into simpler ones, such as fatty acids and glycerol, that can replenish the glycolytic pathway after conversion to dihydroxyacetone phosphate. Another example of molecules that can feed the glycolytic pathway is fructose, which enters the glycolytic pathway at the second step.

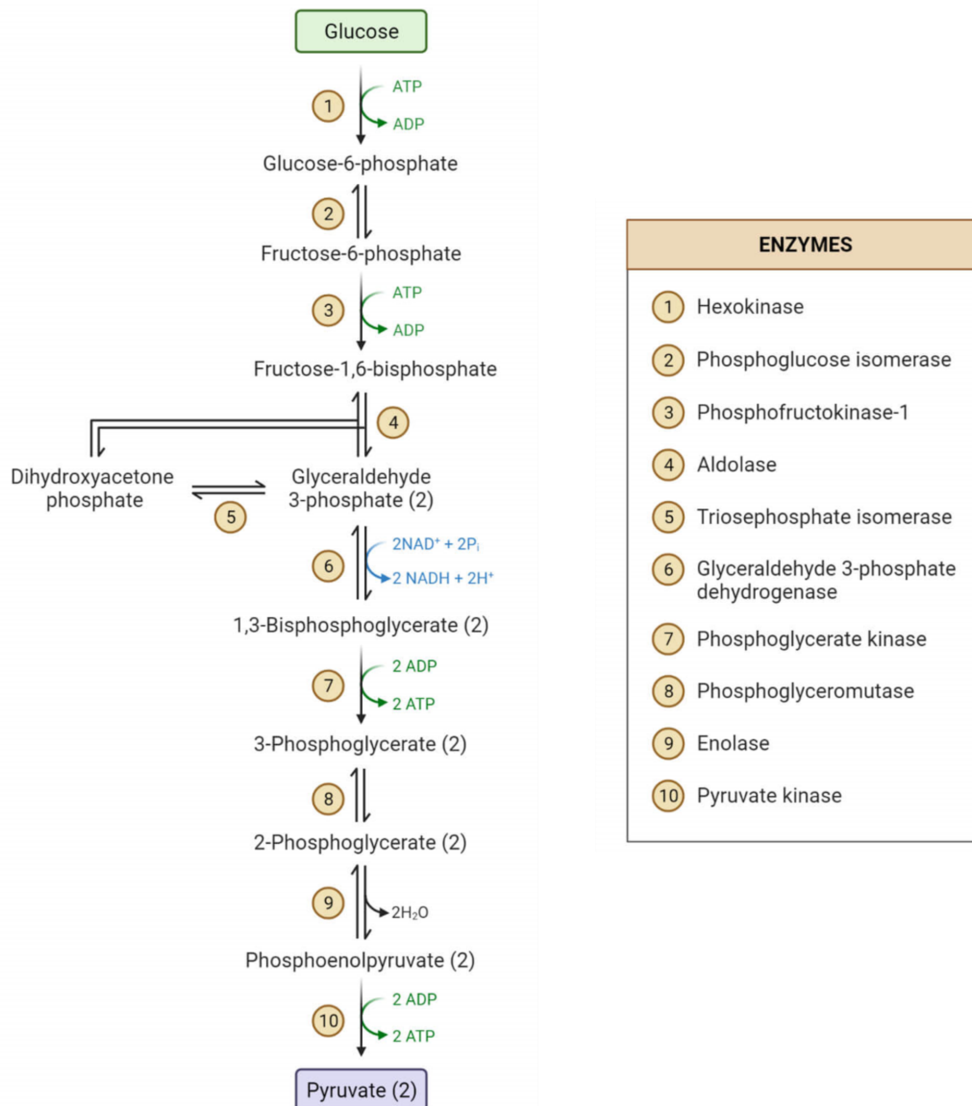


Figure 4.3. Glycolysis pathway.
Glycolysis is a 10-step pathway that reduces one molecule of glucose into 4 ATP, 2 NADH and 2 pyruvates (Taken from https://thebiologynotes.com/glycolysis-10-steps/)

4.2.2 Anaerobic respiration

The fate of pyruvate produced during glycolysis depends in general on oxygen levels. When the oxygen supply is insufficient (anaerobic conditions), pyruvate enters the anaerobic homolactic fermentation pathway in which the pyruvate is reduced into lactate by the action of lactate dehydrogenase and results in the generation of 2 ATP molecules **(Figure 4.4)** (Straub, 1940)**.** During this process, NADH is reduced into NAD^+, which can further be reused by glyceraldehyde 3-phosphate dehydrogenase and allow the glycolytic cycle to proceed in the absence of oxygen. In mammals, lactate is secreted and accumulated in the liver where it is further transformed into glucose through the Cori cycle in a process known as gluconeogenesis **(Figure 4.5)** (Ginsberg, 2010). Indeed, depending on the cellular type, anaerobic respiration has different end products, such as lactate in muscular cells or alcohol and CO_2 in yeast. The common process of pyruvate degradation in absence of oxygen is known as fermentation.

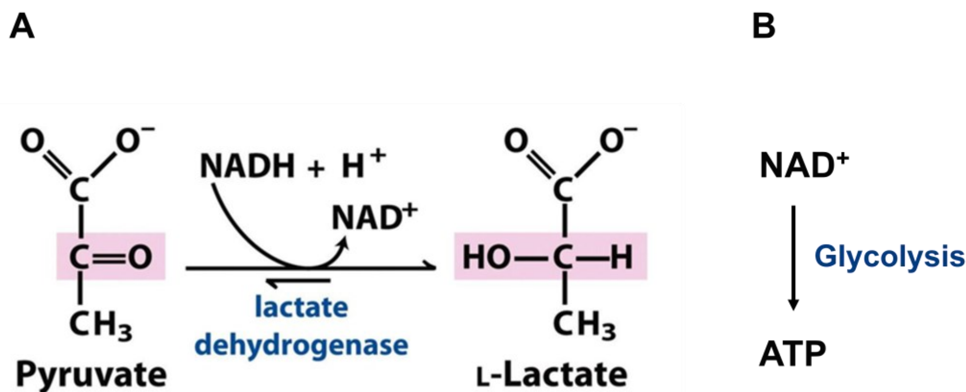


Figure 4.4. Anaerobic homolactic fermentation
(A) The pyruvate under anaerobic conditions is reduced into Lactate through the action of lactate dehydrogenase. One pyruvate molecule produces one molecule of ATP, lactate, and NAD^+. (B) NAD^+ replenish the glycolytic cycle and further produce ATP as mentioned in the section **4.2.1.** (Adapted from Leininger principle of biochemistry fifth edition)

4.2.3 TCA cycle

During aerobic respiration, pyruvate is the major substrate for oxidative metabolism. Pyruvate, produced from different cellular sources, is transported into the mitochondria through the pyruvate carrier, where it is then converted into acetyl-CoA by the action of pyruvate dehydrogenase (PDH). Further down the path, acetyl-CoA enters the TCA cycle, where it is reduced into different high-energy reducing equivalent molecules. The Tricarboxylic Acid Cycle

(known also as Krebs cycle or citric acid cycle) was described by Hans Adolf Krebs (1900 to 1981), who was further awarded with a shared Nobel Prize in physiology and Medicine in 1953 along with Fritz Albert Lipmann, the co-discoverer of coenzyme A. The TCA cycle is a series of eight enzymatic reactions within the mitochondrial matrix, that reduces different organic molecules into the energetic intermediates, such as NADH and FADH, that further produce ATP in the electronic transport chain **(Figure 4.6)**. Depending on cellular needs, the TCA cycle can act as an amphibolic pathway that integrates catabolism of organic molecules (carbohydrates, lipids and proteins), which consumes and regenerates citrate during the cycle or it integrates the anabolism that generates precursors for amino acid synthesis and lipid storage.

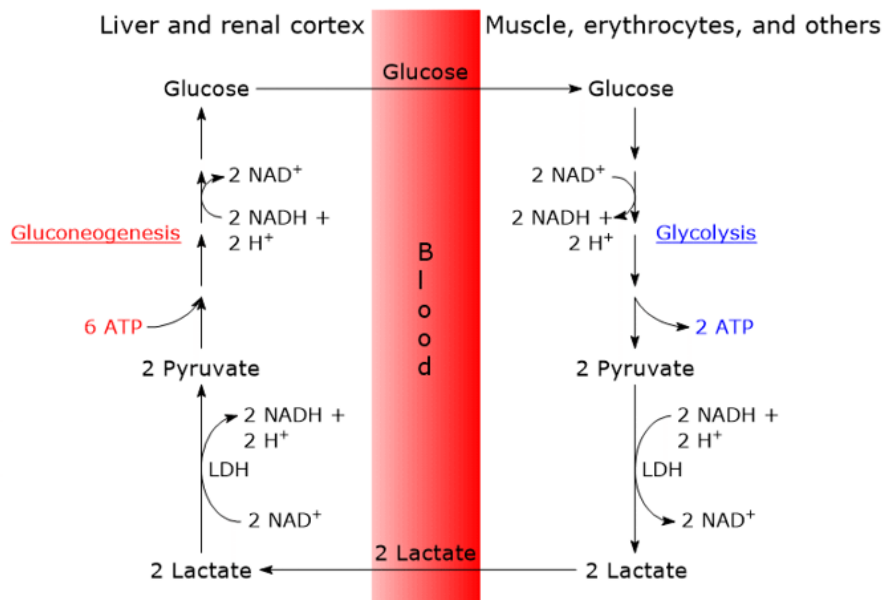


Figure 4.5. Cory cycle.
In mammals, Lactate is transported to the liver and renal cortex where is transformed back into glucose through a process known as gluconeogenesis. The glucose further may be initiated by the glycolytic pathway and in anaerobic conditions produce lactate. (Taken from https://www.tuscany-diet.net/2016/12/18/cori-cycle/)

The TCA cycle is a closed loop that begins with the combination of two-carbon acetyl-CoA molecules with a four-carbon oxaloacetate (OAA) molecule to generate a six-carbon molecule known as citrate by the action of citrate synthase. During the second step, the aconitase enzyme mediates citrate isomerization into isocitrate with an intermediate product known as Cis-aconitate. During the third step, isocitrate is converted into the five-carbon α-ketoglutarate (α-KG), which involves a double oxidative decarboxylation of isocitrate by the action of isocitrate dehydrogenase.

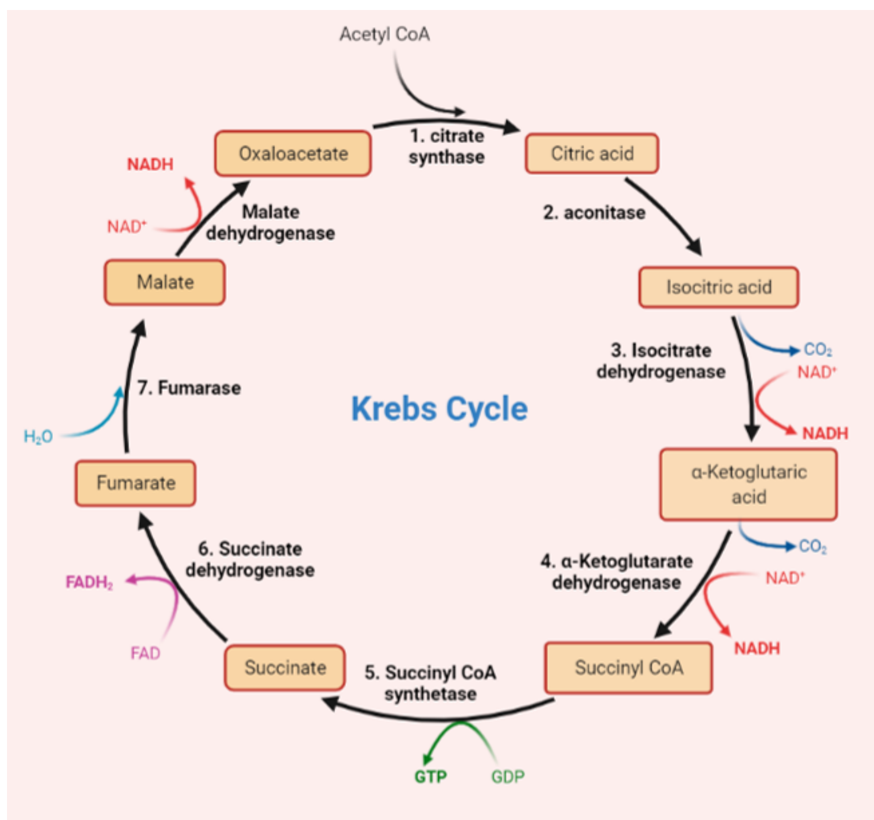


Figure 4.6. TCA cycle.
TCA cycle is a 6-steps pathway that reduces one molecule of Acetyl-CoA into 3 NADH, 1 $FADH^2$ and1 GTP (Taken from https://microbeonline.com/krebs-cycle-steps-and-products/)

During the fourth step, α-ketoglutarate is decarboxylated by of α-ketoglutarate dehydrogenase, forming two CO_2 molecules, two NADH and a four-carbon compound, termed succinyl-CoA. During the fifth step, succinyl-CoA is cleaved into succinate by the action of succinyl-CoA synthase. Here, one guanosine diphosphate (GDP) gets phosphorylated, hence generating guanosine triphosphate (GTP), which further transfers the phosphate group to ADP, leading to ATP generation. During the sixth step, succinate is dehydrogenated by the succinate dehydrogenase complex (SDH), generating fumarate and $FADH_2$. During the seventh step, fumarate is reversibly hydrated to form L-malate by the fumarate hydratase. During the last step, malate is dehydrogenated by L-malate dehydrogenase, generating oxaloacetate and NADH. It is important to note that oxaloacetate replenishes the TCA cycle and starts over the same loop. At this point, the TCA cycle is complete and ends in producing 3 NADH, 1 $FADH_2$ and 1 GTP. Noteworthy, 1 NADH is formed from pyruvate in the oxidative decarboxylation of pyruvate to Acetyl CoA.

4.2.4 Electron transport chain

The central node of energy transfer during aerobic respiration takes place in the mitochondrial electron transport chain (ETC), which employs a panoply of electron transfer reactions, establishing a proton gradient that couples oxidative phosphorylation (OXPHOS) and drives ATP generation through ATP synthase. Noteworthy, the ETC generates ATP and H_2O from different high-energy reducing molecules derived from the catabolic pathways, which include glycolysis, TCA cycle, fatty acid metabolism and amino acid metabolism. Remarkably, the way as to how the ETC produces ATP from different substrates was addressed by Peter Mitchell by linking oxidative phosphorylation with mitochondrial function. In 1961, Peter Mitchell proposed the chemiosmotic theory in which electron transfer occurs from the oxidation of different substrates, driving ATP synthesis in the mitochondrial ETC (Mitchell, 1961).

The ETC is embedded along the several-fold larger inner mitochondrial membrane (cristae) in a complex structure known as respirasome (Gu *et al.*, 2016), which presents a close location to the mitochondrial matrix that provides different elements, such as NADH and FADH2, which supply the electron transfer in the different ETC complexes. The electron transport chain is composed of mobile free electron transfer carriers including ubiquinone, heme and cytochrome c and 5 transmembrane multi-subunit protein complexes: Complex I (NADH: ubiquinone oxidoreductase), Complex II (succinate dehydrogenase), Complex III (coenzyme Q: cytochrome c reductase), Complex IV (cytochrome c oxidase) and Complex V (ATP synthase). The complexes I, III and IV collectively create the proton motive force across the inner mitochondrial membrane, which promotes ATP synthesis through complex V. The respiratory chain complexes create the oxidative phosphorylation system (OXPHOS) **(Figure 4.7)** (Iwata *et al.*, 1998; Sazanov & Hinchliffe, 2006).

Complex I, also named NADH-ubiquinone oxidoreductase, is the first and biggest protein complex in the ETC with the key role of transferring electrons from the mitochondrial matrix to the lipid-soluble carrier ubiquinone (CoQ), with NADH acting as a donor. Complex I is composed of two principal domains: the matrix arm protruding into the matrix and the membrane arm embedded in the inner membrane in which around 45 proteins are present as components of the core subunits. The matrix arm also contains important co-factors, such as flavin mononucleotide (FMN), Fe-S clusters and the final electron-accepting iron-sulphur cluster that deliver electrons to CoQ (Carroll *et al.*, 2006; Efremov & Sazanov, 2011; Sazanov & Hinchliffe, 2006).

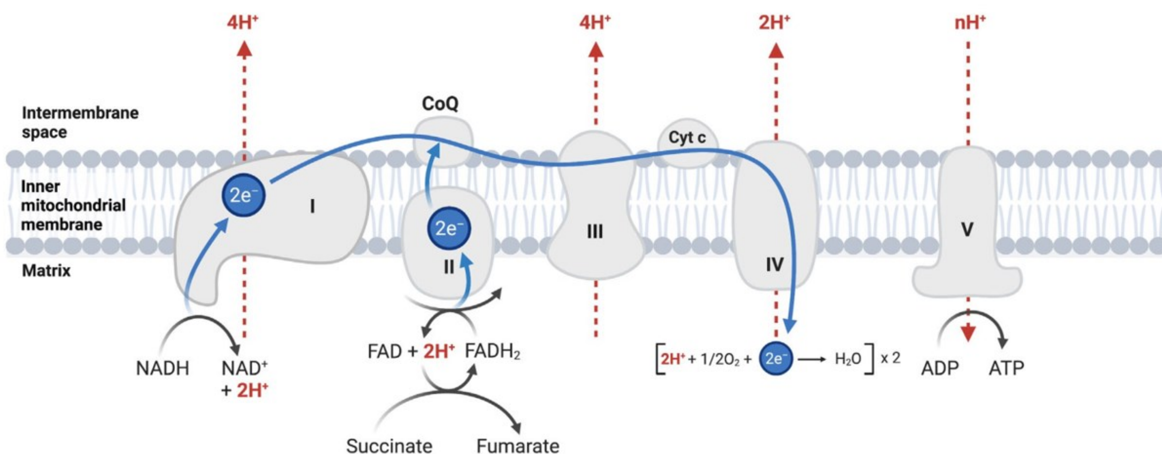


Figure 4.7. Electron transport chain.
The electron transport chain integrates oxidative phosphorylation to produce ATP. There is an electron transfer from NADH and $FADH_2$ that come through the complex I, II, III and IV. The electron transfer is coupled to a proton pump across the inner mitochondrial membrane generating an electrochemical proton gradient that drives ATP production at complex V (Yin & O'Neill, 2021).

In the catalytic site of complex I, the abundantly generated NADH in the mitochondrial matrix donates a pair of electrons that bind the FMN, generating $FMNH_2$. Subsequently, these electrons are transferred into iron-sulphur cluster chains arranged from low to high electronic potential. Next, the CoQ acts as the last electron acceptor in complex I. During the CoQ reduction to ubiquinol (QH_2), the proton pump pumps four protons from the matrix into the intermembrane space (Tan *et al.*, 2015; Wikström & Hummer, 2012). The functioning of Complex I leads to a premature electron leakage to oxygen that generates mitochondrial reactive oxygen species (ROS), which are the primary source of free radicals such as superoxide (Scialò *et al.*, 2017).

Complex II, also named succinate dehydrogenase, is a dual component mechanism that links the TCA cycle with the ETC (Rutter *et al.*, 2010). Complex II is composed of four subunits (Cecchini, 2003; Sun *et al.*, 2005). Two subunits include the membrane-anchor proteins CybL and CybS, which contain the CoQ binding site, and the two other subunits are located on the matrix side of the inner membrane and contain the binding site of the succinate substrate, three Fe-S clusters and a flavoprotein covalently bound to a FAD cofactor. As it was mentioned before, the succinate dehydrogenase catalyzes the oxidation of succinate to fumarate in the TCA cycle. During this process, complex II donates two electrons from succinate to FAD generating $FADH_2$ to ensure electron flow in the respiratory chain. Next, $FADH_2$ transfers the

electrons to Fe-S clusters in a similar fashion as complex I does with CoQ, where Fe-S acts as a final electron acceptor of complex II. Unlike complex I, the electron transport in complex II does not promote proton translocation into the intramembrane space (Bezawork-Geleta *et al.*, 2017; Iverson, 2013). Complex III, also named cytochrome b complex or CoQ-cytochrome c reductase, transfers the electrons carried by QH_2 to cytochrome c. Complex III is a dimeric protein complex with 11 subunits per monomer with cytochrome b and iron-sulphur proteins as its active subunits (Schägger *et al.*, 1986; Yang & Trumpower, 1986). The electron transfer process at complex III involves QH_2 oxidation into ubisemiquinone (QH-), where two electrons are transferred to the Fe-S clusters. This process pumps two more protons per electron from the matrix into the intermembrane space (Trumpower, 2002). Further, the Fe-S clusters transfer those electrons to the mobile electron carrier cytochrome c. Besides complex I, Complex III is another major ROS source in the ETC. Complex IV, also named cytochrome c oxidase, is the last enzyme in the respiratory ETC that transfers electrons from cytochrome c to the terminal electron acceptor O_2. Complex IV is composed of 13 different subunits containing different metal centers, including heme, which transfers electrons to O_2 generating H_2O (Kadenbach & Hüttemann, 2015; Tsukihara *et al.*, 1996). Four electrons from cytochrome c are simultaneously transferred to bind dioxygen. This process releases in total eight protons of which four are used to form two water molecules and the other four are pumped from the matrix into the intermembrane space (Wikstrom, 1977). Complex V, also named F_1F_0-ATP synthase, is the last step in the ETC coupled ATP synthesis during OXPHOS by using the electrochemical gradient, generated by proton pumping across the inner mitochondrial membrane (Yin & O'Neill, 2021). Complex V is composed of two functional domains: F_0 and F_1. The F_0 domain is located in the inner mitochondrial membrane and contains a characteristic c-ring subunit, which is oligomycin sensitive. The F_1 domain is located in the mitochondrial matrix and contains 9 soluble subunits that create the central stalk connecting the F1 head with the F_0 subunit c-ring (Dickson *et al.*, 2006; Jonckheere *et al.*, 2012). The protons pumped from the matrix into the intermembrane space through complexes I, III and IV return to the matrix by crossing F_0, which transfers the stored energy by the proton electrochemical gradient to F_1. The proton exchange induces a conformational change in the F_1F_0 ATP synthase, which favours the ADP phosphorylation into ATP (Jonckheere *et al.*, 2012).

The electrochemical proton gradient generated during the OXPHOX is a set of exergonic reactions that are driven by the difference in the Gibbs free energy. These reactions promote the

conversion of a higher-energy electron donor and acceptor into lower-energy products while electrons are transferred from a lower to higher redox potential. This proton gradient is what principally is responsible for the mitochondrial membrane potential ($\Delta\Psi M$) generation (Zorova *et al.*, 2018). In general, even if cells use the TCA cycle coupled to OXPHOS to produce high ATP yields, glycolysis can still serve as an emergency backup for ATP production due to the faster ATP generation under energetic demands (100 times faster). Importantly, some cells, such as erythrocytes, do not have mitochondria, thus, ATP production relies solely on glycolysis.

4.3 Mitochondria life cycle

Mitochondria are highly dynamic organelles that undergo a series of coordinated and frequent fusion, fission and mitophagy (programmed mitochondrial clearing) cycles over time that maintain their distribution, size, shape and assure mitochondria quality control in a process denominated mitochondrial dynamics. During cell homeostasis, it is imperative that mitochondrial metabolism rapidly adapts to cellular needs to support different cellular processes including energy conduction, cell cycle, immunity, cell death and, most importantly, keep proper mitochondrial quality to prevent dysfunctional mitochondria from causing harmful effects such as oxidative stress, impaired cellular functions or, in superior organisms, to promote different metabolic diseases such as diabetes, Alzheimer's, Parkinson and aging (Devi *et al.*, 2006; Manczak *et al.*, 2006; Simmons *et al.*, 2005; Tanaka *et al.*, 1996). Mitochondria cannot be synthesized de *novo*. Instead, they must arise from pre-existing mitochondria in a process denominated as mitochondrial biogenesis. During this process, new emerging mitochondria increase in number and size, along with lipid synthesis and new ETC complex assemblies. The process by which one mitochondrion is divided into two distinct mitochondria is denominated mitochondrial fission. Also, two independent mitochondria can fuse together to become a single mitochondrion in a process denominated mitochondrial fusion. In the case of a defective mitochondrion that cannot be repaired, the mitochondria enter into a coordinated death program denominated mitophagy. Noteworthy, the mitochondria, rather than exist as a single unit, interact in a complex network that is continuously remodelled through fusion and fission processes that result in either a hyper-fused network with elongated and highly connected mitochondria or a fragmented network characterized by a large number of small round-shape mitochondria **(Figure 4.8)** (Shaw & Nunnari, 2002; Tilokani *et al.*, 2018; Westermann, 2002).

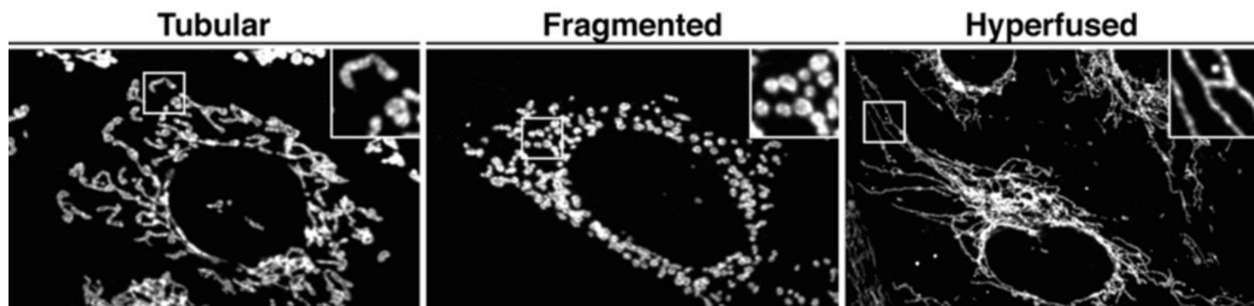


Figure 4.8. Mitochondrial network.
Mitochondrial network shape changes according to cellular needs by a process denominated mitochondrial dynamics. (Tilokani *et al.*, 2018)

4.3.1 Mitochondrial biogenesis

During the mitochondrial evolutionary process, the mitochondrial genome lost and transferred the majority of its genes to the nuclear genome, thereby conserving only 13 protein-encoded genes of the respiratory chain, 22 tRNAs and 2 rRNAs (Kelly & Scarpulla, 2004). When generating new mitochondria, there is a highly regulated program that coordinates the mitochondrial and nuclear genomes by specific signalling modules, transcription factors and gene expression regulators (Austin & St-Pierre, 2012; Dominy & Puigserver, 2013b; Mehta *et al.*, 2017). In this way, mitochondrial biogenesis involves the import of nucleus-encoded proteins and membrane lipids from the cytosol into the mitochondria, along with the amplification of the mitochondrial genome and translation of mitochondrion-encoded proteins followed by their fragmentation via fission.

Several internal and external stimuli trigger mitochondrial biogenesis such as temperature, ATP deprivation, nutrient availability, and growth factors (Scarpulla, 2008). In general, among the specific molecules involved in the induction of mitochondrial biogenesis, the peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α, is denominated as the master regulator of mitochondrial biogenesis by activating mtDNA transcription (Dominy & Puigserver, 2013a; Puigserver *et al.*, 1998; Scarpulla, 2008; Scarpulla, 2011; Wu *et al.*, 1999) and regulating several metabolic pathways such as cellular respiration, thermogenesis, and hepatic glucose metabolism (Diaz & Moraes, 2008; Rasbach & Schnellmann, 2007). PGC-1α expression presents a variety of regulatory mechanisms in accordance with cellular needs. Additionally, previous studies have described that signalling events that lead to mitochondrial biogenesis are coordinated by AMP-activated protein kinase (AMPK), which is the major regulator of energy

metabolism that potentiates the transcriptional activity of PGC-1α (Dominy & Puigserver, 2013a; Jäger *et al.*, 2007). Other signalling axes involved in mitochondrial biogenesis induction include, Calcium/calmodulin-dependent protein kinase type IV (CaMKIV), nitric oxide (NO), Sirtuin 1 (SIRT1), calcineurin, mitogen-activated protein kinase (p38 MAPK) and receptor-interacting protein 140 (RIP140) (Akimoto *et al.*, 2005; Chin *et al.*, 1998; Nisoli *et al.*, 2003; Rodgers *et al.*, 2005; White *et al.*, 2008; Wu *et al.*, 2000). PGC-1α initiates mitochondrial biogenesis upon its activation by either phosphorylation or deacetylation that then activates a series of transcription factors including the nuclear respiratory factor-1 and-2 (NRF-1, NRF-2), the estrogen-related receptor-α (ERR-α), uncoupling proteins (UCP2) and the effector of mtDNA transcription and replication TFAM (Cameron *et al.*, 2016) leading to the increase in transcription of key mitochondrial enzymes involved in OXPHOS and structural components of mitochondria. Noteworthy, TFAM also drives mtDNA replication, which favour mitochondrial fission events (Virbasius & Scarpulla, 1994). Those co-activators have been identified as crucial factors linking external stimuli with mitochondrial biogenesis (Lee *et al.*, 2004). Furthermore, the translation of different mitochondria-encoded genes from the nuclear and the mitochondrial genomes and the levels of mitochondrial proteins are regulated by the translational activator of cytochrome c oxidase 1 (TACO1), which binds the mitochondrial RNA (mRNA)(Yokokawa *et al.*, 2018). The mitochondria-encoded proteins synthesized within the cytosol are issued with a cleavable amino-terminal mitochondrial target signal that promotes its translocation into the mitochondria through the translocase of the outer membrane and translocase of the inner membrane complexes (TIM/TOM) **(Figure 4.9)** (Eliyahu *et al.*, 2010).

4.3.2 Mitochondrial dynamics

The mitochondrial network is constantly moving, changing shape and size by a coordinated process known as mitochondrial dynamics, which is required for a panoply of cellular functions including apoptosis, autophagy flux, energy balance and mitosis (Hyde *et al.*, 2010). Mitochondrial dynamics are characterized by active mitochondrial fusion and fission that facilitate inner and outer membrane fusions and allow the exchange of different molecules such as proteins, metabolites, and mitochondrial DNA, mediated by the action of several dynamin-like GTPases (Liesa *et al.*, 2009; Tilokani *et al.*, 2018).

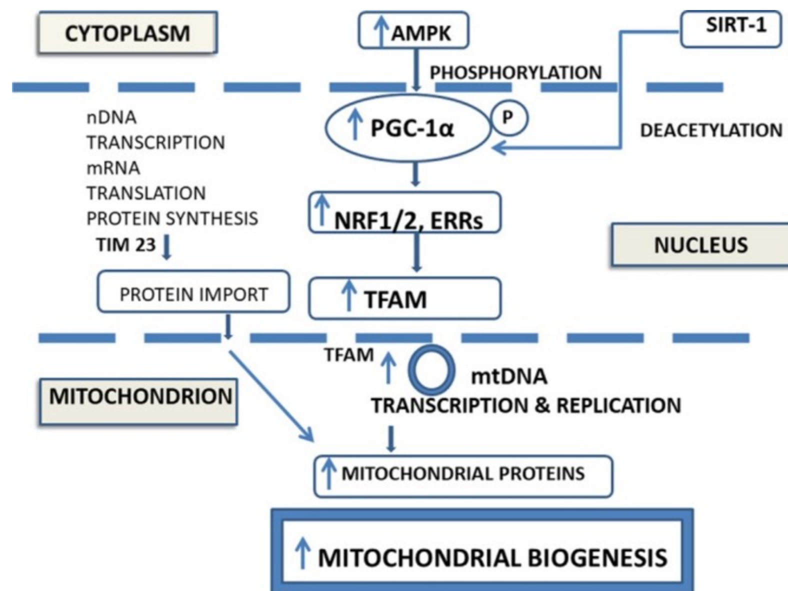


Figure 4.9. Mitochondrial biogenesis.
PGC-1α is activated by AMPK and/or AMPK, which leads to the activation of transcription factors activations that promote transcription and translation of mitochondrial encoded genes from the nuclear and the mitochondrial genome. Mitochondrial proteins are imported to new mitochondria.

Mitochondrial fusion mixes and unifies mitochondrial compartments. It is also characterized by the combination of the inner and outer mitochondrial membranes in a process where the outer and the inner membranes are fused in three coordinated steps: tethering of two adjacent mitochondria, docking of the membranes, which increases the contact area, and fusion of the membranes mediated by GTP hydrolysis **(Figure 4.10)** (Tilokani *et al.*, 2018). During the pro-fusion step, the optic atrophy gene 1 (OPA1) protein located in the inner membrane and the proteins of the family of mitofusins (MFN 1 and MFN 2) located in the outer membrane merge into the same protein complex (Liesa *et al.*, 2009). Then, the MFNs proteins mediate the outer membrane fusion, whereas OPA1 mediates the inner membrane fusion (Meeusen *et al.*, 2004). The fusion process may be a transient event (only outer membrane fusion) or complete (outer and inner membrane fusion) (Hyde *et al.*, 2010).

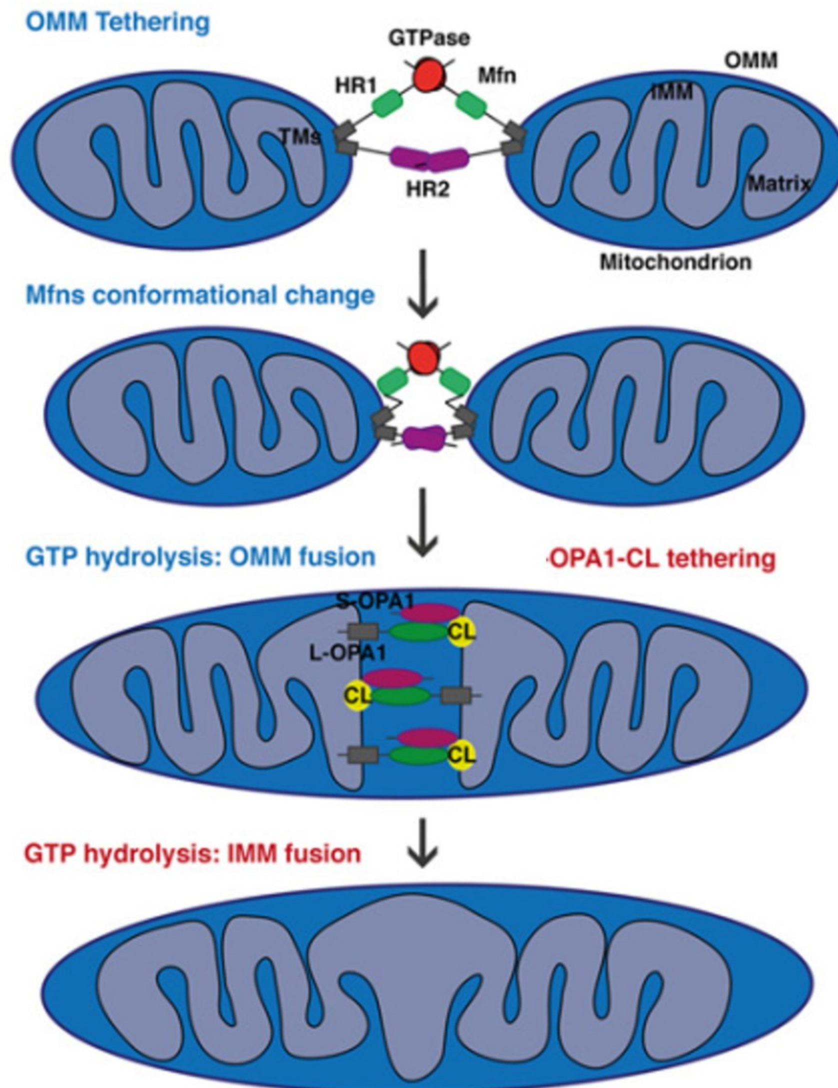


Figure 4.10. Mitochondria fusion.
On the outer membrane of two opposing mitochondria, the proteins HR1, HR2 and Mfns are recruited. Further, there is a conformational change in the complex HRs/MFNs that reduce the distances and leads to mitochondrial docking. There is mitochondria OMM fusion, which is mediated GTP-dependent for its oligomerization. The interaction between OPA1 and cardiolipin (CL) tethers the IMM (Tilokani *et al.*, 2018).

Importantly, it has been shown that the disruption of the MFNs complex is sufficient to prevent mitochondrial fusion (Zorzano, 2009). The mitochondrial fusion factor OPA1 is encoded by a single gene with eight possible transcript variants resulting from alternative splicing. These variants exert different functions such as promoting mitochondrial inner membrane fusion, shaping the mitochondrial cristae that is required during the assembly of the respiratory complex

and the quality control of the mitochondrial cristae morphology (Frezza *et al.*, 2006; Ishihara *et al.*, 2006). OPA1 knock-out mice induce spontaneous apoptotic cell death (Olichon *et al.*, 2003) and present a fragmented mitochondrial network, whereas OPA1 overexpression induces mitochondrial elongation (Waterham *et al.*, 2007). Interestingly, OPA1-dependent inner membrane fusion depends on MFN1 but not on MFN2, suggesting a possible communication between the inner and outer membrane where OPA1 and Mfn1 directly interact with each other (Mattie *et al.*, 2018). The MFNs 1 and 2 present a coiled-coil transmembrane region that mediates tethering between two adjacent mitochondria and are critical to stabilize interactions between mitochondria and the endoplasmic reticulum, which deliver a significant part of the lipids required for the fusion process (de Brito & Scorrano, 2008). MFN 1 and 2 knock-out mice die in gestation due to a defect in embryonic development (Chen *et al.*, 2003). Otherwise, overexpression of MFN2 in cultured cells promotes a small fragmented mitochondrial mass formation, reduced mitochondrial membrane potential, increased glucose oxidation and impaired mitochondrial function, which may lead to apoptotic cell death due to the cyt c release into the cytosol (Huang *et al.*, 2007; Zorzano *et al.*, 2010).

On the other hand, mitochondrial fission constricts mitochondrial compartments and promotes the separation of mitochondrial membranes **(Figure 4.11).** The most relevant identified proteins that mediate mitochondrial fission are the GTPase dynamin-related protein 1 (DRP1), fission 1 homolog protein (hFis1) and the mitochondrial fission factor (Mff) (Suen *et al.*, 2008). Recently, the proteins MiD49 and MiD51, which, in mammals, bind DRP1 also were related with mitochondrial fission (Palmer *et al.*, 2011). DRP1 is a conserved cytosolic protein that is dynamically recruited to mitochondria, where it oligomerizes into spirals at the mitochondria division sites and drives the mitochondrial membrane constriction (Kraus & Ryan, 2017; Liesa *et al.*, 2009). DRP1 function is highly regulated by phosphorylation, sumoylation, ubiquitination, and S-nitrosylation (Chang & Blackstone, 2010; Wakabayashi *et al.*, 2009). DRP1 presents four domains involved in membrane binding and its oligomerization (Fröhlich *et al.*, 2013). During mitochondrial division, DRP1 is recruited to the outer mitochondrial membrane along with ER tubules and actine, which are placed together along the mitochondria and wrap around them, forming a ring-like structure that leads to membrane constriction and further excision in a GTP-dependent manner (Smirnova *et al.*, 2001). Importantly, the protein inverted formin 2 (INF2) and Spire1C mediated actin polymerization during mitochondrial fission, playing a key role in DRP1 recruitment and oligomerization to regulate mitochondrial constriction and division (Korobova *et*

al., 2013; Manor *et al.*, 2015). DRP1 knock-outs promote mutants that inhibit mitochondrial fission, therefore resulting in the drastic elongation of mitochondrial networks into a tubular shape due to ongoing fusion and restricted fission (Ishihara *et al.*, 2009; Wakabayashi *et al.*, 2009). hFis1 and Mff are located in the outer mitochondrial membrane and their function has been proposed in the recruitment of DRP1 to the mitochondrial surface, which does not rely on GTPase activity. Of interest, DRP1, Fis1 and Mff also control peroxisomal fission (Schrader, 2006; Waterham *et al.*, 2007).

4.3.1 Mitophagy

During cellular stress, mitochondrial function may be impaired, which could result in mitochondrial damage and disruption of cellular homeostasis. As a quality control and repairing mechanism, the cell promotes an upregulation of specific mitochondrial chaperones, which favour mitochondrial fission and fusion to restore the impaired mitochondrial function and preserve energy metabolism. If mitochondrial restoration is impossible, the unrepairable mitochondria are removed through a selective autophagy program specific to mitochondria, denominated as mitophagy. Mitophagy is a conserved program that promotes mitochondria engulfment into vesicles coated with the autophagosome marker MAP1 light chain 3 (LC3), which induces lysosomal degradation. Mitophagy is induced through multiple signalling cascades involving ubiquitin-dependent or -independent pathways (Ashrafi & Schwarz, 2013; Khaminets *et al.*, 2016). Also, mitophagy degrades mitochondria through receptor-mediated mechanisms. Importantly, mitophagy impairment promotes dysfunctional mitochondrial function and causes progressive accumulation of defective organelles, leading to cell or tissue damage and is associated with a broad spectrum of pathologies (Palikaras *et al.*, 2017). The most characterized mitophagy pathway is mediated by the phosphatase and tensin homolog-induced kinase 1 (PTEN1), induced putative kinase 1 (PINK1) and E3 ligase Parkin (Parkin), which drive ubiquitin-dependent mitophagy (Pickles *et al.*, 2018).

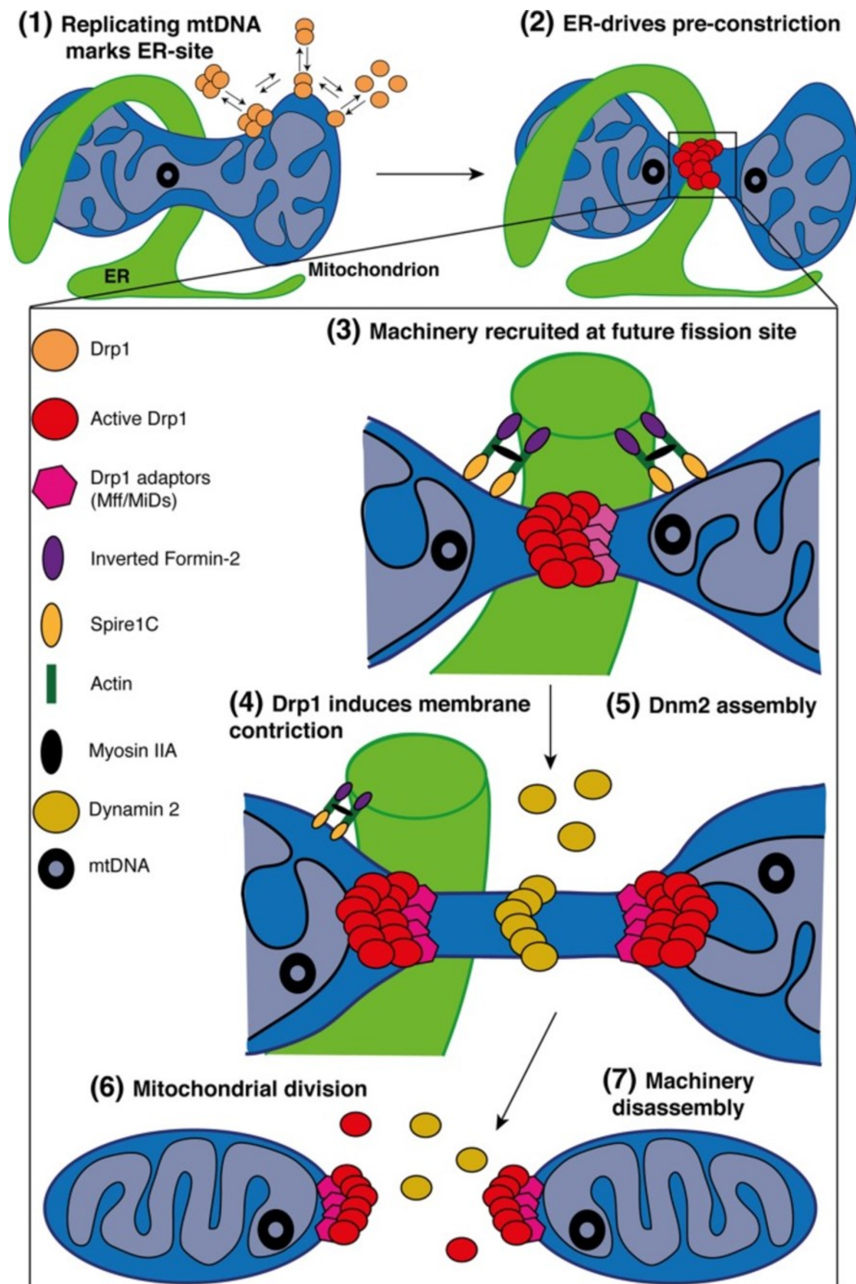


Figure 4.11. Mitochondrial fission.
(1) mtDNA replication set the marks where the mitochondrial will divide, further by ER recruitment. Drp1 oligomers start to aggregate along with ER inducing a mitochondrial membrane pre-constriction. (2) Drp1 is activated and accumulate at ER sites where the pre-constriction occurred. (3, 4,) The inset region showed the different molecules involved in mitochondrial division and its regulation. There is a ring-like structure formation that enhances the pre-existing mitochondrial constriction site in where Drp1 aggregates in the edges of the separated IMM. (5, 6, 7) On the mitochondrial constriction site dynamine 2 is recruited and the final membrane scission occurs, leading to two daughter mitochondria. The fission machinery is disassembly (Tilokani *et al.*, 2018).

Under normal conditions with functional mitochondria, PINK1 is constantly transported into the inner mitochondrial membrane, where it is further cleaved mainly by the mitochondrial processing peptidase (MPP). The remaining transmembrane fragment of PINK1 is then cleaved by the IMS presenilin-associated rhomboid-like (PARL) protease and degraded by ubiquitin proteasome system (UPS) in the cytoplasm (Greene *et al.*, 2012; Meissner *et al.*, 2015). Importantly, cleaved PINK1 fragments bind directly to the Parkin, an E3 ubiquitin ligase, thereby blocking its translocation to the OMM and inhibiting mitophagy induction (Fedorowicz *et al.*, 2014). In mitochondria with impaired membrane potential (ΔΨm), PINK1 is stabilized in the outer mitochondrial membrane (OMM), thus preventing MPP- and PARL-dependent cleavage (Lazarou *et al.*, 2012). Furthermore, PINK1 begins to homodimerize, thus inducing its autophosphorylation and leading Parkin translocation onto the mitochondrial surface. PINK1-dependent phosphorylation alters Parkin conformation, promoting their association on the mitochondrial surface and triggering its E3 ligase activity. Additionally, several OMM proteins undergo a poly-ubiquitination in a parkin dependent-manner that leads to their recognition by mitophagy receptors such as nucleoporin 62/sequestosome 1 (p62/SQSTM1) and BRCA 1 (NBR1) (Chan *et al.*, 2011). The ubiquitinated proteins present a ubiquitin-binding domain that binds light chain 3 (LC3), which mediates the interaction with the phagophore. LC3 is a ubiquitin-like protein covalently attached to phosphatidylethanolamine during autophagosome biogenesis, which allows it to integrate into the growing membrane during cargo recruitment and autophagosome formation (Nakatogawa *et al.*, 2007). In mammalian cells, mitophagy is preceded by mitochondrial fission by the indirect action of PINK1, which triggers dynamin-related protein 1 (DRP1) activity, promoting the fission of large mitochondria into smaller pieces capable of being encapsulated in the autophagosome. Indeed, mitochondria fission acts as a quality control segregation in which dysfunctional mitochondrial regions are released as a material for selective removal by mitophagy **(Figure 4.12)** (Westermann, 2010; Youle & Narendra, 2011).

Mitophagy may also be activated and regulated by several ubiquitin E3 ligases, such as SIAH1, MUL1, Gp78, SMURF1 and ARIH1 (Orvedahl *et al.*, 2011; Villa *et al.*, 2017). Once the ubiquitin E3 ligases are localized on the mitochondrial membrane, they trigger a poli-ubiquitination chain of reactions, which promotes the recruitment of autophagy adaptors such as optineurin (OPTN), p62 and nuclear dot protein 52 (NDP52) as well as autophagic components such as unc-51-like autophagy activating kinase 1 (ULK1), double FYVE domain-containing protein 1 (DFCP1) and WD repeat domain phosphoinositide interacting 1 (WIPI1) protein. They mediate the

phagophore biogenesis and autophagosomal membrane expansion mediated by LC3 (Lazarou *et al.*, 2015). Mitophagy is promoted by different "eat me" signals on the mitochondrial surface designated for clearance, which involves a coordinated regulation of ubiquitination (Palikaras *et al.*, 2018). (Palikaras et al., 2018).

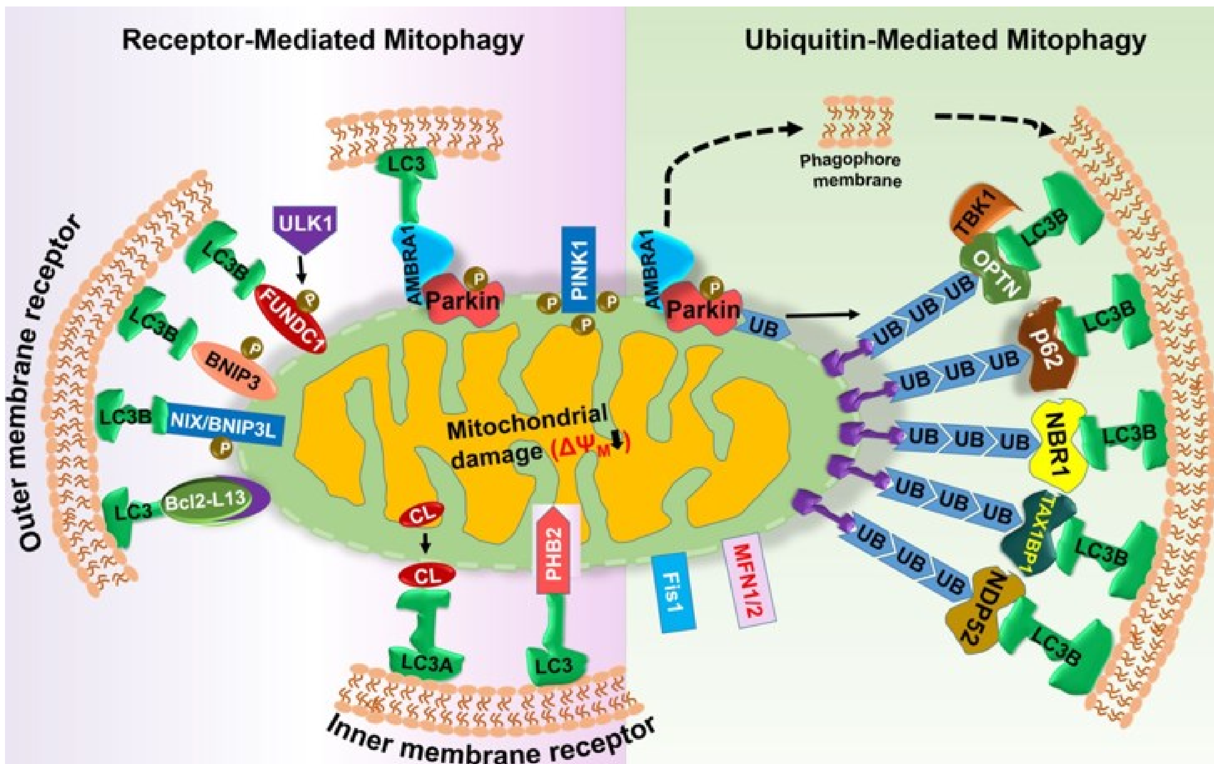


Figure 4.12. Mitophagy.
Induced mitophagy during cellular can be activated for different mechanism. Receptor-mediated mitophagy induction, is mediated for several OMM proteins including Bcl2-L13, NIX, BNIP3, FUNDC1, and AMBRA1 acting as mitophagic receptors and interacts directly with LC3 promoting defective mitochondria degradation. Ubiquitin-mediated mitophagy induction, is mediated for OMM proteins that undergo a poly-ubiquitination process, which promotes recruitment of proteins such us including NDP52, TAX1BP1, NBR1, p62/SQSTM, and OPTN. These proteins acts as mitophagic adaptors that interact with LC3 promoting defective mitochondria degradation (Praharaj *et al.*, 2019). Pink-Parkin dependent mitophagy also involves a poly-ubiquitination leading to mitochondria degradation.

