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# *Leishmania* modulates phagosome functions by altering certain SNAREs in a GP63-dependent pathway

By

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A thesis submitted in partial fulfillment of the requirements of the degree of *Philosophiae Doctor* (PhD) in Immunology and Virology

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**The most beautiful thing we can experience is the mysterious. It is the source of all true art and science.**

**(Albert Einstein)**

# Abstract

*Leishmania* is the parasite responsible for the leishmaniases. Like several other intracellular pathogens, they evolved multiple strategies to escape the immune system. In macrophages, phagosomes mature through sequential fusion and fission events with various compartments of the endocytic pathway. This process is interrupted by *Leishmania* promastigotes when they block aquisition of microbicidal molecules from late endosomes and lysosomes while amastigotes-harboring vacuoles are hybrid compartments composed of both endoplasmic reticulum and endocytic pathway components. The N-ethylmaleimide sensitive factor (NSF) attachment receptor proteins (SNAREs) act in intracellular trafficking pathways such as phagolysosome biogenesis. Inhibition of phagosome remodeling through different virulenece factors including LPG and GP63 is one of the most important strategies by *Leishmania* to survive and replicate in host cells.

In the first project, we showed that *Leishmania* cleaves VAMP8 protein which is one of the vesicular SNAREs on phagosome membranes. We also demonstrated that VAMP8 plays a key role in cross-presentation, which is inhibited by *Leishmania* through GP63-dependent cleavage of VAMP8. It had been already shown that $gp91^{phox}$ is one of the crucial subunits in the NADPH oxidase complex that regulates cross-presentation in dendritic cells. We found that VAMP8 is necessary for the recruitment of $gp91^{phox}$ on the phagosome membrane whereby the NADPH oxidase regulates cross-presentation in dendritic cells.

In the second project, we investigated the effect of *Leishmania* on other SNAREs. It is already known that Sec22b is a regulator for cross-presentation and in this study we showed that Sec22b (ER-SNARE) is excluded from phagosomes harbouring *Leishmania* in a GP63-

dependent manner. Since Sec22b is not cleaved by GP63, this exclusion is likely to be due to the action of GP63 on cognate proteins in the Sec22b complex. We also showed that some Sec22b interacting proteins such as syntaxin 4, -5 and SNAP23 are cleaved by GP63 and play a bactericidal role in macrophages.

Altogether, we show novel mechanisms used by *Leishmania* to escape the immune system through the impairment of cross-presentation by degrading key regulators of vesicular trafficking. Also, we show that *Leishmania* cleaves SNAREs that regulate the bactericidal ability of the macrophage.