Mitophagy is also promoted in a ubiquitin-independent pathway in which mitochondrial proteins act as mitophagy receptors, targeting dysfunctional mitochondria directly to autophagosome degradation. Mitophagy receptors including BCL-2-like protein 13 (BCL2L13), anti-apoptotic FK506-binding protein 8 (FKBP8), BCL2 interacting protein 3 (BNIP3), FUN14 domain-

containing protein 1 (FUNDC1) and NIP3-like protein X (NIX), interact directly with the LC3 proteins, and GABA(A) receptor-associated proteins (GABARAP) through their LIR motifs. That interaction induces autophagosomal membrane expansion and mediates Parkin-independent mitophagy **(Figure 4.12)** (Diwan *et al.*, 2007; Liu *et al.*, 2012; Murakawa *et al.*, 2015; Sandoval *et al.*, 2008; Wei *et al.*, 2017).

4.4 Metabolic reprogramming in macrophage polarization

The essential role of metabolism in controlling a panoply of pathways to support and dictate cellular fate has only been recently explored. In this regard, the metabolism and its link with immune function gave rise to a new field known as immunometabolism in which mitochondria have emerged as its central hub. Mitochondria are fundamental to coordinating a plethora of essential cellular functions to support life as we know it today. Over the last decades, the mitochondrial role is going beyond simple ATP production. Otherwise, mitochondria are playing a principal role in metabolism, homeostasis and in a wide spectrum of cellular functions. For instance, mitochondria play a central role in Ca^{2+} homeostasis, which regulates cell death. Furthermore, mitochondria, under specific stimuli, induce intrinsic apoptosis by releasing cytochrome c into the cytosol (Detmer & Chan, 2007; Tait & Green, 2010; Wang & Youle, 2009). Also, the mitochondrial shape, which is a product of balance between fission and fusion undergoes a profound metabolic change that controls the cell cycle (Horbay & Bilyy, 2016; Karbowski & Youle, 2003; Mandal *et al.*, 2005; Mitra *et al.*, 2009). The mitochondrion is the primary place where protein cofactor biosynthesis, such as Iron–sulphur (Fe/S) and heme-cluster families, which are critical to support the electron transfer reactions, occurs (Lill *et al.*, 2012). Opposite to the mitochondrion's anaplerotic role to replenish metabolic intermediates in the TCA cycle, they also generate biosynthetic precursors for macromolecules such as nucleotides, fatty acids, cholesterol, amino acids and glucose, in a process known as cataplerosis (Ahn & Metallo, 2015; Owen *et al.*, 2002; Spinelli & Haigis, 2018). Likewise, mitochondria have a primordial function in immunity against pathogens and dictate immunological fate by inducing a profound reprograming in cell metabolism (Ganeshan & Chawla, 2014; Mehta *et al.*, 2017; Weinberg *et al.*, 2015).

In this section, due to the importance of mitochondrial biology in cellular function, we will disclose how mitochondrial metabolic reprogramming has a heavy impact on cellular fate during macrophage activation. Macrophages are versatile cells that integrate multiple signalling axes and adopt different functional programs depending on the stimulatory context. Hence, during macrophage activation, there is a dynamic process in which the macrophage undergoes metabolic changes to support its respective phenotype and fulfill its function. (Mantovani *et al.*, 2013; Murray, 2017; Pollard, 2009). As mentioned in chapter 1, M1 macrophages arise in an inflammatory context and are generally associated with immunity against intracellular pathogens and bacteria by employing inflammatory mediators, such as nitric oxide (NO) and reactive oxygen species (ROS). Otherwise, M2 macrophages arise in an anti-inflammatory context and are generally associated with immunity against helminth and tissue repair (Muraille *et al.*, 2014). The M1/M2 classification is a simplified nomenclature based on *in vitro* studies, which only represents two extremes of a broad spectrum of polarized macrophages. However, it is an excellent model to explain how macrophage mitochondria metabolism changes according to their respective subset.

The M1 macrophage's metabolic print displays an enhanced glycolytic flux and a concomitant decrease in mitochondrial oxygen consumption (OXPHOS) compared to unpolarized macrophages **(Figure 4.13)** (Haschemi *et al.*, 2012; Mills & O'Neill, 2014; Mills & O'Neill, 2016). The particular increase in aerobic glycolysis, even in the presence of oxygen, is a distinguishing characteristic of M1 macrophages and is known as the Warburg effect. The increased glycolytic flux and reduced oxidative respiration lead to a broken TCA cycle that is caused by two distinctive processes. The first break point occurs when the citrate is accumulated in the TCA cycle due to isocitrate dehydrogenase (IDH) expression. Citrate accumulation may trigger its export from the mitochondria to the cytoplasm, thus promoting fatty-acid synthesis and histone acetylation. Also, citrate accumulation leads to the generation of cis-aconitate, a citrate intermediate, that is further decarboxylated into itaconate by the action of the immune-responsive gene 1 protein (*Irg1* or *CAD*). *Irg1* is the most up-regulated transcript in M1 macrophages. Importantly, itaconate is a potent anti-inflammatory modulator and has been shown to inhibit the glyoxylate shunt pathway in pathogens such as *M. tuberculosis* and *Legionella,* hence decreasing their viability (Michelucci *et al.*, 2013; Naujoks *et al.*, 2016). Under LPS stimulation, *Irg1* and itaconate are highly produced in macrophages (Lampropoulou *et al.*, 2016). Increased itaconate levels lead to the second break point in the TCA cycle between the

succinate and the fumarate steps in which complex II (succinate dehydrogenase) is partially inhibited. The functional consequence of succinate accumulation is the stabilization and activation of the hypoxia-inducible factor 1-alpha (HIF1α) along with gene expressions of HIF1α stimulated genes such as the pro-inflammatory cytokine IL-1β, solute carrier family 2 member 1 (*SLC2A1*) that encodes for glucose transporter 1 (GLUT 1) and lactate dehydrogenase A (LDHA) enzyme (Tannahill *et al.*, 2013; Wang *et al.*, 2010). As a functional consequence of HIF1α stabilization, there is an enhanced lactate production along with an increased production of pyruvate dehydrogenase kinase (PDK), that in turn, impairs the pyruvate dehydrogenase complex (PDC) activity, thus limiting the acetyl-coA production for the TCA cycle (Kim *et al.*, 2006). Importantly, HIF-1α also interferes with the expression of cytokines by promoting the transcription of IL-1β and constitutes a hallmark of inflammasome activation (Tannahill *et al.*, 2013). Of note, in macrophages under hypoxic conditions, HIF-1α is the key regulator of the metabolic switch from oxidative phosphorylation to glycolysis (Corcoran & O'Neill, 2016). Interestingly, increased succinate levels lead to enhanced reverse electron transport and ROS production from complex I of the ETC, which also promotes HIF1α stabilization (Chouchani *et al.*, 2014). Another consequence of a broken TCA cycle is the NO production from glutamate-derived amino groups through the arginosuccinate shunt present between the fumarate and oxaloacetate steps (Jha *et al.*, 2015). Also, NO inhibits mitochondria respiration through the nitrosylation of iron-sulphur proteins of complex I and cytochrome c oxidase complex (Brown, 1999). The enhanced glycolytic flux in M1 macrophages is also promoted by the up-regulation of the *Pfkfb3* gene that maintains higher levels of fructose 2,6-biphosphate, which is a potent PFK1 stimulator and drives lactate production (De Bock *et al.*, 2013) Additionally, there is a downregulation of the carbohydrate kinase-like protein (CARKL) resulting in a reduction of sedoheptulose- 7-phosphate, which is important during the pentose phosphate pathway and the M1 type development (Galván-Peña & O’Neill, 2014).

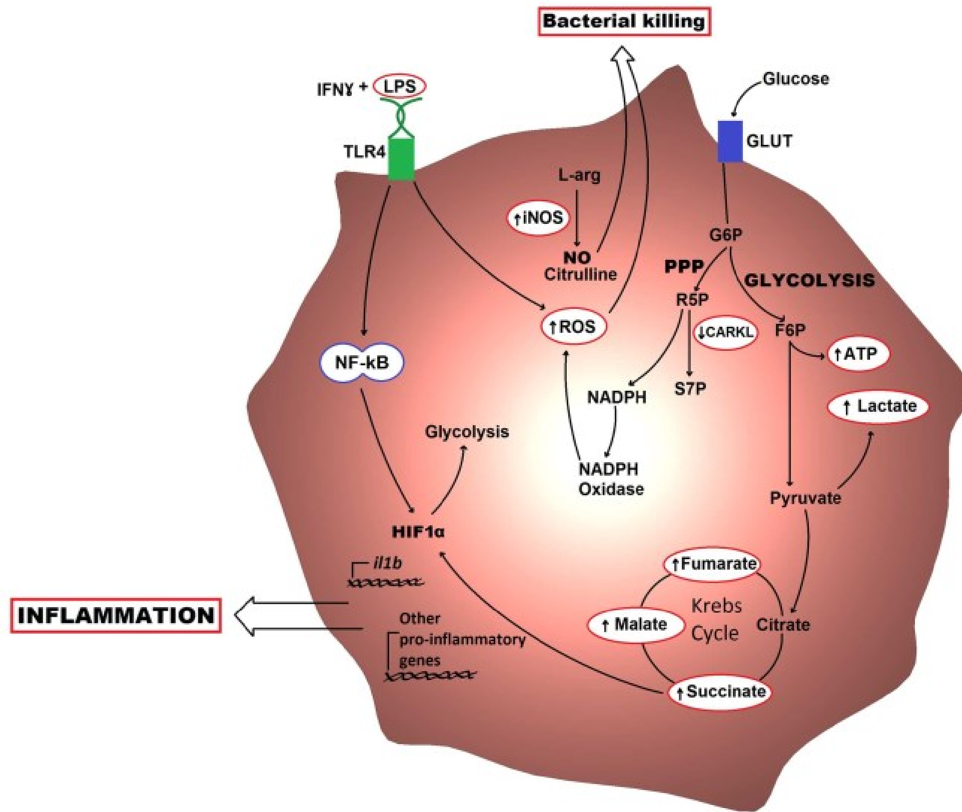


Figure 4.13. M1 macrophages.
M1 macrophages display an enhanced glycolytic flux and a broken TCA cycle, which is required for ROS and NO production during pathogen clearance. (Galván-Peña & O'Neill, 2014).

The M2 macrophage's metabolic print, unlike the one of M1 macrophages, exert reduced glycolytic flux, an intact TCA cycle and an enhanced mitochondrial oxidative phosphorylation coupled to the electron transport chain **(Figure 4.14)** (Jha *et al.*, 2015). M2 polarization generally promotes mitochondrial biogenesis, enhanced glutamine metabolism, and fatty-acid oxidation, which is dependent on cell-intrinsic lysosomal lipolysis to supply and enhance the mitochondrial flux (Huang *et al.*, 2014; Vats *et al.*, 2006). Importantly, enhanced mitochondrial metabolism is highly dependent on the AMPK and mTORC2 signalling axes along with the expression of peroxisome proliferator-activated receptor-γ (PPARγ), the co-activator 1β (PGC1β) and the transcription factor STAT6 (Huang *et al.*, 2016; Mounier *et al.*, 2013; Vats *et al.*, 2006). Upon M2 activation, there is an enhanced external FA uptake, which feeds the mitochondrial oxidative phosphorylation with the energetic intermediator acetyl-coA. As well, FA may be oxidised by the enzyme lysosomal acid lyase (Huang *et al.*, 2014; Sag *et al.*, 2008). Also, M2 macrophage

metabolism promotes the hexosamine pathway that generates uridine diphosphate (UDP)-N-acetylglucosamine; intermediates, which are important during protein glycosylation involved in M2-associated receptors such as mannose receptors (Jha *et al.*, 2015; Sica & Mantovani, 2012). Additionally, the enhanced glutamine metabolism in M2 macrophages is required to replenish the TCA cycle with α-KG. An impairment of the glutamine metabolism induces a dysfunctional M2 subset. For instance, glutamine removal promotes a decrease in the transcriptional signature of the TCA cycle and the downregulation of anti-inflammatory gene markers (Jha *et al.*, 2015). The M2 subset also presents an enhanced induction of arginine metabolism in which the TNF-alpha-induced protein 8-like 2 (TIPE2) has been associated. Hence, TIPE2 negatively regulates Inflammation by switching the arginine metabolism from nitric oxide synthase to arginase production along with polyamines and ornithine (Lou *et al.*, 2014). Contrary to M1, *PFKB3* is over-expressed, resulting in higher levels of PFK2, which promotes a reduction in fructose-2,6-bisphosphate and glycolytic flux levels (Galván-Peña & O'Neill, 2014).

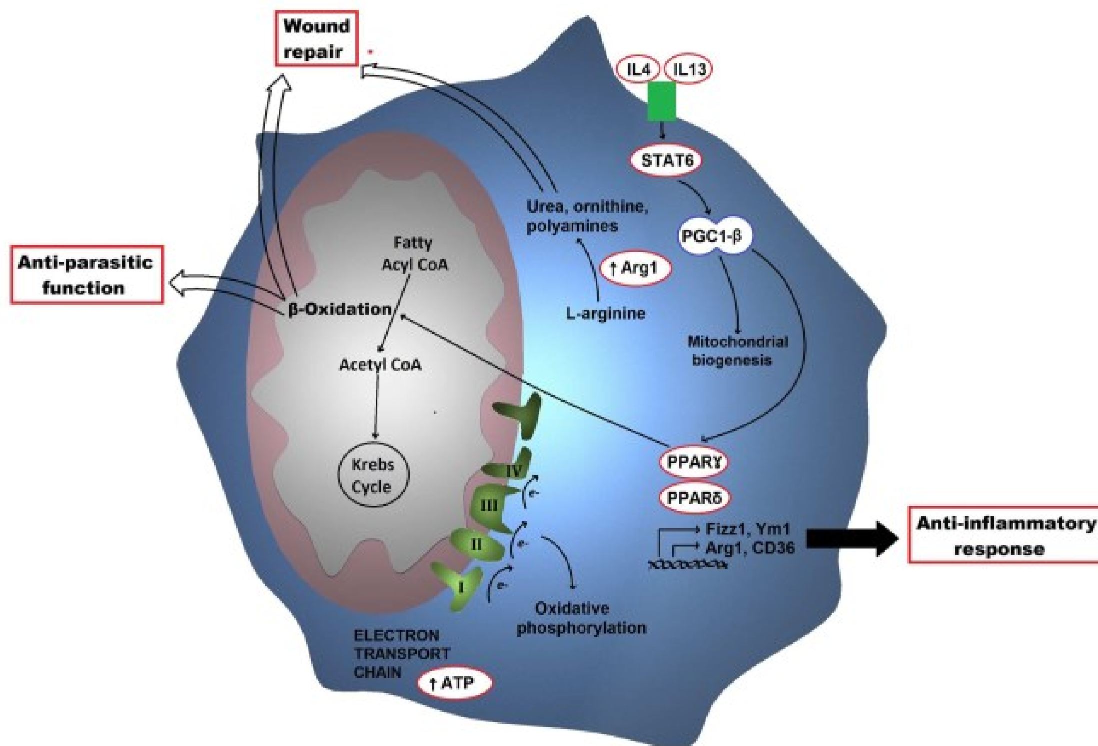


Figure 4.14. M2 macrophages.
M2 macrophages display a decreased glycolytic flux and enhanced OXPHOS, which is required for wound healing and to control helminth infections (Galván-Peña & O'Neill, 2014).

4.5 Host Immune-metabolic interactions with *Leishmania*

Since both mitochondria and metabolism play an important role in the cell's immune fate, it is tempting to assume that pathogens may target mitochondrial function to successfully establish an infection. Thus, the host mitochondria-pathogen interactions may lead to a modulation that might result in a microenvironment change that provides access to nutrients for the pathogen. On top of that, modulation of host cell death pathways to modify its life span or attenuate the host cell's immune response to assure the pathogen replication are other reasons that may prove to be beneficial when targeting mitochondrial function. An interesting and well-studied example of how mitochondria establish a metabolic defence against intracellular pathogens by changing their morphology to efficiently adapt to cellular demands and respond to environmental stimuli such as nutrient deprivation occurs in *Toxoplasma* infection (Tiku et al., 2020). A principal hallmark of *Toxoplasma* infection is the active recruitment of mitochondria to the PV through the secretion of related mitochondrial association factor 1 (TgMAF1) (Kelly *et al.*, 2017). An interesting result of these mitochondria-*Toxoplasma* interactions is the competition for nutrients such as fatty acids (FA). *Toxoplasma* triggers lipophagy that degrades host lipid droplets (LDs) into FA, which is essential for its intracellular development. To counter *Toxoplasma,* host cell mitochondria counteract FA uptake by inducing mitochondrial fusion around the PV and promoting mitochondrial β-oxidation, which reduces the *Toxoplasma* FA uptake (Pernas *et al.*, 2018). Even if there are several examples where mitochondria play an important role during pathogenesis (parasites, viruses and bacteria) (Caradonna *et al.*, 2013; Elesela & Lukacs, 2021; Spier *et al.*, 2019), in this section, we will discuss some key findings describing *Leishmania*-mitochondria interaction.

Manipulation of the host's metabolic program by *Leishmania* represents a strategy to prevent the host immune response and assure the uptake of different metabolites that is required during the colonization process to support parasite differentiation as well as replication and that leads to long-term parasite growth. An excellent example of how *Leishmania* competes with the host, from a metabolic point of view, is during iron scavenging from the host. Iron is an important metabolic element required to support *Leishmania* growth and survival due to its heme auxotrophy (Laranjeira-Silva *et al.*, 2020b). During the colonization, the *Leishmania* amastigote iron uptake is mediated by the transporters LFR1 for Fe^{3+}, LIT1 for $Fe^{2+}$and the heme transporter LHR1 (Flannery *et al.*, 2011; Huynh *et al.*, 2006; Huynh *et al.*, 2012). These transporters are overexpressed due to the restricted iron levels in the PV. In contrast, the host restricts iron

availability within the PV through the expression of natural resistance-associated macrophage protein 1 (NRAMP1), which promotes iron efflux pump recruitment in maturing phagosomes and lysosomes. For instance, LHR1$^{+/-}$ heterozygous mutants were attenuated and presented 50% of the iron pool when compared to wild-type strains in heme-deficient medium. Nevertheless, they present normal differentiation into amastigotes, but they can only replicate in macrophages under iron-supplemented conditions. Also, LHR1-null mutants do not survive at all, suggesting an essential LHR1 role in the promastigotes' survival (Flannery *et al.*, 2013; Huynh *et al.*, 2012). *Leishmania* expresses the last 3 enzymes involved in heme biosynthetic pathway, thus, *Leishmania* can synthetize heme from coproporphyrinogen III (COPROIII), protoporphyrinogen IX (PPGIX) or protoporphyrin IX (PPIX). Interestingly, the transporters that mediate heme or heme precursor internalization to the PV or *Leishmania* remain to be discovered. *Leishmania* is also highly dependent on extracellular amino acid scavenging, such as Arginine, which is essential to support parasite proliferation. It is involved in immune regulation and is important during the metabolic cross-talk between the host and *Leishmania* (Ren *et al.*, 2018). Hence, the role of Arginine has been correlated with *Leishmania* growth mediated by Arginase 1 (Arg-1) activity (Modolell *et al.*, 1995). Metabolic studies using different *Leishmania* species have shown, that in infected macrophages and *in vivo*, enhanced Arg-1 activity, which promotes urea and ornithine generation that, is diverted toward polyamine synthesis rather than NO production by nitric oxide synthase II. The host Arg-1 activity is mediated by a balance between IL-4 and IL-12, which, in *in vivo* models, induces a Th2 response and is associated with alternative macrophage activation. (Iniesta *et al.*, 2005; Kropf *et al.*, 2005; Muxel *et al.*, 2019). Importantly, there is a positive feedback loop in Arg-1 activity mediated by IL-10 (Mandal *et al.*, 2017). In Arginine deprivation conditions, *L. donovani* promotes an over-expression and activity of the *Leishmania* arginine transporter (LdAAP3) as a salvage mechanism, which forces the arginine scavenging (Goldman-Pinkovich *et al.*, 2016). Other amino acid sources, such as glutamine, have been shown to have important relevance in supporting the amastigotes' oxidative phosphorylation and amino acid metabolism. Thus, the glutamine synthetase (GS) from different *Leishmania* species has been identified as a potential antileishmanial target (de Lima *et al.*, 2022; Kumar *et al.*, 2020b; Naderer *et al.*, 2008; Saunders *et al.*, 2014). Controversially, it is essential for the host to control visceral leishmaniasis. For instance, glutamine supplementation has shown to improve miltefosine treatment efficacy for visceral leishmaniasis (Ferreira *et al.*, 2020; Kumar *et al.*, 2017b).

There is a growing number of studies indicating that *Leishmania* targets host cell energy metabolism to create a permissive environment for their replication. For instance, the transcriptional signature of *L. major* infected macrophages showed an enhanced glycolysis rate and lactate production with a concomitant reduction in the pyruvate flux through the tricarboxylic acid (TCA) cycle. Indeed, *Leishmania*-infected macrophages promote the upregulation of several glycolytic enzymes such as hexokinase, pyruvate kinase isozyme M2 and lactate dehydrogenase A (Basu *et al.*, 2020; Rabhi *et al.*, 2012). Also, the bioenergetic profiling of macrophages infected with various *Leishmania* species revealed that there is an induction of glycolysis during the early phases of infection, followed by a switch from glycolysis to OXPHOS during the late phase of infection (Moreira *et al.*, 2015b; Ty *et al.*, 2019). Those metabolic changes suggest that infected macrophages would tend to convert glucose into lactate even in the presence of oxygen to support mitochondrial oxidative phosphorylation. Analysis of the molecular mechanisms, by which *L. infantum* modulates mitochondrial metabolism and the metabolic flux, revealed an essential role for sirtuin 1 (SIRT1) and liver kinase B1 (LKB1)/AMPK signalling axis, which is a key modulator of the PGC-1α activity and inductor of mitochondrial biogenesis. Thus, the abolition of activation of AMPK, SIRT1 or LKB1 in macrophages during infection, sustains the glycolytic flux and it associates with parasite clearance (Moreira *et al.*, 2015b). Noteworthy, the benefit of enhanced mitochondrial biogenesis induced by metformin and AICAR, displaying OXPHOS has been addressed in *L. infantum*, *L. major* and *L. brasiliensis*-infected macrophages where the parasites presented increased growth (Lima *et al.*, 2020; Moreira *et al.*, 2015b; Postat *et al.*, 2018). Likewise, *Leishmania* infection exploits the Myeloid Cell Leukemia 1 (MCL-1) signalling axis in neutrophils and macrophages, which promotes a reduction in the mitochondria-mediated apoptosis by repressing the release of cytochrome c (Akarid *et al.*, 2004; Giri *et al.*, 2016). Interestingly, the AMPK signalling axis is closely related to mTOR, thus, in an energy deprivation context, inhibition of mTORC1 by AMPK dictates parasite fate by regulating mTOR-induced autophagy along with a general repression of host translation, which is crucial during the early time of infection (Jaramillo *et al.*, 2011; Thomas *et al.*, 2018). Also, the importance of mTOR in *L. donovani* fate was dissected in myeloid-restricted HIF-1α knockout mice. HIF-1α deficiency promotes increased lipogenesis through the activation of mTOR and the nuclear translocation of sterol regulatory element-binding protein-1c (SREBP-1c). These alterations are associated with enhanced susceptibility to *L. donovani* infection (Mesquita *et al.*, 2020). Indeed, during *Leishmania* pathogenesis, HIF-1α has been

found to be upregulated and associated with parasite survival (Alonso *et al.*, 2019; Degrossoli *et al.*, 2007; Singh *et al.*, 2012).

In conclusion, *Leishmania*-host immune system interactions are a crucial interplay that dictates parasite fate. Thus, understanding the mechanisms employed by *Leishmania* to alter the host metabolism may eventually lead to the identification of novel targets for leishmaniasis treatment.

CHAPTER 2: HYPOTHESES AND OBJECTIVES

To colonize macrophages, *Leishmania* metacyclic promastigotes employ a panoply of virulence factors, including Lipophosphoglycan (LPG), which impairs different host cell processes and rewires host cell metabolism, hence creating a metabolically-adapted microenvironment required for pathogen replication. Whereas the role of the SIRT1-AMPK axis in mediating *Leishmania*-induced changes in host cell bioenergetics profile has been established, scarce attention has been paid to the nature of both the host cell receptors and *Leishmania* virulence factors involved in macrophage metabolic reprogramming. In this regard, the hypothesis of this thesis is:

In macrophages, *Leishmania donovani* modulates host cell mitochondrial metabolism and function in an LPG-dependent manner.

To address this hypothesis, the following research objectives are proposed.

1. Analyze mitochondrial metabolic flux in *Leishmania donovani*-infected macrophages and the role of LPG therein.
2. Elucidate the role of *Leishmania donovani* LPG in macrophage mitochondrial biogenesis induction.
3. Evaluate the signalling axis involved in macrophage mitochondrial biogenesis induction.

These objectives will be addressed in the primary article (Chapter 3) and extensively discussed in Chapter 4.

CHAPTER 3: PRIMARY ARTICLE

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RESEARCH ARTICLE

Macrophage Mitochondrial Biogenesis and Metabolic Reprogramming Induced by *Leishmania donovani* Require Lipophosphoglycan and Type I Interferon Signaling

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ABSTRACT Pathogen-specific rewiring of host cell metabolism creates the metabolically adapted microenvironment required for pathogen replication. Here, we investigated the mechanisms governing the modulation of macrophage mitochondrial properties by the vacuolar pathogen *Leishmania*. We report that induction of oxidative phosphorylation and mitochondrial biogenesis by *Leishmania donovani* requires the virulence glycolipid lipophosphoglycan, which stimulates the expression of key transcriptional regulators and structural genes associated with the electron transport chain. *Leishmania*-induced mitochondriogenesis also requires a lipophosphoglycan-independent pathway involving type I interferon (IFN) receptor signaling. The observation that pharmacological induction of mitochondrial biogenesis enables an avirulent lipophosphoglycan-defective *L. donovani* mutant to survive in macrophages supports the notion that mitochondrial biogenesis contributes to the creation of a metabolically adapted environment propitious to the colonization of host cells by the parasite. This study provides novel insight into the complex mechanism by which *Leishmania* metacyclic promastigotes alter host cell mitochondrial biogenesis and metabolism during the colonization process.

IMPORTANCE To colonize host phagocytes, *Leishmania* metacyclic promastigotes subvert host defense mechanisms and create a specialized intracellular niche adapted to their replication. This is accomplished through the action of virulence factors, including the surface coat glycoconjugate lipophosphoglycan. In addition, *Leishmania* induces proliferation of host cell mitochondria as well as metabolic reprogramming of macrophages. These metabolic alterations are crucial to the colonization process of macrophages, as they may provide metabolites required for parasite growth. In this study, we describe a new key role for lipophosphoglycan in the stimulation of oxidative phosphorylation and mitochondrial biogenesis. We also demonstrate that host cell pattern recognition receptors Toll-like receptor 4 (TLR4) and endosomal TLRs mediate these *Leishmania*-induced alterations of host cell mitochondrial biology, which also require type I IFN signaling. These findings provide new insight into how *Leishmania* creates a metabolically adapted environment favorable to their replication.

KEYWORDS IFNAR, *Leishmania*, lipophosphoglycan, macrophages, mitochondria

Leishmania is a trypanosomatid parasite responsible for a spectrum of human diseases called leishmaniasis (1). This parasite is transmitted to mammals by phlebotomine sand flies through the inoculation of metacyclic promastigotes, which are internalized by phagocytic cells (2). There, they create specialized parasitophorous vacuoles that support their differentiation and replication as amastigotes (3) Given their auxotrophies for several essential metabolites they must acquire from their host (4–6), *Leishmania* parasites rewire or alter diverse host cell metabolic pathways during the infection process (6, 7). In particular, a growing number of studies indicate that

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Leishmania targets host cell energy metabolism to create an environment permissive to their replication. Hence, studies on the transcriptional signature of macrophages infected with *L. major* suggested an enhanced rate of glycolysis and lactate production with reduced pyruvate flux through the tricarboxylic acid cycle, indicating that infected macrophages would tend to convert glucose into lactate even in the presence of sufficient oxygen to support mitochondrial oxidative phosphorylation (OXPHOS) (8). Bioenergetics profiling of macrophages infected with various *Leishmania* species revealed an induction of glycolysis during the early phases of infection, followed by a switch from glycolysis to OXPHOS during the late phase of infection (9, 10). Analysis of the molecular mechanisms by which *L. infantum* modulates mitochondrial metabolism revealed an essential role for the metabolic sensor AMP-activated protein kinase (AMPK) (9), a key modulator of peroxisome proliferator-activated receptor gamma (PPARγ) coactivator 1α (PGC-1α) activity (11, 12). PGC-1α is a transcriptional coactivator and is the master switch that integrates mitochondrial biogenesis and energy-generating functions of mitochondria with metabolic demands associated with physiological states associated with health and disease (13–16). Hence, PGC-1α controls multiple aspects of mitochondrial biogenesis, including increased mitochondrial number and biogenesis of the OXPHOS system (13), by coordinating the concerted expression of nuclear and mitochondrial genes encoding proteins involved in these processes (16–18).

Macrophages and other phagocytes are highly plastic cells that rapidly adapt their metabolism in response to invading pathogens. It is well documented that the interplay between pathogen-derived molecules and host innate immune receptors contributes significantly to these metabolic changes (19, 20). Hence, exposure of monocytes to bacterial lipopolysaccharide (LPS), which stimulates Toll-like receptor 4 (TLR4), induces an increase in glycolysis and a decrease in OXPHOS, whereas the TLR2 ligand Pam_3CysSK_4 (a synthetic lipopeptide) upregulates both glycolysis and OXPHOS (21). However, studies with live intracellular pathogens revealed a diversity of macrophage metabolic changes consistent with the notion of host-driven and pathogen-driven metabolic rewiring (22). This notion reflects the fact that pathogens produce several effectors and virulence factors that dynamically interact with various host cell innate immune receptors and molecules over the course of infection. Of interest, *Mycobacterium tuberculosis* decreases both glycolysis and OXPHOS in infected macrophages, and recent evidence indicates that type I interferon (IFN) expressed by these cells plays a central role in decreasing macrophage energy metabolism during mycobacterial infection (23, 24).

Whereas the role of the sirtuin 1 (SIRT1)-AMP-activated protein kinase (AMPK) axis in mediating *Leishmania*-induced changes in the host cell bioenergetics profile has been established (9), our knowledge of the nature of both the host cell receptors and *Leishmania* effectors involved in the macrophage metabolic reprogramming remains fragmentary. In the present study, we investigated the mechanisms by which *L. donovani* metacyclic promastigotes alter host cell mitochondrial biology. In particular, we sought to determine the potential role of the parasite cell surface glycolipid lipophosphoglycan (LPG) in this process (25). This virulence factor contributes to the ability of *Leishmania* promastigotes to successfully colonize phagocytic cells and to alter the innate immune response by downmodulating macrophage microbicidal functions, inducing the secretion of cytokines, and shaping the parasitophorous vacuole in which *Leishmania* replicates (25–29). We report that *L. donovani* metacyclic promastigotes induce at least two distinct host cell responses in the context of the modulation of macrophage mitochondrial biogenesis, which are mediated by TLR4 and endosomal TLRs. One is the LPG-dependent enhancement of macrophage mitochondrial mass, increased expression of PGC-1α and of genes associated with the electron transport chain, and stimulation of OXPHOS. The other response is the LPG-independent induction of alpha interferon (IFN-α) expression, which also mediates enhancement of macrophage mitochondrial mass but has no impact on the induction PGC-1α expression or on mitochondrial flux.

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RESULTS

LPG is required for the alteration of host cell bioenergetic metabolism by *L. donovani* metacyclic promastigotes. Whereas previous studies revealed that *Leishmania* promastigotes modulate host macrophage metabolism (8–10, 30), little is known regarding the parasite molecules involved in this process. We therefore sought to determine the potential role of the abundant cell surface virulence glycolipid LPG on the dynamics of host cell mitochondrial function induced by *L. donovani* metacyclic promastigotes. First, we assessed the impact of this glycolipid on the bioenergetic profile of bone marrow-derived macrophages (BMM) infected with either wild-type (WT) *L. donovani* metacyclic promastigotes, an isogenic LPG-defective mutant (Δ*lpg1*), or its complemented counterpart (Δ*lpg1*+*LPG1*) (see Fig. S1A in the supplemental material). We used live cell extracellular flux analysis to determine the oxidative metabolism of infected macrophages by measuring the mitochondrial oxygen consumption rate (OCR) as well as glycolysis through the measurement of the extracellular acidification rate (ECAR) (Fig. 1A). In BMM infected with either WT or Δ*lpg1*+*LPG1 L. donovani* metacyclic promastigotes, we observed an increase in OCR values which peaked at 4 h postinfection and partially declined by 24 h (Fig. 1B). In contrast, in BMM infected with the Δ*lpg1* mutant, OCR remained significantly lower at all time points postinfection, suggesting that LPG is required to stimulate the oxidative metabolism of infected macrophages. For glycolysis, in BMM infected with WT, Δ*lpg1*, and Δ*lpg1*+*LPG1* metacyclic promastigotes, we observed an increased in ECAR values compared to noninfected macrophages at 4 h and 8 h postphagocytosis, corresponding to an increase in the glycolytic flux (Fig. 1C). At 24 h postinfection, whereas the ECAR levels returned to the levels observed in noninfected cells for BMM infected with either WT or Δ*lpg1*+*LPG1* parasites, it remained elevated in BMM infected with Δ*lpg1* metacyclic promastigotes (Fig. 1C). The increase in ECAR was associated with an increased proton efflux rate in infected macrophages, which was significantly higher in BMM infected with Δ*lpg1* promastigotes than in BMM infected with either WT or Δ*lpg1*+*LPG1* promastigotes at 24 h postphagocytosis. (Fig. 1D). These results suggested that *L. donovani* metacyclic promastigotes induce an increase in glycolysis during the early phases of infection, which remains elevated in the absence of LPG. The elevated OCR/ECAR ratio observed for BMM infected with either WT or Δ*lpg1*+*LPG1* parasites indicates that higher OXPHOS takes place in these cells than in BMM infected with Δ*lpg1* parasites (Fig. 1E). To rule out the possibility that the increase in the OCR/ECAR ratio induced by *L. donovani* metacyclic promastigotes was the consequence of a phagocytic stimulus, we measured the OCR/ECAR ratio in BMM fed zymosan. As shown in Fig. S1B, phagocytosis of zymosan caused an important reduction in the basal OCR/ECAR ratio at 1 h, 4 h, 8 h, and 24 h postphagocytosis, indicating a reduction in the oxidative metabolism of BMM. Treatment with LPS induced a similar decrease in the OCR/ECAR ratio (Fig. S1C), consistent with increased glycolysis and reduced OXPHOS (21, 31). Additional control experiments confirmed that free *L. donovani* metacyclic promastigotes by themselves do not contribute to the OCR and ECAR measurements in infected BMM (Fig. S1D and E). To further characterize the process by which *L. donovani* metacyclic promastigotes modulate mitochondrial metabolic flux in BMM, we evaluated additional parameters of mitochondrial function. Hence, we observed an LPG-dependent increase in basal respiration, mitochondrial ATP production, nonmitochondrial oxygen consumption, and proton leak (Fig. S2A to E). In contrast, the observed increase in the spare respiratory capacity, which was calculated by the difference between the maximal OCR determined in the presence of the uncoupler fluoro-carbonyl cyanide phenylhydrazone (FCCP), was independent of LPG (Fig. S2F). Collectively, these results indicate that *L. donovani* metacyclic promastigotes induce an increase in both glycolysis and OXPHOS in infected BMM. Whereas LPG has little role to play in the increase in glycolysis, it contributes to OXPHOS in infected macrophages. Having shown that *L. donovani* metacyclic promastigotes modulate host cell metabolism, we next sought to identify the carbon dependence source to conduct energy production in infected BMM (Fig. 1A).

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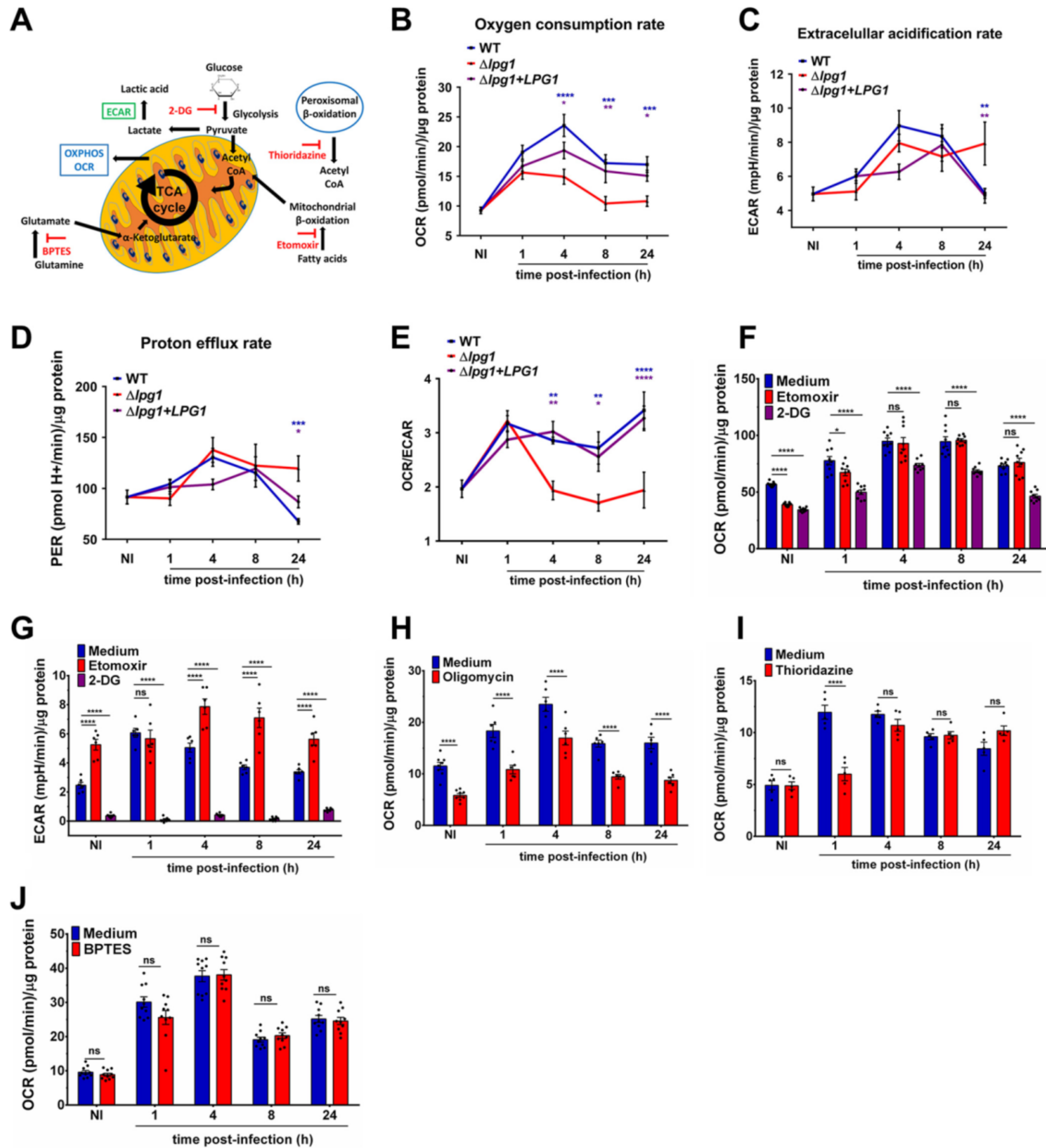

FIG 1 LPG contributes to the alteration of host cell bioenergetic metabolism by *L. donovani* metacyclic promastigotes. (A) Schematic showing the metabolic pathways detailing readouts for OCR, ECAR, and the carbon dependence source using inhibitors for glycolysis, mitochondrial β-oxidation, glutaminolysis, and peroxisomal β-oxidation. (B, C, D, and E) BMM were infected with either WT, Δ*lpg1*, or Δ*lpg1*+*LPG1* *L. donovani* metacyclic promastigotes, and at the indicated time points OCR, ECAR, PER, and the OCR/ECAR ratio were determined. (F and G) BMM were infected with WT *L. donovani* metacyclic promastigotes, and 30 min prior to the readouts for OCR and ECAR, 50 mM 2 deoxy-D-glucose (2-DG) or 4 μm etomoxir was added to the cells. (H, I, and J) BMM were infected with WT *L. donovani* metacyclic promastigotes, and 30 min prior to the readouts for OCR, 1 μM oligomycin, 1 μM thioridazine, or 3 μM BPTES was added to the cells. The readouts in each sample were normalized using the protein concentration, and the measurements were expressed as (OCR, ECAR, and PER)/μg protein. Representative graphic for three independent experiments (F to J). The data are presented as mean values ± SEM from three independent experiments. ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ according to a two-way ANOVA with Dunnett's multiple-comparation test.

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Inhibition of the glycolytic pathway with 2 deoxy-D-glucose (2-DG) 30 min prior to the readouts significantly reduced *L. donovani*-induced OCR and ECAR (Fig. 1F and G). On the other hand, while inhibition of mitochondrial β-oxidation with etomoxir 30 min prior to the readouts had no major impact on OCR (Fig. 1F), we observed a significant increase in ECAR at 4 h, 8 h, and 24 h postphagocytosis (Fig. 1G). These results indicate that *L. donovani* metacyclic promastigotes promote an increase in host cell mitochondrial activity in a glycolytic-dependent manner and that blocking mitochondrial β-oxidation increases extracellular acidification. The reduction of OCR upon inhibition of the glycolytic pathway can partially explain where energy comes from (20% to 40%). Thus, we next sought to identify other carbon sources. Inhibition of ATP synthase (complex V) with oligomycin 30 min prior to the readouts significantly reduced *L. donovani*-induced OCR at 1 h, 4 h, 8 h, and 24 h postphagocytosis (Fig. 1H). Inhibition of peroxisomal β-oxidation with thioridazine 30 min prior to the readouts significantly reduced *L. donovani*-induced OCR at 1 h postphagocytosis and had no major impact on OCR at 4 h, 8 h, and 24 h postphagocytosis (Fig. 1I). On the other hand, inhibition of the glutamine oxidation pathway with BPTES [bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide] 30 min prior to the readouts, had no major impact on OCR in *L. donovani*-infected BMM (Fig. 1J). These results indicate that increased OCR in *L. donovani*-infected macrophages is independent of glutaminolysis, is partially supported by peroxisomal β-oxidation during the first hour postphagocytosis, and is complex V-dependent.

***L. donovani* metacyclic promastigotes stimulate host cell mitochondrial biogenesis in an LPG-dependent manner.** To further investigate the contribution of LPG to the metabolic changes induced in macrophages by *L. donovani* promastigotes, we assessed the impact of this glycolipid on host cell mitochondrial biogenesis. To this end, we used immunofluorescence confocal microscopy to quantify alterations in the mitochondrial network area labeled with the outer mitochondrial membrane receptor Tom20. Compared to uninfected BMM, we observed a doubling in the mitochondrial network area relative to the cell area in BMM infected with either WT or Δ*lpg1*+*LPG1 L. donovani* metacyclic promastigotes for 24 h (Fig. 2A and B and Fig. S3A). In contrast, the mitochondrial network area remained unaltered in BMM infected with Δ*lpg1* metacyclic promastigotes (Fig. 2A and B and Fig. S3A), suggesting that LPG participates in mitochondrial biogenesis induction in BMM infected with *L. donovani* promastigotes. To further demonstrate the impact of LPG on mitochondrial biogenesis, we infected BMM with either WT, Δ*lpg1*, or Δ*lpg1*+*LPG1 L. donovani* metacyclic promastigotes, and we measured the mitochondrial/nuclear (mt/n) DNA ratio by quantitative PCR (qPCR), using two mitochondrion-encoded genes, 16S ribosomal (*MT-16S*) and NADH dehydrogenase 1 (*MT-ND1*) and the nuclear-encoded gene hexokinase-2 (*HK2*) for normalization. As shown in Fig. 2C and Fig. S3B, WT and Δ*lpg1*+*LPG1* metacyclic promastigotes induced a 2-fold increase in the ratio of *MT-16S* and *MT-ND1* relative to *HK2* (mt/n DNA ratio) at 8 h and 24 h postinfection compared to uninfected cells. In contrast, the mt/n DNA ratio remained unaltered in BMM infected with Δ*lpg1* metacyclic promastigotes (Fig. 2C and Fig. S3B), consistent with a requirement for LPG in the induction. An increase in the mt/n DNA ratio was induced at a low parasite-to-macrophage ratio and was not significantly augmented at higher parasite loads (Fig. S3C). This macrophage response was also induced by metacyclic promastigotes from other *Leishmania* species, and the extent and kinetics of increased mitochondrial DNA (mtDNA) content in BMM infected with *L. mexicana*, *L. major*, and *L. amazonensis* metacyclic promastigotes were similar to those observed in BMM infected with *L. donovani* (Fig. S3D).

***L. donovani* metacyclic promastigotes induce the expression of genes associated with mitochondrial biogenesis in an LPG-dependent manner.** Mitochondrial biogenesis is a highly coordinated process that requires the expression of nuclear and mitochondrial genes and is regulated by specific signaling modules, transcription factors, and regulators of gene expression (14, 15, 32). We evaluated the impact of *L. donovani* metacyclic promastigotes and of LPG on the expression kinetics of the genes encoding PGC-1α (*PARGC1A*) and nuclear respiratory factor 1 (*NRF1*), two key regulators of gene expression associated with mitochondrial biogenesis and metabolism (14,

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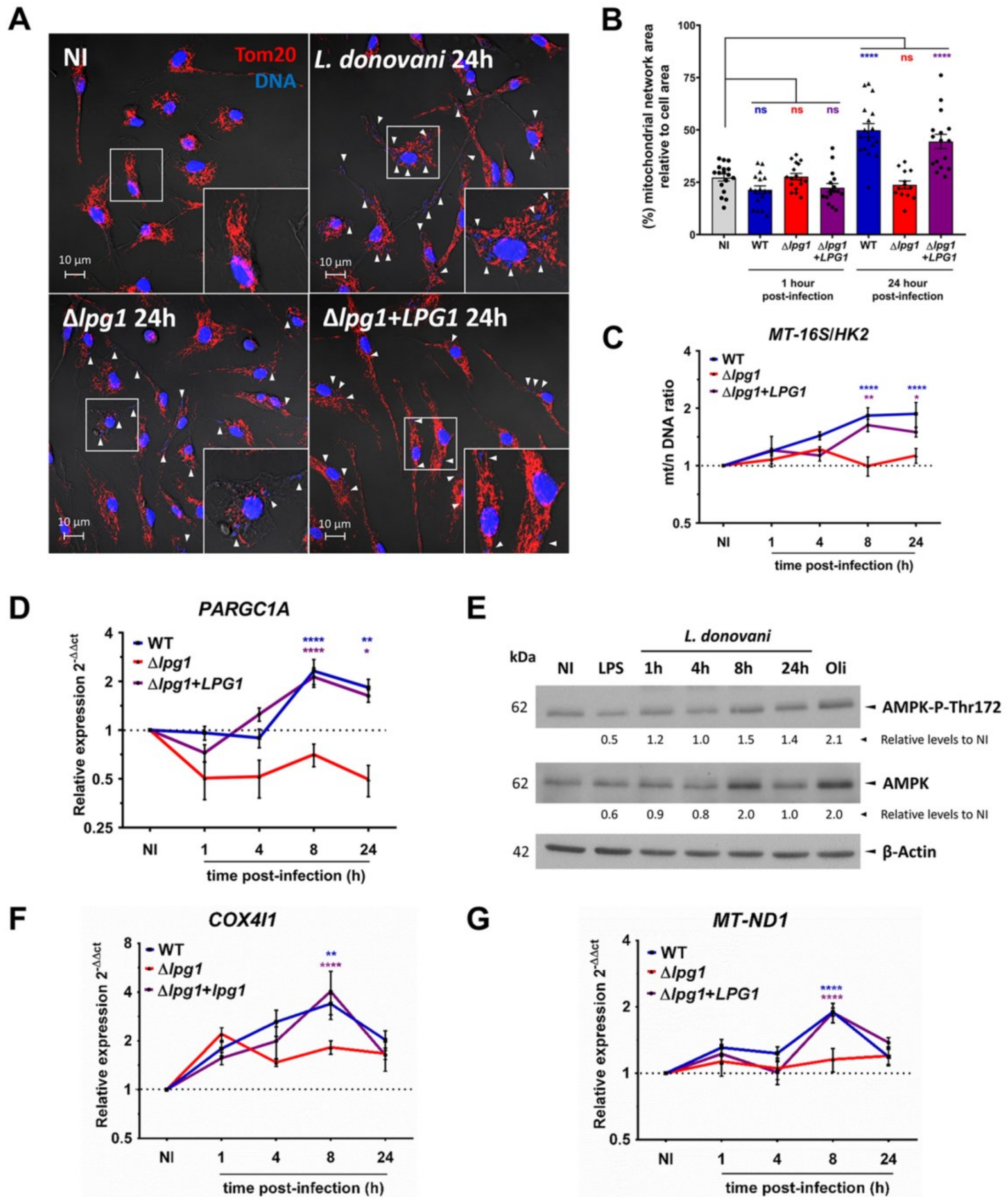

FIG 2 *L. donovani* metacyclic promastigotes stimulate host cell mitochondrial biogenesis in an LPG-dependent manner. (A) Distribution of Tom20 (red) in BMM infected for 24 h with either WT, Δ*lpg1*, or Δ*lpg1*+*LPG1* *L. donovani* metacyclic promastigotes. DAPI staining for DNA is in blue, and 2×-enlarged insets of representative mitochondria regions are shown. (B) Quantification of the mitochondrial network area relative to the cell area. (C) Mitochondrial/nuclear (mt/n) DNA ratio (*MT-16S/HK2*) in BMM infected with either WT, Δ*lpg1*, or Δ*lpg1*+*LPG1* *L. donovani* metacyclic promastigotes. (D) Kinetics of *PARGC1A* expression in BMM infected with either WT, Δ*lpg1*, or Δ*lpg1*+*LPG1* *L. donovani* metacyclic promastigotes. (E) Protein levels of AMPK-P-Thr172 and

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15, 32). We included in our analysis the phosphorylation of AMPK, a kinase which participates in mitochondrial biogenesis by potentiating the transcriptional activity of PGC-1α (11, 14). We observed that both WT and Δ*lpg1*+*LPG1* metacyclic promastigotes induced a 2-fold increase in the expression of *PARGC1A* at 8 h and 24 h postinfection and a 1.5-fold increase in the expression of *NRF1* at 8 h postinfection. In contrast, neither *PARGC1A* nor *NRF1* were induced by the Δ*lpg1* mutant (Fig. 2D, Fig. S3E). Interestingly, *L. donovani* metacyclic promastigotes, but not LPS, activated the phosphorylation of AMPK on Thr172, independently of LPG (Fig. 2E, Fig. S3J). We next assessed the expression of genes encoding components of the electron transport chain and associated with mitochondrial metabolism in infected BMM. We observed that both WT and Δ*lpg1*+*LPG1* metacyclic promastigotes increased by 2- to 3-fold the expression of genes encoded in the host cell nuclear (*COX4I1* and *NDUFA9*) and mitochondrial (*MT-ND1*, *MT-CO1*, *MT-CO2*) genomes (Fig. 2F and G, Fig. S3F to H). In contrast, expression of those genes remained unchanged in BMM infected with Δ*lpg1* metacyclic promastigotes (Fig. 2F and G, Fig. S3F to H). Western blot analyses confirmed the increased levels of mitochondrial proteins COX IV, NDUFA9, and Tom20 in BMM infected for 24 h with WT *L. donovani* metacyclic promastigotes compared to unstimulated BMM (Fig. S3I). These results are consistent with a requirement for LPG in the induction of mitochondrial biogenesis by *L. donovani* promastigotes. We next determined whether LPG is sufficient to stimulate mitochondrial biogenesis. To this end, we incubated BMM with either heat-killed or live *L. donovani* metacyclic promastigotes for 8 h, and we included polystyrene beads as phagocytic controls. As shown in Fig. S4A and B, only live WT *L. donovani* stimulated an increased mt/n (*MT-16S/HK2*) DNA ratio and *PARGC1A* gene expression. Additionally, we fed BMM with either zymosan or LPG-coated zymosan and measured the mt/n (*MT-16S/HK2*) DNA ratio and the expression of *PARGC1A* at 8 h postinternalization. Similar to WT *L. donovani* promastigotes, phagocytosis of LPG-coated zymosan resulted in the rapid redistribution of LPG within BMM (Fig. S4C). However, as shown in Fig. S4D and E, neither zymosan nor LPG-coated zymosan stimulated mitochondrial biogenesis as assessed by measuring the mt/n (*MT-16S/HK2*) DNA ratio and the expression of *PARGC1A* at 8 h postinternalization. To rule out the possibility that serum-opsonization influenced the stimulation of mitochondrial biogenesis by *L. donovani* metacyclic promastigotes, we compared the responses of BMM infected with unopsonized or serum-opsonized WT, Δ*lpg1*, or Δ*lpg1*+*LPG1* *L. donovani* metacyclic promastigotes. As shown in Fig. S5A and B, unopsonized and opsonized WT and Δ*lpg1*+*LPG1* *L. donovani* promastigotes induced a 2-fold increase in the ratio of *MT-16S* relative to *HK2* (mt/n DNA ratio) and a 2- to 4-fold increase in the expression of *PARGC1A* at 24 h postinfection. In contrast, neither unopsonized nor opsonized Δ*lpg1* *L. donovani* promastigotes stimulated an increase in the mt/n DNA ratio and the expression of *PARGC1A*. Collectively, these results support the notion that LPG is essential but not sufficient for the induction of mitochondrial biogenesis and expression of the respiratory chain components by live *L. donovani* metacyclic promastigotes.

Following internalization by macrophages, promastigotes differentiate into amastigotes, which are the mammalian-adapted forms of the parasite that replicate within phagolysosomes (33). To determine whether increased mitochondrial biogenesis induced during the internalization of metacyclic promastigotes is a transient event or persists as the parasites differentiate and replicate, we assessed the mt/n (*MT-16S/HK2*) DNA ratio and the expression of *PARGC1A* up to 72 h following the infection of BMM with *L. donovani* metacyclic promastigotes. As shown in Fig. 3A and B, compared to

FIG 2 Legend (Continued)
AMPK in BMM infected with *L. donovani* metacyclic promastigotes. BMM treated with 2.5 μM oligomycin for 30 min were used as the positive control for AMPK-P-Thr172. β-Actin was used as a loading control. Representative immunoblots for two independent experiments. (F and G) Kinetics of (F) *COX4I1* and (G) *MT-ND1* expression in BMM infected with either WT, Δ*lpg1*, or Δ*lpg1*+*LPG1* *L. donovani* metacyclic promastigotes. The data are presented as means values ± SEM from three independent experiments. ****, $P < 0.0001$; **, $P < 0.01$; *, $P < 0.05$ according to a one-way ANOVA with Dunnett's multiple-comparation test.

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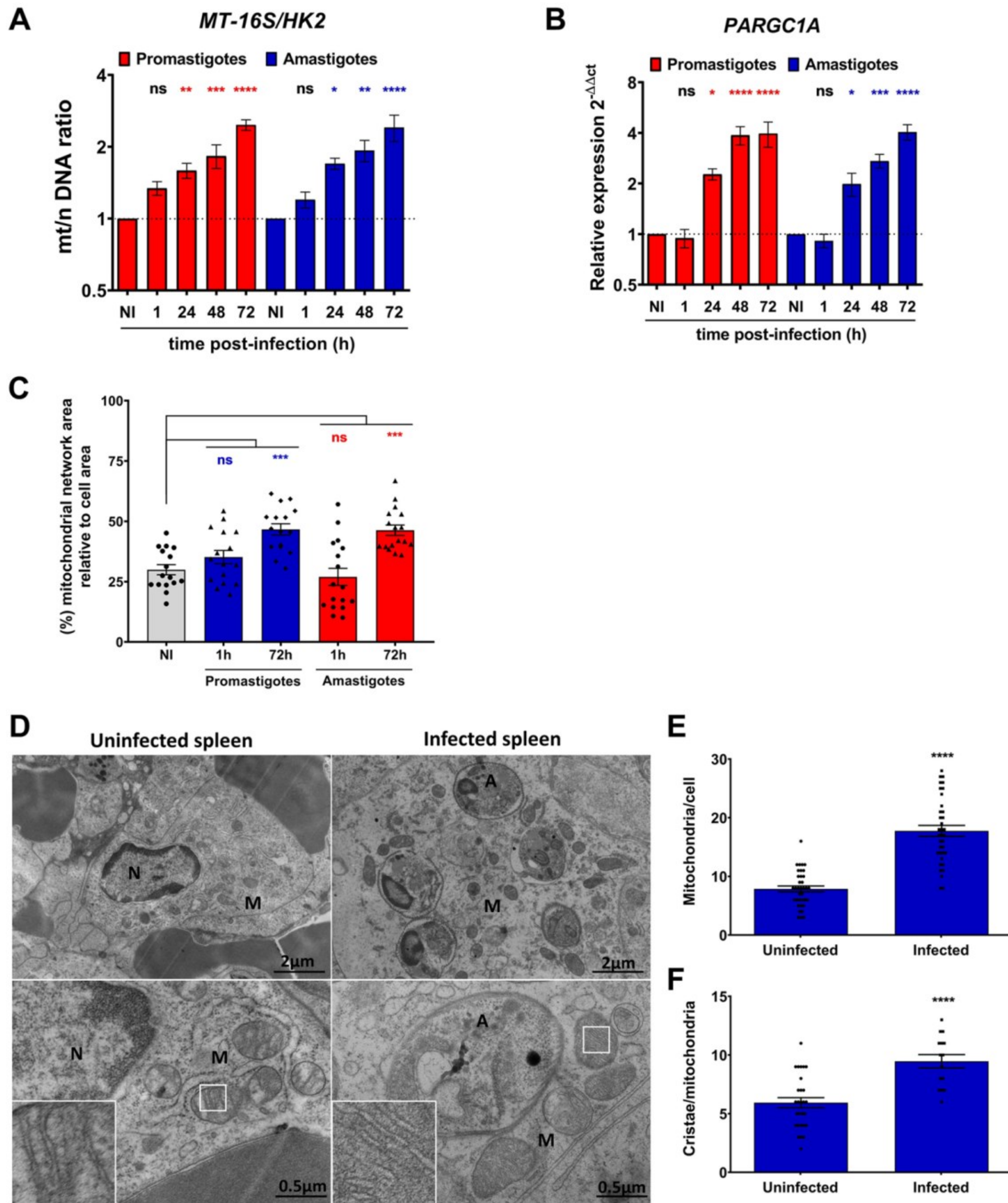

FIG 3 *L. donovani* amastigotes stimulate host cell mitochondrial biogenesis *in vitro* and *in vivo*. (A to C) BMM were infected with either *L. donovani* metacyclic promastigotes or amastigotes, and (A) the mitochondrial/nuclear (mt/n) DNA ratio (*MT-16S/HK2*), (B) the kinetics of *PARGC1A* expression, and (C) the mitochondrial network area relative to the cell area were determined. (D) Electron microscopy assessment of spleen from uninfected and *L. donovani*-infected hamsters. A, M, and N represent amastigotes, mitochondria, and nuclei, respectively. 5×-enlarged insets of representative mitochondrial regions are shown. (E and F) The numbers of mitochondrial (E) and cristae (F) were determined. The data are presented as mean values ± SEM from three independent experiments. (A to C) ****, $P < 0.0001$; **, $P < 0.01$; *, $P < 0.05$ according to a one-way ANOVA with Dunnett's multiple-comparation test. (E and F) ****, $P < 0.0001$ according to an unpaired *t* test with Welch's correction.

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uninfected BMM, a 2-fold increase in the mt/n DNA ratio was maintained in infected BMM up to 72 h, whereas expression of *PARGC1A* was increased by 4-fold at 48 h and 72 h postinfection, at the time when promastigotes are fully differentiated into amastigotes. These results led us to assess mitochondrial biogenesis in BMM infected with *L. donovani* amastigotes isolated from the spleen of infected hamsters. Similar to metacyclic promastigotes, amastigotes induced a 2-fold increase in the mt/n (*MT-16S/HK2*) DNA ratio at 48 h and 72 h postinfection and a 4-fold increase in the expression of *PPARGC1A* at 72 h postinfection compared to uninfected BMM (Fig. 3A and B). These changes in the mt/n (*MT-16S/HK2*) DNA ratio correlated with an increased mitochondrial network area relative to cell area in BMM infected with either promastigotes or amastigotes for 72 h (Fig. 3C, Fig. S4F). As expected, infection with *L. donovani* metacyclic promastigotes did not change the BMM nuclear/nuclear (*Pecam/HK2*) DNA ratio over the course of infection (Fig. S4G). To further investigate the mechanism by which amastigotes stimulate mitochondrial biogenesis, we used amastigotes recovered from BMM infected for 24 h with either WT or Δ*lpg1 L. donovani* metacyclic promastigotes. We infected BMM with either WT or Δ*lpg1 L. donovani* amastigotes, and we assessed the mt/n (*MT-16S/HK2*) DNA ratio and the expression of *PARGC1A* at 24 h postinfection. As shown in Fig. S5C and D, similar to splenic amastigotes, WT amastigotes isolated from infected BMM promoted an increase in the mt/n (*MT-16S/HK2*) DNA ratio and *PARGC1A* gene expression compared to uninfected BMM. In contrast, the mt/n (*MT-16S/HK2*) DNA ratio and *PARGC1A* gene expression remained unaltered in BMM infected with Δ*lpg1* amastigotes compared to uninfected BMM (Fig. S5A and B). Collectively, these results suggest that LPG or a structurally related glycolipid contributes to the induction of mitochondrial biogenesis by *L. donovani* amastigotes. Next, we assessed the impact of *L. donovani* infection on mitochondria in the spleen of infected hamsters. Using transmission electron microscopy, we quantified the number of mitochondria and the number of cristae per mitochondria in cells from the spleens of uninfected and infected hamsters. As shown in Fig. 3D to F, we observed a 2-fold increase in the number of mitochondria per cell in the spleen of infected hamsters compared to cells from uninfected spleens. Importantly, we observed a significant increase in the number of cristae per mitochondrion in infected spleens compared to uninfected spleens. We also observed that *L. donovani*-containing vacuoles are surrounded by mitochondria (Fig. 3D), suggesting a repositioning of these organelles in infected cells. Collectively, these results are consistent with the notion that *Leishmania* induces mitochondrial biogenesis in host cells.

TLRs mediate host cell mitochondrial biogenesis induced by *L. donovani* metacyclic promastigotes. We previously showed that LPG is shed from the surface of internalized promastigotes and traffics out of the parasitophorous vacuole (34). However, we did not observe significant colocalization between LPG and mitochondria at various time points postinfection (Fig. S6), indicating that LPG acts on mitochondria through receptor-mediated signaling pathways. Several TLRs have been implicated in the recognition of *Leishmania* and *Leishmania*-derived components by various immune cell types and in the modulation of host cell responses to infection (35–39). We therefore sought to assess the contribution of TLRs to the induction of mitochondriogenesis triggered by *L. donovani* metacyclic promastigotes. Previous studies revealed that depending on the *Leishmania* species and immune cell types involved, LPG may be recognized by either TLR2, TLR4, or both (40–47). To determine whether these receptors are required for the induction of mitochondrial biogenesis, we infected BMM from WT, *Tlr2*$^{-/-}$, and *Tlr4*$^{-/-}$ mice with *L. donovani* metacyclic promastigotes and at 1 h and 8 h postphagocytosis, we assessed the mt/n (*MT-16S/HK2*) DNA ratio by qPCR. As shown in Fig. 4A, an increase in the mt/n DNA ratio induced by *L. donovani* metacyclic promastigotes was completely abrogated in the absence of TLR4, whereas a partial increase in the mt/n DNA ratio occurred in the absence of TLR2. Next, we evaluated by real-time quantitative PCR (RT-qPCR) the expression kinetics of the *PARGC1A* gene. As shown in Fig. 4B, induction of *PARGC1A* gene expression by *L. donovani* metacyclic promastigotes required TLR4, whereas TLR2 was dispensable. We included in our analyses

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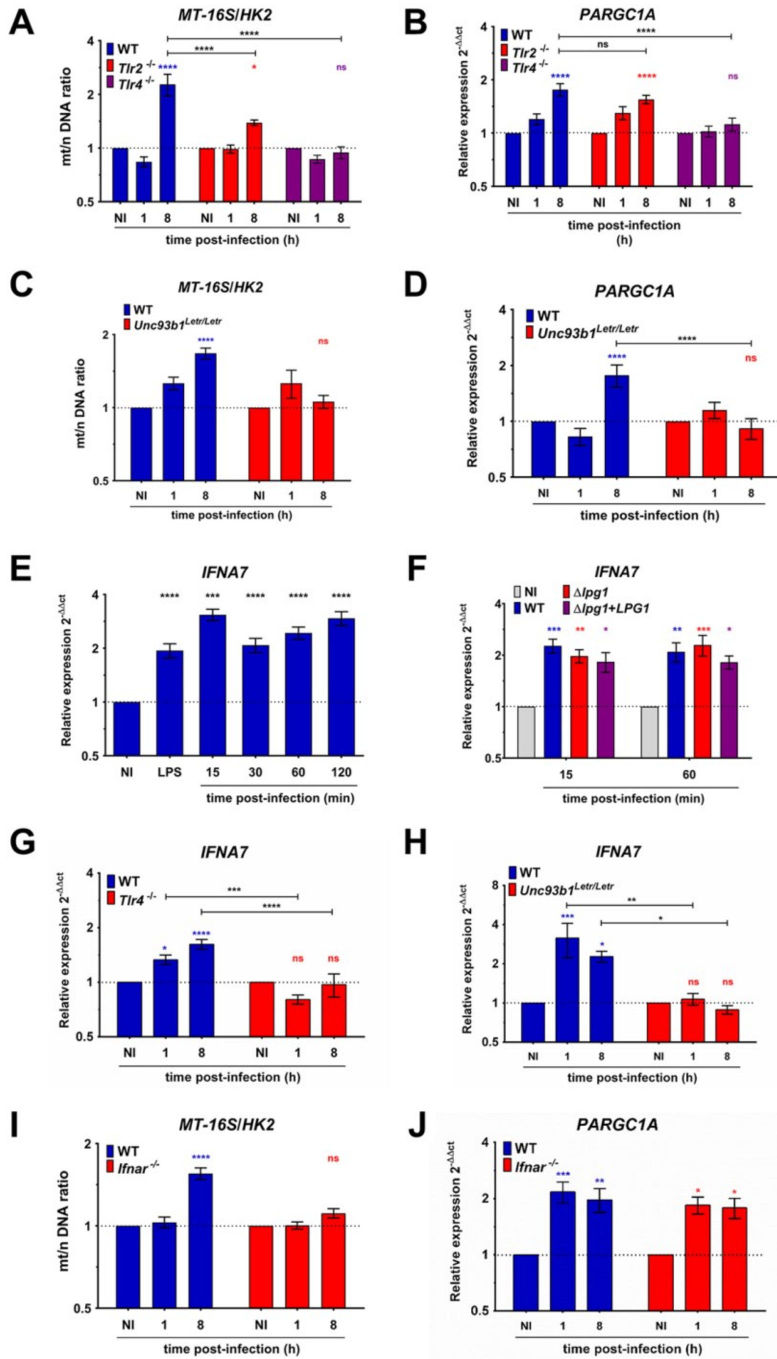

FIG 4 TLRs and the IFN-I signaling axis mediate host cell mitochondrial biogenesis induced by *L. donovani* metacyclic promastigotes. BMM from either WT, $Tlr2^{-/-}$, $Tlr4^{-/-}$, or $Unc93b1^{Letr/Letr}$ mice were infected with WT

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the expression of inducible nitric oxide synthase (iNOS), interleukin-6 (IL-6), and IL-10 using peptidoglycan (PGN) and LPS in BMM from *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ mice as controls for the validation of the TLR-dependent pathways (Fig. S7A and B). These results establish a role for TLR4 in mediating mitochondriogenesis in response to *L. donovani* metacyclic promastigotes. Endosomal TLRs also contribute to host cell responses triggered by *Leishmania* (48–53). To determine the potential role(s) of endosomal TLRs in mitochondriogenesis, we used BMM derived from *Unc93b1*$^{Letr/Letr}$ mice (54). This mutation in the *Unc93b1* gene precludes proper assembly and function of TLR3, TLR7, and TLR9. Expression of IL-6, IL-10, and hypoxia-inducible factor 1α (HIF-1α) is abrogated in BMM from *Unc93b1*$^{Letr/Letr}$ stimulated with poly(I·C) but not with LPS (Fig. S7C and D). As shown in Fig. 4C and D, *L. donovani* metacyclic promastigotes failed to increase the mt/n DNA ratio and *PARGC1A* gene expression in *Unc93b1*$^{Letr/Letr}$ BMM, indicating that similar to TLR4, endosomal TLRs are required for the induction of host cell mitochondrial biogenesis by *L. donovani* promastigotes.

Host cell mitochondrial biogenesis induced by *L. donovani* metacyclic promastigotes requires the type I IFN signaling pathway. Activation of TLRs leads to the production of type I IFNs in response to various pathogens, including *Leishmania* (47, 52, 53, 55–57). Given that type I IFNs were reported to modulate mitochondrial metabolism in plasmacytoid dendritic cells (58), we investigated the possibility that autocrine signaling triggered by these cytokines regulates mitochondrial biogenesis in response to *L. donovani*. Using RT-qPCR, we first obtained evidence that *L. donovani* metacyclic promastigotes rapidly induced the expression of *IFNA7* (which encodes IFN-α), but not of *IFNB1* (which encodes IFN-β) in BMM (Fig. 4E, Fig. S7E). The observation that WT, Δ*lpg1*, or Δ*lpg1*+*LPG1* *L. donovani* metacyclic promastigotes induced similar levels of *IFNA7* gene expression indicated that induction of IFN-α expression is LPG independent (Fig. 4F, Fig. S7F). Expression of *IFNA7* was accompanied by an increase in the secretion of IFN-α by BMM infected with *L. donovani* for 1 h, 8 h, and 24 h compared to uninfected BMM (Fig. S7G). The abolition of *IFNA7* gene expression observed in both *Tlr4*$^{-/-}$ and *Unc93b1*$^{Letr/Letr}$ BMM (Fig. 4G and H) suggested that TLR4 and endosomal TLRs act in concert to mediate the production of type I IFN in response to *L. donovani* metacyclic promastigotes. In contrast, the absence of TLR2 had no effect on the induction of *IFNA7* expression by *L. donovani* promastigotes (Fig. S7H). We next verified whether type I IFN participates in *L. donovani*-induced mitochondriogenesis, by infecting BMM derived from mice lacking the IFN-I receptor (*Ifnar*$^{-/-}$) (59). In the absence of the IFN-I receptor, the *L. donovani*-induced increase of the mt/n (*MT-16S/HK2*) DNA ratio was abrogated (Fig. 4I), whereas induction of *PARGC1A* gene expression remained unaffected (Fig. 4J). Since type I IFN signaling contributes to *L. donovani*-induced mitochondriogenesis, we assessed the contribution of this pathway in the modulation of host macrophage mitochondrial metabolism using live cell extracellular flux analysis. As shown in Fig. 5A to D and Fig. S8A to C, absence of the IFN-I receptor (*Ifnar*$^{-/-}$ BMM) had no effect on the *L. donovani*-induced changes in the various parameters of mitochondrial metabolism examined (OCR, ECAR, PER, OCR/ECAR, basal respiration, mitochondrial ATP production, maximal respiration), as well as on the phosphorylation of AMPK (Fig. 5E). These results indicate that *L. donovani* induces an LPG-independent expression of IFN-α, which acts in an autocrine manner to stimulate an increase in the mt/n DNA ratio.

FIG 4 Legend (Continued)

L. donovani metacyclic promastigotes. (A to D) At the indicated time points, the mt/n DNA ratio (*MT-16S/HK2*) and *PARGC1A* expression were determined. (E) BMM were infected with WT *L. donovani* metacyclic promastigotes, and at the indicated time points *IFNA7* expression was determined. (F) BMM were infected with either WT, Δ*lpg1*, or Δ*lpg1*+*LPG1* *L. donovani* metacyclic promastigotes, and at the indicated time points *IFNA7* expression was determined. (G and H) BMM from either WT, *Tlr4*$^{-/-}$, or *Unc93b1*$^{Letr/Letr}$ mice were infected with WT *L. donovani* metacyclic promastigotes, and at the indicated time points *IFNA7* expression was determined. (I and J) BMM from either WT or *Ifnar*$^{-/-}$ mice were infected with WT *L. donovani* metacyclic promastigotes, and at the indicated time points the (I) mt/n DNA ratio (*MT-16S/HK2*) and (J) *PARGC1A* expression were determined. The data are presented as mean values $\pm$ SEM from three independent experiments. (A to D and F to J) ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ according to a two-way ANOVA with Dunnett's multiple-comparation test. (E) ****, $P < 0.0001$; ***, $P < 0.001$ according to a one-way ANOVA with Dunnett's multiple-comparation test.

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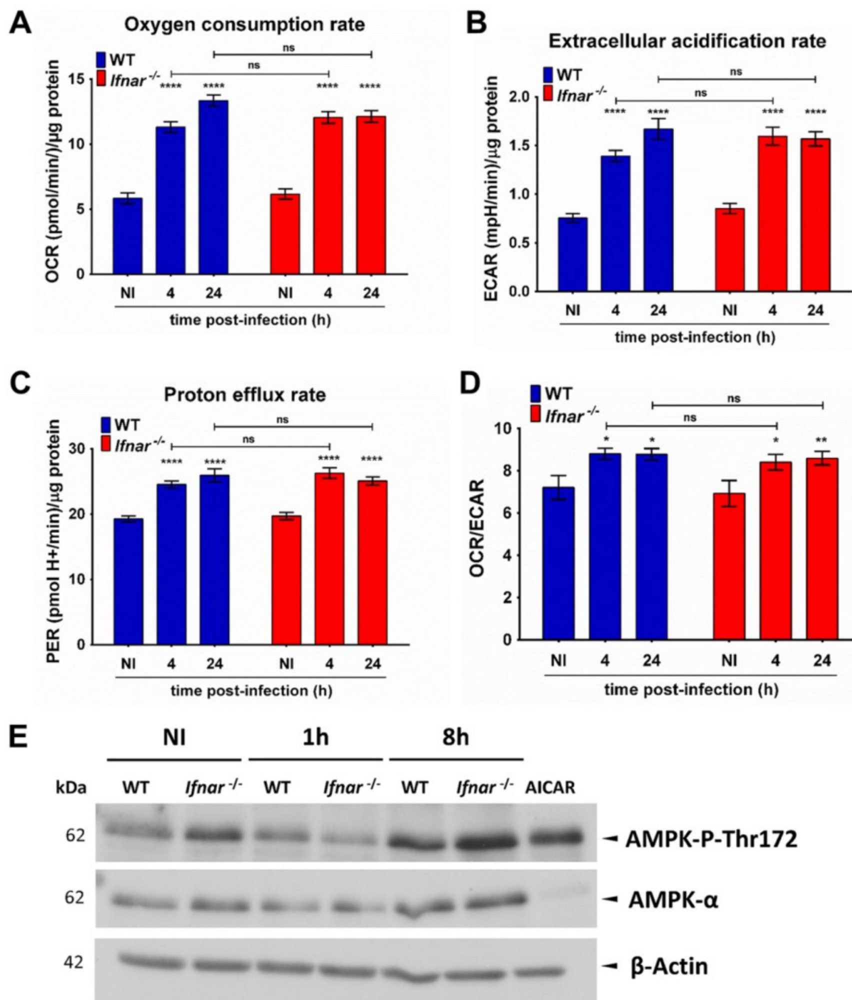

FIG 5 Alteration of host cell bioenergetic metabolism by *L. donovani* metacyclic promastigotes is independent of IFNAR. (A to D) BMM from either WT or *Ifnar* $^{-/-}$ mice were infected with WT *L. donovani* metacyclic promastigotes, and at 4 h and 24 h postinfection the OCR, ECAR, and PER and the OCR/ECAR ratio were determined. The readouts in each sample were normalized using the protein concentration, and the measurements were expressed as (OCR, ECAR and PER)/μg protein. (E) BMM from either WT or *Ifnar*$^{-/-}$ mice were infected with WT *L. donovani* metacyclic promastigotes, and at 1 h and 8 h postinfection the protein levels of AMPK-P-Thr172 and AMPK were determined by Western blot analysis. BMM were treated with 0.1 mM AICAR for 4 h. β-Actin was used as a loading control. Representative immunoblots for two independent experiments. The data are presented as mean values $\pm$ SEM from three independent experiments. (A to D) ****, $P < 0.0001$; **, $P < 0.01$; *, $P < 0.05$ according to a two-way ANOVA with Dunnett's multiple-comparation test.

Mitochondrial biogenesis contributes to the ability of *L. donovani* promastigotes to colonize BMM. We next investigated the biological relevance of LPG-induced mitochondrial biogenesis with respect to the ability of *L. donovani* metacyclic promastigotes to colonize macrophages. Since TLR4, endosomal TLRs, and the IFN-I receptor are essential for the induction of mitochondrial biogenesis (Fig. 4A to D and I to J), we compared the fate of WT *L. donovani* metacyclic promastigotes in wild-type, *Tlr4*$^{-/-}$, *Unc93b1*$^{Letr/Letr}$, and *Ifnar*$^{-/-}$ BMM. Given that TLR2 has no influence on mitochondrial biogenesis, we included *Tlr2*$^{-/-}$ BMM as a control. As shown in Fig. 6A and B, *L.*

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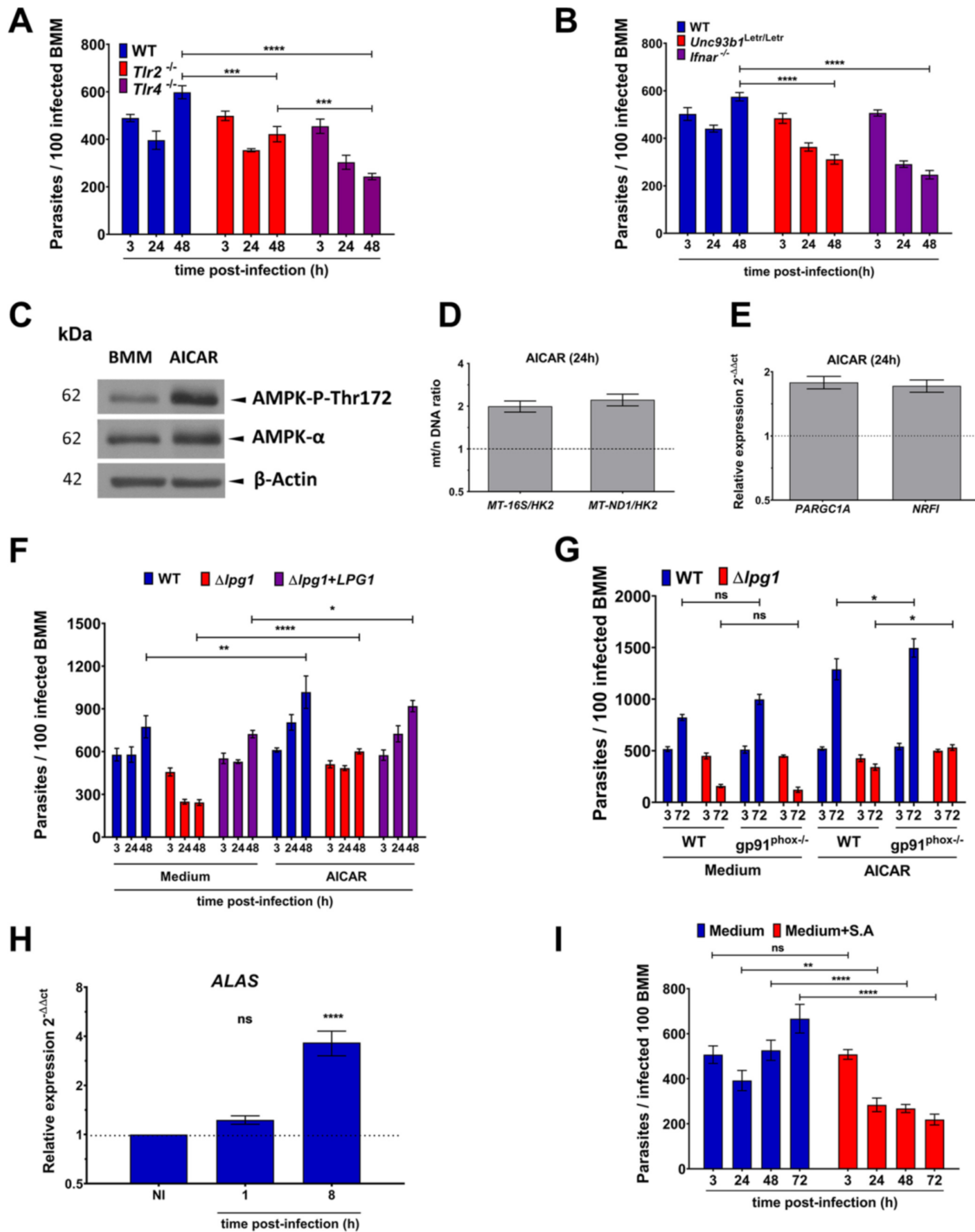

FIG 6 Mitochondrial biogenesis contributes to the ability of *L. donovani* promastigotes to colonize BMM. (A and B) WT, *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$, *Unc93b1*$^{Letr/Letr}$, or *Ifnar*$^{-/-}$ BMM were infected with *L. donovani* metacyclic promastigotes, and parasitemia was assessed at the indicated time points. (C) BMM were treated

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donovani metacyclic promastigotes replicated over 48 h postphagocytosis in wild-type BMM. The absence of TLR2 had no impact on the internalization and subsequent survival of *L. donovani*, although we observed a slight reduction in replication after 48 h in *Tlr2*$^{-/-}$ BMM compared to wild-type BMM. In contrast, survival of *L. donovani* metacyclic promastigotes and their subsequent replication was significantly impaired in the absence of TLR4, endosomal TLRs, or the IFN-I receptor. These results suggested that induction of mitochondrial biogenesis mediated by those TLRs and the IFN-I receptor contributes to the survival and replication of *L. donovani* in BMM. Since events downstream of those TLRs and the autocrine signaling triggered by IFN-α other than mitochondrial biogenesis may affect the outcome of *L. donovani* infection, we determined the fate of *L. donovani* in BMM in which mitochondriogenesis was pharmacologically induced prior to infection. To this end, we infected BMM pretreated with AICAR (60), an activator of AMPK which stimulates mitochondrial biogenesis as assessed by the increased mt/n DNA ratio (*MT-ND1/HK2* and *MT-16S/HK2*), expression of *PARGC1A* and *NRF1*, and phosphorylation of AMPK (Fig. 6C to E). As shown in Fig. 6F, at 24 h and 48 h postinfection, replication of both WT and Δ*lpg1*+*LPG1* metacyclic promastigotes was significantly increased in AICAR-treated BMM compared to unstimulated BMM. Strikingly, whereas the Δ*lpg1* mutant was cleared in unstimulated BMM, there was a significant increase in the parasite burden (3-fold) of this mutant in AICAR-treated BMM at 48 h postinfection compared to unstimulated BMM (Fig. 6F). A recent report indicated that AICAR significantly decreased the production of reactive oxygen species (ROS) in N-formylmethionyl-leucyl-phenylalanine (fMLF)-stimulated macrophages (61). To determine whether the increased survival of the Δ*lpg1* mutant in AICAR-treated BMM was related to impaired ROS production, we compared the fate of WT and Δ*lpg1* *L. donovani* metacyclic promastigotes in BMM from WT and gp91$^{phox-/-}$ mice pretreated or not with AICAR. As shown in Fig. 6G, at 72 h postinfection, the absence of gp91$^{phox-/-}$ had no significant impact on the fate of both WT and Δ*lpg1* *L. donovani* promastigotes in control BMM, whereas we observed a slight increase in the parasite burden in gp91$^{phox-/-}$ BMM pretreated with AICAR for both WT and Δ*lpg1* *L. donovani* metacyclic promastigotes. These results indicate that increased survival of the Δ*lpg1* mutant in AICAR-treated BMM is not related to an inhibition of ROS production. Moreover, these results suggest that pharmacological induction of mitochondrial biogenesis and stimulation of PGC-1α expression creates a metabolically adapted environment favorable to the replication of *L. donovani* and that enables the avirulent *L. donovani* Δ*lpg1* mutant to survive in BMM. These observations led us to explore the possible link between heme synthesis in the context of mitochondrial biogenesis (62) and the ability of *Leishmania* to establish infection. Heme synthesis is associated with increased electron transport chain and with enhanced OXPHOS (63–66). The first biosynthetic step occurs in the mitochondrion and is catalyzed by the 5-aminolevulinic acid synthase (ALAS1), which is the rate-limiting heme biosynthetic enzyme (67). Expression of ALAS1 is tightly regulated and is under the control of NRF-1 and PGC-1α (68). Similar to the induction of these two regulators of gene expression associated with mitochondrial biogenesis and metabolism (Fig. 2D, Fig. S3E), we found that *L. donovani* metacyclic promastigotes induce high *ALAS1* gene expression, as determined by RT-qPCR (Fig. 6H). These results indicate that *L. donovani* stimulates heme biosynthesis during host cell infection. To assess the impact of heme biosynthesis on the ability of *L. donovani* to replicate in BMM, we inhibited the

FIG 6 Legend (Continued)
with 0.1 mM AICAR for 4 h, and the levels of AMPK-P-Thr172 and AMPK were determined by Western blot analysis. β-Actin was used as a loading control. Representative immunoblots for two independent experiments. (D and E) BMM were incubated for 24 h with 0.1 mM AICAR and the (D) mt/n DNA ratio (*MT-16S/HK2* and *MT-ND1/HK2*) and (E) *PARGC1A* and *NRF1* expression were determined. (F) BMM were incubated with 0.1 mM AICAR for 4 h prior to infection with either WT, Δ*lpg1*, or Δ*lpg1*+*LPG1* *L. donovani* metacyclic promastigotes. At the indicated time points (with or without AICAR), parasite burden was assessed. (G) WT or gp91$^{phox-/-}$ BMM were incubated with 0.1 mM AICAR for 4 h prior to infection with either WT or Δ*lpg1* *L. donovani* metacyclic promastigotes. At the indicated time points (with or without AICAR), parasite burden was assessed. (H) BMM were infected with *L. donovani* metacyclic promastigotes, and at the indicated time points *ALAS1* expression was assessed. (I) BMM were infected with *L. donovani* metacyclic promastigotes in the absence or presence 100 μM S.A., and parasite burden was assessed at the indicated time points. The data are presented as means values $\pm$ SEM from three independent experiments. (A, E, and F,) ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ according to a two-way ANOVA with Dunnett's multiple-comparation test.

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second heme biosynthetic enzyme, aminolevulinic acid dehydratase (ALAD), which catalyzes the conversion of 5-aminolevulinic acid into porphobilinogen. To this end, BMM were incubated with 100 μM succinylacetone 4 h prior to infection, and the inhibitor was maintained for 72 h postinfection. At this concentration, succinylacetone had no effect on the growth of *L. donovani* promastigotes *in vitro* (data not shown). As shown in Fig. 6I, inhibition of ALAD prevented the replication of *L. donovani*, indicating that heme biosynthesis during mitochondrial biogenesis contributes to the ability of this parasite to colonize macrophages.

DISCUSSION

In the present study, we investigated the mechanisms by which *L. donovani* metacyclic promastigotes alter host cell mitochondrial biology. We describe an essential role for the virulence glycolipid LPG in the stimulation of OXPHOS and in the induction of mitochondrial biogenesis in infected macrophages. Additionally, we demonstrate that mitochondriogenesis requires the action of type I IFN, which is induced independently of LPG. Coupled to the observation that pharmacological induction of mitochondrial biogenesis increases the permissiveness of macrophages to *L. donovani*, this study supports the notion that mitochondrial biogenesis creates a metabolically adapted environment propitious to the replication of the parasite.

Given the importance of LPG for *Leishmania* promastigotes to colonize their host cells (26–28, 69–72), we sought to determine the potential role of this virulence glycolipid in the modulation of host cell mitochondrial biogenesis and metabolism. As previously reported for *L. infantum* (9), we found that *L. donovani* metacyclic promastigotes stimulate OXPHOS, enhance macrophage mitochondrial mass, and induce the expression of PGC-1α and NRF1 as well as respiratory gene expression. Taking advantage of a genetically and structurally defined *L. donovani* mutant defective in the synthesis of LPG (Δ*lpg1*) and of its complement counterpart (Δ*lpg1*+*LPG1*), we obtained evidence that this molecule is essential for the profound changes in host cell mitochondrial biology induced by *L. donovani* promastigotes. Thus, in addition to its important role in turning off key host defense processes to protect promastigotes against the microbicidal capacity of phagocytic cells (27, 28, 69–72), our work revealed that modification of host cell metabolism represents an important function for LPG. Similar to metacyclic promastigotes, we observed that splenic amastigotes efficiently stimulate mitochondrial biogenesis in BMM and in the spleen of infected hamsters. Previous reports indicated that splenic *L. donovani* amastigotes express reduced levels of LPG compared to promastigotes (73, 74) and that they express LPG-like glycolipids which share several chemical and structural properties with promastigote LPG (74). The observation that Δ*lpg1 L. donovani* amastigotes failed to trigger increase in the mt/n (*MT-16S/HK2*) DNA ratio and in *PARGC1A* gene expression suggests that the levels of LPG or LPG-like glycolipids expressed by amastigotes are sufficient to stimulate mitochondrial biogenesis. Previous reports identified TLR2 and TLR4 as the receptors responsible for the recognition of LPG from various *Leishmania* species (40, 42, 44, 46, 75–77). Our findings indicate that TLR4 is essential for the stimulation of PGC-1α expression and of mitochondrial biogenesis and are consistent with a role for this receptor in the recognition of LPG. However, LPG by itself is not sufficient to stimulate PGC-1α expression and mitochondrial biogenesis, indicating that additional factors are necessary. Hence, we found that mitochondrial biogenesis requires an LPG-independent pathway linked to the expression of type I IFN. Interestingly, this pathway also involves TLR4, suggesting that this receptor plays a dual role in the control of mitochondrial biogenesis. Although we have not investigated the ligand(s) responsible for TLR4-mediated induction of type I IFN, previous studies evidenced a key role for the neutrophil elastase-TLR4 pathway in this process (47). Using BMM from *Unc93b1*$^{Letr/Letr}$ mice (54), we found that in addition to TLR4, induction of mitochondrial biogenesis and of type I IFN expression by *L. donovani* metacyclic promastigotes requires endosomal TLRs. Several studies have highlighted the contribution of these receptors in the host response to various *Leishmania* species (37–39, 41, 48–52,

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78). Recent work revealed that TLR3 is the endosomal TLR that mediates type I IFN expression in response to *L. donovani* (53), consistent with the defective type I IFN expression in *L. donovani*-infected *Unc93b1*$^{Letr/Letr}$ BMM. Little is known concerning the nature of *Leishmania* ligand(s) recognized by TLR3, with the exception of the double-stranded RNA virus LRV1 (48) present in isolates of various *Leishmania* species (79). It is noteworthy that TLR3 contributes to the recognition of *L. donovani* promastigotes despite the fact that these parasites do not harbor double-stranded RNA viruses (41, 53). The nature of the *L. donovani*-derived ligand(s) recognized by TLR3 thus remains to be elucidated. One possibility is that extracellular vesicles containing RNA (80) are released within the parasitophorous vacuoles and activate endosomal TLRs. Alternatively, TLR3 may be activated by RNA released by apoptotic parasites present in metacyclic promastigote populations (81).

In the absence of IFNAR, *L. donovani* promastigotes fail to induce mitochondrial biogenesis, consistent with the notion that type I IFN acts in an autocrine manner in this process. The fact that *L. donovani*-induced mitochondrial biogenesis does not take place in *Ifnar*$^{-/-}$ BMM despite the induction of PGC-1α expression illustrates the complexity of the pathways involved in this process and highlights the multiple roles of PGC-1α in the modulation of energetic metabolism. Interestingly, our results indicate that type I IFN signaling does not play a significant role in the stimulation of OXPHOS and glycolysis in *L. donovani*-infected macrophages. This contrasts with the recent findings with *Mycobacterium tuberculosis* infection, which is characterized by a decrease in both glycolysis and mitochondrial respiration (24). In that study, the authors found that type I IFN is directly responsible for the reduced macrophage energy metabolism during *M. tuberculosis* infection, suggesting that the effects of type I IFN are pathogen and context specific.

Several studies have highlighted a protective role for type I IFN in leishmaniasis (30, 82, 83). However, accumulating evidence indicates that type I IFN signaling also plays a detrimental role for the host, favoring intracellular parasite replication (47, 52, 53, 56, 83–86). Consistently, targeting type I IFN during anti-*Leishmania* drug treatment was shown to improve Th1 cell-mediated immunity (87). Based on our results, it is tempting to speculate that type I IFN contributes to the ability of *Leishmania* to proliferate within its host through the stimulation of mitochondrial biogenesis. In this regard, our results support the notion that induction of mitochondriogenesis by *L. donovani* promastigotes is important for the host cell colonization process. Indeed, in agreement with previous reports (47, 53), survival and replication of *L. donovani* was markedly impaired in TLR4-, endosomal-, and IFNAR-deficient macrophages, in which the parasite fails to induce mitochondrial biogenesis. Additionally, pharmacological induction of mitochondrial biogenesis significantly increased the permissiveness of macrophages to *L. donovani* replication, suggesting that induction of mitochondrial biogenesis creates a metabolically adapted environment propitious to the replication of the parasite. Similar findings were previously reported for *L. infantum*-infected BMM (9). This contrasts with several pathogens, including *Mycobacterium tuberculosis*, *Haemophilus parasuis*, *Staphylococcus aureus*, and *Plasmodium falciparum*, whose survival and replication were impaired by AICAR pretreatment of their host cells (88–90). Strikingly, the avirulent LPG-defective *L. donovani* Δ*lpg1* mutant, which does not induce mitochondrial biogenesis, survived in BMM pretreated with AICAR. This finding suggests that pharmacological stimulation of mitochondrial biogenesis bypasses the requirement for LPG to create conditions favorable to the parasite's development within host cells.

Because heme is an essential cofactor for several enzymes of the electron transport chain, its synthesis plays a central role in mitochondrial biology and in OXPHOS complex formation and function (63–66). Hence, the first and rate-limiting step of heme synthesis occurs in mitochondria and is catalyzed by ALAS1 to generate 5-aminolevulinic acid (67). Expression of ALAS1 is tightly regulated and is under the control of PGC-1α (68), which also controls mitochondrial biogenesis and oxidative metabolism (13–15, 17). Our findings that *L. donovani* promastigotes induce *ALAS1* expression and that pharmacological

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inhibition of the second heme biosynthetic step impaired the ability of *L. donovani* to replicate within host macrophages support the importance of this parasite to induce mitochondrial biogenesis and of OXPHOS for its development within mammalian hosts. Additionally, *Leishmania* is a heme auxotroph that must acquire heme or heme precursors from the host to develop intracellularly (91). Activation of heme biosynthesis upon host cell colonization may thus also serve to fulfill the heme requirement of *Leishmania* (92). Future studies will be required to elucidate this issue.

In sum, we provide novel information on the mechanisms leading to mitochondrial biogenesis and metabolic reprograming in macrophages infected with *L. donovani*. Our results are consistent with the notion of pathogen-specific metabolic rewiring (21), which results from the intricate interplay between complex sets of pathogen molecules and host cell receptors.

MATERIALS AND METHODS

Ethics statement. Animal work was conducted in accordance with protocols 1706-06 and 1706-07, which were approved and defined by the Committee Institutionel de Protection des Animaux of the INRS-Armand-Frappier Santé Biotechnologie. These protocols respect the procedures on animal practice stipulated by the Canadian Council on Animal Care (CCAC).

Animals and parasites. C57BL/6 (JAX stock no. 000664), *Tlr2*$^{-/-}$ ([93], JAX stock no. 004650), *Tlr4*$^{-/-}$ (JAX stock no. 029015), and gp91$^{phox-/-}$ (JAX stock no. 002365) female and male mice were purchased from The Jackson Laboratories. *Ifnar*$^{-/-}$ (59) (kindly provided by Alain Lamarre, Institut National de la Recherche Scientifique) and *Unc93b1*$^{Letr/Letr}$ mice (54) (kindly provided by Salman Qureshi, McGill University) were bred and housed at the Institut National de la Recherche Scientifique animal facility under specific-pathogen-free conditions and used at 8 to 12 weeks of age. Female HsdHan:AURA hamsters of 4 to 6 weeks of age were purchased from Harlan Sprague Dawley, Inc. Promastigotes of *L. donovani* (MHOM/ET/67/Hu3:LV9), *L. major* NIHS (MHOM/SN/74/Seidman), *L. amazonensis* LV79 (MPRO/BR/72/M 1841), and *L. mexicana* (MNYC/BZ/62/M379) were cultured in *Leishmania* medium (M199 medium supplemented with 10% heat-inactivated fetal bovine serum [FBS] [HyClone], 100 μM hypoxanthine, 10 mM HEPES, 5 μM hemin, 3 μM biopterin, 1 μM biotin, penicillin [100 U/mL], and streptomycin [100 μg/mL]) at 26°C. The isogenic *L. donovani* Δ*lpg1* mutant (71) was cultured in M199 medium supplemented with hygromycin (100 μg/mL), and its complemented counterpart *L. donovani* Δ*lpg1*+*LPG1* (71) was cultured in M199 medium supplemented with hygromycin (100 μg/mL) and zeocin (100 μg/mL). Amastigotes of *L. donovani* (MHOM/ET/67/Hu3:LV9) were isolated from the spleens of hamsters infected 8 to 12 weeks earlier with 1.5×10^8 amastigotes by intraperitoneal inoculation (94). To isolate *in vitro* amastigotes, AICAR-pretreated BMM were infected with either WT or Δ*lpg1 L. donovani* for 24 h and were lysed for 5 min in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.05% SDS.

Macrophage culture and infections. Marrow was extracted from the femurs and tibias of 8- to 12-week-old male and female mice and differentiated for 7 days into bone marrow-derived macrophages (BMM) in Dulbecco's modified Eagle's medium with glutamine (DMEM; Thermo Fisher Scientific) containing 10% heat-inactivated fetal bovine serum (FBS; HyClone), 10 mM HEPES, pH 7.4, penicillin (100 IU/mL) and streptomycin (100 μg/mL) and supplemented with 15% (vol/vol) L929 cell-conditioned medium as a source of colony-stimulating factor-1 (CSF-1) in a 37°C incubator with 5% CO_2. BMM were made quiescent by culturing them in DMEM without CSF-1 for 24 h prior to infection or pharmacological treatments. For infections, metacyclic promastigotes were enriched from late stationary-phase cultures using Ficoll gradients, as previously described (95). Complement opsonization of metacyclic promastigotes, heat-killed metacyclic promastigotes, polystyrene beads, zymosan, and LPG-coated zymosan was performed prior to macrophage internalization through incubation in Hank's balanced salt solution (HBSS) containing 10% C5-deficient serum from DBA/2 mice for 30 min at 37°C. Adherent BMM were then incubated at 37°C with metacyclic promastigotes or particles, and after 3 h of incubation, noninternalized parasites were removed by washing three times with warm HBSS. Since LPG may reduce the phagocytosis of *Leishmania* promastigotes, we ensured that similar levels of infection were achieved by infecting BMM at multiplicities of infection (MOIs) of 7:1 for WT parasites, 6:1 for the Δ*lpg1* mutant, and 8:1 for the Δ*lpg1*+*LPG1*. A particle-to-cell ratio of 7:1 was used for heat-killed parasites, polystyrene beads, and zymosan or LPG-coated zymosan per BMM unless otherwise specified. For infection or treatments shorter than 3 h, noninternalized parasites or particles were removed by washing three times with warm HBSS at the specified time postphagocytosis and immediately processed. Intracellular parasitemia was assessed at the indicated time point by counting the number of parasites per 100 infected BMM upon staining with the Hema 3 staining kit. For pharmacological treatments, BMM were incubated with the following compounds for the indicated time points: 100 ng/mL LPS (*Escherichia coli*, strain 0127: B8, Sigma) for 6 h, 0.1 mM 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR, Sigma) for 4 h and 24 h, 10 μg/mL PGN (BioChemika) for 18 h, 10 μg/mL poly(I·C) (Sigma) for 6 h, and 100 μM succinyl acetone (S.A., Sigma) for 4 h.

Confocal immunofluorescence microscopy. BMM were seeded in 24-well plates containing microscope coverslips (Fisher Scientific) and infected with either WT, Δ*lpg1*, or Δ*lpg1*+*LPG1 L. donovani* metacyclic promastigotes or were fed zymosan and LPG-coated zymosan and amastigotes for the indicated time points. Cells were washed with phosphate-buffered saline (PBS), fixed with 3.7% paraformaldehyde

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(PFA) for 30 min, and then permeabilized in 0.1% Triton X-100 for 5 min. Then, the samples were blocked in 10% bovine serum albumin for 1 h. Cells were incubated for 1 h using an anti-Tom20 rabbit polyclonal antibody (1:200) and mouse monoclonal antibody (CA7AE, Cedarlane). BMM were next incubated with an appropriate combination of secondary antibodies (anti-rabbit Alexa Fluor 568, 1:500, and anti-IGM Alexa Fluor 488, 1:500) for 1 h. Macrophage and promastigote nuclei were stained with DAPI (Molecular Probes). Coverslips were washed three times with PBS after every step, and all steps were performed at room temperature. Analyses of Tom20 distribution were performed on a LSM780 confocal microscope (Carl Zeiss Microimaging) using Plan Apochromat ×63 oil-immersion differential interference contrast (DIC) (NA 1.64) objective, and images were acquired in sequential scanning mode. Images were processed with ZEN 2012 software. At least 30 cells per condition were analyzed using Icy image analysis software, and statistical differences were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's posttests (three groups). Data were considered statistically significant when P was <0.05, and graphs were plotted with GraphPad Prism 5.

Quantitative PCR analysis. Total RNA from BMM was isolated using an RNeasy minikit (Qiagen) according to the manufacturer's protocol. RNA (2 μg) was reverse transcribed using Oligo(dT) 12-18 primer (Invitrogen), and real-time quantitative PCR (RT-qPCR) experiments were performed in independent biological replicates (at least 3 replicates); reactions were run in at least duplicate for each sample using iTaq Universal SYBR green supermix (Bio-Rad) on a Stratagene mx3005p real-time PCR system using 10 ng cDNA. Gene expression changes were analyzed using the comparative threshold cycle (CT) method ($\Delta\Delta CT$) (96). Relative mRNA amounts were normalized to the Rps29 gene and expressed as the fold increase compared to noninfected controls. To determine the mitochondrial/nuclear (mt/n) DNA ratio, total BMM DNA was extracted using a DNeasy blood and tissue kit. Quantitative PCR (qPCR) experiments were performed in independent biological replicates (at least 3 replicates), and reactions were run at least in duplicates for each sample using iTaq Universal SYBR green supermix (Bio-Rad) on a Stratagene mx3005p real-time PCR system using 10 ng DNA. The amount of mtDNA present per nuclear genome was determined using the comparative CT method ($\Delta\Delta CT$) (96). Relative mtDNA amounts were normalized to the hexokinase gene and expressed as the fold increase compared to noninfected controls. The DNA and RNA concentrations were determined by optical density at 260 nm (OD_{260}) measurement using a NanoDrop spectrophotometer. The complete list of primers used is shown in Table S1.

Western blot analysis. Prior to lysis, adherent BMM were placed on ice and washed 3 times with PBS containing 1 mM sodium orthovanadate and 5 mM 1,10-phenanthroline (Sigma). Cells were scraped in the presence of lysis buffer containing 1% NP-40, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8), 10 mM 1,10-phenanthroline, and phosphatase and protease inhibitors (Roche). After 24 h of incubation at −70°C, lysates were centrifuged for 30 min, and the soluble phase was collected. After protein quantification, 20 μg of protein was boiled (100°C) for 5 min in SDS sample buffer and migrated in SDS-PAGE gels. Proteins were transferred onto Hybond-ECL membranes (Amersham Biosciences), blocked for 1 h in Tris-buffered saline (TBS) 1×-0.1% Tween containing 5% BSA, and incubated with primary antibodies (diluted in TBS 1×-0.1% Tween containing 5% BSA) overnight at 4°C and then with suitable horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Membranes were incubated in ECL (GE Healthcare), and immunodetection was achieved via chemiluminescence. Densitometric analysis of Western blot bands was done using ImageJ software. Primary antibodies were directed against total AMPKα (23A3, Cell Signaling), AMPKα phosphorylated at Thr172 (2531, Cell Signaling), Tom20 (Abcam), NDUFA 9 (Abcam), antiphosphoglycan (Galβ1,4Manα1-PO_4) mouse monoclonal antibody (CA7AE, Cedarlane), anti-*L. donovani* aldolase rabbit polyclonal antibody (kind gift from A. Jardim), and β-actin (Cell Signaling).

ELISA. IFN-α levels in supernatants were measured using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol. Each treatment was tested in duplicate. A mouse IFN-α SimpleStep ELISA kit (catalog [cat.] no. ab252352) was purchased from Abcam (Burlingame, CA, USA).

Electron microscopy. Uninfected and *L. donovani*-infected spleens were recovered, and 2-mm^2 samples were fixed with 1.3% (wt/vol) osmium tetroxide in collidine buffer for 1 h. The dehydration process was done in 25%, 50%, 75%, and 95% solutions of acetone/ethanol in water for 30 min each, followed by two changes of pure acetone/ethanol for 30 min each. Then, the samples were immersed for 16 to 18 h in Spurr:acetone (1:1). After that, the samples were immersed in two successive baths of Spurr mixtures for 2 h each. The samples were cut into smaller pieces, placed in BEEM capsules, filled using SPURR mixtures, and left to stand at room temperature for 18 h. Then, the filled capsules were placed at 60° to 65°C for 30 h to polymerize the resin. The polymerized resins were cut into ultrathin sections on an ultramicrotome and put onto a Formvar- and carbon-covered copper 200-mesh grid. The samples were stained with uranyl acetate in 50% ethanol for 15 min, followed by lead citrate for 5 min. Image acquisitions were made using an electronic microscope (Hitachi H-7100) with an AMT camera.

Metabolism assays. The bioenergetic profile of *L. donovani*-infected BMM was analyzed using an XF-96 extracellular flux analyzer (Seahorse Bioscience). The oxygen consumption rate (OCR), extracellular acidification rate (ECAR), and proton efflux rate (PER) were determined at the indicated time postinfection. BMM were seeded at 8×10^4 cells/well in 100 μL of DMEM in XF-96 cell culture plates, and after an overnight incubation period, cells were infected with WT, Δ*lpg1*, and Δ*lpg1*+*LPG1 L. donovani* metacyclic promastigotes. One hour before the defined times of infection, the cells were washed and the medium was changed to XF medium (buffered DMEM supplemented with 4.5 g/L glucose, 2% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin). The real-time measurement of the bioenergetic profile was obtained under basal conditions and in response to oligomycin (1 μM), fluoro-carbonyl cyanide phenylhydrazone (FCCP, 2 μM), rotenone (1 μM), and antimycin A (1 μM). The nonmitochondrial respiration was obtained by subtracting the rotenone/antimycin A values. The spare respiratory capacity (SRC) was

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obtained by subtracting FCCP from basal OCR values, and the glycolytic capacity was defined as the variation between oligomycin and basal ECAR values. The procedure used in the experiments was established according to the manufacturer's (Seahorse) instructions. Glucose dependence was assessed using 2-deoxyglucose (2-DG, 50 mM), mitochondrial β-oxidation dependence was assessed using etomoxir (4 μM), glutaminolysis dependence was assessed using BPTES (3 μM), and peroxisomal β-oxidation dependence was assessed using thioridazine (1 μM). In all cases, the treatments were done 30 min prior the readouts of the specific time point.

Statistics and reproducibility. GraphPad Prism 6 software was used to generate the graphs and statistical analyses. All experiments were conducted three independent times, unless otherwise specified in the figure legends. Methods for statistical tests, exact value of *n*, and definition of error bars are indicated in the figure legends; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. All immunoblots and images shown are representative of these independent experiments.

Data availability. All data generated or analyzed during this study are included in this published article and its supplemental information files.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, TIF file, 0.9 MB.
FIG S2, TIF file, 1.3 MB.
FIG S3, TIF file, 1.7 MB.
FIG S4, JPG file, 0.7 MB.
FIG S5, JPG file, 0.9 MB.
FIG S6, JPG file, 0.8 MB.
FIG S7, JPG file, 0.9 MB.
FIG S8, JPG file, 0.7 MB.
TABLE S1, TIF file, 1.1 MB.

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We declare no competing interests.

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Fig S1

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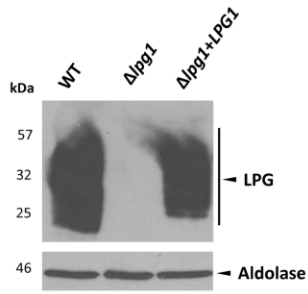

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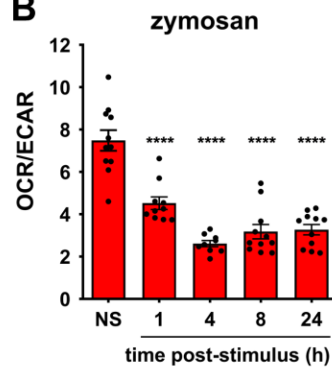

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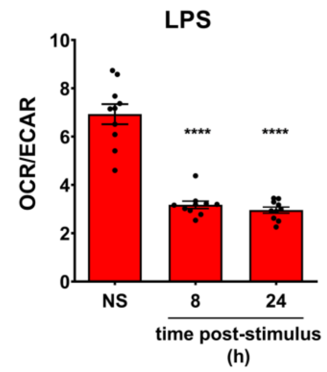

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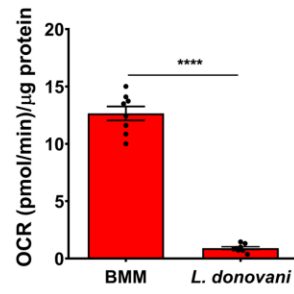

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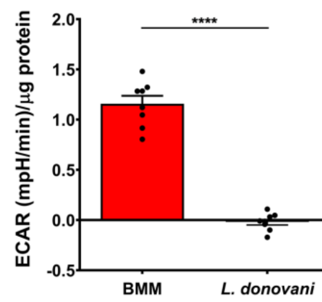

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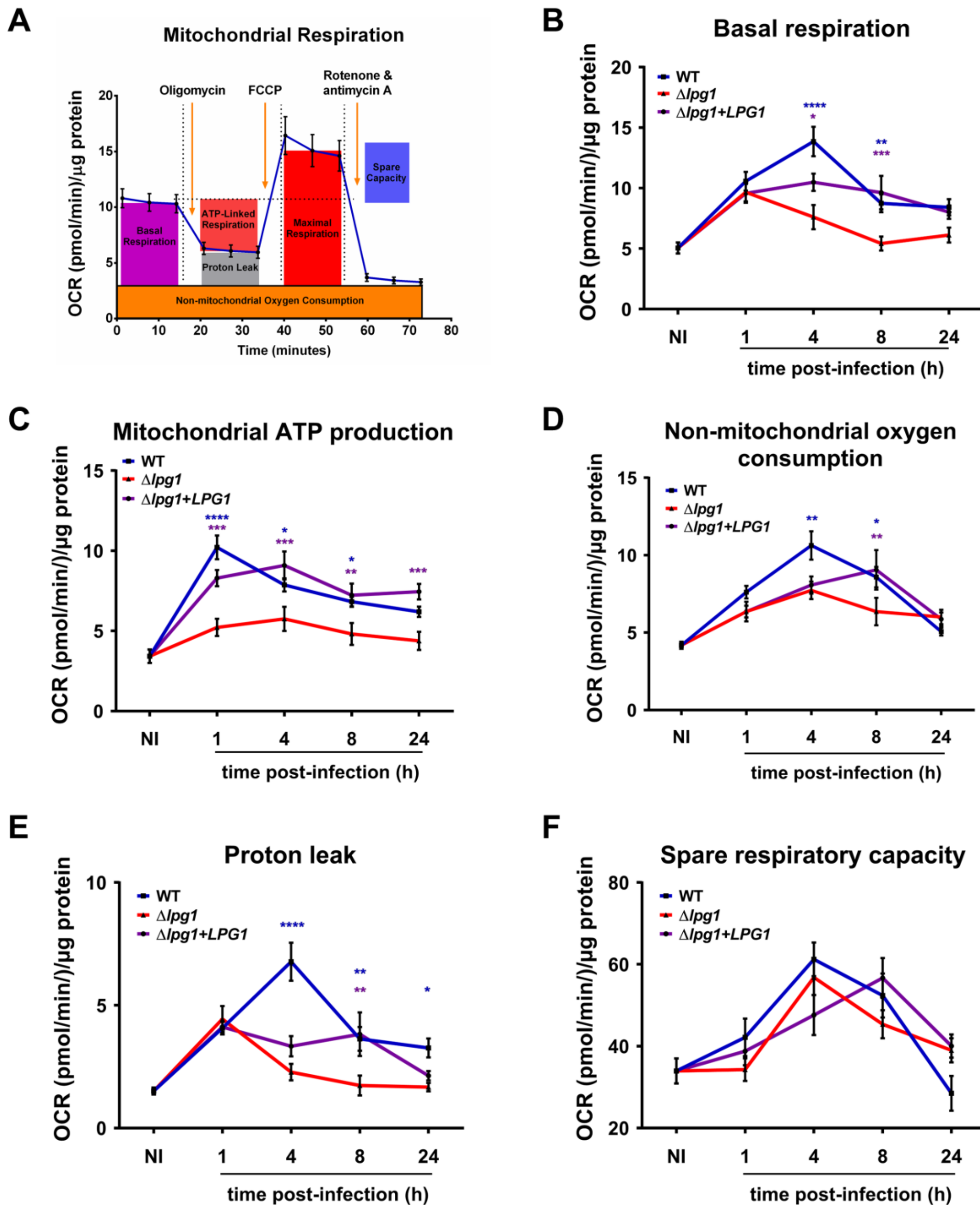

Fig S3

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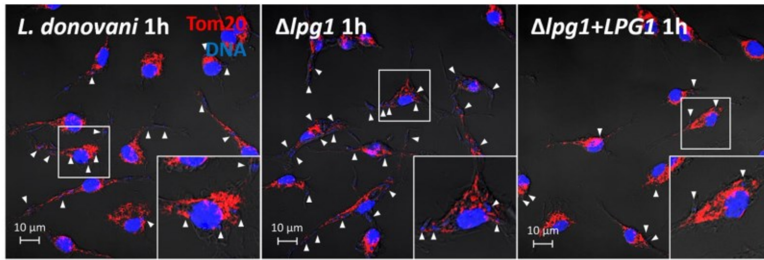

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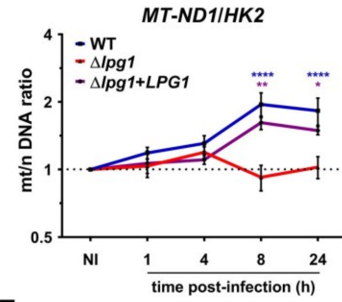

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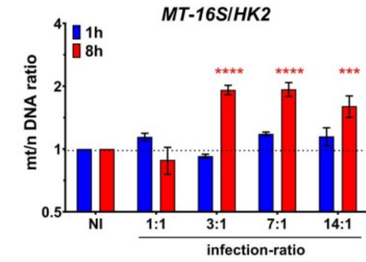

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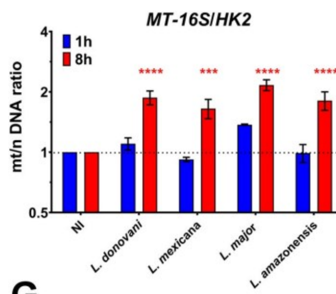

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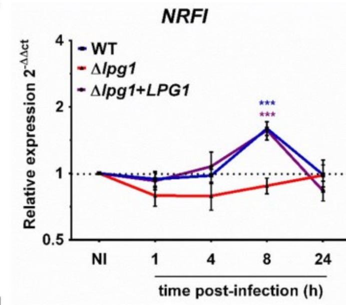

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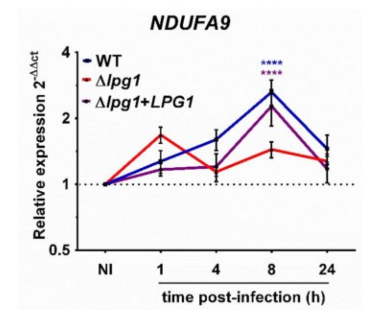

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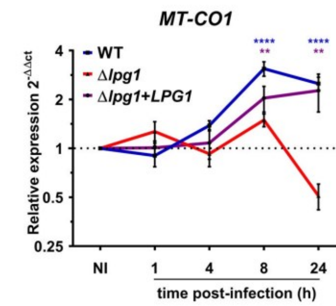

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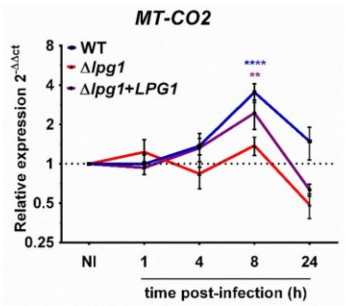

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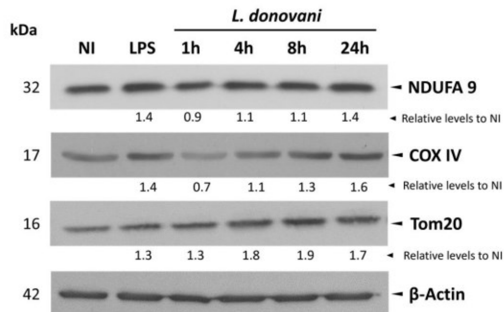

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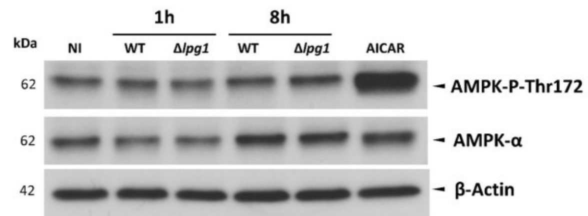


Fig S4

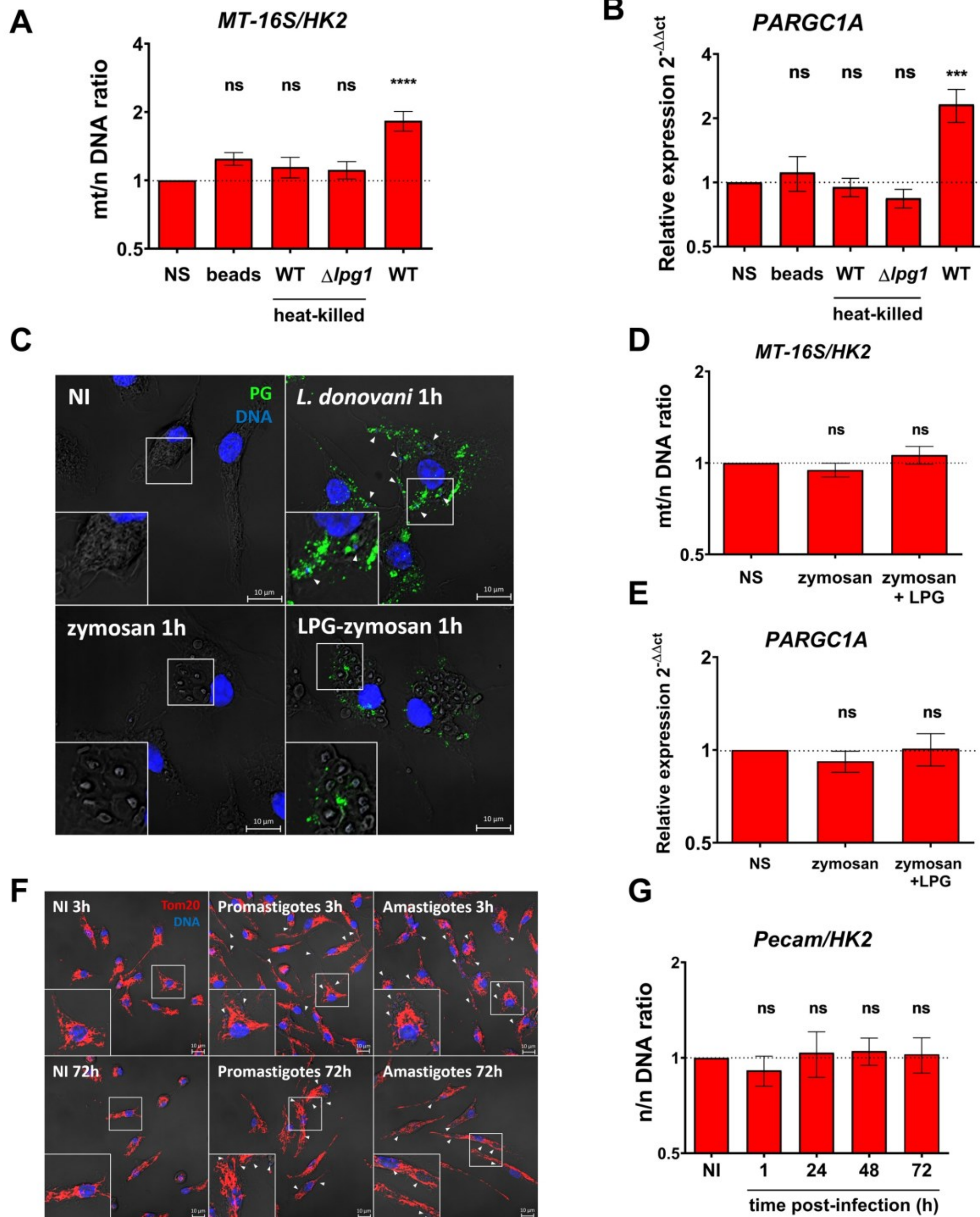
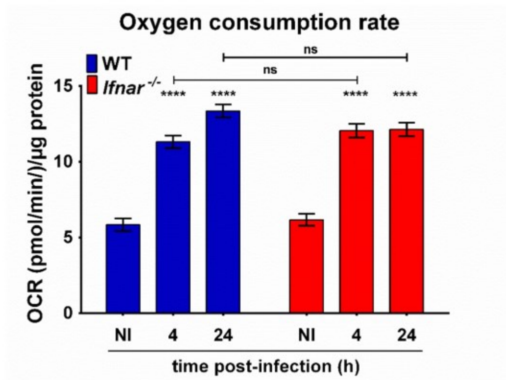


Fig 5

A



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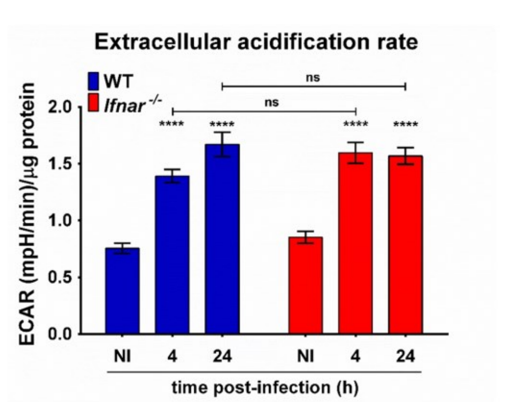

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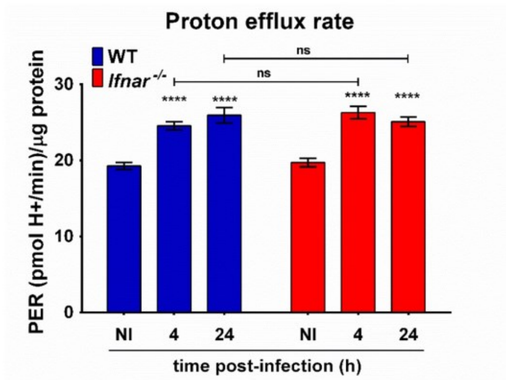

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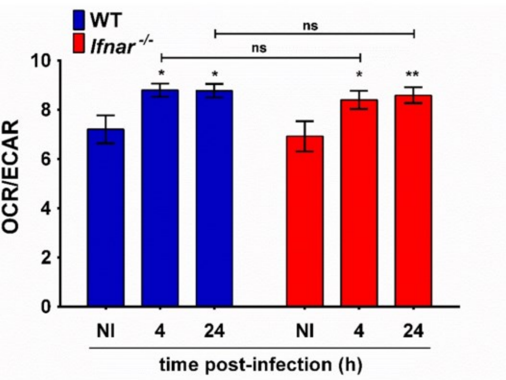

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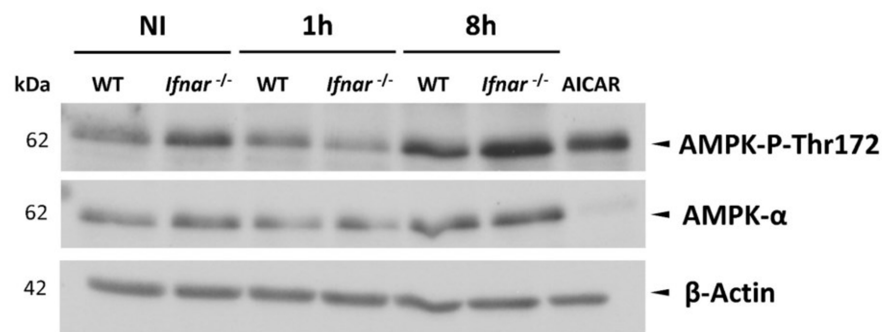


Fig S6

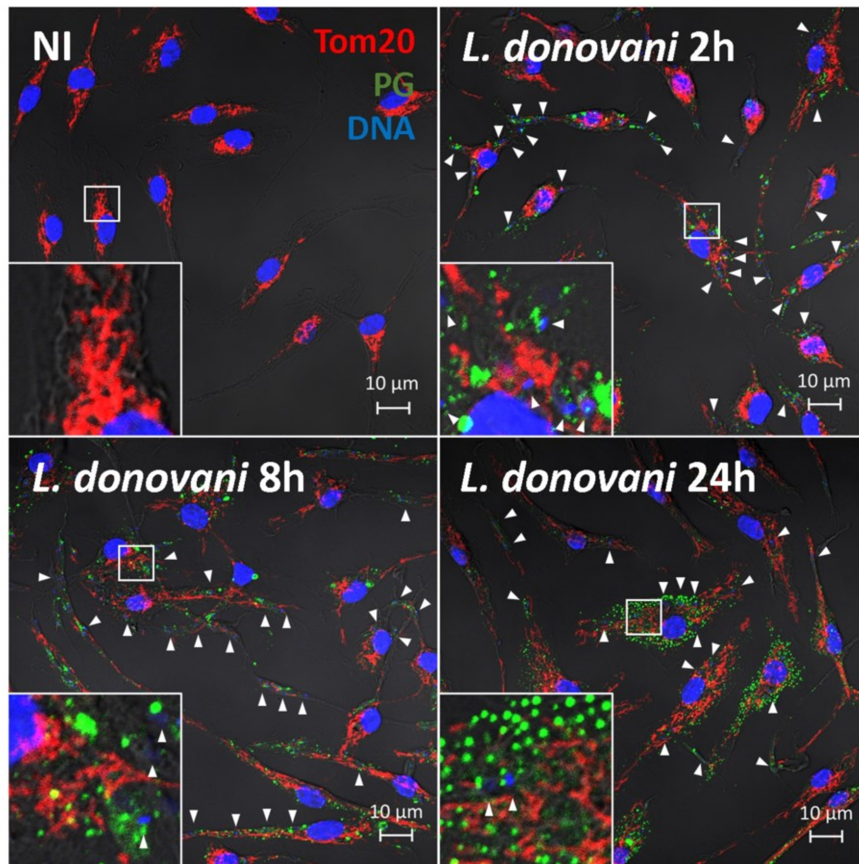


Fig S7

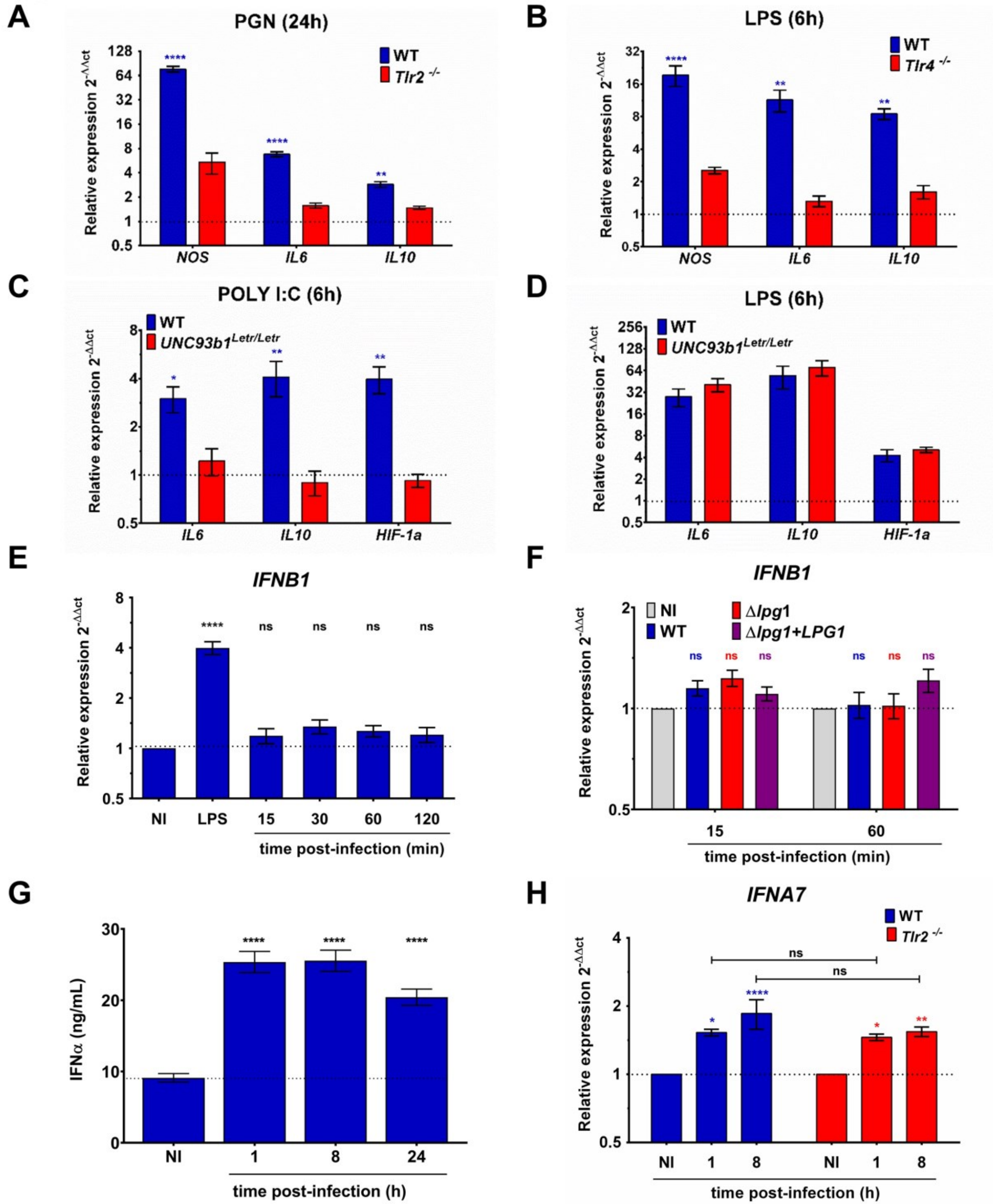
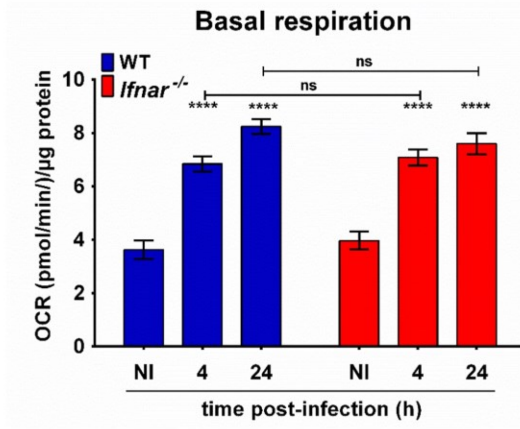
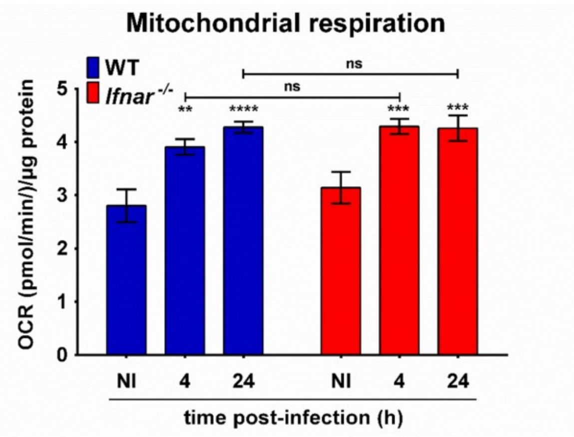
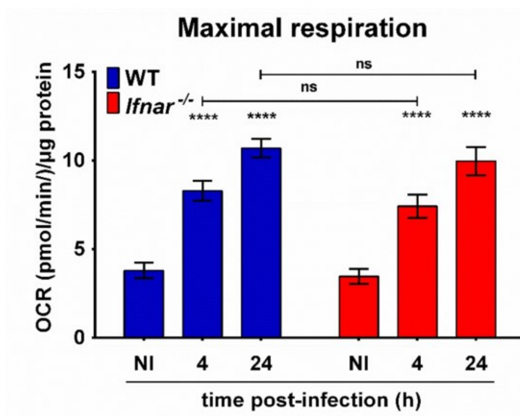


Fig S8







S1 table

RT-qPCR	Forward Sequence	Reverse Sequence
Rps29	CACCCAGCAGACAGACAAACTG	GCACTCATCGTAGCGTTCCA
PARGC1A	AGCCGTGACCACTGACAACGAG	GCTGCATGGTTCTGAGTGCTAAG
NRF1	CCATCTATCCGAAAGAGACAGC	GGGTGAGATGCAGAGTACAATC
COX4I1	TGGCAAGAGAGCCATTTCTACT	GTGGGGAAAGCATAGTCTTCAC
NDUFA9	TGGGAGAGAAGGAAGTGAGAAG	CACAGCAAGAAACCAACGATAA
MT-ND1	CAGGCCCTTGGACACATAGT	GTCCACTGCGTACATCCACA
MT-CO1	GTGCTGGGGCAGTGCTGGAG	TGGGGCCTGAGTAGCCCGTG
MT-CO2	CCACTTCAAGGGAGTCTGGA	AGTCATCTGCTACGGGAGGA
IFNA7	CATCTGCTGCTTGGGATGGAT	TTCCTGGGTCAGAGGAGGTTC
IFNB1	TCAGAATGAGTGGTGGTTGC	GCACTTTCAAATGCAGTAGATTCA
NOS	CAAGATGCGTGGAAACTACC	TTGAGAATGGATGCGAAGG
IL6	ACAACCACGGCCTTCCCTACTT	CACGATTTCCCAGAGAACATGTG
IL10	TAAGGCTGGCCACACTTGAG	GTTTTCAGGGATGAAGCGGC
HIF-1A	CCACAGGACAGTACAGGA	TCAAGTCGTGCTGAATAATACC
ALAS1	TGTGATGAGTTGATGACCAGG	TGGGCTTGAGCAGCCTCTT
qPCR	**Forward Sequence**	**Reverse Sequence**
HK2	GCCAGCCTCTCCTGATTTTAGTGT	GGGAACACAAAAGACCTCTTCTGG
Pecam	ATGGAAAGCCTGCCATCATG	TCCTTGTTGTTCAGCATCAC
MT-16S	CCGCAAGGGAAAGATGAAAGAC	TCGTTTGGTTTCGGGGTTTC
MT-ND1	CCTATCACCCTTGCCATCAT	GAGGCTGTTGCTTGTGTGAC

CHAPTER 4: DISCUSSION OF THE PRIMARY ARTICLE

5 DISCUSSION AND CONCLUSIONS

5.1 Mitochondria at the core of immunometabolism

The immune system plays a critical role in pathogen defence, from the detection of invasive pathogens and to their elimination. During the past centuries, several researchers have elegantly dissected the mechanisms employed by different immune cells to exert their respective functions under homeostasis or during a pathogenic challenge. Nowadays, there are well-understood mechanisms by which the immune system detects PAMPs and/or DAMPs through a set of germline-encoded PRRs, by paracrine action or mediated by cytokines. Recognition of those molecules leads to a panoply of cellular responses depending on the nature of the ligand and the immune cells involved in their detection. Several studies of different cell types involved in immunity have displayed a heterogeneous metabolic signature, which initially was associated only with the support of biosynthetic and energetic pathways and it was considered to be specific to each cellular type at a given time. Indeed, the metabolic print of pro-inflammatory or anti-inflammatory polarized cells, such as macrophages, revealed completely different metabolic programs, which fulfill their specific needs. During the recent decades, metabolism and mitochondrial function are regarded as something beyond ATP production. Moreover, they are rising as a central node of immunometabolism, thus playing an essential role in controlling the fate of immune cells and facilitating their effector functions (Mehta *et al.*, 2017). The critical role of mitochondrial metabolism is being intensively explored in macrophages, due to its plasticity during the polarization process. Importantly, the crucial role of the Krebs cycle in macrophages discloses high regulatory capacity for metabolites such as succinate, α-ketoglutarate, citrate, and itaconate during macrophage activation. As the prominent immunologist and pioneer in immunometabolism research, Luke O'Neill, once said, , “the Krebs cycle has emerged as the central immunometabolic hub of the macrophage” (Ryan & O'Neill, 2020).

Strikingly, since mitochondrial function plays a critical role in immune cell fate by altering their metabolism, it is appealing to assume that pathogens have evolved and developed different mechanisms to target and hamper mitochondrial function and successfully establish an infection. Thus, the host mitochondria-pathogen interaction can be dynamically altered during infection according to the host’s status and the employment of virulence factors by the pathogen. This interface may induce metabolic microenvironmental changes that could provide nutrient access

for the pathogen or, on the contrary, mount a host metabolic defence against the pathogen. Host metabolic modulation may also induce changes in the host cell's life span (death pathways) or alter the host's immune response, which may assure pathogen replication or clearance. During pathogenic encounters, modulation of mitochondrial function and metabolism has been identified as key virulent strategies employed by different intracellular pathogens such as *Trypanosoma*, *Toxoplasma* and *Leishmania* (Caradonna *et al.*, 2013; Hos *et al.*, 2017; Moreira *et al.*, 2015b; Pernas *et al.*, 2018). At the host-*Leishmania* metabolic interface, one of the pioneering articles published by Rabhi and colleagues (Rabhi *et al.*, 2012) deciphered by transcriptomic analysis, the existence of host metabolic alterations, which involved host glycolysis enhancement through increased glycolytic enzymes transcriptions and accompanied by energetic and redox impairment. Additionally, Moreira *et al* (Moreira *et al.*, 2015b) further described that the enhanced glycolytic program in *L. infantum* infected macrophages is a transient metabolic program that is further reprogramed towards enhanced mitochondrial respiration through the activation of the SIRT1-AMPK axis. This in turn has a significant impact on parasite survival. Whereas the role of the SIRT1-AMPK axis has been established in mediating *Leishmania*-induced changes in host cell bioenergetics profile, scarce attention has been paid to the nature of both the host and *Leishmania* factors involved in the macrophage metabolic reprogramming.

Due to the importance of host cell receptors and *Leishmania* virulence factors in modulating immunity, we sought to characterize the mechanisms by which *L. donovani* metacyclic promastigotes alter host cell mitochondrial biology and identify its impact on the parasite fate. Here, we describe the essential role of the glycolipid LPG in the stimulation of OXPHOS and the induction of mitochondrial biogenesis in infected macrophages. Additionally, we demonstrate that mitochondriogenesis requires the action of endosomal TLRs, TLR4 and type-I IFN. Importantly, we observed IFNα production independently of LPG presence, which is linked to mitochondrial biogenesis induction. Coupled with the observation that pharmacological induction of mitochondrial biogenesis increases the permissiveness of macrophages to *L. donovani* and leads to LPG mutants' survival in macrophages, it supports the notion that mitochondrial biogenesis creates a metabolically-adapted environment propitious to the replication of the parasite.

5.2 Energetic metabolisms at the interface between *Leishmania* and macrophages

Given the importance of LPG for *Leishmania* promastigotes to colonize their host cells (de Carvalho *et al.*, 2019; Desjardins & Descoteaux, 1997; Franco *et al.*, 2012; Lodge & Descoteaux, 2006; Matte *et al.*, 2021; Späth *et al.*, 2003; Vinet *et al.*, 2009), we sought to initially determine the potential role of this virulence glycolipid in the modulation of host cell metabolic flux by taking advantage of a genetically and structurally defined *L. donovani* mutant defective in the synthesis of LPG *(*Δ*lpg1*) and its complemented counterpart *(*Δ*lpg1+LPG1*). We found that *L. donovani* metacyclic promastigotes promote a rapid increase in OCR/ECAR ratio from 1 hour to 24 hours post-infection, indicating that infected macrophages tend to conduct ATP production by OXPHOS. Importantly, we obtained evidence that LPG is essential to sustain enhanced OXPHOS that is induced by *L. donovani* promastigotes starting at 4 hours post-infection. The observation that Δ*lpg1* partially increases OCR and promotes ECAR and PER enhancement compared to WT parasites suggests that Δ*lpg1* tends to sustain ATP conduction through lactate production rather than OXPHOS. This observation was further strengthened by observing reduced mitochondrial ATP production and basal OCR in Δ*lpg1*-infected macrophages compared to WT. Contrary to the findings observed with *L. infantum* infected cells (Moreira *et al.*, 2015b), we did not observe the Warburg effect (decreased OCR/ECAR ratio) at early-times post infection. This difference could be explained by the use of enriched metacyclic promastigotes, which are known to enter the macrophage without triggering NADPH oxidase and respiratory burst in the PV compared to procyclic promastigotes (Sehgal *et al.*, 1993; Ueno *et al.*, 2009). Also, it can be explained by a differential host metabolic reprogramming induced by *L. donovani* and *L. infantum*. Indeed, we observed enhanced OXPHOS at later times post-infection as previously reported for *L. infantum, L. amazonensis and L. donovani* (Moreira *et al.*, 2015b; Ty *et al.*, 2019). Increases in OXPHOS generally promote the oxidation of different carbon sources such as glucose, glutamine and fatty acids among others to support the energetic demand (Spinelli & Haigis, 2018). Here, we observed that enhanced OXPHOS in *L. donovani*-infected macrophages was highly dependent on glycolysis coupled to mitochondrial ATP production, highlighting the role of those metabolic pathways in driving mitochondrial ATP production. Other carbon sources, such as glutaminolysis and mitochondria β-oxidation, were not involved in the ATP production in *L. donovani*-infected macrophages, suggesting the independence of these metabolic pathways to support mitochondrial ATP production.

Interestingly, inhibition of peroxisomal β-oxidation significantly reduced OCR at 1 hour post-infection. This could be related to the initial phagocytosis process that involves lipid degradation and transfer into the PV during its biogenesis, which in part is mediated by autophagy (Frank *et al.*, 2015; Matte *et al.*, 2016). Surprisingly, we observed enhanced ECAR in *L. donovani*-infected macrophages after blocking mitochondria β-oxidation using the etomoxir inhibitor. This result suggested that lipids degradation are important for mitochondrial ATP production or could suggest oxidative stress after the inhibition of the carnitine palmitoyl-transferase 1a (CPT1a), even if we used a low concentration of the inhibitor (4μM) to prevent off-target action as it was observed during the T-cell differentiation process or during IL-4-mediated macrophage polarization, which inhibits the adenine nucleotide translocase and disturbed the coenzyme A homeostasis using etomoxir 10μM and 200μM respectively (Divakaruni *et al.*, 2018; O'Connor *et al.*, 2018). In perspective, it will be interesting to block the metabolic pathways described abode, prior to *Leishmania* infection and analyze their survival and replication.

Control experiments confirmed that FCCP 2 μM induces the maximal respiratory capacity in macrophages. Importantly, free *L. donovani* metacyclic promastigotes by themselves do not contribute significative to the OCR or ECAR measurements. Also, macrophages fed with zymosan particles exert an important decrease in OCR/ECAR ratio, indicating a reduction in the macrophages' OXPHOS, similar to the one observed in LPS-stimulated macrophages, which is consistent with increased glycolysis and reduced OXPHOS. Enhanced glycolysis and decreased OXPHOS in macrophages fed with zymosan particles or stimulated with LPS-, are characteristic metabolic prints observed in the M1 polarized macrophages that are required to exert their microbicidal duties (Lachmandas *et al.*, 2016; van Teijlingen Bakker & Pearce, 2020). Interestingly, we observed a lower OXPHOX and enhanced ECAR in Δ*lpg1*-infected macrophages compared to WT parasites. This could indicate that *L. donovani* infection may drive a macrophage polarization towards the M2 subset, which is associated to be permissive for parasite replication; whereas Δ*lpg1* drives a response closer to the M1 subset, which leads to parasite clearance (Muxel *et al.*, 2018; Tomiotto-Pellissier *et al.*, 2018). Nevertheless, macrophage polarization and the role of LPG in this context deserve to be further evaluated. Also, it will be interesting to evaluate whether macrophages primed with LPS, which drive a reduction in OXPHOS, can reverse their phenotype towards OXPHOS when infected with *L. donovani,* due to their capacity to promote mitochondrial biogenesis.

Metabolic flux analyses make it possible to determine that *L. donovani*-infected macrophages promote OXPHOS enhancement through glycolysis and mitochondrial ATP production, as a specific consequence of host-pathogen interaction. Also, in addition to the important role of LPG in sabotaging the host key defence processes to protect the promastigotes from the microbicidal response of phagocytic cells, our work revealed that modification of host cell metabolism represents an important function for LPG. In order to better understand the mechanism by which *L. donovani* modulates mitochondrial metabolism, additional complementary analysis of the carbon dependence source in Δ*lpg1*-infected macrophages is required. Also, enhanced OXPHOS may increase different glycolytic and TCA intermediates, which also merits further analysis. So far, in our initial studies, we observed increased glycolytic and TCA metabolites in *L. donovani*-infected macrophages, which may in part explain the enhanced OXPHOS **(Figure 5.1)**.

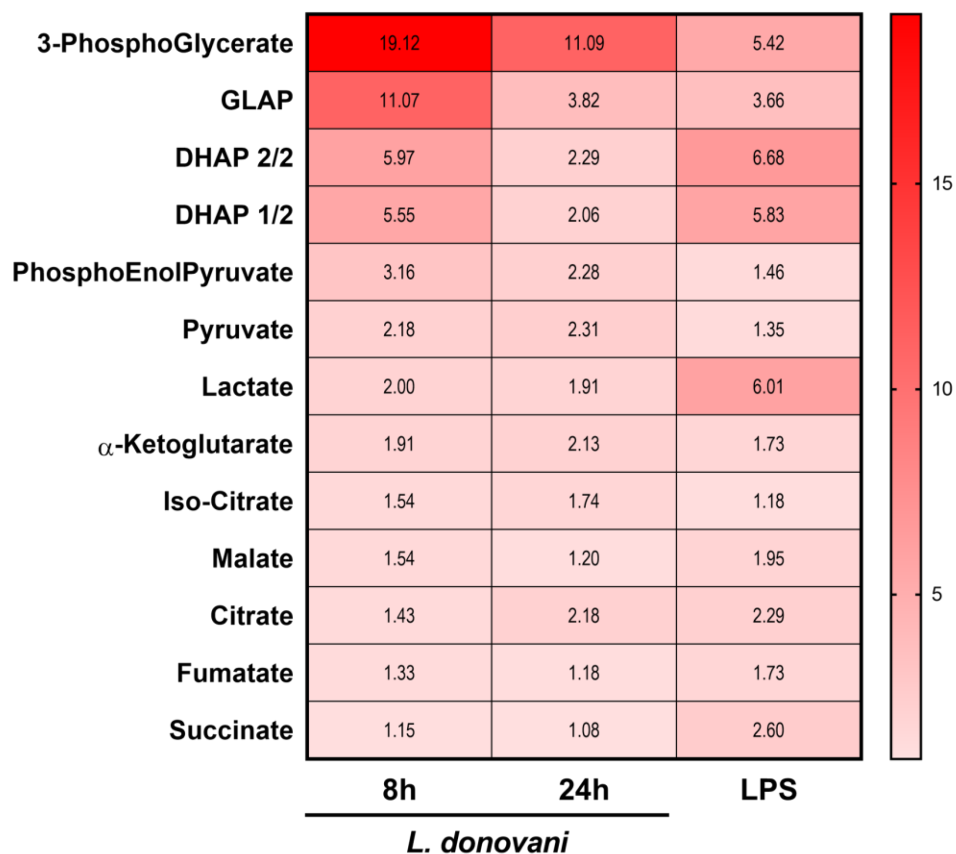


Figure 5.1. Metabolic print of *L. donovani* infected macrophage.
Macrophages were infected for 8 and 24 hours and the metabolic print was analyzed by GC-MS. LPS was used as a control for enhanced lactate production.

5.3 Host mitochondrial biogenesis: a common process during *Leishmania* pathogenesis

Depending on cell-energetic demands, the activation of different metabolic programs to support cellular needs is required. There exists a catabolic program that drives a highly efficient ATP production that is usually mediated by OXPHOS (Martínez-Reyes & Chandel, 2020). The process mentioned above is supported through the promotion of inner mitochondrial membrane rearrangement for the proper optimization of the surface area where the respiratory complex is assembled and through mitochondrial biogenesis induction. Mitochondrial biogenesis is a highly coordinated process that requires the expression of nuclear and mitochondrial genes and that is regulated by specific signalling modules, transcription factors and regulators of gene expression (Austin & St-Pierre, 2012; Dominy & Puigserver, 2013b; Mehta *et al.*, 2017). Our results reveal that *L. donovani* promotes an increase in the host mitochondrial network area relative to the cell area in an LPG-dependent manner. Interestingly, we did not observe bystander effects promoting either increases or decreases in the mitochondrial network from uninfected macrophages in the same cultures from WT or Δ*lpg1* infected macrophages respectively, indicating a specific response associated with regards to the host-parasite interaction. We did not observe apparent changes in the mitochondrial network morphology in infected macrophages. Nevertheless, the analysis for mitochondrial fission and fusion could be explained by how mitochondrial dynamic events shape the mitochondrial network as it was described for *Toxoplasma* and *Trypanosoma* that both promote fusion events, leading to enlarged mitochondrial networks around the vacuoles harboring intracellular parasites (Lentini *et al.*, 2018; Pernas *et al.*, 2018). As previously reported for other trypanosomatid members such as *L. infantum* and *T. cruzi* (Moreira *et al.*, 2015a; Wan *et al.*, 2016), we also found that *L. donovani* metacyclic promastigotes promote macrophage mitochondrial mass enhancement. Importantly, we observed that Δ*lpg1*-infected macrophages failed to promote mitochondrial biogenesis. Macrophage mitochondrial biogenesis induction was observed with a low parasite-to-macrophage ratio and was not further increased by higher infection ratio. Also, this macrophage response was induced by other *Leishmania* species, suggesting that mitochondrial biogenesis is a common host response induced by *Leishmania* and that LPG is required for host mitochondrial biogenesis induction that is independent of species specific LPG structure recognition (Descoteaux & Turco, 1999; McConville *et al.*, 1995).

In accordance with the increase in host mitochondrial biogenesis, we observed *NRF1* and *PPARGC1A* overexpression, two key regulatory proteins associated with mitochondrial biogenesis that drive the transcription of several components of the mitochondrial electron transport chain (Austin & St-Pierre, 2012; Dominy & Puigserver, 2013b; Mehta *et al.*, 2017). Hence, in *L. donovani*-infected macrophages, we observed overexpression of different protein-encoded genes from the mitochondrial and the nuclear genomes (Scarpulla, 2011; Scarpulla *et al.*, 2012). Contrary to *L. donovani*-infected macrophages, Δ*lpg1*-infected macrophages did not promote *NRF1*, *PPARGC1A* expression, nor the overexpression of protein-encoded genes of the mitochondrial electron transport chain. Noteworthy, these observations may explain why Δ*lpg1*-infected macrophages displayed reduced OXPHOS compared to WT-infected macrophages and strengthen the notion that LPG is essential for the profound changes in host cell mitochondrial biology that is induced by *L. donovani* promastigotes. To further decipher the signalling axis involved in host mitochondrial biogenesis induction in *L. donovani*-infected macrophages, we analyzed the energetic sensor AMPK, which participates in mitochondrial biogenesis by potentiating the transcriptional activity of PGC-1α (Dominy & Puigserver, 2013b; Jäger *et al.*, 2007). We observed an increased expression of AMPK and its subsequent activation as phospho-AMPK on Thr172 in *L. donovani*-infected macrophages. Likewise, we observe similar AMPK and phospho-AMPK levels in Δ*lpg1*-infected macrophages indicating that AMPK activation is LPG-independent. Therefore, the Δ*lpg1*-infected macrophages' incapacity to promote mitochondrial biogenesis should be explained by the modulation of the signalling axis at another point. Presumably, it may occur up-stream of PGC-1α and down-stream of AMPK. Also, other factors such as the receptor-interacting protein 140 (RIP140) could act as a negative regulator of mitochondrial biogenesis as it was reported to have opposite effects on mitochondrial biogenesis compared to PGC-1α (Christian *et al.*, 2005; White *et al.*, 2008). We conclude that increases in *PPARGC1A* and AMPK activation induce a positive control over mitochondria biogenesis in *L. donovani*-infected macrophages (Dominy & Puigserver, 2013a). Complementary experiments evaluating other mitochondrial biogenesis regulators such as mitochondrial transcription factor A (TFAM), the transcription factor Yin Yang 1 (YY1), estrogen-related receptors (ERRs), and CaMKIV in the context of *L. donovani* infection, will further strengthen the knowledge during the mitochondrial metabolism modulation.

In macrophages, phagocytosis promotes different metabolic changes according to the nature of the stimulation (Morioka *et al.*, 2018; Pavlou *et al.*, 2017). Here, we validate our results, by

comparing with specific controls, that *Leishmania*-induced mitochondria modulation is a specific response during the host-pathogen interaction rather than responses caused by the phagocytosis process. Hence, by using inert bead particles and heat-killed parasites to feed macrophages, we did not observe mitochondrial biogenesis induction, nor *PPARGC1A* overexpression compared to live parasites. Thus, we exclude the possibility that phagocytosis per se promotes mitochondrial biogenesis and indicate that the profound host metabolic change is required for parasite survival. Similar results were observed for heat-killed *L. infantum (Moreira et al., 2015b)*. Also, to rule out the possibility that serum-opsonization influenced the stimulation of mitochondrial biogenesis by *L. donovani*, we infected macrophages with unopsonized or serum-opsonized *L. donovani* metacyclic promastigotes and we did not observe differences in the LPG-dependent increase in the mitochondrial mass, nor in the *PPARGC1A* expression indicating that WT and Δ*lpg1* macrophage responses are not influenced by serum opsonization. This supports our previous reports indicating that in LPG absence, the metalloprotease GP63 acts as a primary acceptor for C3 deposition (Brittingham *et al.*, 1995; Russell & Wright, 1988), and that LPG-deficient parasites are efficiently opsonized with C3 and that both WT and LPG-deficient *L. donovani* enter macrophages predominantly through CR3 (Lodge & Descoteaux, 2006). LPG is known to reduce macrophage uptake by inhibiting phagocytosis (Vinet *et al.*, 2011). To normalize the infection levels between the different *L. donovani* strains, we adjust the initial MOI to ensure a similar initial parasite load of 5-7 parasites per macrophages at 3 h post-infection. Hence, we had to use an MOI of 7:1 for *L. donovani* WT, an MOI of 6:1 for *L. donovani* Δ*lpg1*, and an MOI of 8:1 for *L. donovani* Δlpg1+ *LPG1*. Importantly, we used C5-deficient serum (from DBA/2 mice) before infecting macrophages to prevent loss in the Δ*lpg1* viability due to their susceptibility to the complement attack.

The incapacity of Δ*lpg1* infected macrophages to promote mitochondrial biogenesis raised the following question: does LPG by itself induce mitochondrial biogenesis? In our analysis, we observed that like *L. donovani*-infected macrophages, macrophages fed with LPG-coated zymosan particles promote LPG release within the macrophage. Contrary to *L. donovani*-infected macrophages, LPG-coated zymosan does not promote mitochondrial biogenesis nor *PPARGC1A* overexpression. Together, these results indicate that LPG is essential but not sufficient for host mitochondrial biogenesis induction and *PPARGC1A* expression by *L. donovani* metacyclic promastigotes. Following internalization by macrophages, promastigotes differentiate into amastigotes, which are forms of parasite specifically adapted to the mammalian- host cells

that replicate within phagolysosomes (Chang & Dwyer, 1976). Then, we evaluated whether increased mitochondrial mass induced during the first 24 hours of infection with metacyclic promastigotes is a transient event or persists as the parasites differentiate and replicate whilst also taking into consideration the fact that amastigotes downregulate LPG levels. Similar to metacyclic promastigotes, we observed that splenic amastigotes promote an enhanced mitochondrial network area relative to the cell area, and efficiently stimulate mitochondrial biogenesis and *PPARGC1A* expression in macrophages. Importantly, *in vivo* analyses revealed an increase in the mitochondrial abundance, which displayed more complex mitochondrial cristae in the spleen of infected hamsters. To further validate that enhanced mitochondrial mass is a specific response caused by *Leishmania*-macrophage interaction, we compared the expression of two nuclear genes that do not encode mitochondrial proteins. In this regard, we did not observe changes in the nuclear/nuclear DNA ratio in *L. donovan*i-infected macrophages compare with the enhanced mitochondrial/nuclear DNA ratio observed in *L. donovan*i-infected macrophages. Those results lead us to conclude that mitochondrial biogenesis is a common host process induced by *Leishmania.*

Previous reports indicated that splenic *L. donovani* amastigotes express 120-fold fewer LPG molecules per cell compared to promastigotes (McConville & Blackwell, 1991) (McNeely and Doyle 1996) and that they express LPG-like glycolipids, which share several chemical and structural properties with promastigote LPG (McNeely and Doyle 1996). The observation that ∆*lpg1 L. donovani* amastigotes failed to trigger mitochondrial biogenesis suggests that LPG levels or LPG-like glycolipids expressed by amastigotes are sufficient to stimulate mitochondrial biogenesis. Also, there is evidence that mitochondria present more complex cristae arrangements indicating that mitochondria are more active, which results in enhanced ATP production, thus agreeing with our previous result where we observed enhanced mitochondrial mass and augmentation in the OXPHOS (den Brave & Becker, 2020; Lobo-Jarne & Ugalde, 2018). Interestingly, we did not observe a mitochondrial network surrounding the PV via immunofluorescence microscopy; however, electron microscopy revealed direct contact between amastigotes and the mitochondria **(Figure 5.2),** which may indicate a membrane contact site that is used to promotes metabolite exchange as it was previously described for *Toxoplasma* and *Trypanosoma* (Caradonna *et al.*, 2013; Pernas *et al.*, 2018).

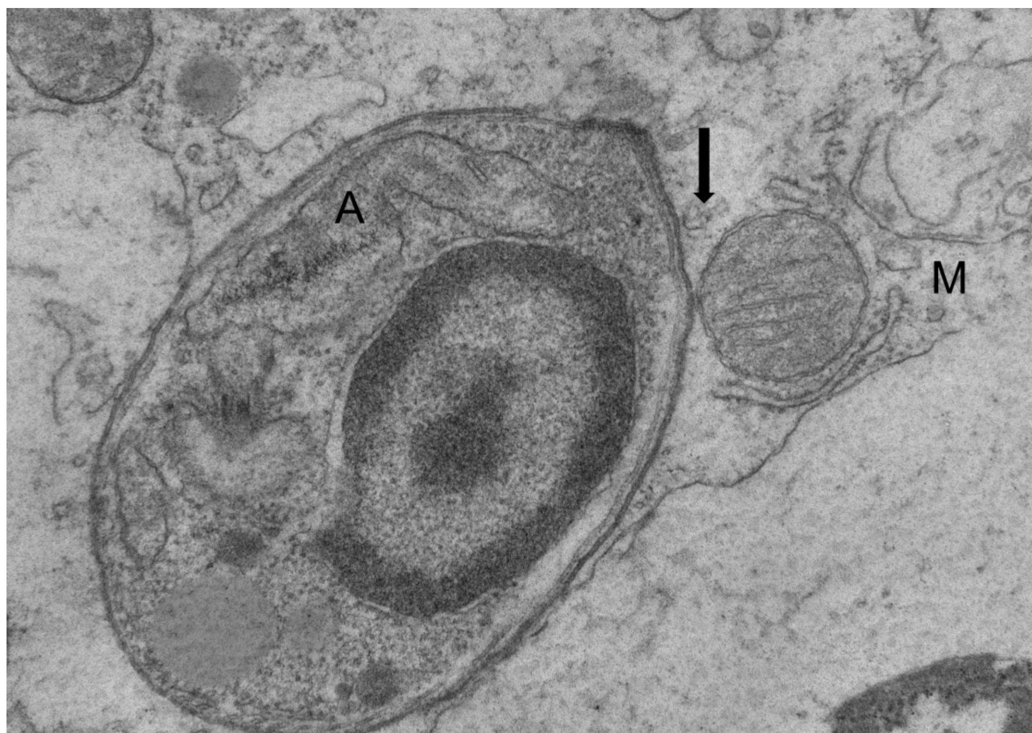


Figure 5.2. Mitochondria-*Leishmania* contact site

Electron microscopy image showing the contact site between mitochondria and *L. donovani* amastigote. The sample was obtained from an infected spleen in hamster. A: amastigote and M: mitochondria

Mitochondrial mass represents the balance between biogenesis and elimination of damaged mitochondria, which is largely mediated by mitophagy (Ashrafi & Schwarz, 2013; Dominy & Puigserver, 2013a). Induction of mitophagy involves a cascade of signalling events that include the mitochondrial recruitment of Parkin, and an E3 ubiquitin ligase (Matsuda, 2016). It was thus of interest to determine whether mitophagy occurs in response to enhanced OXPHOS in *L. donovani*-infected macrophages. Contrary to CCCP-stimulated macrophages, *L. donovani*-infected macrophages did not result in the disruption of the mitochondrial network nor the redistribution of Parkin from a diffuse location to punctate structures surrounding the mitochondrial clusters (Referred to as Tom20/Parkin puncta accumulation) **(Figure 5.3)**. Those observations indicate that *L. donovani* does not promote mitophagy-parkin dependent pathways in macrophages, suggesting that mitochondrial biogenesis is largely responsible for the increased mitochondrial mass observed in infected macrophages. This contrasts with other microorganisms such as *Listeria, influenza A* and *Coxsackievirus B* that promote mitophagy to spread into another cell (Sin *et al.*, 2017; Yoshizumi *et al.*, 2014; Zhang *et al.*, 2019).

Complementary experiments co-labelling mitochondria with the autophagosome marker LC3 will further validate whether there is mitophagy induction in a parkin-independent response in macrophages. Also, it will be interesting to infect CCCP-stimulated macrophages and evaluate whether *L. donovani* may reduce mitophagy induction.

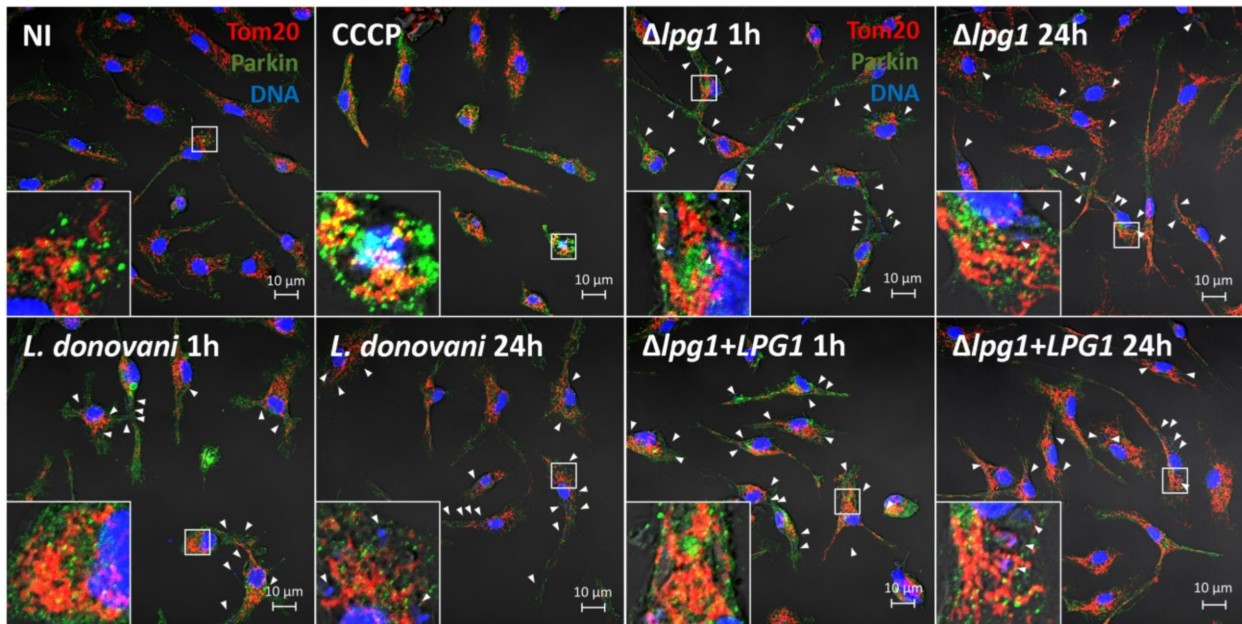


Figure 5.3. Parkin/Tom20 puncta accumulation

Confocal microscopy images of *L. donovani, Δlpg1 and Δlpg1+LPG1* infected macrophages for 1 h and 24 h, macrophages stimulated 3 h with carbonyl cyanide m-chlorophenyl hydrazine (CCCP) 25μM and uninfected macrophages. Tom20 is shown in red, DNA is shown in blue, Parkin is shown in green and yellow arrows indicate parasites. 10X-enlarged insets of representative Parkin/Tom20 puncta accumulation are shown.

Mitochondrial structural analysis and the participation of several factors involved in mitochondrial biogenesis make it possible to determine that macrophages infected with *L. donovani* promastigotes or amastigotes promote mitochondrial biogenesis. Although LPG plays an essential role in the stimulation of host macrophage mitochondrial biogenesis, by itself, it is not sufficient to promote mitochondrial biogenesis. Interestingly, the fact that long term-infections with promastigotes and amastigotes maintained enhanced mitochondrial mass, indicates that LPG is essential for the induction of mitochondrial biogenesis during promastigote infection and that LPG or LPG-like glycolipids. expressed by amastigotes are sufficient to stimulate mitochondrial biogenesis.

5.4 Signalling axis involved in mitochondrial biogenesis

We previously showed that LPG is shed from the surface of internalized promastigotes and traffics out of the parasitophorous vacuole (Arango Duque *et al.*, 2019a). However, our findings indicate that LPG does not interact directly with the mitochondrial network, suggesting that LPG may act on mitochondria through receptor-mediated signalling pathways. Previous reports identified TLR2 and/or TLR4 as the receptors responsible for LPG recognition from *L. major, L infantum, L mexicana* and *L. braziliensis* (Becker *et al.*, 2003; de Veer *et al.*, 2003; Dos Santos *et al.*, 2016; Kavoosi *et al.*, 2010; Rojas-Bernabé *et al.*, 2014; Srivastava *et al.*, 2013; Vieira *et al.*, 2019). Our results indicate that TLR4 is essential for the stimulation of *PPARGC1A* expression and mitochondrial biogenesis, which is consistent with the role of this receptor in the recognition of LPG. However, as we showed before, LPG by itself is not sufficient to stimulate *PPARGC1A* expression or mitochondrial biogenesis, indicating that aside from TLR4, additional factors are required to promote this response. TLR activation leads to type-I IFNs production in response to different pathogens, including *Leishmania* **(Figure 5.4)** (Blasius & Beutler, 2010; Dias *et al.*, 2019; Dias *et al.*, 2022; Oosenbrug *et al.*, 2020; Sacramento *et al.*, 2020; Silva-Barrios *et al.*, 2016). Given that type-I IFNs were reported to modulate mitochondrial metabolism in plasmacytoid dendritic cells (Wu *et al.*, 2016), we investigated the possibility of autocrine signalling that would be triggered by these cytokines to regulate mitochondrial biogenesis in response to *L. donovani*. Hence, we found that mitochondrial biogenesis requires the expression of IFNα, which is produced independently of LPG recognition. Interestingly, this pathway also involves TLR4, suggesting that this receptor plays a dual role in the control of mitochondrial biogenesis. Although we have not investigated the ligand(s) responsible for TLR4-mediated induction of type-I IFN, previous studies evidenced a key role for the neutrophil elastase-TLR4 pathway in this process (Dias *et al.*, 2019). Interestingly, we observed that TLR2 activation is independent of *PPARGC1A* expression and IFNα production, indicating that TLR2 is not responsible for mitochondrial biogenesis in *L. donovani*-infected macrophages.

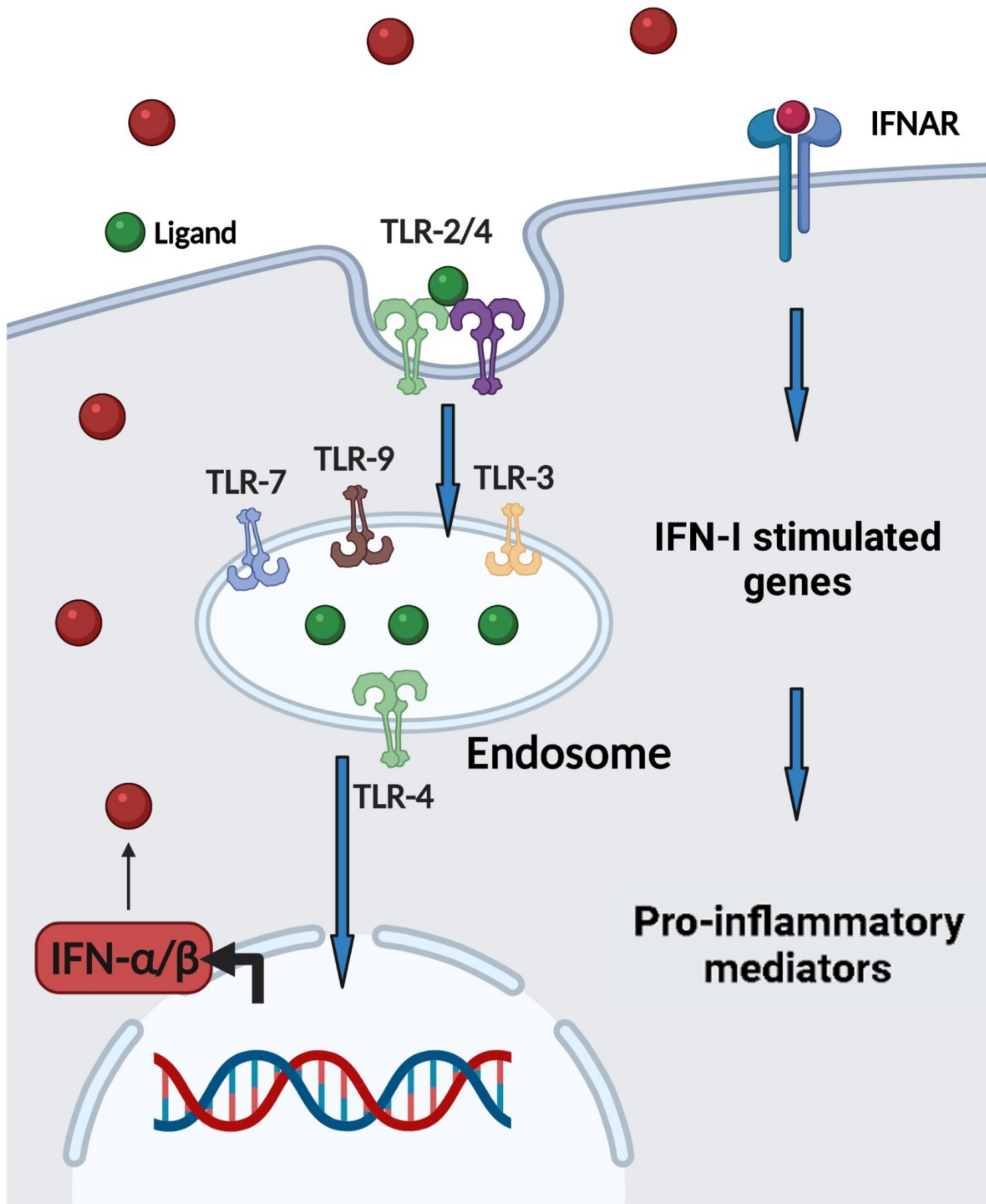


Figure 5.4. Type I IFN signalling axis

Upon TLR2, TLR4 and/or endosomal TLRs bind their respective ligands, there are a set of transduction signals that promote type I IFN production. Further, type I IFN acts in a autocrine fashion and promote expression of IFN I-stimulated genes. Image created with BioRender.

Leishmania is an intravacuolar parasite that interacts with endosomal TLRs. Several studies have highlighted the contribution of these receptors in the host response to various *Leishmania* species (Chauhan *et al.*, 2017; Dos-Santos *et al.*, 2016; Flandin *et al.*, 2006; Franco *et al.*, 2017; Ives *et al.*, 2011; Regli *et al.*, 2020; Schamber-Reis *et al.*, 2013; Schleicher *et al.*, 2007; Shukla *et al.*, 2021; Silva-Barrios *et al.*, 2016). Using macrophages from *Unc93b1$^{Letr/Letr}$* mice (Lafferty *et al.*, 2014), we found that induction of mitochondrial biogenesis and of type-I IFN expression by *L. donovani* metacyclic promastigotes requires endosomal TLRs. Recent work revealed that TLR3 is the endosomal TLR that mediates type-I IFN expression in response to *L. donovani* (Dias *et al.*, 2022), which is consistent with the defective type-I IFN expression in *L. donovani*-infected *Unc93b1$^{Letr/Letr}$* macrophages. Little is known about the nature of *Leishmania* ligand(s) recognized by TLR3, except for the double-stranded RNA virus LRV1 (Ives *et al.*, 2011) present in isolates of various *Leishmania* species such as *L. guyanensis* (Cantanhede *et al.*, 2021). Noteworthy, TLR3 contributes to the recognition of *L. donovani* promastigotes even though these parasites do not harbour double-stranded RNA viruses (Dias *et al.*, 2022; Flandin *et al.*, 2006). The nature of the *L. donovani*-derived ligand(s) recognized by TLR3 thus still remains to be elucidated. However, it raises the hypothesis that extracellular vesicles containing RNA are released within the parasitophorous vacuoles and activate endosomal TLRs (Lambertz *et al.*, 2015). Alternatively, TLR3 may be activated by RNA released by apoptotic parasites present in metacyclic promastigote populations (van Zandbergen *et al.*, 2006). Also, mitochondrial biogenesis impairment in *L. donovani Unc93b1$^{Letr/Letr}$* infected macrophages raises the hypothesis that mitochondrial dynamic events may be reduced due to the absence of TLR7, which was previously linked with MARCH5, a molecule present in a loop that regulates mitochondrial fission (Shi *et al.*, 2011). Another attractive hypothesis is that the absence of IFNα and the impairment of mitochondrial biogenesis in *L. donovani Unc93b1$^{Letr/Letr}$* infected macrophages is due to the absence of TLR9, which is involved in the recognition of DNA-CpG motifs and induces type-I IFN production. *Leishmania* genome (nDNA and mtDNA) is rich in CpG motifs, which could be a possible TLR9 ligand. Thus, *L. donovani* infected macrophages from TLR9 deficient mice deserve further analysis. Even if we did not observe an enhancement of mitochondrial clearing (mitophagy), it would be of interest to evaluate whether host mitochondrial genomic material is released to the cytoplasm and further recognized by the receptor cGAS-STING. Besides, host mtDNA could also act as a ligand for TLR9 promoting type-I IFN production in both scenarios.

Noteworthy, *Leishmania* species harbouring the LRV1 virus may be an attractive model to investigate the role of IFN-I in the mitochondrial metabolic flux due to the link between IFN-I and mitochondria biogenesis. Also, *L. donovan*i-infected macrophages from TLR2-, TLR4- and endosomal TLRs- deficient mice deserve further analysis to decipher the role of these TLRs in the mitochondrial metabolic flux reprogramming induced by *Leishmania*.

In the absence of type-I interferon, *L. donovani* promastigotes fail to induce mitochondrial biogenesis, consistent with the notion that IFNα acts in an autocrine manner in this process. These results are in accordance with the fact that TLR signalling has reciprocal control over mitochondrial dynamics during an inflammatory context (Escoll & Buchrieser, 2019; Lachmandas *et al.*, 2016; O'Neill & Pearce, 2016). The fact that *L. donovani*-induced mitochondrial biogenesis does not take place in *Ifnar*$^{-/-}$ macrophages despite the induction of PGC-1α expression, illustrates the complexity of the pathways involved in this process and highlights the multiple roles of PGC-1α in the modulation of energetic metabolism. Interestingly, our results indicate that type-I IFN signalling does not play a significant role in stimulating OXPHOS and glycolysis in *L. donovani*-infected macrophages. This contrasts with the recent findings of *Mycobacterium tuberculosis* infections, which are characterized by a decrease in both glycolysis and mitochondrial respiration (Olson *et al.*, 2021). In that study, the authors found that type-I IFN is directly responsible for the reduced macrophage energy metabolism during *M. tuberculosis* infection, suggesting that the effects of type-I IFN are pathogen- and context-specific. Enhanced OXPHOS in macrophages that does not promote mitochondrial biogenesis could be explained by a rearrangement of the inner mitochondrial membrane (cristae) towards a more optimized respirasome complex assembly, which is known to be mediated by PGC-1α (Cantó & Auwerx, 2009; Handschin *et al.*, 2005; Rodgers *et al.*, 2005; Scarpulla, 2011).

Here, we linked the role of IFNα and type-I IFN receptor during mitochondrial biogenesis induction in *L. donovani*-infected macrophages. Further experiments should be addressed to strengthen the autocrine role in mitochondrial biogenesis through IFNα by blocking Ifnar$^{-/-}$ receptor with IFNα antibody, and to determine whether IFNβ is playing a similar role during mitochondrial biogenesis, considering the fact that IFNβ is reported to be produced during *Leishmania* infection (Dias *et al.*, 2019; Dias *et al.*, 2022; Schleicher *et al.*, 2018). Also, it would be interesting to evaluate whether by complementing with recombinant IFNα *L. donovani* infected macrophages from TLR4 or endosomal TLRs deficient mice, may lead to the induction of mitochondrial biogenesis.

5.5 Importance of type I IFN and mitochondrial biogenesis for *Leishmania*

Initial studies have highlighted the protective role of IFN-γ during *Leishmania* pathogenesis when coupled with pentavalent antimonial treatments (Badaro *et al.*, 1990; Squires *et al.*, 1993). Further, several studies have revealed a protective role for type-I IFN in leishmaniasis (Diefenbach *et al.*, 1998; Jaramillo *et al.*, 2011; Silva-Barrios & Stäger, 2017b). However, accumulating evidence indicates that type-I IFN signalling also plays a detrimental role for the host, thus favouring intracellular parasite replication, which indicates that there are much more factors that dictate parasite fate (Dias *et al.*, 2019; Dias *et al.*, 2022; Rossi *et al.*, 2017; Sacramento *et al.*, 2020; Silva-Barrios *et al.*, 2016; Silva-Barrios & Stäger, 2017b; Vivarini Ade *et al.*, 2011; Xin *et al.*, 2010). Based on our results, we propose that type-I IFN contributes to the ability of *Leishmania* to proliferate within its host through the stimulation of mitochondrial biogenesis. In this regard, our results support the notion that induction of mitochondriogenesis by *L. donovani* promastigotes is important for the host cell colonization process. Indeed, in agreement with previous reports (Dias *et al.*, 2019; Dias *et al.*, 2022), survival and replication of *L. donovani* was markedly impaired in TLR4-, endosomal-, and IFNAR-deficient macrophages, which did not induce mitochondrial biogenesis nor IFNα production. Importantly, survival and replication of *L. donovani* were slightly reduced in TLR2-deficient macrophages in which we observed mitochondrial biogenesis induction along with IFNα production. Thus, here we hypothesized that the reduction in parasite survival is linked to it's incapacity to induce host mitochondrial biogenesis. Hence, events downstream of those TLRs and the autocrine signalling triggered by IFNα may affect the outcome of *L. donovani* infection, other than mitochondrial biogenesis. We decided to analyze the importance of mitochondrial biogenesis on the fate of *Leishmania* in primed macrophages where mitochondrial biogenesis was induced prior to the infection. Remarkably, pharmacological induction of mitochondrial biogenesis using AICAR, significantly increased the permissiveness of macrophages to *L. donovani* replication, suggesting that induction of mitochondrial biogenesis creates a metabolically adapted environment propitious to the replication of the parasite. Similar findings were previously reported for *L. infantum*-infected BMMs (Moreira *et al.*, 2015a). This contrasts with other pathogens, including *Mycobacterium tuberculosis*, *Haemophilus parasuis*, *Staphylococcus aureus*, and *Plasmodium falciparum*, whose survival and replication was impaired by AICAR pre-treatment of their respective host cells (Bulusu *et al.*, 2011; Kumar *et al.*, 2016; Shen *et al.*, 2019), strongly indicating that mitochondrial modulation is pathogen-dependent. Strikingly, the

avirulent LPG-defective *L. donovani* Δ*lpg1* mutant, which does not induce mitochondrial biogenesis and does not survive well within host cells, survived and replicated in a slightly higher rate in macrophages pretreated with AICAR. This finding suggests that pharmacological stimulation of mitochondrial biogenesis bypasses the requirement for LPG to create conditions required for the parasite's development within host cells. Macrophages stimulated with AICAR may trigger different responses aside from mitochondrial biogenesis inductions due to its pleiotropic activities, which may affect parasite replication. For instance, a recent report indicated that AICAR significantly decreased the production of reactive oxygen species (ROS) in fMLF-stimulated macrophages (Nassif *et al.*, 2022). Thus, we included in our analysis a control experiment to sort out the possibility that Δ*lpg1* mutant survived in AICAR-primed macrophages due a ROS interference by using macrophages from gp91$^{phox-/-}$ mice. We did not observe a difference in the parasite replication in gp91$^{phox-/-}$ compared to WT macrophages. These findings strengthen the hypothesis that mitochondrial biogenesis is a metabolic adaptation to support parasite replication rather than an interference within the macrophage's effector functions.

A hallmark of mitochondrial biogenesis is the enhanced iron metabolism, which is needed to support heme requirements for ATP production. Heme is an essential cofactor for several enzymes of the electron transport chain, its synthesis plays a central role in mitochondrial biology as well as OXPHOS complex formation and function (Kalainayakan *et al.*, 2018; Ogura *et al.*, 2011; Sohoni *et al.*, 2019; Sugiyama *et al.*, 2014). Thus, we sought to address whether enhanced mitochondrial biogenesis modulates iron metabolism. Hence, the first and rate-limiting step of heme synthesis occurs in mitochondria and is catalyzed by ALAS1 to generate 5-aminolevulinic acid (May *et al.*, 1995). The expression of ALAS1 is tightly regulated and is under the control of PGC-1α (Handschin *et al.*, 2005), which also controls mitochondrial biogenesis and oxidative metabolism (Austin & St-Pierre, 2012; Dominy & Puigserver, 2013b; Scarpulla, 2011; Scarpulla *et al.*, 2012). Our findings that *L. donovani* promastigotes induce *ALAS1* expression and that pharmacological inhibition of the second heme biosynthetic step impaired the ability of *L. donovani* to replicate within host macrophages support the importance of this parasite to induce mitochondrial biogenesis and of OXPHOS for its development within mammalian hosts. Additionally, *Leishmania* is a heme auxotroph that must acquire heme or heme precursors from the host to develop itself intracellularly (Laranjeira-Silva *et al.*, 2020a). Activation of heme biosynthesis upon host cell colonization may thus also serve to fulfill the

Leishmania heme requirement (Orrego *et al.*, 2019). Future studies will be required to elucidate the *Leishmania* heme uptake and the role of LPG in this process.

5.6 Can mitochondrial metabolism be targeted to prevent leishmaniasis?

Leishmaniasis is a neglected tropical parasitic disease that represents a health problem in the tropic, subtropic, and southern European countries. There is no prophylactic or preventive vaccine, thus, leishmaniasis is traditionally treated by using pentavalent antimonials, paromomycin and amphotericin B, which present difficult parental administration, high cost, toxic effects and, as recently reported by the WHO, leads to the significant increase of drug-resistant strains. Therefore, it is imperative to increase our efforts to find new antileishmanial targets and compounds to restrict intracellular amastigote growth. Nowadays, multi-screening of molecules with potential antileishmanial activity that is able to prevent parasite replication are being conducted (Yazdanparast *et al.*, 2014). Also, different parasite molecules involved in the scavenging of different metabolites from the host are showing interesting targets of opportunity to prevent parasite growth (Flannery *et al.*, 2011; Huynh *et al.*, 2012; Kumar *et al.*, 2020b; Kushawaha *et al.*, 2012). Strikingly, identification of metabolic events that govern immune cell function are providing new therapeutic targets that may eventually lead to immune and inflammatory disease treatments as well as pathogenic infections treatments. Thus, TCA cycle intermediates are showing promising results during inflammatory diseases. For instance, the cell-permeable dimethyl fumarate targets GAPDH and aerobic glycolysis and induces anti-inflammatory effects showing a potential treatment for Relapsing-Remitting Multiple Sclerosis and Psoriasis (Kornberg et al., 2018). Also, the use of the cell-permeable 4-Octyl itaconate and Di-methyl itaconate showed in a murine model to be protective against sepsis and psoriasis, respectively (Bambouskova et al., 2018; Mills et al., 2018). Therefore, using molecules based on Krebs cycle intermediates and their derivatives is gaining importance as a promising therapeutic approach in inflammatory diseases.

The complex host-*Leishmania* interface presents a fine line between parasite replication or clearance, where *Leishmania* co-opts different host organelles and metabolites as a mechanism to ensure its survival. In the past years, the importance of host-pathogen interaction with the metabolic context is rising as an attractive concept to develop novel pathogen treatments

(Kumar *et al.*, 2017a; Ryan & O'Neill, 2020; Singh & Sundar, 2014). Therefore, targeting the host cell processes that facilitate parasite growth may be a viable alternative to treating leishmaniasis. Consistently, pioneer works have evaluated the effect of IFN-γ along with pentavalent antimony therapy during pathogenesis where they observed parasite clearing (Badaro *et al.*, 1990; Squires *et al.*, 1993). More recently, targeting type-I IFN during anti-*Leishmania* drug treatment was shown to improve Th1 cell-mediated immunity, which improves anti-parasitic immunity in VL patients (Kumar *et al.*, 2020a). Therefore, altering the metabolic host responses is a promising new therapeutic approach for leishmaniasis.

Here, we showed the relevance of IFNα during the host mitochondrial biogenesis induction and its importance for parasite replication, highlighting the fact that type-I IFNs are a promising approach to control parasite replication. Also, we determined that mitochondrial biogenesis is critical to support *Leishmania* growth within the macrophage. Thus, it is tempting to assume that targeting mitochondrial biology may affect *Leishmania* replication. Previously, it was shown that inhibition of AMPK activation by using compound C promotes parasite clearing in *L. infantum (Moreira et al., 2015b)*. However, we determined that compound C prevents *L. donovani* promastigote growth in culture (data not shown). We speculate that restricting mitochondrial biogenesis induction with other molecules such as doxycycline may prevent parasite growth as it was previously reported by the groups of both Akulinina and Masmoudi *et al* (Akulinina *et al.*, 2021; Masmoudi *et al.*, 2008). Other promising metabolites such as itaconate are showing important bactericidal properties (Michelucci *et al.*, 2013; Naujoks *et al.*, 2016) and present a higher regulation of mitochondrial metabolism. However, further studies analyzing itaconate effect on *Leishmania* growth must be evaluated.

5.7 General conclusion and final remarks

In summary, we provide novel information on the mechanisms leading to mitochondrial biogenesis and metabolic reprogramming in macrophages infected with *L. donovani*. We conclude that *L. donovani* metacyclic promastigotes induce five distinct host cell responses in the context of the modulation of macrophage mitochondrial biogenesis. The first one is the LPG-dependent enhancement of macrophage mitochondrial mass, increased expression of PGC-1α and genes associated with the electron transport chain, and stimulation of OXPHOS. The second is the TLR4 and endosomal TLRs dependence for mitochondrial biogenesis induction. The third is the LPG-independent induction of IFNα expression, which also mediates the induction of macrophage mitochondrial biogenesis, but has no impact on the induction of PGC-1α expression or mitochondrial flux. The fourth is the importance of mitochondrial biogenesis for *Leishmania* fate. Finally, the fifth one is the enhanced heme biosynthesis, which supports parasite replication **(Figure 5.5)**. Our results are consistent with the notion of pathogen-specific metabolic rewiring (Lachmandas *et al.*, 2016), which results from the intricate interplay between complex sets of pathogen molecules and host cell receptors. Additionally, we suggest that the complex nutritional requirements of amastigotes have contributed to the necessity for these parasites for heme scavenging, which may be supported by enhanced mitochondrial mass.

These findings provide new insight into how *Leishmania* creates a metabolically adapted environment, favourable for its replication and opens several subjects to keep dissecting the role of mitochondrial metabolism at the interface with *Leishmania*.

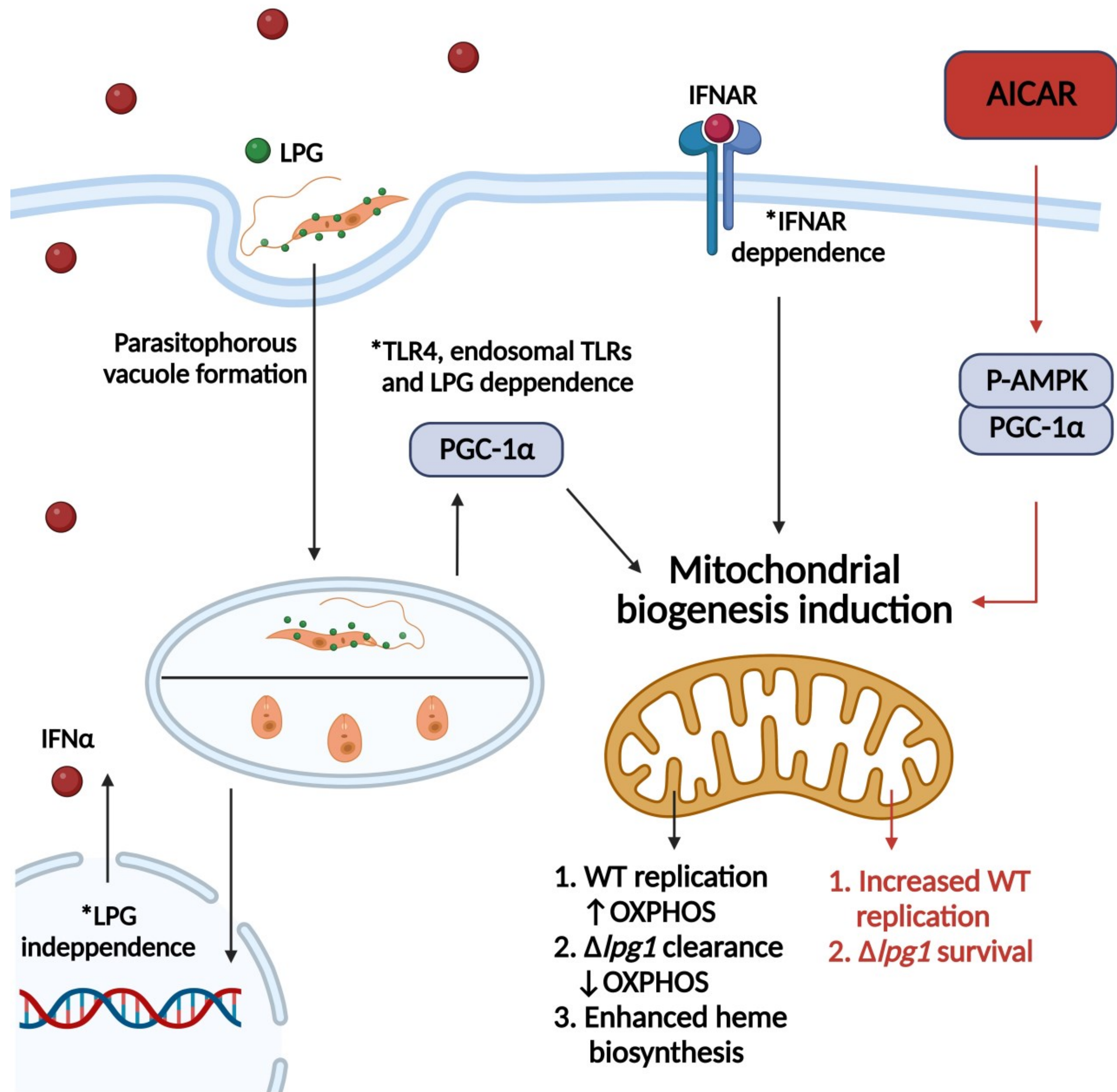


Figure 5.5. Graphical respresentation sumarizing the present findings.

Model of how *L. donovani* modulates macrophage mitochondrial function. (I) *L. donovani* internalization leads to a parasitophorous vacuole formation and differentiates into amastigote. (II) There is IFNα induction in an LPG-independent fashion. (III) There is PGC-1α induction in an LPG-, TLR4-, endosomal TLRs- and IFNAR-dependent fashion, which lead to mitochondrial biogenesis induction. (IV) *L. donovani* WT proliferates in macrophages, promotes enhanced OXPHOS and augments heme biosynthesis, whereas *L. donovani* Δ*lpg1* is cleared and promotes a decreased macrophage OXPHOS. (V) Induction of mitochondrial biogenesis with AICAR enhances *L. donovani* WT replication and allows *L. donovani* Δ*lpg1* to survive. Image created with BioRender.

CHAPTER 5: RÉSUMÉ COMPLET EN FRANÇAIS

6 RÉSUMÉ

6.1 Introduction et objectifs de recherche

Leishmania ssp. sont des parasites protozoaires et les agents étiologique de la leishmaniose, une maladie transmise par un vecteur. *Leishmania* a un cycle de vie digénétique, au cours duquel le parasite est transmis à différents hôtes, incluant les mammifères, lors du repas de sang de mouches de sable phlébotomes femelles. Des parasites, sous leur forme promastigote métacyclique, sont inoculés au nouvel hôle et ciblera les cellules phagocytaires mononucléaires, plus particulièrement les macrophages. À ce moment-là, ils créent une vacuole parasitophore (VP) nécessaire à leur différenciation en amastigote pour ultimement pouvoir s'y répliquer. Considérant leur auxotrophie pour plusieurs métabolites essentiels, les parasites *Leishmania* redirigent, réorganisent ou altèrent divers processus et voies de signalisation de leur cellule hôte au cours de l'infection. Plusieurs études ont d'ailleurs montré que *Leishmania* cible le métabolisme énergétique de la cellule hôte afin de créer un environnement permissif à sa réplication. Des études portant sur la signature transcriptionnelle de macrophages infectés par *L. major* avaient d'ailleurs montré une augmentation de la glycolyse ainsi que de la production de lactate combiné à une réduction du flux de pyruvate au cycle d'acide tricarboxylique, indiquant que les macrophages infectés ont tendance à convertir le glucose en lactate même en présence de suffisamment d'oxygène pour supporter la phosphorylation oxydative mitochondriale (OXPHOS). Le profile bioénergétique des macrophages infectés avec différentes souches de *Leishmania* révèle une induction de la glycolyse pendant les premiers moments de l'infection, suivi par une conversion de la glycolyse vers l'OXPHOS lors de la phase tardive de l'infection. L'analyse de mécanismes moléculaires employés par *L. infantum* pour moduler le métabolisme mitochondrial a révélé un rôle essentiel du senseur métabolique AMPK (adenosine monophosphate-activated protein kinase), un régulateur clé de l'activité de PGC-1α. PGC-1α est un coactivateur de la transcription et est responsable d'intégrer la biogénèse et les fonctions de génération d'énergie mitochondriales selon la demande métabolique associée à différents stades physiologiques. PGC-1α contrôle donc de multiples aspects de la biogénèse mitochondriale, incluant l'augmentation du nombre de mitochondries et la biogénèse du système OXPHOS en coordonnant l'expression des gènes nucléaires et mitochondriaux codant pour les protéines impliquées dans ces processus.

Les macrophages et autres phagocytes sont des cellules ayant une grande plasticité et sont rapidement capable d'adapter leur métabolisme en réponse à une invasion par un pathogène. Il est très bien connu dans la littérature que l'interaction entre les molécules de pathogènes et les récepteurs cellulaires gouvernant le système immunitaire inné contribue significativement à ces changements métaboliques. Cependant, les études utilisant différents pathogènes intracellulaires ont révélé une panoplie de changements métaboliques chez les macrophages infectés induits qui semblent induits par ces pathogènes. Cette notion reflète le fait que ces pathogènes produisent plusieurs molécules capables d'interagir dynamiquement avec les récepteurs immunitaires cellulaires et autres molécules clés de l'hôte au cours de l'infection.

Au cours du processus de colonisation du macrophage, *Leishmania* emploie plusieurs facteurs de virulence, incluant le glycolipide lipophosphoglycane (LPG). LPG contribue à l'habilitée de *Leishmania* (sous sa forme promastigote) à coloniser les cellules phagocytaires. Plus précisément, LPG aide à altérer la réponse du système immunitaire inné en réduisant les fonctions microbicides, en induisant la sécrétion de cytokines et en construisant la vacuole parasitophore. *Leishmania* crée ainsi un microenvironnement parfaitement adapté nécessaire pour sa réplication. Ces interactions sont cruciales pour déterminer la réussite de l'établissement de l'infection par le parasite.

Sachant que le rôle de la voie SIRT1-AMPK dans la modulation bioénergétique de la cellule hôte a déjà été établi, nos connaissances sont cependant limitées quant à la nature des facteurs cellulaires et parasitaires impliqués. Au cours de cette étude, nous avons investigué le mécanisme employé par lequel les promastigotes métacycliques de *L. donovani* altèrent les fonctions mitochondriales du macrophage. Plus précisément, nous avons intenté de déterminer le rôle potentiel du glycolipide de surface parasitaire LPG (lipophosphoglyane) lors du processus infectieux. L'hypothèse principale de cette étude est donc : Chez les macrophages, *Leishmania donovani* modifie le métabolisme et les fonctions mitochondriales de l'hôte de façon LPG-dépendante.

Pour adresser cette hypothèse, ces objectifs ont été établis:

1. Analyser le flux métabolique mitochondrial chez les cellules infectées par *Leishmania donovani* et le rôle de LPG à cet égard.
2. Élucider le rôle du LPG de *Leishmania donovani* au niveau de l'induction de la biogénèse mitochondriale.
3. Évaluer la voie de signalisation impliquée dans l'induction de la biogénèse mitochondriale chez le macrophage.

Ces objectifs sont adressés en détails dans l'article principal (Chapitre 3) et discuté extensivement au Chapitre 4.

6.2 Méthodologie

Afin d'élucider les possibles mécanismes gouvernant l'interaction *Leishmania*-macrophage, des macrophages dérivés de la moelle osseuse (BMM) infectés soit par *L. donovani*, un mutant *L. donovani* génétiquement modifié afin qu'il ne synthétise pas LPG *(*Δ*lpg1*) ou son homologue complémenté (Δ*lpg1+LPG1*). De plus, l'ultrastructure mitochondriale pendant l'infection a été évaluée *in vivo*. Des macrophages déficients pour TLR4, TLR2, TLRs endosomaux et IFNAR ont aussi été utilisés afin de valider la relation entre les récepteurs de l'hôte et la reprogrammation mitochondriale. Finalement, l'importance de cette reprogrammation pour le sort de *Leishmania* a été élucidée.

6.3 Résultats

Des études antérieures ont révélé que les promastigotes de *Leishmania* modulent le métabolisme des macrophages de l'hôte. Cependant, peu est connu sur les molécules parasitaires impliquées dans ce processus. Ainsi, dans un premier temps, nous avons analysé le rôle potentiel de l'abondant facteur de virulence LPG (lipophosphoglycane) dans les changements bioénergétiques induits chez les macrophages infectés par *L. donovani.* Chez les macrophages infectés par des promastigotes métacycliques de *L. donovani* sauvages ou Δ*lpg1* + *LPG1*, nous avons observé une augmentation du ratio OCR/ECAR, qui atteint son apogée à 4

h post-infection et a partiellement diminué à 24 h. En revanche, dans les macrophages infectés par le mutant Δ*lpg1*, le ratio OCR/ECAR est resté significativement inférieur à tous les moments post-infection, ce qui suggère que LPG est nécessaire pour stimuler le métabolisme oxydatif des macrophages infectés. Après avoir montré que les promastigotes métacycliques de *L. donovani* modifient le métabolisme des cellules hôtes, nous avons ensuite cherché à identifier la source de carbone qui conduit la production d'énergie chez les macrophages infectés. L'inhibition de la voie glycolytique avec le 2 désoxy-d-glucose (2-DG) a réduit de manière significative le taux de consommation d'oxygène mitochondrial induit par *L. donovani* (OCR) et le taux d'acidification extracellulaire (ECAR).

Même si l'inhibition de la β-oxydation mitochondriale avec l'étomoxir n'a pas eu d'impact majeur sur l'OCR, nous avons observé une augmentation significative de l'ECAR à 4 h, 8 h et 24 h post-infection. L'inhibition de l'ATP synthase (complexe V) par l'entremise de l'oligomycine a considérablement réduit l'OCR induit par *L. donovani*. L'inhibition de la β-oxydation peroxysomale avec la thioridazine a significativement réduit l'OCR induit par *L. donovani* 1 h après la phagocytose des parasites. D'autre part, l'inhibition de la voie d'oxydation de la glutamine avec BPTES n'a eu aucun impact majeur sur l'OCR dans les BMM infectés par *L. donovani*. Ces résultats indiquent donc que l'augmentation de l'OCR dans les macrophages infectés par *L. donovani* est indépendante de la glutaminolyse et de la β-oxydation mitochondriale, est cependant partiellement soutenue par la β-oxydation peroxysomale au cours de la première heure post-phagocytose et est dépendante de la glycolyse et du complexe V mitochondrial.

Pour étudier plus en détail la contribution de LPG aux changements métaboliques induits chez le macrophage par les promastigotes de *L. donovani*, nous avons évalué l'impact de ce glycolipide sur la biogenèse mitochondriale des cellules hôtes. Chez les macrophages infectés par des promastigotes métacycliques de *L. donovani* sauvages ou Δ*lpg1*+*LPG1*, nous avons observé que le réseau mitochondrial double par rapport à l'aire cellulaire 24 h post-infection. En revanche, la taille du réseau mitochondrial est restée inchangée dans les macrophages infectés par les promastigotes métacycliques *L. donovani* Δ*lpg1*, ce qui suggère que LPG participe à l'induction de la biogenèse mitochondriale dans les macrophages infectés. Ensuite, pour démontrer l'impact de LPG sur la biogenèse mitochondriale, nous avons mesuré le ratio ADN mitochondrial/nucléaire (mt/n) par qPCR en utilisant deux gènes codés par les mitochondries, le 16S ribosomal (*MT-16S*) et NADH déshydrogénase 1 (*MT-ND1*) qui seront comparés au gène

nucléaire Hexokinase-2 (*HK*2). Chez les macrophages infectés par des promastigotes métacycliques de *L. donovani* sauvage ou Δ*lpg1+LPG1*, nous avons observés que le ratio de *MT-16S* et *MT-ND1* relativement à *HK2* double (ratio d'ADN mt/n) comparativement aux cellules non-infectées. De plus, le ratio ADN mt/n est resté inchangé chez les macrophages infectés par des promastigotes métacycliques Δ*lpg1*, ce qui suggère l'importance de LPG dans l'induction de la biogenèse mitochondriale.

La biogénèse mitochondriale est un processus coordonné qui nécessite l'expression de gènes nucléaires et mitochondriaux, qui sont eux-mêmes régulés par des molécules de signalisation spécifiques, des facteurs de transcription et des régulateurs d'expression géniques. Par exemple, *PPARGC1A* et *NRF1* sont deux régulateurs clés de l'expression génique associés au métabolisme et à la biogénèse mitochondriale. Lors de nos analyses, nous avons observés que les promastigotes métacycliques de *L. donovani* sauvages et Δ*lpg1+LPG1* induisent tous les deux une expression deux fois plus élevée de *PPARGC1A* et 1,5 fois plus élevée de *NRF1* chez les macrophages infectés. À l'opposé, aucun changement d'expression n'a été observé, ni pour *PPARGC1A* ou *NRF1,* chez les macrophages infectés par les promastigotes métacycliques *L. donovani* Δ*lpg1.* À noter que les promastigotes métacycliques de *L. donovani* activent la phosphorylation de l'AMPK sur le Thr172, qui participe à la biogenèse mitochondriale en potentialisant l'activité transcriptionnelle de PGC-1α. Une augmentation des régulateurs de la biogenèse mitochondriale a été observée en analysant l'expression des gènes codés par les génomes nucléaires et mitochondriaux de la cellule hôte. Une augmentation des niveaux de protéines mitochondriales, de manière LPG-dépendante, a également été observée. Ces résultats démontrent l'importance de LPG dans l'induction de la biogenèse mitochondriale par les promastigotes de *L. donovani*. Nous avons ensuite déterminé si LPG seulement est suffisant pour stimuler la biogenèse mitochondriale. Semblable à ce qui est observé avec les promastigotes sauvages de *L. donovani*, la phagocytose de particules de zymosan recouverte de LPG a entraîné la redistribution rapide de LPG dans le macrophage. Cependant, ni le zymosan seul ni le zymosan enrobé de LPG n'ont stimulé la biogenèse mitochondriale telle qu'évaluée en mesurant le rapport ADN mt/n et l'expression de *PPARGC1A,* indiquant que LPG est essentiel mais pas suffisant par lui-même pour induire l'augmentation de la biogenèse mitochondriale et de l'expression des composants de la chaîne respiratoire par *L. donovani*.

Après l'internalisation dans les macrophages, les promastigotes se différencient en amastigotes. Cette forme du parasite est adaptée aux mammifères et se réplique dans les phagolysosomes.

Afin de déterminer si l'augmentation de la biogenèse mitochondriale induite lors de l'internalisation des promastigotes métacycliques est un événement transitoire ou persistant lorsque les parasites se différencient et se répliquent, nous avons évalué le rapport ADN mt/n et l'expression de *PPARGC1A* jusqu'à 72 h post-infection chez les macrophages infectés par des promastigotes métacycliques de *L. donovani* et par des amastigotes de *L. donovani* fraichement isolés de la rate de hamsters infectés. Comparativement aux macrophages non-infectés, nous avons observé une augmentation significative du rapport ADN mt/n et de l'expression de *PPARGC1A* dans les macrophages infectés jusqu'à 72 h, au moment où les promastigotes sont complètement différenciés en amastigotes. En effet, de façon similaire aux promastigotes métacycliques, les amastigotes ont induit une augmentation significative du rapport d'ADN mt/n (*MT-16S/HK2*) et de l'expression de *PPARGC1A* jusqu'à 72 h après l'infection, par rapport aux macrophages non-infectés. Également, nous avons observé que le nombre de mitochondries par cellule provenant de rates de hamsters infectés a doublé par rapport aux cellules provenant de rates non-infectées. Nous avons aussi observé une augmentation significative du nombre de crêtes par mitochondrie dans les cellules des rates infectées par rapport aux rates non-infectées. Nous avons en outre noté que les vacuoles contenant *L. donovani* sont entourées de mitochondries, suggérant un repositionnement stratégique de ces organelles dans les cellules infectées. Pour étudier plus en détail le mécanisme par lequel les amastigotes stimulent la biogenèse mitochondriale, nous avons utilisé des amastigotes collectés de macrophages infectés pendant 24 h avec des promastigotes métacycliques sauvages ou Δ*lpg1* de *L. donovani*. Nous avons infecté des macrophages avec ces amastigotes sauvages ou Δ*lpg1* et nous avons observé que, comme les amastigotes spléniques, les amastigotes sauvages isolés de macrophages infectés favorisaient une augmentation du rapport ADN mt/n et de l'expression de *PPARGC1A* par rapport aux BMM non infectés. En revanche, le rapport ADN mt/n et l'expression de *PPARGC1A* sont restés inchangés dans les macrophages infectés par les amastigotes Δ*lpg1* par rapport aux macrophages non-infectés. Collectivement, ces résultats suggèrent que LPG ou un glycolipide structurellement apparenté contribue à l'induction de la biogenèse mitochondriale par les amastigotes de *L. donovani*.

Nous avons précédemment montré que LPG est excrété de la surface des promastigotes internalisés et sort de la vacuole parasitophore. Cependant, nous n'avons pas observé de colocalisation significative entre le LPG et les mitochondries, ce qui indique que le LPG agit indirectement sur les mitochondries via des voies de signalisation médiées par les récepteurs.

Pour déterminer si les récepteurs TLRs (Toll-like receptors) sont nécessaires à l'induction de la biogenèse mitochondriale, nous avons infecté des macrophages murins provenant de souris mutantes pour *Tlr2-/-, Tlr4-/-* et les TLRs endosomaux (*Unc93b1*$^{Letr/Letr}$) avec des promastigotes métacycliques de *L. donovani*. Nous avons observé que l'augmentation du ratio ADN mt/n et de l'expression de *PPARGC1A* induite par les promastigotes métacycliques de *L. donovani* était complètement supprimée en l'absence de TLR4 et de TLR endosomaux, alors qu'une augmentation partielle du rapport ADN mt/n et de l'expression de *PPARGC1A* se produisait en l'absence de TLR2. Ces résultats établissent un rôle pour le TLR4 et les TLR endosomaux dans la médiation de la mitochondriogénèse en réponse à une infection par les promastigotes métacycliques de *L. donovani*.

L'activation des TLR conduit à la production d'IFN de type I en réponse à divers agents pathogènes, dont *Leishmania*. Nous avons donc étudié la possibilité que la signalisation autocrine déclenchée par ces cytokines régule la biogenèse mitochondriale en réponse à *L. donovani*. Nous avons déterminé que les promastigotes métacycliques de *L. donovani* induisaient des niveaux similaires d'expression d'*IFNA7,* et ce, de manière indépendante de LPG. Il est important de noter que l'abolition de l'expression d'*IFNA7* observée à la fois dans les macrophages *Tlr4-/-* et les TLRs endosomaux (*Unc93b1*$^{Letr/Letr}$) a suggéré que TLR4 et les TLR endosomaux agissent de concert pour médier la production d'IFN de type I en réponse aux promastigotes métacycliques de *L. donovani*. En revanche, l'absence de TLR2 n'affecte pas l'induction de l'expression d'*IFNA7* par les promastigotes de *L. donovani*. De plus, nous avons observé qu'en absence du récepteur IFN-I (IFNAR), l'augmentation normalement induite par *L. donovani* du ratio mt/n ADN était absente, alors que l'induction de l'expression de *PPARGC1A* restait inchangée. Étonnamment, l'absence du récepteur IFN-I n'a pas affecté les changements induits par *L. donovani* dans le métabolisme mitochondrial (rapport OCR/ECAR), ni la phosphorylation de l'AMPK. Ces résultats indiquent que *L. donovani* induit une expression de l'IFNα, indépendante de LPG, qui agit de manière autocrine pour stimuler une augmentation du rapport ADN mt/n.

Nous avons ensuite étudié l'importance de la biogenèse mitochondriale induite par LPG concernant la capacité des promastigotes métacycliques de *L. donovani* à coloniser le macrophage. Considérant que TLR4, les TLR endosomaux et le récepteur IFN-I sont essentiels à l'induction de la biogenèse mitochondriale, nous avons comparé le sort des promastigotes métacycliques sauvages de *L. donovani* dans les macrophages sauvages, *Tlr4-/-*,

Unc93b1$^{Letr/Letr}$ et *Ifnar-/-*. Étant donné que TLR2 n'influence pas la biogenèse mitochondriale, nous avons inclus Tlr2-/- BMM comme contrôle. Nous avons observé que les promastigotes métacycliques de *L. donovani* se répliquaient dans les macrophages de type sauvage. L'absence de TLR2 n'a eu aucun impact sur l'internalisation et la survie ultérieure de *L. donovani*, bien que nous ayons observé une légère réduction de la réplication dans les macrophages Tlr2-/- par rapport aux sauvages. En revanche, la survie des promastigotes métacycliques de *L. donovani* et leur réplication ultérieure étaient significativement altérées en absence de TLR4, de TLR endosomaux ou du récepteur IFN-I. Ces résultats suggèrent que l'induction de la biogenèse mitochondriale médiée par ces TLRs et le récepteur IFN-I contribuent à la survie et à la réplication de *L. donovani* dans le macrophage.

Il est également important de considérer que certains événements autre que la biogenèse mitochondriale en aval des TLRs et de la signalisation autocrine déclenchée par l'IFNα peuvent affecter l'issue de l'infection par *L. donovani.* Nous avons donc évalué la croissance de *L. donovani* dans des macrophages chez lesquels la mitochondriogénèse a été pharmacologiquement induite avant l'infection. Ceci a été fait avec AICAR, un activateur de AMPK qui stimule la biogenèse mitochondriale. Nous avons observé que la réplication des promastigotes métacycliques sauvages et Δ*lpg1+LPG1* était significativement augmentée dans les macrophages traités à l'AICAR par rapport aux macrophages non-stimulés. Curieusement, alors que le mutant *Δlpg1* a été éliminé dans les macrophages non-stimulés, il y a eu une augmentation significative de la charge parasitaire (3 fois) de ce mutant dans les macrophages traités à l'AICAR par rapport à ceux non-stimulés. Pour déterminer si l'augmentation de la survie du mutant Δ*lpg1* dans les macrophages traités par AICAR était liée à une altération de la production de ROS (due à l'effet pléiotrope de AICAR), nous avons comparé la croissance des promastigotes métacycliques sauvages et Δ*lpg1* dans des macrophages murins sauvages et gp91$^{phox-/-}$, prétraitées ou non avec AICAR. Nous avons observé que la mutation de gp91$^{phox-/-}$ n'avait pas d'impact significatif sur le sort des promastigotes *L. donovani* sauvages ou Δ*lpg1* au niveau des macrophages témoins non-traités. Cependant, nous avons observé une légère augmentation de la charge parasitaire dans les macrophages gp91$^{phox-/-}$ prétraité avec AICAR, autant pour *L. donovani* sauvages que Δ*lpg1*. Ces résultats indiquent que la survie accrue du mutant Δ*lpg1* dans les macrophages traités à l'AICAR n'est pas liée à une inhibition de la production de ROS. De plus, ces résultats suggèrent que l'induction pharmacologique de la biogenèse mitochondriale et la stimulation de l'expression de PGC-1α créent un environnement

métaboliquement adapté favorable à la réplication de *L. donovani* et qui permet au mutant *L. donovani* Δ*lpg1* avirulent de coloniser le macrophage.

Ces observations nous ont amenés à explorer le lien possible entre la synthèse de l'hème dans le cadre de la biogenèse mitochondriale et la capacité de *Leishmania* à établir une infection. La synthèse de l'hème est associée à une chaîne de transport d'électrons accrue et à une OXPHOS améliorée. La première étape de la synthèse se produit dans la mitochondrie et est catalysée par l'acide 5-aminolévulinique synthase (ALAS1), qui est l'enzyme biosynthétique de l'hème limitant la vitesse de production. L'expression d'ALAS1 est étroitement régulée et est sous le contrôle de NRF-1 et PGC-1α. Semblable à l'induction de ces deux régulateurs de l'expression génique associés à la biogenèse et au métabolisme mitochondriaux, nous avons constaté que les promastigotes métacycliques de *L. donovani* induisent une expression plus élevée d'*ALAS1*. Ces résultats indiquent que *L. donovani* stimule la biosynthèse de l'hème lors de l'infection de la cellule hôte. Pour évaluer l'impact de la biosynthèse de l'hème sur la capacité de *L. donovani* à se répliquer dans les macrophages, nous avons inhibé avec 100 μM de succinylacétone la deuxième enzyme biosynthétique de l'hème, l'acide aminolévulinique déshydratase (ALAD). Celle-ci catalyse la conversion de l'acide 5-aminolévulinique en porphobilinogène. Nous avons observé que l'inhibition de l'ALAD empêchait la réplication de *L. donovani*, indiquant que la biosynthèse de l'hème au cours de la biogenèse mitochondriale contribue à la capacité de ce parasite à coloniser le macrophage.

6.4 Discussion

Dans cette étude, nous avons étudié les mécanismes par lesquels les promastigotes métacycliques de *L. donovani* modifient la biologie mitochondriale des cellules hôtes. Nous décrivons le rôle essentiel du facteur de virulence, le glycolipide LPG, dans la stimulation de l'OXPHOS et l'induction de la biogenèse mitochondriale chez les macrophages infectés. De plus, nous démontrons que la mitochondriogénèse nécessite l'action de l'IFN de type I, qui est induite indépendamment de LPG. Considérant que l'induction pharmacologique de la biogenèse mitochondriale augmente la permissivité des macrophages à *L. donovani*, cette étude soutient l'idée que la biogenèse mitochondriale crée un environnement métaboliquement adapté propice à la réplication du parasite.

Compte tenu de l'importance de LPG pour la colonisation des cellules hôtes par *Leishmania*, nous avons cherché à déterminer le rôle potentiel de ce glycolipide de virulence dans la modulation de la biogenèse et du métabolisme mitochondriaux. Comme décrit précédemment pour *L. infantum*, nous avons constaté que les promastigotes métacycliques de *L. donovani* stimulent l'OXPHOS, augmentent la masse mitochondriale des macrophages et induisent l'expression de PGC-1α et NRF1, ainsi que l'expression des gènes de la chaîne respiratoire. À l'aide d'un mutant génétiquement et structurellement défini de *L. donovani* déficient pour la synthèse de LPG *(Δlpg1*) et de son homologue complémenté *(Δlpg1+LPG1*), nous avons obtenu la preuve que cette molécule est essentielle aux modifications de la biologie mitochondriale de la cellule hôte induite par les promastigotes de *L. donovani*. Ainsi, en plus de son rôle important dans le désamorçage des processus clés de défense de l'hôte qui protège les promastigotes contre les fonctions microbicides des cellules phagocytaires, nos travaux ont révélé que la modification du métabolisme des cellules hôtes représente une fonction primordiale de LPG. Comme pour les promastigotes métacycliques, nous avons observé que les amastigotes spléniques stimulent efficacement la biogenèse mitochondriale dans les macrophages et dans la rate des hamsters infectés. Le fait que les amastigotes de *L. donovani Δlpg1* ne parviennent pas à déclencher une augmentation du rapport ADN mt/n et de l'expression de *PPARGC1A* suggère que les niveaux de LPG ou de glycolipides de type LPG exprimés par les amastigotes sont suffisants pour stimuler la biogenèse mitochondriale.

Des études antérieures ont identifié TLR2 et TLR4 comme les récepteurs responsables de la reconnaissance du LPG de diverses espèces de *Leishmania*. Nos résultats indiquent que TLR4 est essentiel pour la stimulation de l'expression de PGC-1α et que la biogenèse mitochondriale est cohérente avec le rôle de ce récepteur dans la reconnaissance du LPG. Cependant, le LPG en lui-même n'est pas suffisant pour stimuler l'expression de PGC-1α et la biogenèse mitochondriale, ce qui indique que des facteurs supplémentaires sont nécessaires. Par conséquent, nous avons constaté que la biogenèse mitochondriale nécessite une voie indépendante du LPG liée à l'expression de l'IFNα. En effet, cette voie implique également TLR4, suggérant que ce récepteur joue un double rôle dans le contrôle de la biogenèse mitochondriale. Bien que nous n'ayons pas étudié le ou les ligands responsables de l'induction de l'IFN de type I médiée par TLR4, des études antérieures ont mis en évidence un rôle clé de la voie neutrophile élastase-TLR4 dans ce processus. À l'aide de macrophages de souris *Unc93b1*$^{Letr/Letr}$, nous avons constaté qu'en plus du TLR4, l'induction de la biogenèse

mitochondriale et de l'expression de l'IFNα par les promastigotes métacycliques de *L. donovani* nécessite des TLR endosomaux. Plusieurs études ont mis en évidence la contribution de ces récepteurs dans la réponse de l'hôte face à diverses espèces de *Leishmania*. Des travaux récents ont révélé que TLR3 est le TLR endosomal qui assure la médiation de l'expression d'IFN de type I en réponse à *L. donovani*, ce qui correspond à l'expression défectueuse d'IFNα dans les macrophages *Unc93b1*$^{Letr/Letr}$ infectés par *L. donovani*. On sait peu de choses sur la nature du ou des ligands de *Leishmania* reconnus par TLR3, à l'exception du virus à ARN double brin LRV1 présent dans les isolats de diverses espèces de *Leishmania*. Il est à noter que TLR3 contribue à la reconnaissance des promastigotes de *L. donovani,* même si ces parasites ne sont pas infectés par un virus à ARN double brin. La nature du ou des ligands dérivés de *L. donovani* reconnus par TLR3 reste donc à être élucider.

En l'absence d'IFNAR, les promastigotes de *L. donovani* ne parviennent pas à induire la biogenèse mitochondriale, conformément à l'idée que l'IFNα agit de manière autocrine dans ce processus. Le fait que la biogenèse mitochondriale induite par *L. donovani* n'ait pas lieu dans les macrophages *Ifnar-/-* malgré l'induction de l'expression de PGC-1α illustre la complexité des voies impliquées dans ce processus et met en évidence les multiples rôles de PGC-1α dans la modulation du métabolisme énergétique. Curieusement, nos résultats indiquent que la signalisation de l'IFNα ne joue pas un rôle significatif dans la stimulation d'OXPHOS et de la glycolyse chez les macrophages infectés par *L. donovani*. Ceci contraste avec les découvertes récentes lors d'infection à *Mycobacterium tuberculosis*, qui se caractérise par une diminution à la fois de la glycolyse et de la respiration mitochondriale, suggérant que les effets de l'IFN de type I sont spécifiques à l'agent pathogène et au contexte.

Plusieurs études ont mis en évidence un rôle protecteur pour l'IFN de type I dans la leishmaniose. Cependant, de plus en plus de preuves indiquent que la signalisation de l'IFN de type I joue également un rôle préjudiciable pour l'hôte, favorisant la réplication intracellulaire du parasite. En effet, il a été démontré que le ciblage de l'IFN de type I pendant le traitement anti-*Leishmania* améliore l'immunité lié à la réponse cellulaire Th1. Sur la base de nos résultats, il est tentant de supposer que l'IFNα contribue à la capacité de *Leishmania* à proliférer au sein de son hôte via la stimulation de la biogenèse mitochondriale. À cet égard, nos résultats appuient l'idée que l'induction de la mitochondriogénèse par les promastigotes de *L. donovani* est importante pour le processus de colonisation des cellules hôtes. En effet, en cohérence avec les travaux précédents, la survie et la réplication de *L. donovani* étaient nettement altérées dans les

macrophages déficients pour TLR4, pour les TLRs endosomaux et IFNAR, dans lesquels le parasite ne parvient pas à induire la biogénèse mitochondriale. De plus, l'induction pharmacologique de la biogénèse mitochondriale a considérablement augmenté la permissivité des macrophages à la réplication de *L. donovani*, ce qui suggère que l'induction de la biogenèse mitochondriale crée un environnement métaboliquement adapté propice à la réplication du parasite. Des résultats similaires ont déjà été rapportés pour les macrophages infectés par *L. infantum*. Cela contraste avec plusieurs agents pathogènes, notamment *Mycobacterium tuberculosis*, *Haemophilus parasuis*, *Staphylococcus aureus* et *Plasmodium falciparum*, dont la survie et la réplication sont altérées si leurs cellules hôtes est prétraitées avec AICAR. De manière frappante, le mutant avirulent *L. donovani* Δ*lpg1* déficient en LPG, qui n'induit pas la biogenèse mitochondriale, a colonisé des macrophages prétraités avec AICAR. Cette découverte suggère que la stimulation pharmacologique de la biogénèse mitochondriale contourne la nécessité de créer des conditions favorables au développement du parasite dans les cellules hôtes de manière LPG-dépendante.

Parce que l'hème est un cofacteur essentiel pour plusieurs enzymes de la chaîne de transport d'électrons, sa synthèse joue un rôle central dans la biologie mitochondriale et la fonctionnalité du complexe OXPHOS. Par conséquent, la première étape de la synthèse de l'hème, qui limite la vitesse de production, se produit dans les mitochondries et est catalysée par ALAS1 pour générer de l'acide 5-aminolévulinique. L'expression d'ALAS1 est étroitement régulée et est sous le contrôle de PGC-1α, qui contrôle également la biogénèse mitochondriale et le métabolisme oxydatif. Nos découvertes selon lesquelles les promastigotes de *L. donovani* induisent l'expression d'ALAS1 et que l'inhibition pharmacologique de la deuxième étape de biosynthèse de l'hème altèrent la capacité de *L. donovani* à se répliquer dans les macrophages hôtes confirment l'importance pour ce parasite d’induire la biogénèse mitochondriale et l’OXPHOS pour son développement chez les mammifères. De plus, *Leishmania* est auxotrophe pour l’hème et doit donc l’acquérir de l'hôte pour se développer de manière intracellulaire. L'activation de la biosynthèse de l'hème lors de la colonisation des cellules hôtes peut donc également servir à répondre aux besoins en hème de *Leishmania*. Des études futures seront nécessaires pour élucider cette question.

6.5 Conclusion

En résumé, nous fournissons de nouvelles informations sur les mécanismes conduisant à la biogénèse mitochondriale et à la reprogrammation métabolique dans les macrophages infectés par *L. donovani*. Nous concluons que les promastigotes métacycliques de *L. donovani* induisent cinq réponses cellulaires distinctes chez son hôte en modulant la mitochondriogénèse des macrophages. Le premier est l'augmentation de la masse mitochondriale des macrophages de manière dépendante de LPG, l'augmentation de l'expression de PGC-1α et des gènes associés à la chaîne de transport d'électrons, et la stimulation d'OXPHOS. Le second est la dépendance des TLR4 et des TLR endosomaux pour l'induction de la biogenèse mitochondriale. Le troisième est l'induction de l'expression de l'IFNα, de manière indépendante de LPG, qui médie également l'induction de la biogénèse mitochondriale des macrophages, mais n'a aucun impact sur l'induction de l'expression de PGC-1α ou du flux mitochondrial. Le quatrième est l'importance de la biogenèse mitochondriale pour le destin de *Leishmania* et le cinquième est la biosynthèse augmentée de l'hème, qui soutient la réplication du parasite. De plus, nous suggérons que les besoins nutritionnels des amastigotes contribuent à la nécessité de l'hème pour ces parasites, ce qui peut être soutenu par une masse mitochondriale accrue. Nos résultats sont cohérents avec la notion de recâblage métabolique spécifique à un agent pathogène, qui résulte de l'interaction complexe entre des ensembles de molécules pathogéniques et des récepteurs de la cellule hôte.

APPENDIX 1: Complementary publications

7 Publication in related fields

7.1 VAMP3 and VAMP8 Regulate the Development and Functionality of Parasitophorous Vacuoles Housing *Leishmania amazonensis*

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Article context and author contributions: In this publication, we evaluated the role of host cell soluble N-ethylmaleimide-sensitive-factors during the parasitophorous vacuole formation and their importance on the parasite fate. In this article, I have made the colocalization of SNAREs with LAMP1 on the membranes of communal PVs harbouring *L. amazonensis*. The conducted experiments are showed in figure 2.

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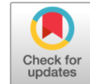

VAMP3 and VAMP8 Regulate the Development and Functionality of Parasitophorous Vacuoles Housing *Leishmania amazonensis*

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ABSTRACT To colonize mammalian phagocytic cells, the parasite *Leishmania* remodels phagosomes into parasitophorous vacuoles that can be either tight-fitting individual or communal. The molecular and cellular bases underlying the biogenesis and functionality of these two types of vacuoles are poorly understood. In this study, we investigated the contribution of host cell soluble *N*-ethylmaleimide-sensitive-factor attachment protein receptor proteins to the expansion and functionality of communal vacuoles as well as the replication of the parasite. The differential patterns of recruitment of soluble *N*-ethylmaleimide-sensitive-factor attachment protein receptor to communal vacuoles harboring *Leishmania amazonensis* and to individual vacuoles housing *L. major* led us to further investigate the roles of VAMP3 and VAMP8 in the interaction of *Leishmania* with its host cell. We show that whereas VAMP8 contributes to the optimal expansion of communal vacuoles, VAMP3 negatively regulates *L. amazonensis* replication, vacuole size, as well as antigen cross-presentation. In contrast, neither protein has an impact on the fate of *L. major*. Collectively, our data support a role for both VAMP3 and VAMP8 in the development and functionality of *L. amazonensis*-harboring communal parasitophorous vacuoles.

KEYWORDS *Leishmania*, SNARE, cross-presentation, macrophage, parasitophorous vacuole

Leishmania is the protozoan parasite responsible for a spectrum of diseases termed leishmaniasis. Shortly after inoculation into a mammalian host by an infected sand fly, promastigote forms of the parasite are taken up by host phagocytes. To colonize these cells, promastigotes subvert their microbicidal machinery by targeting signaling pathways and altering intracellular trafficking (1–3) and create a hospitable niche that will allow their differentiation and replication as mammalian-stage amastigote forms (4, 5). Most *Leishmania* species replicate in tight-fitting individual parasitophorous vacuoles (PVs), with the exception of species of the *Leishmania mexicana* complex, which replicate in spacious communal PVs. For tight-fitting individual PVs, the replication of the parasites entails vacuolar expansion and fission, yielding two individual PVs containing one parasite each. In contrast, communal PVs occupy a large volume within infected cells and contain several amastigotes. These two different lifestyles imply that *Leishmania* uses distinct strategies to create the space needed for its replication within infected cells.

The biogenesis and expansion of communal PVs are accomplished through the acquisition of membrane from several intracellular compartments (4). Hence, shortly after phagocytosis, phagosomes harboring *Leishmania amazonensis* fuse extensively with host cell late endosomes/lysosomes and secondary lysosomes (6, 7), consistent with

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the notion that communal PVs are highly fusogenic (8). Homotypic fusion between *L. amazonensis*-containing PVs also occurs, but its contribution to PV enlargement remains to be further investigated (9, 10). The interaction of these PVs with various subcellular compartments indicates that the host cell membrane fusion machinery is central to the biogenesis and expansion of communal PVs and is consistent with a role for soluble *N*-ethylmaleimide-sensitive-factor attachment protein receptor (SNARE) proteins in this process (11). In this regard, the roles of a number of macrophage SNAREs and their regulators have been studied in the context of *Leishmania* infection, including Sec22b, syntaxin 5 (STX5), syntaxin 18, synaptotagmin V (Syt V), synaptotagmin IX, VAMP3, and VAMP8 (12–19). The latter two SNAREs, VAMP3 and VAMP8, are mainly associated with the endocytic pathway. They regulate fusion between endosomes and lysosomes and contribute to a number of cellular processes, including phagolysosome biogenesis and function (14, 20–22), exocytosis and secretion (21, 23–25), and antimicrobial and canonical autophagy (15, 26–29). Hence, *Leishmania*-harboring communal PVs interact with the host cell endoplasmic reticulum (ER), and disruption of the fusion machinery associated with the ER-Golgi intermediate compartment (ERGIC) was shown to inhibit parasite replication and PV enlargement (16–18, 30). PV size and parasite replication were also shown to be controlled by LYST (31), a protein associated with the integrity of lysosomal size and quantity (32), and by the scavenger receptor CD36, possibly through the modulation of fusion between the PV and endolysosomal vesicles (33). Interestingly, the V-ATPase subunit d2, which does not participate in phagolysosome acidification, was recently shown to control the expansion of *L. amazonensis*-harboring PVs through its ability to modulate membrane fusion (34). In addition to regulating the biogenesis of phagolysosomes, trafficking and fusion events play a critical role in the acquisition by phagosomes of the capacity to process antigens for presentation to T cells (35–37). In the case of *Leishmania*-harboring PVs, investigations of their immunological properties revealed that the processing of antigens for the activation of T cells may take place, although the efficiency can be attenuated by the parasite (14, 38–48).

How parasites of the *L. mexicana* complex coopt host cell processes to create and maintain hospitable communal PVs is poorly understood. In the case of *Leishmania* species living in tight-fitting individual PVs, two abundant components of the promastigote surface coat modulate PV composition and properties: the glycolipid lipophosphoglycan (LPG) and the zinc metalloprotease GP63. LPG contributes to the ability of *Leishmania donovani*, *L. major*, and *L. infantum* to colonize phagocytes (49–51) by reducing phagosome fusogenicity toward late endosomes and lysosomes, impairing the assembly of the NADPH oxidase, and inhibiting phagosome acidification (49, 50, 52–56). In contrast, LPG is not required for infection of macrophages or mice by *L. mexicana* (57), suggesting that LPG has little impact on the formation and properties of communal PVs. GP63 contributes to the properties and functionality of tight-fitting PVs by targeting components of the host membrane fusion machinery, including the SNAREs VAMP8 and Syt XI, both of which regulate microbicidal and immunological properties of phagosomes (13, 14). We also previously reported that the episomal expression of GP63 increases the ability of an *L. mexicana* Δ*cpb* mutant to replicate in macrophages and to generate larger communal PVs (12). This correlated with the exclusion of the endocytic SNARE VAMP3 from the PV, suggesting a potential role for this component of the host cell membrane fusion machinery in the regulation of communal PV biogenesis.

In the present study, we sought to further elucidate the contribution of host cell SNAREs to the biology of communal PVs. We provide evidence that the endocytic SNAREs VAMP3 and VAMP8 regulate the development and functionality of communal PVs and impact the growth of *L. amazonensis*.

RESULTS

Differential association of SNAREs with PVs harboring *L. major* and *L. amazonensis*. To study the host cell machinery associated with the development of *Leishmania*-harboring communal PVs, we first compared the kinetics of recruitment

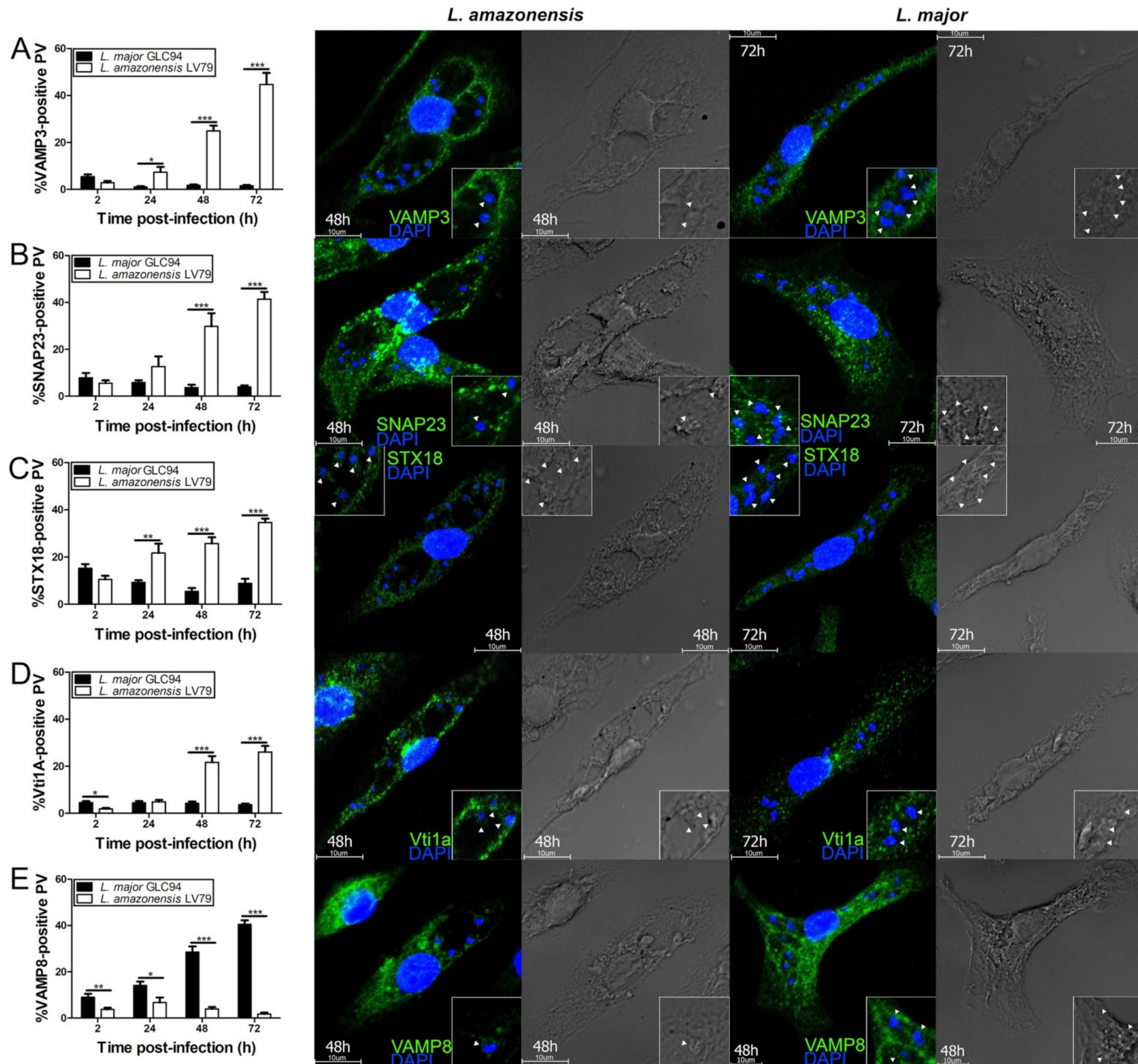

FIG 1 Differential recruitment of SNAREs to *Leishmania*-harboring PVs. BMMs were infected with *L. major* GLC94 or *L. amazonensis* LV79 promastigotes, and the presence of VAMP3 (A), SNAP23 (B), STX18 (C), Vti1A (D), and VAMP8 (E) in PVs was assessed and quantified by confocal immunofluorescence microscopy at 2, 24, 48, and 72 h postphagocytosis. SNAREs are shown in green, and DNA is shown in blue. Data are presented as the means ± standard errors of the means (SEM) of values from three independent experiments. Representative images from 3 experiments are shown. Insets display the PV area. For L. amazonensis, arrowheads indicate recruitment, while for L. major arrowheads indicate the absence of recruitment. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

and trafficking of host SNAREs to tight-fitting individual and communal PVs. To this end, we infected bone marrow-derived macrophages (BMMs) with either *L. major* strain GLC94 or *L. amazonensis* strain LV79 and used confocal immunofluorescence microscopy to assess the fate of SNAREs associated with various host cell compartments up to 72 h postinfection. As shown in Fig. 1A, we found a gradual increase in the proportion of *L. amazonensis* LV79-harboring communal PVs positive for the recycling endosomal vesicular (v)-SNARE VAMP3, whereas the proportion of VAMP3-positive tight-fitting PVs harboring *L. major* GLC94 remained below 5%. We observed a similar selective recruitment pattern around communal PVs harboring *L. amazonensis* LV79 for the plasma membrane-associated target (t)-SNARE SNAP23 (Fig. 1B), the ER t-SNARE syntaxin

18 (Fig. 1C), and the *trans*-Golgi t-SNARE Vti1A (Fig. 1D). Lysosome-associated membrane protein 1 (LAMP1), which we used to define lysosomal features (58), also accumulated mainly on communal PVs containing *L. amazonensis* LV79 (see Fig. S1A in the supplemental material). In contrast, the late endosomal v-SNARE VAMP8 was gradually recruited to tight-fitting PVs containing *L. major* but not to communal PVs containing *L. amazonensis* LV79 (Fig. 1E). In the case of the early endosomal t-SNARE syntaxin 13, we found that this SNARE was associated with only a small subset (10 to 15%) of PVs harboring either *L. major* GLC94 or *L. amazonensis* LV79 (Fig. S1B). To provide further evidence that the v-SNARE VAMP3 and the t-SNAREs SNAP23, syntaxin 18, and Vti1A are recruited to the membrane of communal PVs containing *L. amazonensis* LV79, we assessed their colocalization with LAMP1, which is also recruited to these PVs. As shown in Fig. 2, these SNAREs colocalize with LAMP1 at the PV membrane. In contrast, we observed little colocalization between the t-SNARE VAMP8 and LAMP1. These results support the notion that in contrast to *L. major*, *L. amazonensis* recruits components from both the host cell endocytic and the secretory pathways for the development and maintenance of communal PVs (4, 30).

VAMP3 and VAMP8 contribute to the control of *L. amazonensis* infection. Given the differential patterns of association of the endocytic v-SNAREs VAMP3 and VAMP8 with tight-fitting individual and communal PVs, we used them as exemplars to further investigate the development of these PVs. We first investigated the impact of these two SNAREs on parasite replication. To this end, we infected wild-type (WT), *Vamp3*$^{-/-}$, and *Vamp8*$^{-/-}$ BMMs with either *L. major* GLC94 or *L. amazonensis* LV79, and at various time points, we assessed the parasite burden and PV surface area. In the case of VAMP8, we previously reported that its absence had no impact on the replication of *L. major* GLC94 (15). Similarly, the absence of VAMP8 had no effect on the replication of *L. amazonensis* LV79 up to 72 h postinfection (Fig. 3A). Strikingly, VAMP3-deficient BMMs were more permissive to the replication of *L. amazonensis* LV79, with a 50% increase in the number of parasites per infected macrophage at 72 h postinfection (Fig. 3B). Moreover, PVs harboring *L. amazonensis* LV79 were significantly larger in the absence of VAMP3 (Fig. 3C). In contrast, the absence of VAMP3 had no impact on the survival and replication of *L. major* GLC94 over a period of 72 h postinfection (Fig. 3B). These results indicate a role for VAMP3 in the control of *L. amazonensis* LV79 replication and communal PV expansion.

To gain insight into the control of *L. amazonensis* LV79 replication and PV size by VAMP3, we asked whether its absence impacted the mobilization to PVs of other known regulators of membrane fusion and PV expansion. We chose SNAP23, which forms complexes with VAMP3 during phagocytosis and negatively regulates phagosome maturation (59, 60); VAMP8, which can replace VAMP3 (24); and the V-ATPase, whose membrane-embedded V0 domain interacts with SNAREs and regulates membrane fusion as well as the expansion of communal PVs (34, 61). We infected WT and *Vamp3*$^{-/-}$ BMMs with *L. amazonensis* LV79, and at various time points, we assessed the recruitment of these molecules to PVs up to 72 h postinfection. As shown in Fig. 4A and B, the absence of VAMP3 resulted in an impaired enrichment of SNAP23 around PVs and had no impact on the recruitment of VAMP8. In the case of the a3 subunit of the V-ATPase, we observed an increasing proportion of PVs containing *L. amazonensis* LV79 positive for ATP6V0a3 from 24 h to 72 h postinfection in the absence of VAMP3 (Fig. 4C).

Virulent strains of *L. amazonensis* were previously shown to induce larger communal PVs (62). Given our data indicating that VAMP3 regulates PV size, we sought to compare PVs harboring *L. amazonensis* strains with different levels of virulence for the recruitment of VAMP3. To this end, we infected BMMs with either *L. amazonensis* strain LV79 or *L. amazonensis* strain PH8, which displayed higher virulence than strain LV79 in an experimental model of murine cutaneous leishmaniasis (63). Using confocal immunofluorescence microscopy, we compared the kinetics of the VAMP3 association with PVs in BMMs infected for various times with either *L. amazonensis* LV79 or PH8.

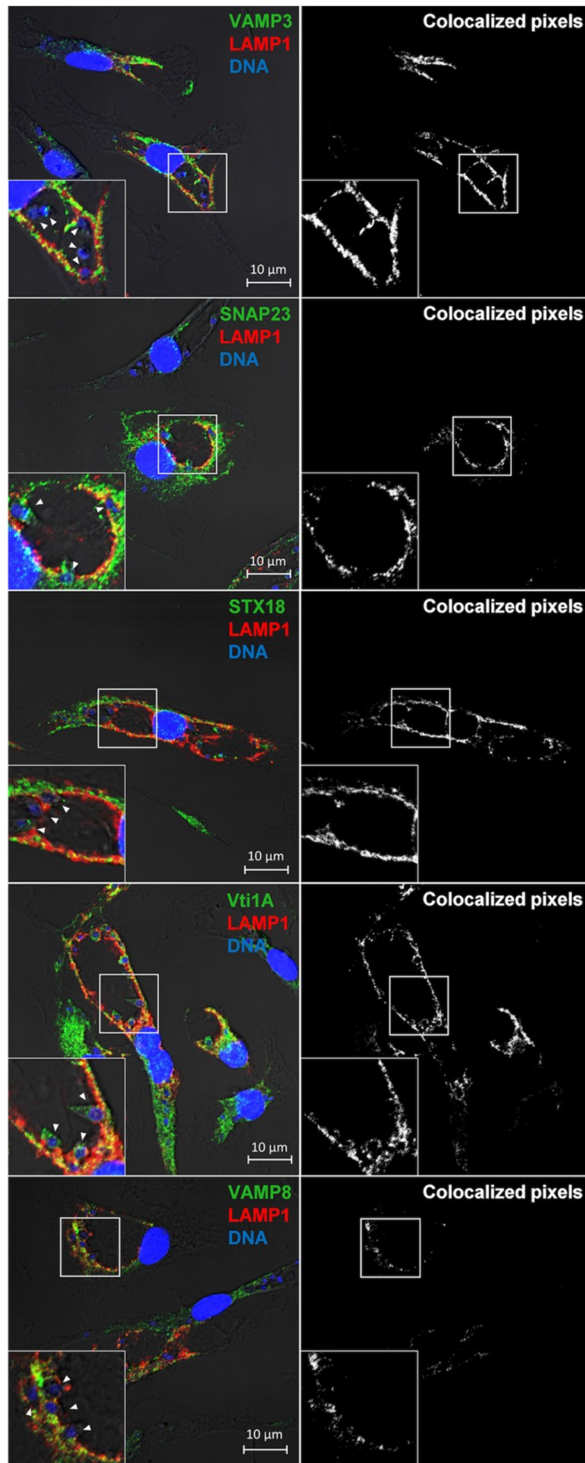

FIG 2 Colocalization of SNAREs with LAMP1 on the membranes of communal PVs harboring *L. amazonensis*. BMMs were infected with *L. amazonensis* LV79 promastigotes, and at 72 h postphagocytosis, the colocalization (white pixels) of LAMP1 (red) with the SNAREs VAMP3, SNAP23, STX18, Vti1A, and VAMP8 (all in green) in PVs was assessed and quantified by confocal immunofluorescence microscopy. DNA is in blue. Representative images from 3 experiments are shown. Insets display the PV area.

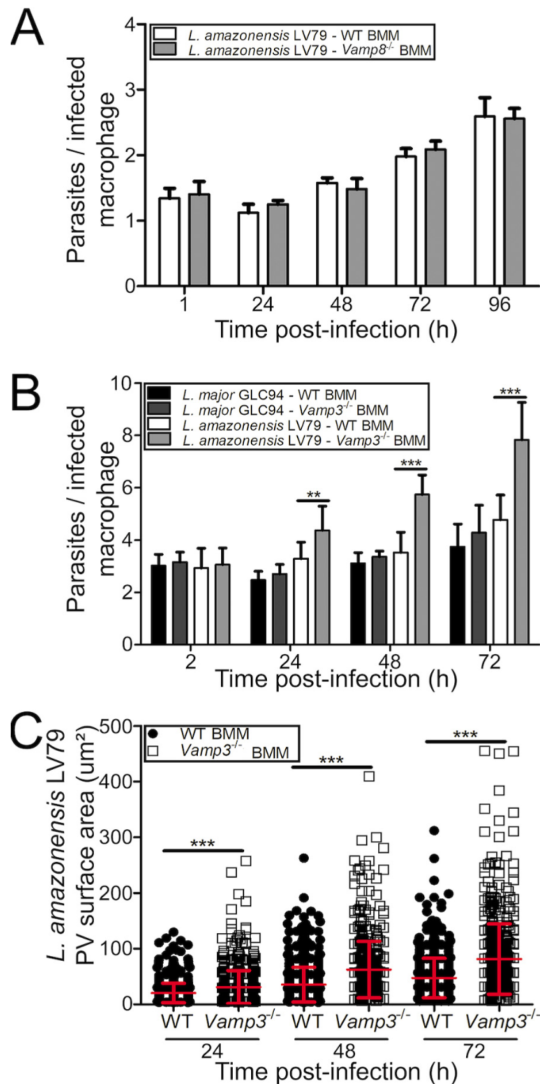

FIG 3 VAMP3 negatively regulates the replication of *L. amazonensis* LV79 and PV expansion. WT, *Vamp8*$^{-/-}$, and *Vamp3*$^{-/-}$ BMMs were infected with *L. major* GLC94 or *L. amazonensis* LV79 promastigotes, and at various time points after phagocytosis, parasite replication and PV size were assessed. (A) Quantification of *L. amazonensis* LV79 promastigote replication in WT and *Vamp8*$^{-/-}$ BMMs at 1, 24, 48, 72, and 96 h postinfection. Data are presented as the means $\pm$ SEM of values from three independent experiments. (B) Quantification of *L. major* GLC94 and *L. amazonensis* LV79 burdens in WT or *Vamp3*$^{-/-}$ BMMs at 2, 24, 48, and 72 h postinfection. Data are presented as the means $\pm$ SEM of values from three independent experiments. **, $P \leq 0.01$; ***, $P \leq 0.001$. (C) Quantification of PV sizes in WT and *Vamp3*$^{-/-}$ BMMs infected with *L. amazonensis* LV79 at 2, 24, 48, and 72 h postinfection. Data are presented as a cloud with means $\pm$ standard deviations (SD) of values from three independent experiments for a total of 450 PVs. ***, $P \leq 0.001$.

Remarkably, the data shown in Fig. 5A revealed that in contrast to PVs harboring *L. amazonensis* LV79, there was no significant association of VAMP3 with PVs containing *L. amazonensis* PH8. Evidence that VAMP3 and VAMP8 may exert overlapping functions within the endocytic pathway and that both SNAREs can substitute for each other (24) led us to assess the fate of VAMP8 during infection of BMMs with both strains of *L. amazonensis*. Unexpectedly, we observed a significant recruitment of VAMP8 to *L. amazonensis* PH8-harboring PVs at 48 h and 72 h postinfection, in contrast to PVs harboring *L. amazonensis* LV79 (Fig. 5B). Together, these findings suggest that the recruitment of

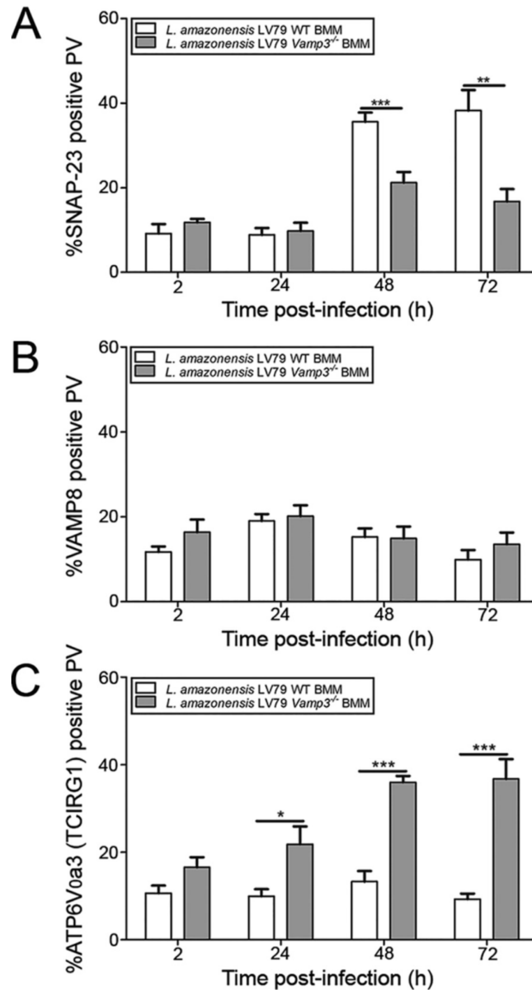

FIG 4 The absence of VAMP3 alters the recruitment of SNAP23 and TCIRG1 to PVs harboring *L. amazonensis* LV79. WT and *Vamp3*$^{-/-}$ BMMs were infected with *L. amazonensis* LV79 promastigotes, and the presence of SNAP23 (A), VAMP8 (B), and ATP6V0a3 (C) in PVs was assessed and quantified by confocal immunofluorescence microscopy at 2, 24, 48, and 72 h postphagocytosis. Data are presented as the means ± SEM of values from three independent experiments.

VAMP3 and VAMP8 to *L. amazonensis*-harboring communal PVs is strain dependent. Given these strain-dependent differences in the association of VAMP3 and VAMP8 with communal PVs, we assessed the impacts of these two SNAREs on the replication of both strains of *L. amazonensis* and PV expansion. We infected WT, *Vamp3*$^{-/-}$, and *Vamp8*$^{-/-}$ BMMs with either strain of *L. amazonensis*, and we determined the parasite burden and PV size at various time points after phagocytosis. As shown in Fig. 5C, whereas the absence of VAMP8 had no impact on the replication of *L. amazonensis* LV79, it significantly restricted the replication of strain PH8 at 24, 48, and 72 h postphagocytosis. These results suggest that VAMP8 participates in fusion events required for the replication of *L. amazonensis* PH8 and are consistent with its recruitment to PVs harboring this strain (Fig. 5B). Interestingly, for both *L. amazonensis* strains, PV size was reduced in the absence of VAMP8 (Fig. 5D), indicating a role for this SNARE in the regulation of PV expansion. In addition, these results indicate that there is no direct correlation between PV size and parasite replication. As depicted in Fig. 5E, VAMP3-deficient BMMs were more permissive than WT BMMs for the replication of both *L. amazonensis*

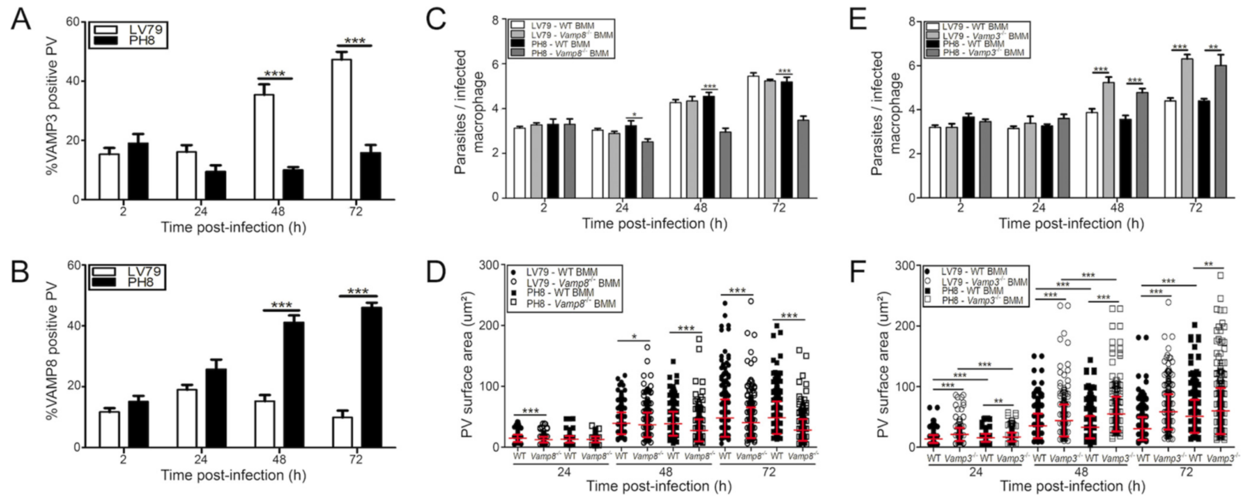

FIG 5 Strain-specific differences in the recruitment of VAMP3 and VAMP8 to PVs harboring *L. amazonensis*. (A and B) WT BMMs were infected with either *L. amazonensis* LV79 or *L. amazonensis* PH8 promastigotes, and the presence of VAMP3 (A) and VAMP8 (B) was assessed and quantified by confocal immunofluorescence microscopy at 2, 24, 48, and 72 h postphagocytosis. Data are presented as the means ± SEM of values from three independent experiments. ***, $P \leq 0.001$. (C and D) WT and *Vamp8*$^{-/-}$ BMMs were infected with either *L. amazonensis* LV79 or *L. amazonensis* PH8 promastigotes, and at various time points after phagocytosis, the parasite burden and PV size were assessed. (C) Parasite burden. Data are presented as the means ± SEM of values from three independent experiments. *, $P \leq 0.05$; ***, $P \leq 0.001$. (D) PV surface area. Data are presented as a cloud with means ± SD of values from three independent experiments for a total of 450 PVs. *, $P \leq 0.05$; ***, $P \leq 0.001$. (E and F) WT and *Vamp3*$^{-/-}$ BMMs were infected with either *L. amazonensis* LV79 or *L. amazonensis* PH8 promastigotes, and at various time points after phagocytosis, the parasite burden and PV size were assessed. (E) Parasite burden. Data are presented as the means ± SEM of values from three independent experiments. **, $P \leq 0.01$; ***, $P \leq 0.001$. (F) PV surface area. Data are presented as a cloud with means ± SD of values from three independent experiments for a total of 450 PVs. **, $P \leq 0.01$; ***, $P \leq 0.001$.

strains. Additionally, for both strains, the absence of VAMP3 led to the formation of larger PVs at 48 h and 72 h postinfection (Fig. 5F), consistent with a role for this SNARE in the control of PV size and *L. amazonensis* replication. The data presented in Fig. 1 to 5 are summarized in Table 1.

VAMP3 negatively regulates antigen cross-presentation. Antigen presentation mediated by major histocompatibility complex (MHC) class I antigens, or cross-presentation, is a phagosome function essential for the activation of $CD8^{+}$ T cells by antigen-presenting cells (36, 64–66). A number of SNAREs, including VAMP8, Sec22b, and SNAP23, were previously shown to contribute to the phagosomal recruitment of the cross-presentation molecular machinery (14, 22, 67, 68). Given the impacts of VAMP3 on the replication of both strains of *L. amazonensis* and on PV size, we investigated whether the absence of VAMP3 influences antigen cross-presentation in infected dendritic cells. To this end, we generated *L. major* strain GLC94 and *L. amazonensis* strains

TABLE 1 Association of SNAREs with PVs harboring *Leishmania* strains in WT, *Vamp3*$^{-/-}$, and *Vamp8*$^{-/-}$ BMMs[a]

	Association								
	WT BMMs			*Vamp3*$^{-/-}$ BMMs			*Vamp8*$^{-/-}$ BMMs		
SNARE or PV feature	*L. major*	LV79	PH8	*L. major*	LV79	PH8	*L. major*	LV79	PH8
SNAP23	−	++	ND	ND	−	ND	ND	ND	ND
VAMP3	−	++	−	ND	ND	ND	ND	ND	ND
VAMP8	++	−	++	ND	−	ND	ND	ND	ND
STX13	−	−	ND	ND	ND	ND	ND	ND	ND
STX18	−	+	ND	ND	ND	ND	ND	ND	ND
Vti1A	−	+	ND	ND	ND	ND	ND	ND	ND
LAMP1	−	++	ND	ND	ND	ND	ND	ND	ND
ATP6V0a3	ND	−	ND	ND	++	ND	ND	ND	ND
PV size	ND	+	++	ND	++	+++	ND	+	+
Replication	++	++	++	++	+++	+++	++	++	−

[a]ND, not determined. − indicates absence, + to +++ indicate relative increase.

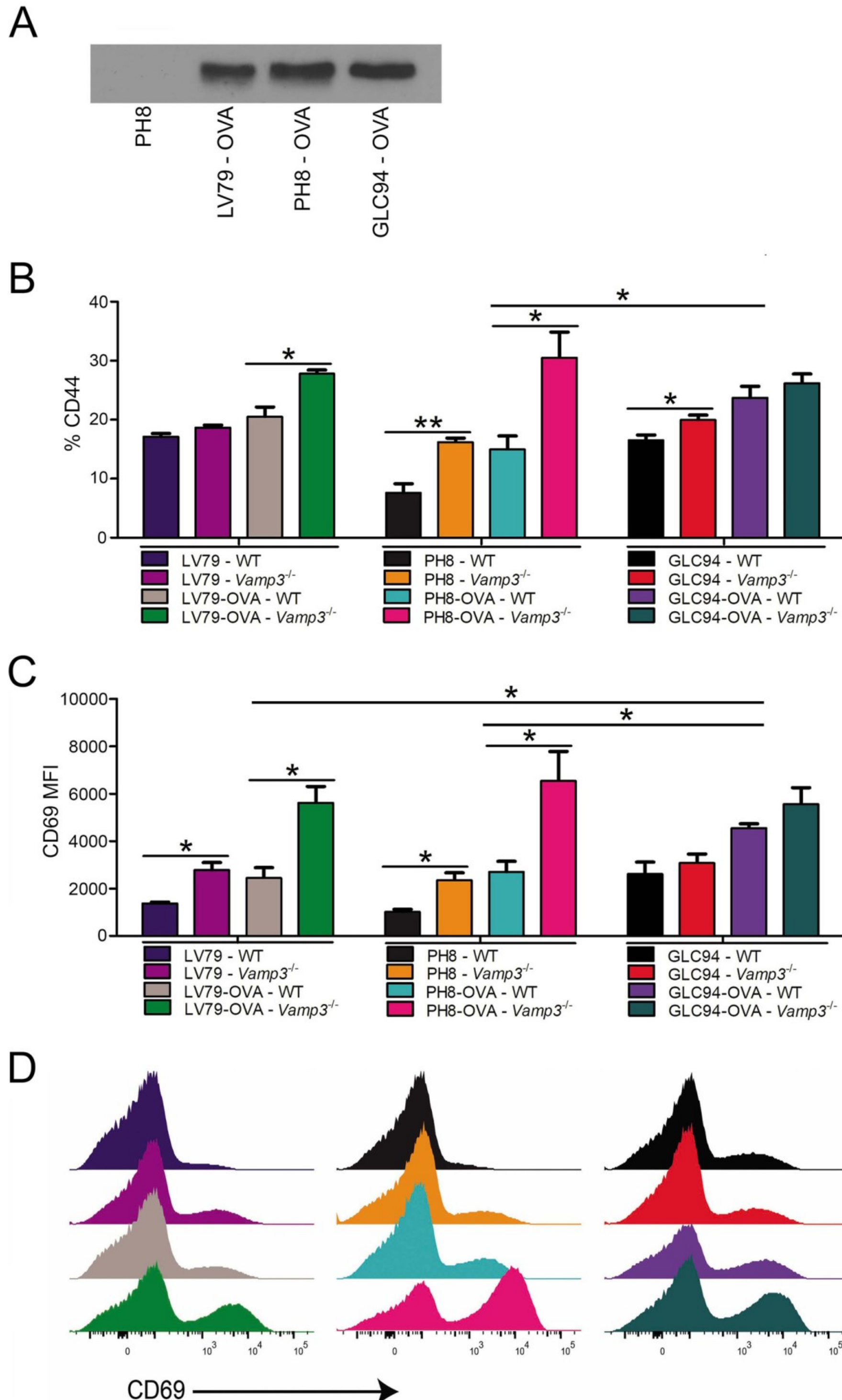

FIG 6 VAMP3 negatively regulates antigen cross-presentation from communal PVs harboring *L. amazonensis*. (A) The expression of ovalbumin by *L. amazonensis*-OVA LV79, *L. amazonensis*-OVA PH8, and *L. major*-OVA GLC94 was assessed by Western blotting. A blot representative of results from two experiments is shown. WT and *Vamp3*$^{-/-}$ BMDCs were infected with either *L. major* GLC94, *L. amazonensis* LV79, and *L. amazonensis* PH8 or *L. major*-OVA GLC94, *L. amazonensis*-OVA LV79, and *L. amazonensis*-OVA PH8 for 48 h. (B to D) Infected and

(Continued on next page)

LV79 and PH8 expressing ovalbumin (OVA) at similar levels (Fig. 6A) and used them to infect WT and *Vamp3*$^{-/-}$ bone marrow-derived dendritic cells (BMDCs) for 48 h prior to adding OVA-specific OT-I CD8^{+} T cells (69). At this time point, both of the *L. amazonensis* strains expressing OVA (*L. amazonensis*-OVA strains) were present in enlarged PVs, whereas *L. major*-OVA replicated in individual PVs (Fig. S2A). Moreover, the absence of VAMP3 had no significant impact on the replication of *L. major* and *L. amazonensis* in BMDCs (Fig. S2B). We then assessed cross-presentation by measuring the expression of the T cell activation markers CD69 and CD44 on OT-I T cells following exposure to *L. amazonensis*-OVA- and *L. major*-OVA-infected WT and *Vamp3*$^{-/-}$ BMDCs. We observed significantly higher CD44^{+} cell frequencies (Fig. 6B) and CD69 median fluorescence intensities (MFIs) (Fig. 6C and D) for OT-I T cells exposed to *Vamp3*$^{-/-}$ BMDCs infected with *L. amazonensis*-OVA than for WT BMDCs infected with *L. amazonensis*-OVA. In contrast, the absence of VAMP3 had no significant impact on the activation of OT-I T cells exposed to BMDCs infected with *L. major*-OVA. The increased cross-presentation by *Vamp3*$^{-/-}$ BMDCs infected with *L. amazonensis* was not caused by increased MHC class I expression, as shown by the SIINFEKL peptide-loading control experiment (Fig. S3A and B). Of note, we observed significantly lower CD44^{+} cell frequencies and CD69 MFIs for OT-I T cells exposed to WT BMDCs infected with *L. amazonensis*-OVA than for those infected with *L. major*-OVA. Collectively, our results indicate that VAMP3 negatively regulates the ability of communal PVs harboring *L. amazonensis* to cross-present antigens in BMDCs.

DISCUSSION

The biogenesis and functionality of phagosomes hinge on intracellular vesicular trafficking and membrane fusion events mediated by SNAREs (11, 14, 65, 67, 70, 71). In this study, we compared and analyzed the kinetics of recruitment/enrichment and trafficking of host cell SNAREs to tight-fitting individual PVs induced by *L. major* and to large communal PVs induced by *L. amazonensis*. Our results revealed differences in the components of the host cell membrane fusion machinery associated with these two types of PVs, consistent with the notion that tight-fitting individual PVs and large communal PVs differ in their capacities to interact with host cell compartments. Moreover, we obtained evidence that both VAMP3 and VAMP8 regulate the development and functionality of *L. amazonensis*-harboring communal PVs.

Previous studies revealed that although VAMP3 plays no major role in phagocytosis (72), this SNARE contributes to host defense against infections by regulating the delivery of tumor necrosis factor (TNF) at the nascent phagocytic cup through focal exocytosis and by contributing to the formation of autophagosomes during xenophagy (20, 21, 28). Moreover, VAMP3 may be coopted by vacuolar pathogens to create and develop their intracellular replicative niches. Hence, in macrophages infected with *Yersinia pseudotuberculosis*, VAMP3 participates in the formation of single-membrane LC3-positive vacuoles containing the bacteria, although it is not known whether VAMP3 influences *Yersinia* replication (27). Using host cells coexpressing enhanced green fluorescent protein (EGFP)-VAMP3 and tetanus toxin (which cleaves VAMP3), Campoy and colleagues obtained evidence that VAMP3 is involved in the biogenesis and enlargement of *Coxiella burnetii* replicative vacuoles by mediating the fusion of these replicative vacuoles with multivesicular bodies (73). In the case of *Brucella melitensis*, although infection increases VAMP3 expression in the mouse macrophage cell line J774, its silencing had no effect on the survival and replication of the bacteria, indicating that VAMP3 is not essential for the biogenesis and expansion of *Brucella*-containing vacuoles (74). In contrast, knockdown experiments revealed that the recruitment of VAMP3 and other SNAREs to chlamydial inclusions plays an important role in

FIG 6 Legend (Continued)

control uninfected BMDCs were incubated with OT-1 T cells, and cross-presentation was assessed by measuring the expression of the activation markers CD44 (B) and CD69 (C and D) on OT-I T cells by flow cytometry. Graphs show data from 1 representative experiment of 6 independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$.

controlling the expansion of the inclusion membrane as well as *Chlamydia* replication (75–77). For *Leishmania*, our results revealed no role for VAMP3 in the replication of *L. major*, which resides in individual tight-fitting PVs. Conversely, we found that VAMP3 is gradually recruited to *L. amazonensis* LV79-harboring PVs starting at 24 h postinfection, when PV expansion becomes noticeable. Unexpectedly, we observed increased replication of *L. amazonensis* and increased PV size in the absence of VAMP3, suggesting that this SNARE may be part of a complex that controls the development of communal PVs, possibly by negatively regulating membrane fusion events, as previously reported for synaptotagmin XI, VAMP8, and VAMP7 during phagocytosis (78, 79). Of interest, a negative regulatory role for VAMP3 has recently been reported in platelets, where the absence of VAMP3 led to enhanced platelet spreading and clot retraction (80). In this context, it was proposed that the absence of VAMP3 could increase the formation of other SNARE complexes and thus increase the exocytosis events needed for spreading. One may thus envision that the absence of VAMP3 may favor the formation of SNARE complexes that lead to increased fusion events required for PV expansion. It is also possible that VAMP3 acts as an inhibitory SNARE (i-SNARE) (81), where it would compete with and substitute for a fusogenic subunit, thereby inhibiting fusion and limiting PV expansion. Of interest, a recent study revealed that the phosphorylation of SNAP23 on Ser95 negatively regulates phagocytosis and phagosome maturation (60). Since the absence of VAMP3 altered the recruitment of SNAP23 to PVs, one may consider the possibility that the improper localization of SNAP23 may impair its phosphorylation on Ser95 and, hence, reduce its inhibitory function. Alternatively, VAMP3 may mediate the delivery of antimicrobial cargo to PVs harboring *L. amazonensis*, thereby limiting parasite replication and PV expansion. Regardless of the mechanism, our study revealed a novel role for VAMP3 in the control of communal PV expansion and *L. amazonensis* replication, highlighting the fact that depending on the pathogen, a molecule involved in regulating vesicular trafficking and membrane fusion events may either favor or impair pathogen growth, as recently described for Rab11 in the context of *Legionella pneumophila* infection (82). Further studies will be aimed at elucidating the underlying mechanism(s).

VAMP8 participates in host defense against infection in diverse ways. It mediates the fusion of antimicrobial and canonical autophagosomes with lysosomes, which is essential for autophagic degradation (26). Depending on the cell type, VAMP8 exerts opposite functions during phagocytosis. Hence, it negatively controls the phagocytosis of *Escherichia coli* in dendritic cells (78) but is required for the entry of *Salmonella* in HeLa cells (83). Our previous work revealed that whereas VAMP8 has no influence on the survival and replication of *L. major*, it modulates cross-presentation and LC3-associated phagocytosis by regulating the phagosomal recruitment of NOX2 (14, 15). Here, we obtained evidence that the absence of VAMP8 leads to the reduced expansion of communal PVs harboring *L. amazonensis*. This suggests that VAMP8 participates in the recruitment of membrane required for the expansion of communal PVs by mediating their interactions with endosomes/lysosomes and/or by mediating homotypic fusion among communal PVs. The observation that a reduction of PV size in the absence of VAMP8 impacted the replication of *L. amazonensis* PH8, but not that of *L. amazonensis* LV79, is intriguing and suggests that *L. amazonensis* replication does not absolutely correlate with PV expansion. Hence, whether parasite growth is the main signal governing PV expansion remains a lingering question (42) for which there have been little data to provide a clear answer. It will thus be of interest to further investigate the nature of the host factors and parasite effectors that modulate PV expansion. The different fates of *L. amazonensis* LV79 and PH8 in *Vamp8*$^{-/-}$ macrophages illustrate the perils of drawing conclusions based on experiments performed with a single *Leishmania* strain or isolate (84). In line with this, it will be important to compare the localization/mobilization of SNAREs in macrophages infected with diverse strains of *Leishmania* species that replicate in individual PVs since we used only one *L. major* strain in the present study.

The cross-presentation of microbial peptides on MHC class I molecules is an important host defense mechanism aimed at deploying $CD8^+$ T cell responses against intracellular pathogens, including *Leishmania* (64, 65, 85–89). Previous studies revealed that phagosomes acquire, through a series of interactions with other organelles, the machinery required to become self-sufficient for antigen cross-presentation (35–37). However, the involvement of the various vesicular trafficking pathways in this process remains to be fully understood (64, 66). To date, a number of SNAREs associated with these pathways have been shown to regulate the trafficking events involved in the acquisition of the phagosomal cross-presentation machinery, including the ER/ERGIC SNARE Sec22b (67) and the endocytic SNAREs VAMP8 and SNAP23 (14, 22). Of interest, Nair-Gupta and colleagues (22) observed no reduction of either Toll-like receptor (TLR)-regulated cross-presentation or phagosomal MHC class I recruitment in BMDCs derived from *Vamp3*$^{-/-}$ mice. This is in contrast to our finding that the absence of VAMP3 increases the level of cross-presentation by *L. amazonensis*-harboring communal PVs. In their study, Nair-Gupta and colleagues used *E. coli* expressing OVA and lipopolysaccharide (LPS)-coated beads to assess the impact of SNAREs on cross-presentation (22), illustrating the complex regulation of this process. Although the underlying mechanism remains to be elucidated, it is possible that the absence of VAMP3 altered SNAP23 phosphorylation, which was shown to regulate cross-presentation, possibly through the stabilization of SNARE complexes (22). Further characterization of communal PVs induced by *L. amazonensis* and the role of VAMP3 in their formation may therefore yield novel information on the process of antigen cross-presentation in the context of cells infected with a pathogen residing in communal vacuoles. Interestingly, we observed that the absence of VAMP3 had no impact on the replication of *L. amazonensis* in BMDCs, in contrast to BMMs, where we found increased *L. amazonensis* replication in the absence of VAMP3. Whether these differences are related to intrinsic phagosomal properties in BMMs and BMDCs, such as the levels of NADPH oxidase activity and acidification (90, 91), is an issue that deserves further investigation.

We provide evidence that both VAMP3 and VAMP8 participate in the development and functionality of *L. amazonensis*-harboring communal PVs. Whereas VAMP3 has detrimental impacts on parasite replication, PV size, and antigen cross-presentation, VAMP8 contributes to PV expansion but does not affect replication. In both cases, the exact mechanisms remain to be elucidated. This is an interesting issue since both VAMP3 and VAMP8 have previously been shown to exert overlapping functions, and they can substitute for each other (24). Depending on the cell type and the intracellular compartment, both SNAREs form complexes with SNAP23 and various syntaxins. To shed more light on the biology of *L. amazonensis*-harboring PVs, future studies will be aimed at identifying the *trans*-SNARE complexes formed by VAMP3 and VAMP8 during the biogenesis and expansion of communal PVs. Whether *Leishmania*-derived molecules, such as the inclusion proteins of *Chlamydia* (75), interact with host SNAREs is also an important issue that will be addressed in future studies to unravel the mechanism by which *Leishmania* species coopt the host cell membrane fusion machinery to create, expand, and maintain their PVs. Finally, since phagosomes play a central role in innate and adaptive immunity, a better understanding of the biology of communal PVs containing *L. amazonensis* may provide new insights into the mechanisms used by this parasite to develop in a communal PV and evade the immune system, which may be useful for the design of future interventions to prevent or treat infection.

MATERIALS AND METHODS

Ethics statement. Experiments involving mice were done as prescribed by protocol 1406-02, which was approved by the Comité Institutionnel de Protection des Animaux of the Institut National de la Recherche Scientifique (INRS). These protocols respect procedures on good animal practice provided by the Canadian Council on Animal Care. *Vamp8*$^{-/-}$ mice were obtained from Wan Jin Hong (A Star Institute, Singapore), *Vamp3*$^{-/-}$ mice were provided by Sidney W. Whiteheart, and OT-I mice were purchased from the Jackson Laboratory. All mice were bred and housed at the INRS animal facility under specific-pathogen-free conditions and used at 6 to 12 weeks of age.

Antibodies. Rabbit polyclonal anti-VAMP3, anti-VAMP8, anti-SNAP23, anti-syntaxin 13, and anti-syntaxin 18 and guinea pig polyclonal anti-Vti1b antibodies were obtained from Synaptic Systems (SySy). The rat monoclonal anti-LAMP1 antibody was developed by J. T. August (clone 1D4B) and obtained through the Developmental Studies Hybridoma Bank at the University of Iowa and the National Institute of Child Health and Human Development. Fluorescence-activated cell sorter (FACS) analyses were performed with fluorochrome-conjugated antibodies against CD3-phycoerythrin (PE)-Cy7 (clone 14S-2C11; BD Bioscience), CD8-Pacific Blue (PB) (clone 53-6.7; BD Bioscience), CD44-allophycocyanin (APC) (clone IM7; BD Bioscience), and CD69-PE (clone H1.2F3; eBioscience).

Bone marrow-derived macrophages and dendritic cells. We used *Vamp8*$^{-/-}$ (24) and *Vamp3*$^{-/-}$ (92) mice, which were maintained on a mixed C57BL/6-129/Sv/J background, and wild-type mice were matched littermates. Bone marrow-derived macrophages (BMMs) were differentiated from the bone marrow of 6- to 8-week-old mice. Cells were differentiated in complete medium (Dulbecco's modified Eagle's medium [DMEM; Life Technologies] supplemented with L-glutamine [Life Technologies], 10% heat-inactivated fetal bovine serum [FBS; Gibco], 10 mM HEPES [Bioshop] at pH 7.4, and antibiotics [Life Technologies]) containing 15% (vol/vol) L929 cell-conditioned medium as a source of macrophage colony-stimulating factor (M-CSF) at 37°C in a humidified incubator with 5% CO_2 for a week. To render BMMs quiescent prior to experiments, cells were transferred to 6- or 24-well tissue culture microplates (TrueLine) and kept for 16 h in complete DMEM without L929 cell-conditioned medium. Bone marrow-derived dendritic cells (BMDCs) were differentiated from the bone marrow of 6- to 8-week-old mice. Cells were differentiated in complete medium (RPMI 1640 [Life Technologies] supplemented with 10% heat-inactivated FBS, 10 mM HEPES [Bioshop] at pH 7.4, and antibiotics [Life Technologies]) containing 10% (vol/vol) granulocyte-macrophage colony-stimulating factor (GM-CSF) at 37°C in a humidified incubator with 5% CO_2 for a week. Sixteen hours prior to infection, nonadherent cells were transferred to 96-well tissue culture microplates (TrueLine) and kept in RPMI 1640 containing 10% heat-inactivated FBS and 5% (vol/vol) GM-CSF.

Parasite strains and culture. The *Leishmania* strains used in this study were *L. major* GLC94 (MHOM/TN/95/GLC94 zymodeme MON25, obtained from L. Guizani-Tabbane, Institut Pasteur de Tunis) (93), *L. amazonensis* LV79 (MPRO/BR/72/M1841, obtained from the American Type Culture Collection), and *L. amazonensis* PH8 (IFLA/BR/67/PH8, obtained from the American Type Culture Collection). Promastigotes were obtained from lesion-derived amastigotes and were cultured in *Leishmania* medium (medium 199 [Sigma-Aldrich] with 10% heat-inactivated FBS, 40 mM HEPES at pH 7.4, 100 μM hypoxanthine, 5 μM hemin, 3 μM biopterin, 1 μM biotin, and antibiotics) in an incubator at 26°C. Promastigotes expressing a secreted form of OVA (*L. major*-OVA and *L. amazonensis*-OVA) were generated by electroporating the pKS-NEO SP:OVA construct, which encodes a fusion protein containing the signal peptide of the *L. donovani* 3′ nucleotidase-nuclease fused to a portion of the OVA protein (positions 139 to 386) containing both MHC class I $OVA_{257-264}$- and class II $OVA_{323-339}$-restricted epitopes (94) (kindly provided by Alain Debrabant, FDA). Transfected parasites were grown in *Leishmania* medium supplemented with 50 μg/mL G418.

Infection of macrophages. Promastigotes in late stationary phase were opsonized with C5-deficient serum from DBA/2 mice prior to infections. Phagocytosis was synchronized by incubating macrophages and parasites at 4°C for 10 min and spinning at 167 $\times$ *g* for 1 min. Internalization was then triggered by transferring the plates to 34°C. At 2 h postinfection, macrophages were washed twice with complete DMEM to remove noninternalized parasites. Cells were then prepared for confocal immunofluorescence microscopy.

Confocal immunofluorescence microscopy. Cells that adhered to coverslips were fixed with 2% paraformaldehyde (Canemco and Mirvac) for 40 min and blocked/permeabilized for 17 min with a solution containing 0.05% saponin, 1% bovine serum albumin (BSA), 6% skim milk, 2% goat serum, and 50% FBS, followed by a 2-h incubation with primary antibodies and subsequent incubation with suitable secondary antibodies in phosphate-buffered saline (PBS) for 45 min (anti-rabbit Alexa Fluor 488 and anti-rat Alexa Fluor 568; Molecular Probes) and 4′,6-diamidino-2-phenylindole (DAPI) in PBS for 15 min (Life Technologies). Three washes in PBS took place after every step. After the final washes, Fluoromount-G (Southern Biotechnology Associates) was used to mount coverslips onto glass slides, and coverslips were sealed with nail polish (Sally Hansen). Macrophages were visualized with an LSM780 microscope with a 63$\times$ objective (Carl Zeiss Microimaging), and images were taken in sequential scanning mode. Image analysis and vacuole size measurements were performed with ZEN 2012 software. The vacuole size measurements were accomplished via the closed Bezier tool of ZEN 2012 that calculates the surface of a selected area on DAPI-stained coverslips (see Fig. S4 in the supplemental material for examples).

Lysis, SDS-PAGE, and Western blotting. Adherent macrophages in 6-well plates were washed with PBS containing 1 mM sodium orthovanadate and 10 mM 1,10-phenanthroline (Roche) on ice prior to lysis. Cells were then scraped into lysis buffer containing 1% Nonidet P-40 (Caledon), 50 mM Tris-HCl (pH 7.5) (Bioshop), 150 mM NaCl, 1 mM EDTA (pH 8), 10 mM 1,10-phenanthroline, and phosphatase and protease inhibitors (Roche). The lysates were left on ice for 10 min and then stored at −70°C. Lysates were thawed on ice, centrifuged for 10 min to remove insoluble matter, and then quantified. Ten micrograms of the samples was boiled (100°C) for 6 min in SDS sample buffer, migrated in 10% SDS-PAGE gels, and then transferred onto Hybond-ECL membranes (Amersham Biosciences). The membranes were subsequently blocked for 1 h in 1$\times$ Tris-buffered saline (TBS)–0.1% Tween containing 5% skim milk, incubated overnight at 4°C with primary antibodies (diluted in 1$\times$ TBS–0.1% Tween containing 5% BSA), and incubated for 1 h at room temperature with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies. Three washes took place after every step. The membranes were finally incubated in ECL reagent (GE Healthcare), and immunodetection was achieved via chemiluminescence.

Antigen cross-presentation. BMDCs were infected for 48 h with WT *L. amazonensis* LV79-OVA, *L. amazonensis* PH8-OVA, or *L. major* GLC94-OVA promastigotes or with promastigotes not expressing OVA. Cells were then washed and fixed for 5 min at 23°C with 1% (wt/vol) paraformaldehyde, followed by three washes in complete medium containing 0.1 M glycine. OT-I T cells were enriched from splenocytes of OT-I mice by magnetically activated cell sorting (MACS) using a $CD8^+$ T cell isolation kit (Miltenyi Biotech) as previously described (95). They were then added to the culture for 16 h. The SIINFEKL peptide was used as a control for T cell activation and expansion. Antigen cross-presentation was assessed by measuring the surface expression modulation of CD69 and CD44 within the $CD3^+$ $CD8\alpha^+$ $V\alpha2^+$ population as markers for T cell activation. Cells were analyzed after fixation with 2% (wt/vol) paraformaldehyde using a BD LSR Fortessa flow cytometer (Becton, Dickinson). Samples were analyzed with FlowJo software.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 3 MB.

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We declare no conflicts of interest.

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7.2 Cell-intrinsic Wnt4 ligand regulates mitochondrial oxidative phosphorylation in macrophages

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Article context and author contributions: In this publication, we evaluated the role of Wtn4 in macrophage polarization and in the energetic metabolism during stimulation with TLR agonist (LPS) and during infection with *Leishmania donovani*. In this article, I have evaluated the *Leishmania donovani* fate in Wnt4$^{\Delta/\Delta}$ and WT infected macrophages and the importance of mitochondrial β-oxidation therein. The conducted experiments are showed in figure 7.

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Cell-intrinsic Wnt4 ligand regulates mitochondrial oxidative phosphorylation in macrophages

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Macrophages respond to their environment by adopting a predominantly inflammatory or anti-inflammatory profile, depending on the context. The polarization of the subsequent response is regulated by a combination of intrinsic and extrinsic signals and is associated with alterations in macrophage metabolism. Although macrophages are important producers of Wnt ligands, the role of Wnt signaling in regulating metabolic changes associated with macrophage polarization remains unclear. Wnt4 upregulation has been shown to be associated with tissue repair and suppression of age-associated inflammation, which led us to generate Wnt4-deficient bone marrow–derived macrophages to investigate its role in metabolism. We show that loss of Wnt4 led to modified mitochondrial structure, enhanced oxidative phosphorylation, and depleted intracellular lipid reserves, as the cells depended on fatty acid oxidation to fuel their mitochondria. Further we found that enhanced lipolysis was dependent on protein kinase C–mediated activation of lysosomal acid lipase in Wnt4-deficient bone marrow–derived macrophages. Although not irreversible, these metabolic changes promoted parasite survival during infection with *Leishmania donovani*. In conclusion, our results indicate that enhanced macrophage fatty acid oxidation impairs the control of intracellular pathogens, such as *Leishmania*. We further suggest that Wnt4 may represent a potential target in atherosclerosis, which is characterized by lipid storage in macrophages leading to them becoming foam cells.

Macrophages possess multiple functions, ranging from pathogen clearance and antigen presentation to T lymphocytes to tissue remodeling and immune suppression (1–3). By analogy with the cytokine responses generated, macrophages have been long divided into two main categories: classically activated proinflammatory macrophages (M1) and alternatively activated anti-inflammatory macrophages (M2) (4–7). These two differentiation profiles have also been characterized by their diverging cellular metabolism (8–10). M1 macrophages upregulate glycolytic enzymes and preferentially use glucose as their main energy source, resulting in ATP production through the conversion of pyruvate to lactate (9, 11). In contrast, M2 macrophage metabolism is supported by high mitochondrial activity and oxidative phosphorylation (OXPHOS), fueled at least in part by fatty acid oxidation (FAO) (12, 13).

Macrophage polarization is regulated by a combination of intrinsic and extrinsic signals, such as cytokines, growth factors, and microbial products. Wnt signaling is known for its pleiotropic effects in cell fate decisions during development and tissue repair, but although macrophages are known to express several Wnt ligands, the role of individual Wnt proteins in macrophage polarization is not well established (14). The prototypical canonical ligand that promotes β-catenin translocation into the nucleus, Wnt3a, increases arginase expression in primary macrophages after bacterial infection and inhibits the secretion of proinflammatory cytokines (15, 16). Conversely, Wnt5a, which is usually associated with β-catenin–independent noncanonical Wnt signaling, promotes inflammatory responses *via* the transcription factor NF-κB to ensure immune surveillance (17), and both Wnt5a and NF-κB expression are increased upon macrophage exposure to mycobacteria (16). However, the role of Wnt signaling in macrophage metabolism has not been investigated in depth.

We have focused our study on Wnt4, a mostly noncanonical ligand (18, 19), whose expression is upregulated in lung macrophages upon injury to promote tissue repair (20). Wnt4 overexpression in bone marrow was shown to inhibit age-associated inflammation (21), while its deletion from dendritic cells impacts their differentiation and promotes the development of type 2 immunity in response to the hookworm parasite *Nippostrongylus brasiliensis* (19). We thus hypothesized that Wnt4 could also contribute to the metabolic reprogramming of bone marrow–derived macrophages (BMDMs).

We show that Wnt4-deficient BMDMs display reduced AKT (Thr308) and ERK1/2 phosphorylation but increased ATP levels, which can be attributed to an enhanced mitochondrial OXPHOS activity. Furthermore, we identify FAO as a principal mechanism involved in the increase in mitochondrial activity. However, while Wnt4-deficient macrophages respond more

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strongly to lipopolysaccharide (LPS)/M1-type stimulation, their altered FA metabolism favors replication of the protozoan parasite *Leishmania donovani*. Wnt4-mediated regulation of macrophage metabolism and mitochondrial activity thus appear important for the control of intracellular pathogens.

Results

Wnt4 promotes AKT (Thr308) and ERK signaling

To elucidate the importance of *Wnt4* in macrophages, we generated conditional knock-out mice in which *Wnt4* is deleted from most macrophages and granulocytes by LysM-Cre–mediated excision (*Wnt4*$^{\Delta/\Delta}$ mice) (22, 23). These mice present no overt alterations in myeloid differentiation *in vivo* (24). We isolated BM cells from *Wnt4*$^{\Delta/\Delta}$ and Cre^{-} littermate control mice, and we obtained comparable numbers of *Wnt4*$^{\Delta/\Delta}$ and control BMDM after 1 week in culture (Fig. 1, *A* and *B*). While *Wnt4* deletion was highly efficient in culture (Fig. 1*C*), *Wnt4*$^{\Delta/\Delta}$ and control BMDM expressed similar levels of the macrophage surface marker F4/80 (Fig. 1*D*), suggesting that Wnt4 deficiency did not significantly alter BMDM differentiation from BM progenitors. There was no difference in β-catenin phosphorylation (Fig. 1*E*) or in the activation of c-Jun N-terminal protein kinase (JNK) (Fig. 1, *F* and *G*), suggesting that the deletion of Wnt4 did not alter the balance between canonical and JNK-dependent noncanonical signaling in the absence of other exogenous ligands.

Macrophage function is regulated not only by JNK but also other members of the mitogen-activated protein kinase (MAPK) family, such as the extracellular signal–regulated kinases 1 and 2 (ERK1/2) (25, 26). Unlike JNK, there was a notable decrease in ERK1/2 phosphorylation in *Wnt4*$^{\Delta/\Delta}$ BMDM (Fig. 1,*H* and *I*). *Wnt4*$^{\Delta/\Delta}$ BMDM also showed a decreased level of AKT phosphorylation on Thr308 (Fig. 1, *J* and *K*), suggesting that these signal transduction pathways

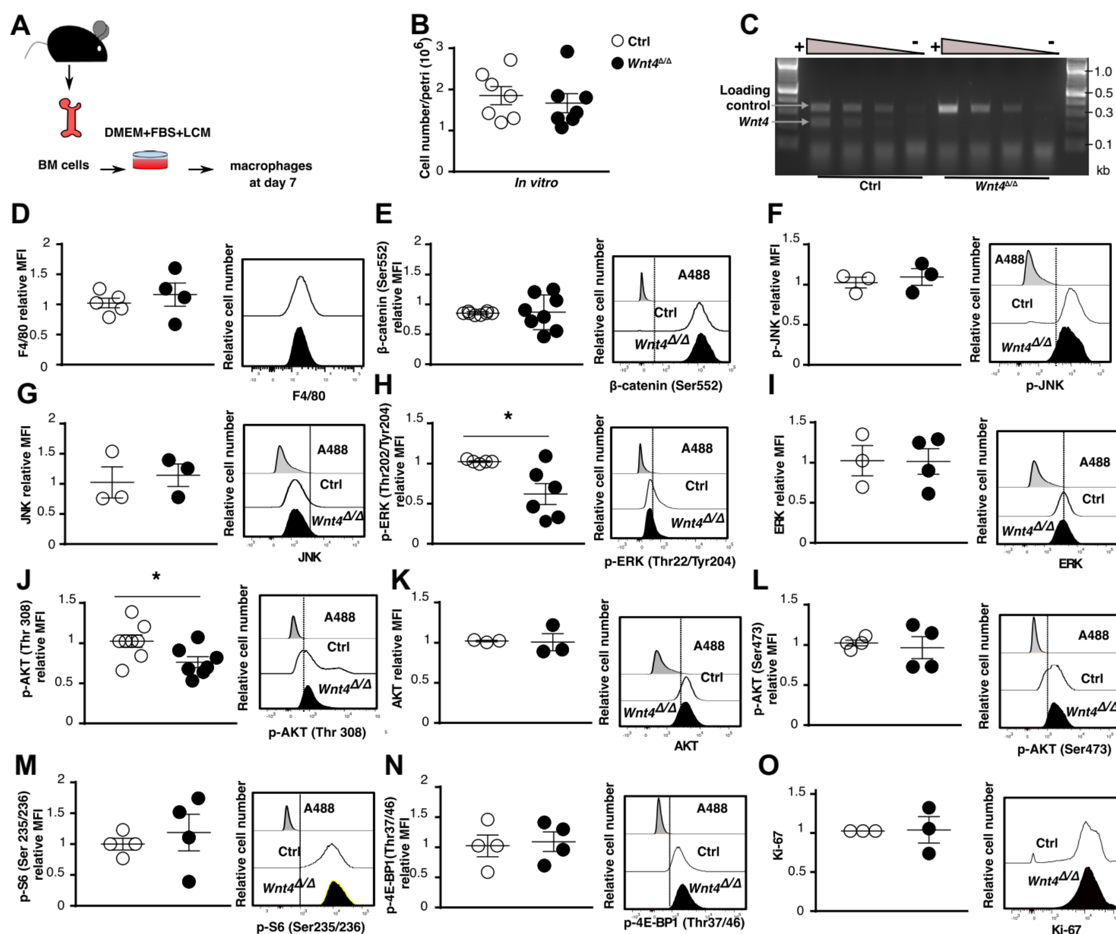

Figure 1. Endogenous Wnt4 promotes AKT (Thr308) and ERK1/2 phosphorylation in macrophages. *A*, bone marrow–derived macrophage (BMDM) differentiation. *B*, number of macrophages collected after 7 days of differentiation in culture. *C*, PCR analysis of decreased DNA concentration extracted from mouse tail sample. *D–O*, relative mean fluorescence intensity (MFI) of F4/80 (*D*), phospho-β-catenin (Ser522) (*E*), phospho-JNK (Thr183/Tyr185) (*F*), JNK (*G*), phospho-ERK1/2 (Thr202/Tyr204) (*H*), ERK1/2 (*I*), phospho-AKT (Thr308) (*J*), AKT (*K*), phospho-AKT (Ser473) (*L*), phospho-S6 (Ser235/Ser236) (*M*), phospho-4EBP-1 (Thr37/Thr46) (*N*), and Ki-67 (*O*) in unstimulated BMDM. The histograms represent compiled data from three to eight animals per group (mean + SEM). *$p < 0.05$ (two-tailed, paired Student's *t* test).

are altered in these cells. Nevertheless, phosphorylation of the mTORC2-dependent site in AKT (Ser473) (Fig. 1*L*), as well as that of the mTORC1 downstream effectors S6 (Ser235/236) (Fig. 1*M*) and 4EBP1(Thr37/46) (Fig. 1*N*) was not affected in $Wnt4^{\Delta/\Delta}$ BMDM, indicating that Wnt4 regulates ERK1/2 and AKT signaling independently from the mTORC axis.

Considering the dual roles of ERK1/2 and AKT in cell proliferation and metabolism (27–29), we further evaluated the proliferative state of $Wnt4^{\Delta/\Delta}$ BMDM using Ki-67. Ki-67 expression was not significantly changed in $Wnt4^{\Delta/\Delta}$ BMDM compared to control (Fig. 1*O*), which together with the comparable cell counts (Fig. 1*B*) suggests that Wnt4 does not affect BMDM proliferation. However, impaired ERK1/2 and AKT (Thr308) activation prompted us to further investigate the functional consequences of Wnt4 deletion on BMDM metabolism.

Wnt4 deficiency increases ATP production through OXPHOS

To address the impact of Wnt4 on macrophage metabolism, we first investigated their ATP levels. $Wnt4^{\Delta/\Delta}$ BMDM displayed higher intracellular ATP levels (Fig. 2*A*) but a comparable ADP/ATP ratio relative to control cells (Fig. 2*B*), suggesting that this increase in ATP is not the consequence of alterations in ATP consumption. In addition, intracellular ATP was decreased to similar levels in both genotypes upon inhibition of the ATP synthase with oligomycin (Fig. 2*A*). As these

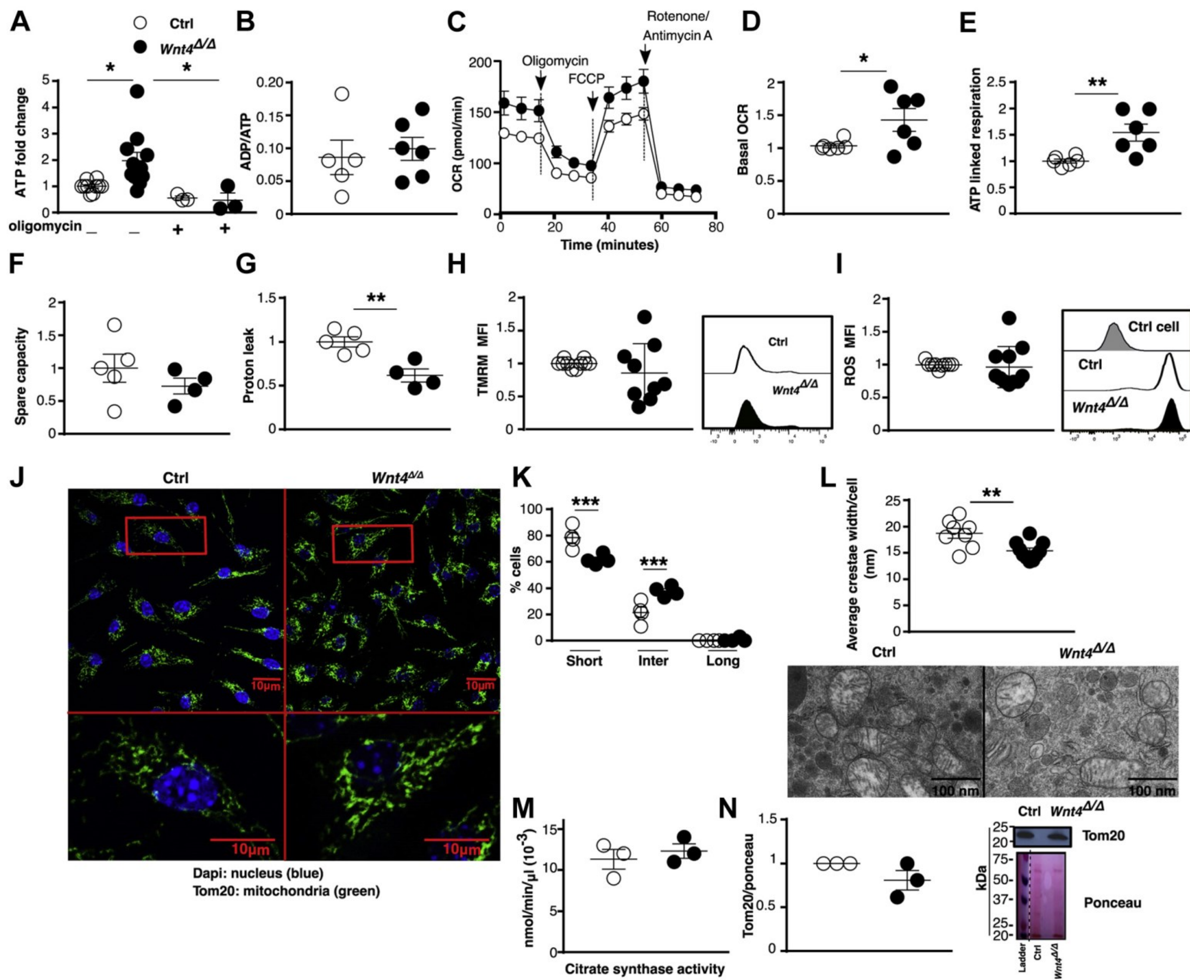

Figure 2. Wnt4 deletion increases mitochondrial energy metabolism. *A*, intracellular ATP oligomycin. *B*, ATP/ADP ratio. *C*, representative oxygen consumption rate (OCR) curves. *D*, basal OCR normalized to control. *E*, ATP linked-respiration (OCR after oligomycin injection—basal OCR). *F*, spare respiratory capacity (OCR after FCCP injection—basal OCR). *G*, proton leak (OCR after oligomycin injection—OCR after rotenone/antimycin A injection). *H*, measure of mitochondrial membrane potential as determined by relative TMRM MFI. *I*, mitochondrial ROS production as measured by relative Mitosox MFI. *J*, confocal microscopy images of BMDM staining for mitochondria (green: TOM20, blue: nucleus, 60×). *K*, analysis of mitochondrial length, represented as proportion of BMDM with predominantly short, intermediate, or long mitochondria. *L*, mitochondrial cristae width, represented as average width/cell from 10 individual cells per condition as measured from electron microscopy images. Representative transmission electron microscopy images are shown below (20,000). *M*, intracellular citrate synthase activity. *N*, quantification and representative immunoblot for TOM20. The histograms represent compiled data from three to six animals per group (mean + SEM). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (two-tailed, unpaired Student's *t* test (for two groups) or one-way ANOVA multiple comparisons). MFI, mean fluorescence intensity; TMRM, tetramethylrhodamine methyl ester; BMDM, bone marrow–derived macrophage.

data suggest that OXPHOS is increased in *Wnt4*$^{\Delta/\Delta}$ BMDM, we measured oxygen consumption rates (OCRs) in control and *Wnt4*$^{\Delta/\Delta}$ BMDM. Consistent with *Wnt4*$^{\Delta/\Delta}$ BMDM having increased OXPHOS activity, basal OCR was significantly increased in these cells (Fig. 2, *C* and *D*). ATP-linked respiration was also increased in *Wnt4*$^{\Delta/\Delta}$ BMDM (Fig. 2*E*), further supporting a role for OXPHOS in the elevated cellular ATP levels observed in *Wnt4*$^{\Delta/\Delta}$ cells. On the other hand, the spare respiratory capacity (a measure of the ability of mitochondria to respond to an increased energy demand) (Fig. 2*F*) was similar between the two genotypes while proton leak (a measure of proton diffusion across the inner membrane) (Fig. 2*G*) was decreased in *Wnt4*$^{\Delta/\Delta}$ BMDM. In addition, mitochondrial membrane potential and mitochondrial ROS levels in *Wnt4*$^{\Delta/\Delta}$ BMDM were comparable to controls (Fig. 2,*H* and *I*). Altogether, our results indicate that mitochondria in *Wnt4*$^{\Delta/\Delta}$ cells have increased flux through the electron transport chain and ATP synthase without major impairment in mitochondrial function.

We then determined if the functional changes we observed in *Wnt4*$^{\Delta/\Delta}$ BMDM were associated with changes in mitochondrial structure or mass. We first stained mitochondria in control and *Wnt4*$^{\Delta/\Delta}$ BMDM macrophages for the mitochondrial outer membrane protein TOM20 and imaged them by confocal microscopy (Fig. 2*J*). While control BMDM had on average very short mitochondria, we observed a significant increase in intermediate mitochondria in *Wnt4*$^{\Delta/\Delta}$ BMDM (Fig. 2*K*). Mitochondrial elongation and increased OXPHOS can be associated with changes in cristae structure, the folds of the inner membrane where the electron transport chain resides. We thus used electron microscopy to evaluate cristae width in control and *Wnt4*$^{\Delta/\Delta}$ BMDM. *Wnt4*$^{\Delta/\Delta}$ BMDM had tighter cristae than their control counterparts (Fig. 2*L*), suggesting improved OXPHOS efficiency. On the other hand, there was no difference in mitochondrial mass as measured by citrate synthase activity (Fig. 2*M*) or TOM20 immunoblotting (Fig. 2*N*). In sum, these findings indicate that Wnt4 controls mitochondrial activity without altering mitochondrial mass.

Lipolysis is enhanced in Wnt4$^{\Delta/\Delta}$ *macrophages*

As our results suggest that the increased OCR and ATP levels observed in *Wnt4*$^{\Delta/\Delta}$ BMDM is the consequence of increased metabolic flow through OXPHOS rather than a major change in mitochondrial structure, we then analyzed the potential substrates supporting OXPHOS in these cells. *Wnt4*$^{\Delta/\Delta}$ BMDM showed a substantial decrease in lactate production (Fig. 3*A*), which was associated with a smaller but significant reduction in glucose consumption (Fig. 3*B*). While these results are consistent with the greater OXPHOS activity, we observed they also suggest that glucose is not the major source of metabolic intermediates supporting enhanced mitochondrial activity in *Wnt4*$^{\Delta/\Delta}$ BMDM. In contrast, there was a drastic reduction in the lipid droplets present in *Wnt4*$^{\Delta/\Delta}$ BMDM relative to control cells, as measured by Oil Red O staining (Fig. 3, *C* and *D*), suggesting that fatty acids (FAs) could be fueling the increased OXPHOS in these cells.

Lipid droplets store triglycerides that must be hydrolyzed to liberate the FA used in mitochondrial ß-oxidation. As lysosomal acid lipase (LAL) is one of the key enzymes that cells use to liberate FA from lipid droplets (30, 31), we measured its activity in control and *Wnt4*$^{\Delta/\Delta}$ BMDM. Consistent with the reduction in lipid droplets, LAL activity was increased 2-fold in *Wnt4*$^{\Delta/\Delta}$ BMDM as compared to controls (Fig. 4*A*). However, this increase is neither due to an increase in *Lipa* gene expression (Fig. S1*A*) nor to an increase in the overall lysosomal content as we did not observe any change in the activity of the lysosomal protease Cathepsin B (Fig. 4*B*) or the expression of the lysosomal membrane protein LAMP-1 (Fig. 4*C*). Moreover, LAL inhibition restored cytosolic lipid content in *Wnt4*$^{\Delta/\Delta}$ BMDM (Fig. 4, *D* and *E*). Consistent with a specific role for LAL, the cytosolic neutral lipase activity was similar between *Wnt4*$^{\Delta/\Delta}$ and control BMDM (Fig. 4*F*), and the genes for the enzymes responsible for this activity, *Lipe* and *Pnpla2*, were expressed at very low levels but similar levels in both genotypes (Fig. S1, *B* and *C*). Altogether these data indicate that enhanced LAL activity is responsible of FA generation in *Wnt4*$^{\Delta/\Delta}$ cells.

As autophagy has been implicated in LAL-dependent lipid metabolism in macrophages (32), we assessed autophagy flux with the membrane autophagosome marker LC3. Although we observed a decrease in the number of LC3 puncta in *Wnt4*$^{\Delta/\Delta}$ BMDM that was partially rescued with bafilomycin treatment (Fig. 4,*G* and *I*), there was no significant increase in the ratio of LC3 puncta (treated/untreated) between *Wnt4*$^{\Delta/\Delta}$ BMDM and controls (Fig. 4*H*), indicating that the autophagic flux was not enhanced in *Wnt4*$^{\Delta/\Delta}$ BMDM. Put together, these data point toward increased lipid degradation by LAL in *Wnt4*$^{\Delta/\Delta}$ BMDM, irrespective of alterations in autophagy.

Wnt4$^{\Delta/\Delta}$ *BMDM shows increased mitochondrial β-oxidation*

Decreased glucose consumption and decreased lipid storage support the hypothesis of FAO as a major source of energy in *Wnt4*$^{\Delta/\Delta}$ BMDM. To more directly evaluate the relative importance of each carbon source, we inhibited glycolysis with 2-deoxy-d-glucose (2-DG) or FA transport into mitochondria using etomoxir. We then measured OCR in otherwise unstimulated and nonpolarized macrophages, similar to Figure 2. Control BMDM showed very little alteration in their basal OCR in response to blocking either one of the two pathways (Fig. 5, *A*–*C*), likely reflecting their relatively low level of metabolic activity in the absence of stimulation (33). Consistent with ß-oxidation providing the extra carbon source to fuel OXPHOS in *Wnt4*$^{\Delta/\Delta}$ cells, etomoxir significantly decreased basal OXPHOS in these cells. Similarly, etomoxir significantly reduced ATP-linked OCR in *Wnt4*$^{\Delta/\Delta}$ but not in control BMDM (Fig. 5*D*), and it also increased the spare capacity of *Wnt4*$^{\Delta/\Delta}$ BMDM but not of control cells (Fig. 5*E*). Moreover, *Wnt4*$^{\Delta/\Delta}$ BMDM challenged with palmitate showed a significant decrease in OCR upon etomoxir treatment while control BMDM was not affected (Fig. 5*F*). This coincided with an increase in Oil Red O staining in etomoxir-treated *Wnt4*$^{\Delta/\Delta}$ BMDM, restoring their lipid droplets to control levels (Fig. 5,

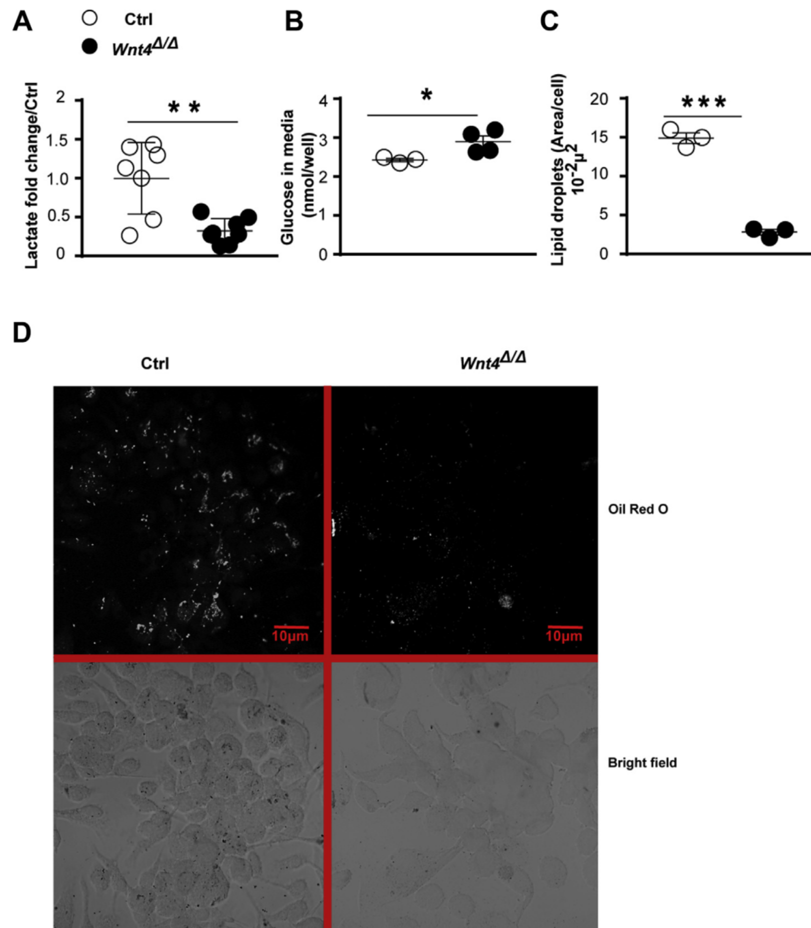

Figure 3. Wnt4 deletion decreases glycolysis and intracellular lipid storage. *A*, total lactate (intracellular + extracellular). *B*, glucose remaining in culture media. *C*, quantification of lipid droplets as determined by the area of Oil Red O staining per cell quantified using ImageJ. *D*, representative confocal microscopy images showing Oil Red O signal in BMDM (60X). The histograms represent compiled data from three to eight animals per group (mean + SEM). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (two-tailed, unpaired Student's *t* test). BMDM, bone marrow–derived macrophage.

G and *H*) and further supports a role for ß-oxidation in the metabolic changes observed in *Wnt4*$^{\Delta/\Delta}$ BMDM. Treatment with 2-DG also somewhat decreased basal OCR in *Wnt4*$^{\Delta/\Delta}$ BMDM (Fig. 5, *A–C*), suggesting that glucose can also contribute to their enhanced OXPHOS. However, the expression of *Pdh1*, an enzyme required to commit pyruvate to the TCA cycle, was not enhanced in *Wnt4*$^{\Delta/\Delta}$ BMDM (Fig. S1*D*), suggesting no major changes in pyruvate handling by *Wnt4*$^{\Delta/\Delta}$ BMDM. Altogether, these results indicate that the loss of Wnt4 stimulates the usage of lipids as an important source of energy.

To better establish how Wnt4 regulates lipid metabolism, we evaluated putative signaling pathways downstream of Wnt4. While JNK phosphorylation (Fig. 1, *E* and *F*) and AKT-dependent β-catenin phosphorylation were not altered in *Wnt4*$^{\Delta/\Delta}$ BMDM (Fig. 1*D*), the classical β-catenin-dependent target genes *c-Myc* and *Ccnd1* (*Axin2* was not expressed in BMDM) were downregulated in *Wnt4*$^{\Delta/\Delta}$ BMDM (Fig. S1, *E* and *G*). As the noncanonical protein kinase C (PKC)/Ca^{2+} pathway negatively regulates TCF/β-catenin-dependent gene expression without impacting intracellular β-catenin levels (34) and inhibits ERK1/2 (35), our results suggest that the absence of Wnt4 promotes the activation of this PKC pathway. Since PKC activity has also been associated with LAL induction during monocyte differentiation into macrophage (36), we evaluated the impact of PKC inhibition on LAL activity in *Wnt4*$^{\Delta/\Delta}$ BMDM. While PKC inhibition had no impact on control BMDMs, LAL activity in *Wnt4*$^{\Delta/\Delta}$ BMDM was reduced to control levels upon PKC inhibition (Fig. 5*I*), suggesting that the enhanced lipolysis observed in the absence of Wnt4 is PKC-dependent. Considering the decrease in AKT (Thr308) phosphorylation observed in *Wnt4*$^{\Delta/\Delta}$ BMDM (Fig. 1*I*), we also evaluated the contribution of the PI3K/PTEN axis. To our surprise, LAL activity was further enhanced in *Wnt4*$^{\Delta/\Delta}$ BMDM upon PTEN inhibition (Fig. 5*J*). While we cannot exclude a potential contribution of AKT downstream of PI3K upon PTEN inhibition, it is possible that the increase in PI3K activity triggered by PTEN inhibition promotes PKC activation

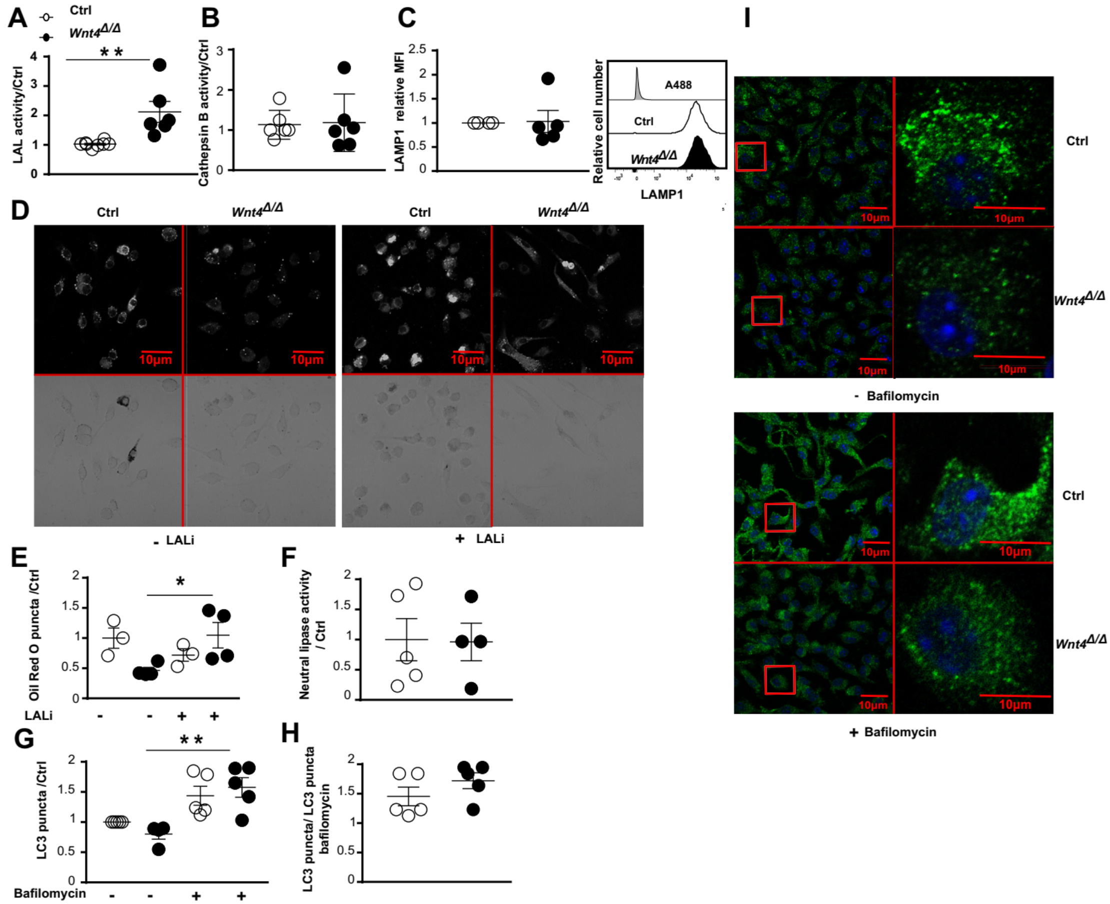

Figure 4. LAL promotes fatty acid usage in *Wnt4*$^{\Delta/\Delta}$ BMDM. *A*, lysosomal acid lipase (LAL) activity, normalized to controls. *B*, cathepsin B activity, normalized to controls. *C*, quantification of lysosomes as determined by relative LAMP-1 MFI. *D*, representative confocal microscopy images showing Oil Red O signal in BMDM in the absence and the presence of a LAL inhibitor (LALi)(60×). *E*, quantification of lipid droplets as determined by the area of Oil Red O staining per cell quantified using ImageJ. *F*, neutral lipase activity, normalized to controls. *G*, quantification of LC3 puncta per cell ± bafilomycin (100 nmol) with ImageJ software. *H*, quantification of the LC3puncta/LC3 puncta + bafilomycin ratio to evaluate autophagy flux. *I*, representative confocal microscopy images of BMDM, staining for autophagosomes (*green*: LC3, *blue*: nucleus, 60×). *$p < 0.05$, **$p < 0.01$ (two-tailed, unpaired Student's *t* test (two groups) or one-way ANOVA multiple comparisons). BMDM, bone marrow–derived macrophage; MFI, mean fluorescence intensity.

via PDK-1 (37, 38). In sum, these results strongly suggest that PKC and PI3K signaling regulate lipolysis in *Wnt4*$^{\Delta/\Delta}$ BMDM.

Wnt4 is not required for the inflammatory response induced by LPS stimulation

Thus far, we have established that *Wnt4*$^{\Delta/\Delta}$ BMDMs have higher ATP levels, mostly as a consequence of increased mitochondrial FAO, which has been generally associated with macrophage polarization to an M2 profile (30, 39). However, flow cytometry analysis revealed no significant differences in the expression of M1 (CD86, MHCII) or M2 cell surface markers (CD206) between unstimulated *Wnt4*$^{\Delta/\Delta}$ and control BMDM (Fig. 6, *A*–*C*). There was also no difference in cathepsin B activity (Fig. 4*B*), the most abundant lysosomal protease (40) whose activity has been shown to be increased in M2 macrophages (41). In summary, the metabolic differences in *Wnt4*$^{\Delta/\Delta}$ BMDM did not appear to result in an inherent bias in unstimulated cells.

LPS is a toll-like receptor 4 agonist that is widely used to promote the secretion of proinflammatory cytokines by macrophages (42). Moreover, LPS-treated macrophages reduce their oxygen consumption and adopt a strongly glycolytic profile (10). We thus stimulated *Wnt4*$^{\Delta/\Delta}$ and control BMDM with LPS to determine if the metabolic differences in *Wnt4*$^{\Delta/\Delta}$ BMDM were reversible. There was a strong suppression of OCR in LPS-treated *Wnt4*$^{\Delta/\Delta}$ BMDM, with basal OCR decreasing even slightly below levels detected in LPS-treated controls (Fig. 6, *D* and *E*). Unsurprisingly, this also corresponded to a significant decrease in ATP-linked respiration (Fig. 6*F*), indicating that LPS inhibits mitochondrial activity in both *Wnt4*$^{\Delta/\Delta}$ and control BMDM. Similarly, extracellular acidification rate was increased to similar levels in LPS-treated *Wnt4*$^{\Delta/\Delta}$ and control BMDM, suggesting an increase in lactate

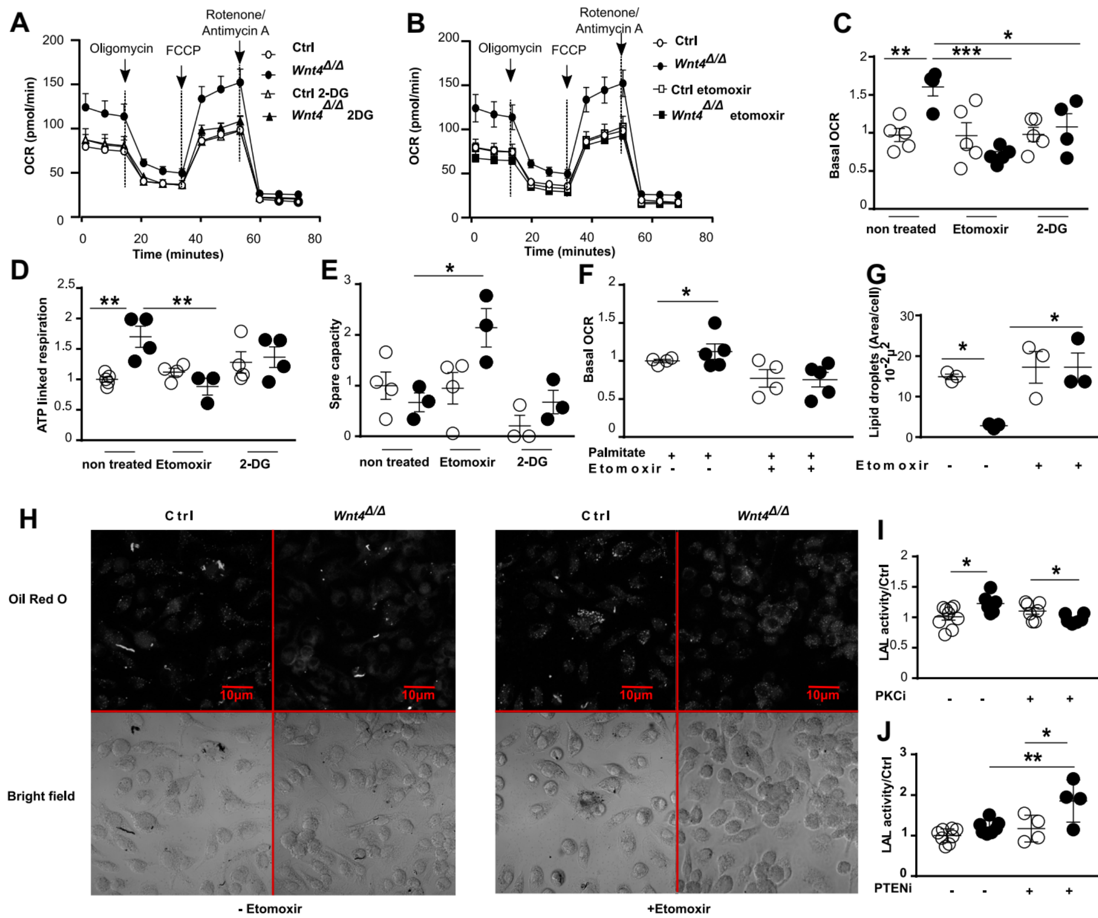

Figure 5. Wnt4 deletion enhances β-oxidation. *A* and *B*, representative oxygen consumption rate (OCR) curves for BMDM pretreated or not with 2-DG (5 mM) (*A*) to block glycolysis or etomoxir (250 μM) (*B*) to block β-oxidation. *C–E*, quantification of basal OCR (*C*), ATP-linked respiration (*D*), and spare respiratory capacity (*E*) with and without 2-DG or etomoxir. *F*, quantification of basal OCR in BMDM exposed to palmitate in the absence or the presence of etomoxir. *G*, quantification of Oil Red O positive area per cell ± etomoxir. *H*, representative confocal microscopy images of Oil Red O signal in BMDM ± etomoxir (60×). *I–J*, lysosomal acid lipase (LAL) activity in BMDM treated with a PKC inhibitor (5 μM) (*I*) or a PTEN inhibitor (100 nM) (*J*), normalized to controls. The histograms represent compiled data from six animals per group (mean ± SEM). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (ordinary one-way ANOVA, multiple comparisons). 2-DG, 2-deoxy-d-glucose; BMDM, bone marrow–derived macrophage; PKC, protein kinase C.

production (Fig. 6*G*). These data indicate that the LPS-induced metabolic switch to glycolysis is not impaired in *Wnt4*$^{Δ/Δ}$ BMDM.

To further evaluate the inflammatory potential of *Wnt4*$^{Δ/Δ}$ BMDM, we measured nitric oxide (NO) and TNFα production in culture supernatants with and without LPS stimulation. *Wnt4*$^{Δ/Δ}$ BMDM produced slightly more NO (Fig. 6*H*) and similar levels of TNFα (Fig. 6*I*) upon LPS stimulation as compared to their normal counterparts. Furthermore, *Wnt4*$^{Δ/Δ}$ and control BMDM showed a comparable expression of M1 (iNOS) and M2 (arginase-1) markers following LPS/IFN-γ and IL-4/IL-13/IL-10-mediated polarization, respectively (Fig. S1, *H* and *I*). These data confirm that the capacity of *Wnt4*$^{Δ/Δ}$ BMDM to respond to a strong proinflammatory stimulus was not negatively affected by the metabolic alterations seen at steady state.

Wnt4 deficiency contributes to the ability of L. donovani promastigotes to colonize BMDM

Metabolic alterations in *Wnt4*$^{Δ/Δ}$ BMDM did not prevent their polarization or LPS-induced glycolytic switch. If anything, the response of *Wnt4*$^{Δ/Δ}$ BMDM was even stronger than controls (Fig. 6, *E* and *H*). To evaluate if *Wnt4*$^{Δ/Δ}$ BMDM was predisposed to respond more strongly to other stimuli, we investigated their response in a more physiologically relevant context, following a parasitic infection.

Macrophages are the principal hosts of the intracellular parasite *Leishmania* and are indispensable for their survival and replication (43). Importantly, macrophage polarization toward an M2 profile promotes parasite growth (44, 45). Here, we compared the fate of *L. donovani* metacyclic promastigotes in wildtype and *Wnt4*$^{Δ/Δ}$ BMDM. There was no significant difference in parasite uptake 6h postinfection between

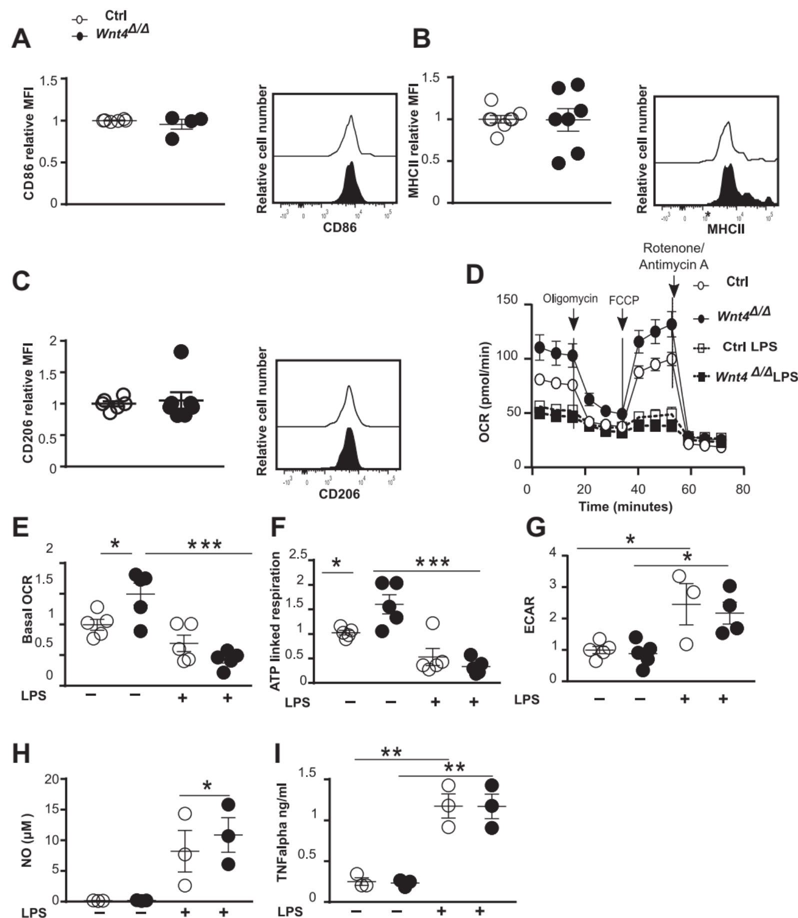

Figure 6. Wnt4$^{Δ/Δ}$ BMDM mount strong metabolic and proinflammatory responses to LPS. *A–C*, expression of cell surface markers in BMDM. Relative mean fluorescence intensity (MFI) of CD86 (M1 marker) (*A*), MHCII (M1 Marker) (*B*), and CD206 (M2 Marker) (*C*). *D*, representative oxygen consumption rate (OCR) curves for BMDM pretreated or not with LPS (100 ng/ml). *E–G*, quantification of basal OCR (*E*), ATP-linked respiration (*F*), and extracellular acidification rate (ECAR) (*G*) in BMDM ± LPS. *H*, measure of nitric oxide (NO) in culture supernatants after a 48 h stimulation with LPS. *I*, secretion of TNFα in culture supernatant from cells treated as in *H*. The histograms represent compiled data from three to eight animals per group (mean + SEM). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (two-tailed, unpaired Student's *t* test (two groups) or one-way ANOVA multiple comparisons). BMDM, bone marrow–derived macrophage; LPS, lipopolysaccharide.

Wnt4$^{Δ/Δ}$ and control BMDM (Fig. 7, *A* and *B*). However, parasite replication was increased in *Wnt4$^{Δ/Δ}$* BMDM over time (Fig. 7, *A* and *B*). These results indicate that although the metabolic alterations observed in the absence of Wnt4 were not irreversible, *Wnt4$^{Δ/Δ}$* BMDM was more permissive to infection and favored parasite replication. To determine the functional impact of metabolic changes in *Wnt4$^{Δ/Δ}$* BMDM on parasite replication, we treated macrophages with etomoxir to inhibit β-oxidation. Pretreatment with etomoxir had no impact on parasite uptake but resulted in decreased parasite numbers at 72 h in both control and *Wnt4$^{Δ/Δ}$* BMDM (Fig. 7, *B* and *C*). However, etomoxir did not fully rescue *Wnt4$^{Δ/Δ}$* BMDM, suggesting that enhanced β-oxidation is not the only mechanism responsible of the impaired parasite control in *Wnt4$^{Δ/Δ}$* BMDM. Altogether, our results indicate that although the metabolic alterations present in the absence of Wnt4 were not irreversible, enhanced β-oxidation rendered *Wnt4$^{Δ/Δ}$* BMDM more permissive to infection and favored parasite replication.

Discussion

The physiological role of individual Wnt ligands remains enigmatic in a large number of situations due to their often promiscuous signaling. Although the most studied ligands are widely used as prototypes of canonical (Wnt3a) and noncanonical (Wnt5a) Wnt signaling, individual Wnt proteins are often able to activate more than one signaling pathway, depending on cell type and receptor availability (46, 47). We report here a new role for the (mostly) noncanonical ligand Wnt4 in regulating BMDM metabolism. Our results show that Wnt4 regulates mitochondrial ATP production and efficiency without impacting mitochondrial mass. We also demonstrate that PKC-dependent LAL activation results in decreased lipid

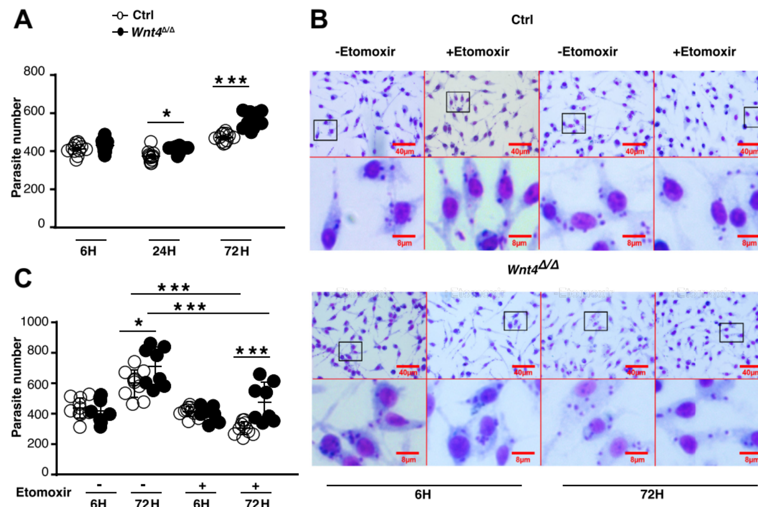

Figure 7. Wnt4 deletion promotes *Leishmania donovani* survival. *A*, parasite numbers per 100 cells as counted from Giemsa-colored slides. *B*, representative microscopy images of infected BMDM at different time points following *L. donovani* infection. *C*, parasite numbers per 100 cells in *L. donovani*-infected cells treated or not with etomoxir, as counted from Giemsa-colored slides. The histograms represent compiled data from five animals per group (mean ± SEM), *$p < 0.05$, ***$p < 0.001$ (two-way ANOVA, multiple comparisons). BMDM, bone marrow–derived macrophage.

storage and provides an important fuel for mitochondria in *Wnt4*$^{\Delta/\Delta}$ BMDM. Wnt4-deficient BMDM was not irreversibly polarized and remained responsive to metabolic reprogramming with LPS. However, enhanced mitochondrial activity and β-oxidation predisposed *Wnt4*$^{\Delta/\Delta}$ BMDM to infection with the intracellular parasite *L. donovani*, in line with the tenet that macrophage metabolism influences their response to pathogens.

Canonical Wnt/β-catenin signaling is well established in reprogramming tumor cell metabolism toward glycolysis and lactate production instead of mitochondrial OXPHOS (48–51). However, these metabolic trends do not necessarily hold true in nonmalignant context; in fact, stimulation with canonical Wnt ligands increases FAO activity and β-oxidation enzymes in osteoblasts, in contrast to noncanonical ligands, such as Wnt4 (52, 53). It also results in decreased adipogenesis and lipid accumulation in brown adipocytes (53, 54). Our analysis of *Wnt4*$^{\Delta/\Delta}$ macrophages revealed increased FAO and mitochondrial activity, concomitant with enhanced lipid degradation and decreased lipid storage, thus presenting striking similarities with osteoblasts or adipocytes responding to canonical Wnt signaling. Wnt4 is generally considered a noncanonical Wnt ligand, and its absence could thus result in disinhibition of canonical signals. However, we did not observe significant changes in intracellular phospho-Ser552-β-catenin staining between *Wnt4*$^{\Delta/\Delta}$ and control macrophages at steady state, and the expression of classical canonical Wnt target genes was downregulated in *Wnt4*$^{\Delta/\Delta}$ cells. Instead, we showed that the enhanced lipolysis in *Wnt4*$^{\Delta/\Delta}$ BMDM was dependent on PKC activity and could be further enhanced by the inhibition of PTEN, suggesting that the lack of Wnt4 may promote disinhibition of the noncanonical PKC/Ca^{2+} pathway.

To our knowledge, there are no prior reports on the role of individual Wnt ligands in macrophage metabolism. However, Wnt signaling has been associated with metabolic diseases such obesity, diabetes, and atherosclerosis (55–57). More specifically in macrophages, expression of the canonical Wnt pathway co-receptor low-density lipoprotein (LDL) receptor–related protein was increased after incubation with LDL, and LDL receptor–related protein 5 promoted cholesterol ester accumulation and macrophage transformation to foam cells *in vitro*. LDL-treated macrophages also upregulated canonical Wnt target genes, suggesting an active role for Wnt signaling in this process (58). Conversely, the Wnt antagonist soluble Frizzled-related protein SFRP5 improved glucose tolerance and insulin sensitivity as well as attenuated weight gain in mice on high-fat diet at least in part by inhibiting Wnt5a-dependent activation of inflammatory macrophages *in vivo* (59, 60). Finally, the deletion of β-catenin in macrophages and myeloid cells increases inflammatory responses in macrophages and enhanced the size of atherosclerotic plaques in LDL receptor–deficient mice (61). Wnt4-deficient macrophages displayed an enhanced capacity to degrade lipids, which tempts us to speculate that inhibiting Wnt4 in macrophages could attenuate the impact of high-fat diet on metabolic disorders and atherosclerosis.

ERK and AKT promote the Warburg effect or the preferential generation of ATP *via* lactate production in cancer cells (26, 62–65) by increasing glucose uptake and promoting the activation of glycolytic enzymes (66, 67) (26, 68, 69). Consequently, the attenuated AKT and ERK1/2 activity in *Wnt4*$^{\Delta/\Delta}$ BMDM should favor OXPHOS instead of lactate production, which is consistent with our analysis. *Wnt4*$^{\Delta/\Delta}$ BMDM consumes less glucose, and blocking FAO with etomoxir not only restored intracellular lipid droplets but also reverted *Wnt4*$^{\Delta/\Delta}$

BMDM mitochondrial activity and increased their spare respiratory capacity, indicating a preferential use of lipids as energy source. Enhanced lipid degradation in *Wnt4*$^{\Delta/\Delta}$ cells was chiefly dependent on the activity of lysosomal lipase LAL, with only modest contribution of neutral lipases, such as the adipose triglyceride lipase (ATGL/PNPLA2). Although ATGL is highly expressed in tissue macrophages and its deficiency results in significant lipid droplet accumulation and diminished macrophage function (70, 71), *Pnpla2* was detected at relatively low levels in BMDM in our study, and there was no difference in neutral lipase activity between *Wnt4*$^{\Delta/\Delta}$ and control BMDM.

Wnt4$^{\Delta/\Delta}$ BMDM possessed longer mitochondria with tighter cristae structure, two important determinants of enhanced mitochondrial OXPHOS efficiency (72, 73), but there were no significant changes in the spare respiratory capacity, mitochondrial membrane potential, ROS levels, or mitochondrial mass. These results are consistent with dynamic changes in mitochondrial usage and efficiency as observed upon altered nutrient availability and do not indicate alterations that would permanently rewire mitochondrial function. Taken together, our results indicate that Wnt4 attenuates OXPHOS, likely by regulating mitochondrial connectivity as well as by repressing lipolysis and FAO.

Lipids are important for macrophage function, including phagocytosis, functional polarization, and production of inflammatory mediators (13, 74, 75). Although FAO activity is largely associated with an anti-inflammatory profile (76), *Wnt4*$^{\Delta/\Delta}$ BMDMs are not irreversibly committed. They remain entirely capable of responding to a strong proinflammatory stimulus, such as LPS, and reducing their mitochondrial activity and shifting to glycolysis (76). Their metabolic switch to M_{LPS} was further corroborated by the production of inflammatory mediators, such as NO and TNFα. It should be noted, however, that the BMDM differentiation environment can promote inflammatory polarization (77). Proteomic studies highlighted that the L929 supernatant used here contains secreted factors involved in the regulation of inflammation, such as MIF that modulate the secretion of inflammatory cytokines/interleukins (TNF-α, IFN-γ, IL-2, IL-6, and IL-8) (78) and osteopontin that upregulates IL-12 production (79). Indeed, BMDM differentiated with L929 and stimulated with LPS secrete more TNF-α, IL-6, and IFN-β compared to M-CSF differentiation alone (77), which could potentially influence the polarization we have observed here.

Nevertheless, *Wnt4*$^{\Delta/\Delta}$ BMDM was more permissive to infection by *L. donovani*, and pretreatment of macrophages with etomoxir resulted in decreased parasite replication, demonstrating that elevated β-oxidation in *Wnt4*$^{\Delta/\Delta}$ cells was at least partially responsible. Macrophages are the natural mammalian host cells for *Leishmania* parasites, and the pathogen has developed multiple strategies to evade their microbicidal effects (80, 81). One such strategy *in vivo* is the generation of monocyte-derived myeloid cells with altered function that will promote parasite growth (82, 83). Our results are well in line with this theory and demonstrate that the metabolic alterations in *Wnt4*$^{\Delta/\Delta}$ BMDM have functional consequences. *L. donovani* infection has also been shown to decrease Wnt5a expression in BMDM at gene and protein levels. Conversely, exogenous Wnt5a decreased parasite burden *via* activation of Rac/Rho GTPases, while Wnt5a knockdown by siRNA prior to infection increased parasite survival (84). Our results phenocopy the impact of Wnt5a deletion on parasite replication. More importantly, our data connect Wnt signaling, β-oxidation, and mitochondrial activity in *L. donovani* survival, thus highlighting the importance of macrophage metabolism to the outcome of host–parasite interactions.

In conclusion, our results identify a cell-intrinsic role for Wnt4 in regulating macrophage metabolism. Wnt4 deficiency disturbs energy homeostasis by increasing mitochondrial ATP levels mainly through FAO. Although Wnt4-deficient macrophages demonstrate a strong proinflammatory response to LPS, they were more susceptible to support the growth of an intracellular pathogen. These results demonstrate that noncanonical Wnt4 signaling regulates macrophage function and modulates their metabolism in a context-dependent manner. Further mechanistic and metabolic investigations may be helpful to identify how the Wnt4 pathway could be best harnessed to promote the control of intracellular infections or to modulate macrophage function in the context of metabolic disorders.

Experimental procedures

Experimental animals

B6.129P2-Lyz2$^{tm1(cre)Ifo}$/J (LysMCre) mice were purchased from The Jackson Laboratory. Mice with a *Wnt4* conditional allele have been described elsewhere (85) and were originally a kind gift from S. Vainio (Oulu University, Finland). Mice were bred and housed under specific pathogen-free conditions in sterile ventilated racks at the animal facility of INRS-Centre Armand-Frappier Santé Biotechnologie. All procedures were approved by the Comité institutionnel de la protection des animaux of the INRS and were conducted in accordance with the Canadian Council on Animal Care guidelines. Only female mice were used for the following experiments.

Flow cytometry analysis

BM was harvested by flushing tibias and femurs with PBS/0.1% BSA/0.5 mmol EDTA using a 25-gauge needle. To analyze BMDM, the following antibodies were used: anti-CD11c, anti-Ly6C, anti-F4/80, anti-MHCII, anti-CD206, anti-CD86 (BD Biosciences). For intracellular staining, surface-stained BM cells were fixed and permeabilized using the Foxp3 staining kit (eBioscience) and then incubated with the following primary antibodies: Arg1 (R&D Systems, PE-conjugated), iNOS (eBioscience, APC-conjugated), p-AKT(Thr308) (1/100), p-AKT (Ser473) (1/100), β-catenin (Ser552) 1/200), p-ERK (Thr202/Tyr204)(1/100), ERK (1/100), AKT (1/100), p-JNK (1/100), JNK (1/100), p-S6 (Ser235/236), p-4E-BP1 (Thr37/46) (all from Cell Signaling Technologies), or Lamp1 (1/800) (Abcam), overnight at 4 °C; or conjugated Ki-67 (FITC) for 1 h at room temperature. Unconjugated

antibodies were detected with an Alexa 488-conjugated $F(ab')_2$ fragment against rabbit IgG (Molecular Probes). The stained cells were analyzed on a four-laser BD LSRFortessa cell analyzer (BD Biosciences) and analyzed using FACS DiVa (v. 8.1) or FlowJo (v. 10.1) software.

Cell culture

BM was flushed with 5 ml Hank's Balanced Salt Solution (HBSS), centrifuged at 1236*g* or 7 min, and the pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) supplemented with 10% fetal bovine serum (FBS Premium, Wisent Bioproducts) at 5×10^6 cell/ml. 1.5×10^6 cells were seeded in a nonadherent Petri dish and cultured in DMEM supplemented with 10% FBS and 20% conditioned medium from L929 fibroblasts as previously described (86). The differentiation medium was refreshed on day 5, and adherent BMDMs were collected with Trypsin/EDTA solution on day 7 and analyzed by flow cytometry or replated in DMEM/10% FBS for further analyses. LPS was purchased from Sigma-Aldrich and used at a final concentration of 100 ng/ml. Oligomycin was (2 µM) (Sigma Aldrich) was added for 1 h at 37 °C. For autophagy induction, BMDMs were replated in Earle's Balanced Salt Solution (Life Technologies) for 2 h at 37 °C with or without Bafilomycin (100 nM).

Immunofluorescence

2×10^5 cells were seeded overnight on uncoated coverslips at 37 °C. The cells were first fixed with 4% paraformaldehyde for 15 min at room temperature and then washed three times with PBS. Fixed BMDM were permeabilized with PBS/0.1% Triton X-100 solution, for 2 min, then blocked with PBS/0.1% Triton/1% BSA for 10 min. The cells were incubated for 1 h at room temperature with primary antibodies: anti-LC3 (1/100) (Cell Signaling Technologies) or anti-Tom20 (1/100) (Abcam), washed three times with PBS, and incubated with Alexa 488-conjugated secondary antibody (1/1000) for 30 min at room temperature. Finally, the coverslips were washed and mounted on the microscope slides with ProLong Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific). Images were taken using a LSM 780 confocal microscope with 60× oil objective.

Enzymatic activity

Cathepsin B activity: Cells were collected with Trypsin with 0.25% EDTA, washed with PBS 1×, and collected by centrifugation at 200*g* for 5 min, at 4 °C. Cells were lysed by buffer containing Tris-HCL 50 mM, NaCl 150 mM, EDTA 1 mM, and Triton X-100 (0.5%) and centrifuged at 18,300*g* for 12 min at 4 °C. Supernatants were collected, and proteins were dosed with Bradford Kit (Bio-Rad). Proteins (10 µg) were diluted in 100 µl of 100 mM Hepes, pH 6.0, 150 mM NaCl, 2 mM DTT, and 5 mM EDTA in the presence of a 5 µM concentration of the cathepsin B substrate zRR-AMC (Sigma-Aldrich). Samples were incubated for 30 min at 37 °C, and fluorescence was measured (excitation/emission 360/440 nm) using a Cytation5 Cell Imaging Multi-Mode Reader.

LAL activity was measured as described (87) by diluting 20 µg of proteins from each samples treated with GF109203X (PKC inhibitor) at 5 µM for 1 h at 37 °C or with bpV(pic) (PTEN inhibitor) at 1 µM for 2 h at 37 °C or controls in 100 µl of reaction buffer (100 mM sodium acetate, pH 4.0, 1% (v/v) Triton X-100, and 0.5% (w/v) cardiolipin) in the presence of 0.345 mM 4-methylumbelliferone (Sigma-Aldrich). Samples were incubated for 1 h at 37 °C. Fluorescence was measured (excitation/emission 360/440 nm) using a Cytation5 Cell Imaging Multi-Mode Reader.

Citrate synthase: Citrate Synthase Activity Colorimetric Assay Kit (BioVision, Catalog # K318–100), following manufacturer's instructions.

ATP and lactate assays

2×10^5 cells were grown in 96-well plates. Cellular ATP was measured by ATP using Cell Titer Glow kit (Promega), and total lactate was measured using Lactate Colorimetric/Fluorometric (BioVision) Assay Kit and was measured in triplicate following the protocol provided.

Elisa TNFα

Supernatants were collected from Wnt4Δ/Δ BMDM and control cultures upon 6 h stimulation with LPS and at 6 h, 24 h, and 72 h postinfection with *L. donovani*. 50 µl from each condition were added in duplicates, and TNFα levels were measured using Mouse TNF-alpha Quantikine ELISA Kit (R&D SYSTEMS, Catalog # MTA00B) as per manufacturer's instructions.

Nitric oxide quantification

Supernatants were collected from $Wnt4^{\Delta/\Delta}$ BMDM and control cultures upon 24 h stimulation with LPS and at 6 h, 24 h, and 72 h postinfection with *L. donovani*. 100 µl from each condition were added in duplicates to 96-well plates. Nitrate levels were measured by the Greiss reaction, as described in (88, 89).

Glucose consumption

Supernatants were collected from Wnt4Δ/Δ BMDM and control cultures. 50 µl from each condition were added in duplicates to 96-well plates, and glucose levels were determined using Glucose Colorimetric/Fluorometric Assay Kit (BioVision, Catalog # K606–100) as per manufacturer's instructions.

MitoSOX

BMDMs were collected and stained with the MitoSOX Red mitochondrial superoxide indicator reagent (Invitrogen) at a final concentration of 5 µM, and the cells were incubated for 30 min at 37 ºC, after which they were washed and analyzed by for flow cytometry.

Tetramethylrhodamine methyl ester

BMDMs were collected and stained with tetramethylrhodamine methyl ester (Thermo Fisher) at a final concentration 0.5 nM, and the cells were incubated for 30 min at 37 ºC, after which they were washed and analyzed by for flow cytometry.

Extracellular flux analysis

BMDMs were seeded at 4 × 10^4 cells on Seahorse XF96 cell culture microplates (Agilent) and treated with etomoxir (250 μM) and 2-DG (5 mM) for 30 min at 37 °C. Medium was changed with Seahorse XF DMEM medium, pH 7.4 (Agilent) supplemented with 10 Mm glucose, 1 Mm pyruvate, and 2 mM glutamine, and cells were incubated for 1 h at 37 °C with no CO2. Mito stress kit (Agilent) was used, and XF analysis was performed using the XFe-96 analyzer (Seahorse Bioscience) as per manufacturer's instructions. All reagents provided by Sigma-Aldrich.

qRT-PCR

Cells were collected from BMDM differentiation after 7 days in culture. The manufacturer's protocol was followed for total RNA extraction. RNAEasy columns (Qiagen) were used to concentrate and clean the preparation. High-capacity cDNA reverse transcription kit (Applied Biosystems) was used to convert total RNA to cDNA. TaqMan custom PreAmp kit (Applied Biosystems) was used to pre-amplified the cDNA product. Quantitative RT-PCR was performed using TaqMan reagents and assays (TBP; and B2m; as an internal control, from Applied Biosystems) on Stratagene M x x3000P instrument. Relative quantification of Wnt4 was determined by using the ΔΔCT method.

L. donovani *culture and infections*

Promastigotes of L. *donovani* (MHOM/ET/67/Hu3:LV9) freshly differentiated from splenic amastigotes were cultured in Leishmania medium (M199 medium supplemented with 10% heat-inactivated FBS (Hyclone), 100 μM hypoxanthine, 10 mM Hepes, 5 μM hemin, 3 μM biopterin, 1 μM biotin, penicillin (100 I.U./ml), and streptomycin (100 μg/ml)) at 26 °C. For BMDM infections, metacyclic promastigotes were isolated at 1400 RPM in 15-ml Falcon conical centrifuge tubes containing 1 ml of 40% Ficoll (GE Healthcare) at the bottom, overlaid by 2 ml of a single gradient of 10% Ficoll and overlay 1 × 10^8 promastigotes from the late stationary growth phase in 2 ml of nonsupplemented DMEM (90). Complement opsonization of metacyclic promastigotes was performed prior to infections by incubating the parasites in HBSS containing 10% serum from DBA/2 mice for 30 min at 37 °C. BMDMs were then incubated at 37 °C with metacyclic promastigotes (parasite-to-macrophage ratio of 5:1). After 3 h of incubation, noninternalized parasites were washed 3× with warm HBSS. The time points are described in each experiment. Infection levels were assessed by microscopic examination of infected cells after Giemsa staining with the Hema 3 system (Fisher Scientific).

Western blot

Cells were collected with trypsin with 0.25% EDTA, washed with PBS 1×, and collected by centrifugation at 200*g* for 5 min, at 4 °C, cells were lysed by buffer containing Tris-HCL 50 mM, NaCl 150 mM, EDTA 1 mM, and Triton 0.5%, and centrifuge at 18,300*g* for 12 min, at 4 °C. Supernatants were collected, and phosphatase (NAF 0.3 mM) inhibitor was added. Proteins were dosed with Bradford Kit (Biorad). Prior to electrophoresis, samples were mixed with loading buffer to obtain 1× and 5% β-mercaptophenol (62.5 mM Tris-HCl pH 6.5, 2.5% SDS, 10% glycerol, 0.01% bromophenol blue) and incubated at 95 °C for 5 min. Proteins (50 μg) were added to each well, then resolved on SDS-PAGE followed by wet-transfer to PVDF membranes. Detection was done by immunoblotting using the indicated antibody, Tom20 (Abcam1/1000). The membrane was developed on the automatic film processor machine (AFP ImageWorks).

Statistical analysis

Each value represents at least three independent experiments. Two-tailed Student *t* test or ordinary one-way ANOVA or two-way ANOVA were used as indicated in figure legends to determine statistical significance. A p value $<$ 0.05 was considered significant.

Data availability

All data presented in the manuscript are contained within the manuscript. Additional information will be shared upon reasonable request to the corresponding author, Krista Heinonen. krista.heinonen@inrs.ca

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Supporting information—This article contains supporting information, providing additional characterization of Wnt4-deficient BMDM.

Author contributions—M. T., M. G., and K. M. H. conceptualization; M. T. formal analysis; M. T., H. A. investigation; M. T. visualization; M. T. writing–original draft; A. D., M. G., and K. M. H. resources; A. D., M. G., and K. M. H. writing–review & editing; K. M. H. funding acquisition; K. M. H. supervision.

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Abbreviations—The abbreviations used are: BMDM, bone marrow–derived macrophage; DMEM, Dulbecco's modified Eagle's medium; ERK1/2, extracellular signal–regulated kinases 1 and 2; FA, fatty acid; FAO, fatty acid oxidation; FBS, fetal bovine serum; HBSS, Hank's Balanced Salt Solution; JNK, c-Jun N-terminal protein kinase; LAL, lysosomal acid lipase; LPS, lipopolysaccharide; M1, classically activated proinflammatory macrophages; M2, alternatively activated anti-inflammatory macrophages; MAPK, mitogen-

activated protein kinase; OXPHOS, oxidative phosphorylation; OCR, oxygen consumption rates.

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7.3 Understanding TGEV–ETEC Coinfection through the Lens of Proteomics: A Tale of Porcine Diarrhea

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COMMENTARY

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Understanding TGEV–ETEC Coinfection through the Lens of Proteomics: A Tale of Porcine Diarrhea

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Porcine diarrhea and gastroenteritis are major causes of piglet mortality that result in devastating economic losses to the industry. A plethora of pathogens can cause these diseases, with the transmissible gastroenteritis virus (TGEV) and enterotoxigenic *Escherichia coli* K88 (ETEC) being two of the most salient. In the December 2017 issue of *Proteomics Clinical Aplications*, Xia and colleagues used comparative proteomics to shed light on how these microbes interact to cause severe disease [1]. The authors discovered that TGEV induces an epithelial-mesenchymal transition-like phenotype that augments cell adhesion proteins mediating the attachment of ETEC to intestinal epithelial cells. Moreover, coinfection was found to modulate several host proteins that could bolster pathogen persistence. Importantly, the authors observed that ETEC suppresses the production of inflammatory cytokines induced by TGEV, which may in turn promote the long-term survival of both microbes.

Enteric infections affecting pigs are widespread, and cause a decrease in feed conversion and performance that ultimately result in high financial losses. Among these ailments, diarrhea and gastroenteritis are some of the most important as they cause high morbidity and mortality.[2] The small intestine of a pig is a major site of nutrient absorption. Similar to the colon, this organ harbors a diverse microbiota that is pivotal to digestion and nutrient absorption. Although most of these microbes have a symbiotic relationship with the host, some can cause extensive harm.[2,3]

Escherichia coli is a Gram-negative, facultatively anaerobic bacterium that is usually harmless and aids digestion.[3,4] However, several *E. coli* strains have evolved toxins that cause extensive pathology in the gut. Indeed, *E. coli* is the most important cause of diarrhea in young swine.[4] Of particular interest is enterotoxigenic *E. coli* K88 (ETEC), a noninvasive type that adheres to the microvilli of intestinal epithelial cells. These bacteria release toxins that evoke gastrointestinal hypersecretion of electrolytes and water. The diarrhea and vomiting that ensues causes dehydration, stunted growth, and death. Profuse diarrhea and gastroenteritis are also caused by the transmissible gastroenteritis virus (TGEV), which is a coronavirus that survives the acidic pH of the stomach and the proteolytic enzymes of the duodenum.[5] It multiplies in the cell lining of the small intestine resulting in the loss of absorptive cells and villous atrophy.[5,6] TGEV spreads rapidly, causes outbreaks involving large numbers of pigs, and often leads to 100% mortality in young piglets. Diarrhea is often caused by coinfecting pathogens that synergize to cause severe disease. For instance, humans with diarrhea have been found to be coinfected with rotaviruses and *E. coli* or *Giardia*.[7] Furthermore, infection by multiple microbes has also been reported to cause purulent diarrhea in swine.[8] This raises many questions concerning the pathogenesis of coinfections. In a recent issue of *Proteomics Clinical Applications*, Xia and colleagues[1] unveiled that TGEV infection augments the attachment of ETEC to intestinal cells, thence altering host cell homeostasis and the production of inflammatory cytokines (**Figure 1**).

Since both TGEV and ETEC infect and attach to a pig's intestinal epithelium, a suitable enterocytic cell line of porcine origin is required to study coinfection in the laboratory. In this regard, the IPEC-J2 cell line is an appropriate model since they are morphologically differentiated cells derived from the small intestine of young piglets.[9] Using these cells, Xia and colleagues investigated the dynamics of coinfection by TGEV and ETEC.[1,10] The authors showed that TGEV is able to grow[1] and persist[10] in IPEC-J2 cells. Importantly, the authors found that a preexisting TGEV infection augmented ETEC attachment to intestinal cells. This observation raised the possibility that there might be a survival advantage for either pathogen during coinfection. To that effect, Xia and colleagues examined viral mRNA and protein expression and found that both decrease when ETEC is present. Why is coinfection beneficial for the bacterium but seemingly unfavorable for the virus? To gain important insight into how TGEV promotes ETEC attachment, Xia et al. undertook a comparative proteomics approach where they employed LC–MS/MS coupled to iTRAQ to study cells infected with TGEV, ETEC, or both.[1] Relative to noninfected cells, TGEV infection modulated the expression of 77 proteins. Of particular interest was integrin-$\alpha 5$, a matrix macromolecule known for binding fibronectin and stimulating angiogenesis. Adhesion molecules such as integrins, cadherins, and selectins have been found to facilitate bacterial attachment and invasion.[11] Xia and colleagues demonstrated that integrin-$\alpha 5$ mRNA and protein expression increased upon TGEV

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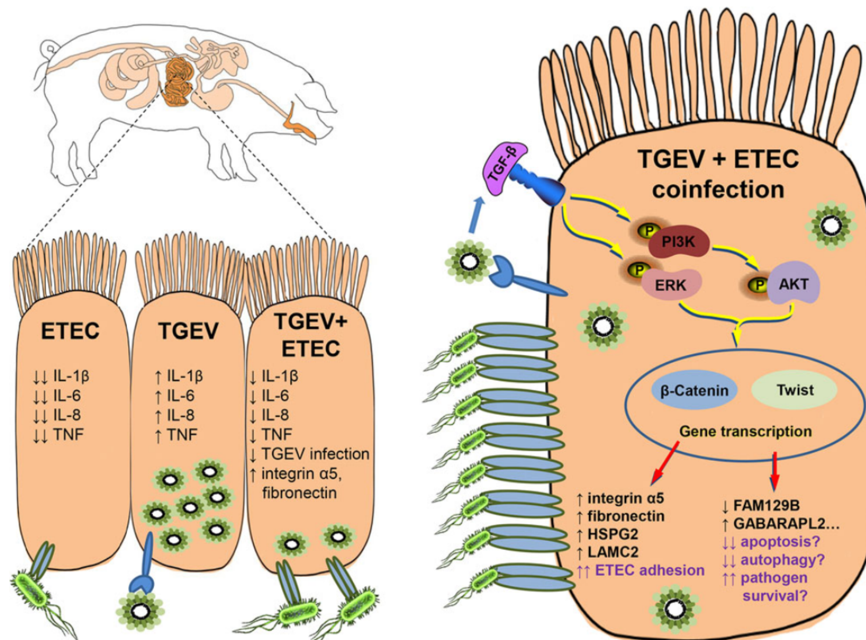

Figure 1. TGEV and ETEC elicit context-dependent host cell responses. Infection of intestinal epithelial cells by TGEV induces a PI3K/Akt-dependent EMT phenotype that elicits plasmalemmal expression of proteins such as integrin-α5. Contrary to ETEC, TGEV augments the production of inflammatory molecules. Increased levels of integrin-α5 promote the attachment of ETEC to epithelial cells, which quells the TGEV-induced production of inflammatory cytokines. Coinfection by TGEV and ETEC also modulates the expression of many proteins involved in homeostasis and host defence, which could ultimately worsen disease severity by enhancing long-term pathogen survival.

infection, and even more saliently so upon coinfection with ETEC. These findings were validated by flow cytometry, which revealed increased cell surface expression of integrin-α5 on infected cells. In order to show a causal link between integrin-α5 and increased ETEC adhesion to TGEV-infected cells, the authors employed peptide agonists or inhibitors of integrin-α5. When integrin-α5 is inhibited on TGEV-infected IPEC-J2 cells, ETEC attachment decreases significantly; the opposite is observed upon treatment with an integrin-α5 agonist.[1] In an accompanying study, Xia and colleagues further elucidated how TGEV induces integrin-α5.[10] There, the authors established that TGEV evoked the production of TGF-β, which in turn induced EMT through the PI3K/Akt pathway. This TGEV-induced EMT was found to augment the expression of vimentin, fibronectin, and integrin-α5 in IPEC-J2 cells and pig intestines[10] (Figure 1). Additionally, the proteome of TGEV–ETEC coinfected cells revealed higher expression of ECM-related proteins HSPG2 and LAMC2.

TGEV causes severe intestinal inflammation.[5,6] Xia and colleagues expanded upon this by reporting that this viral infection induces production of proinflammatory cytokines TNF, IL-1β, IL-6, and IL-8 in IPEC-J2 cells[1,10] (Figure 1). Those cytokines contribute to immune cell infiltration at the infection site[12] and may lead in viral clearance. Upon coinfection with ETEC, the authors found that the bacterium lowered cytokine levels by at least 40%.[1] This finding can be explained by the fact that ETEC lowers TGEV replication,[1] and may—by itself—induce apoptosis.[13] As observed with other pathogens,[12] one can hypothesize that a decreased inflammatory response might prevent removal of TGEV and ETEC from the gut. The question still remains on whether ETEC promotes the long-term survival of TGEV. From the proteome of TGEV–ETEC coinfected cells reported by the authors, one can see an increase in FAM129B,[1] a negative regulator of apoptosis that is overexpressed in cancer cells.[14] Also exclusive to TGEV–ETEC coinfection was a decrease in GABARAPL2,[1] a protein that is essential to autophagy.[15] Decreased autophagy could prevent the elimination of TGEV from the intestine. Since increased viral persistence could translate into augmented transmission and greater financial losses, future experiments could investigate whether the presence of ETEC improves the long-term fitness of TGEV in vitro and in vivo. Future investigations may also evaluate the roles of FAM129B and GABARAPL2 in TGEV–ETEC infection; their knockdown could hamper the survival of TGEV and ETEC in the porcine gut.

In sum, Xia and colleagues convincingly showed that TGEV promotes ETEC adhesion to intestinal epithelial cells.[1,10] The coinfection that ensues lowers the production of proinflammatory molecules and may modulate—to the pathogens' advantage—the expression of host proteins involved in homeostasis and microbial clearance (Figure 1).

Abbreviations

EMT, epithelial-mesenchymal transition; ETEC, enterotoxigenic *Escherichia coli* K88; FAM129B, family with sequence similarity 129 member B; GABARAPL2, GABA(A) receptor-associated protein like 2;

HSPG2, heparan sulfate proteoglycan 2; IPEC-J2, intestinal columnar epithelial cells; iTRAQ, isobaric tags for relative and absolute quantification; LAMC2, laminin gamma 2; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; TGEV, transmissible gastroenteritis virus

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

cytokines, diarrhea, epithelial-mesenchymal transition, host-pathogen interactions, proteomics, TGEV–ETEC coinfection

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