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Albert Descoteaux

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# List of abbreviations

**AP:** acid phosphatase

**AP-1:** activator protein-1

**APCs:** antigen presenting cells

**Arf6:** ADP-ribosylation factor 6

**Arp2/3:** actin related protein 2/3

**ATP:** Adenosine triphosphate

**BMM:** bone marrow-derived macrophage

**BNIP1:** BCL2/adenovirus E1B 19kDa interacting protein 1

**CLIP:** Class II-associated invariant chain peptide

**COS:** CV-1 (simian) in Origin, and carrying the SV40 genetic material

**CPs:** Cystein proteases

**CR:** Complement receptor

**DC-SIGN:** dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin

**4E-BP1:** eIF4E-binding protein 1

**EEA1**: Early endosome antigen 1

**eIF4F:** eukaryotic initiation factor 4F

**ER:** Endoplasmic reticulum

**ERAAPs:** ER aminopeptidase associated with antigen processing

**ERAD:** endoplasmic reticulum-associated protein degradation

**ERGIC:** ER-Golgi intermediate compartment

**ERS-24:** Endoplasmic Reticulum SNARE of 24 kD

**FAD:** flavine adenine dinucleotide

**GEFs:** guanine nucleotide exchange factor

**GDI:** Guanine nucleotide dissociation inhibitor

**GILT:** Gamma-interferon-inducible lysosomal thiolreductase

**GIPL**: Glycoinositolphospholipid

**GP63:** Glycoprotein 63

**GPI:** Glycosylphosphatidylinositol

**GTPase:** Guanosine triphosphatase or guanosine triphosphate phosphohydrolase

**IL:** interleukin

**ITAM:** immunoreceptor tyrosine activation motif

**JAK/STAT:** Janus kinase/signal transducer and activator of transcription

**JNK:** c-Jun N-terminal kinase

**LAMPs**: Lysosomal-associated membrane proteins

**LCM:** L929 cell-conditioned medium

**LE:** Late endosome

**LPS**: Lipopolysaccharide

**LPG**: Lipophosphoglycan

**MARKS:** myristoylated alanine-rich C kinase substrate

**MFR:** mannose-fucose receptor

**MHC I:** major histocompatibility complex I

**MHC II:** major histocompatibility complex II

**miR-122:** microRNA-122

**MIIC:** MHC-II-containing compartments

**MRP:** MARKS-related proteins

**MSG**: major surface glycoprotein

**mTOR:** mammalian/mechanistic target of rapamycin

**M6PR**: mannose-6-phosphate receptor

**NADPH:** nicotinamide adenine dinucleotide phosphate

**Nck:** non-catalytic region of tyrosine kinase adaptor protein 1

**NF-κB:** Nuclear Factor kappa B

**NK cell:** natural killer cell

**NLR:** nucleotide-binding oligomerization domain receptors

**NLR:** Nod-like receptors

**NO:** nitric oxide

**NSF:** *N*-ethylmaleimide-sensitive factor

**PAMPs**: pathogen-associated molecular patterns

**PKC:** protein kinase C

**PG:** Phosphoglycan

**PMN**: polymorphononuclear cells

**PPGs:** Proteophosphoglycans

**PRRs:** pattern recognition receptors

**PSP**: promastigote surface protease

**PS**: Phosphatidylserine

**PTP:** protein tyrosine phosphatase

**Rab:** Ras-related in brain

**Rac-1:** Ras-related C3 botulinum toxin substrate 1

**RAD50:** DNA repair protein

**RINT-1:** RAD50-interacting protein 1

**ROS:** Reactive oxygen species

**SH3:** Src homology 3 domains

**SHP-1:** Src homology region 2 domain-containing phosphatase-1

**sp:** species

**Sly 1:** Suppressor of loss of yeast Rab GTPase protein 1 (YPT1)

**SNAP:**soluble NSF-attachment protein

**Stx:** syntaxin

**SNAREs:** soluble *N*-ethylmaleimide-sensitive factor-attachment protein receptors

**TCPTP:** T cell protein tyrosine phosphatase

**TGF-β:** Transforming growth factor beta

**TGN:** *trans*-Golgi network

**TLR:** toll-like receptor

**TNF:** tumor necrosis factor-alpha

**TNF-R1:** TNF-α receptor-1

**t-SNARE:** target-associated soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors, also known as Q-SNARE.

**VAMP:** vesicle-associated membrane protein

**VASP:** Vasodilator-stimulated phosphoprotein

**v-SNARE:** vesicle-associated soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors, also known as R-SNARE.

**Vti 1b:** vesicle transport through interaction with the t-SNARE homologue 1b

**WHO:** World Health Organization

**WASP:** Wiscott-Aldrich syndrome protein

**YPT1 :** yeast Rab GTPase protein 1

**Zym:** Zymosan

# Introduction

# 1. Phagocytosis

Despite the complexity of phagocytosis and its varations in different phagocytic cells, the process in general follows a series of sequential events that happen as follows: particle internalization through interaction of specific receptors on the surface of phagocytes, actin polymerization at the site of ingestion, actin detachment after phagosome internalization and at the end, phagosome maturation by fusion and fission events with components of the endocytic pathway (Figure 1) (Vieira, Botelho et al. 2002, Deschamps, Echard et al. 2013).

Phagocytes are a group of cells with a wide range of receptors that recognize and internalize harmful foreign particles or dead and apoptotic cells. The terminology of their name comes from two Greek words "*phagein*", "to eat" and "*-cyte*", "cell". Phagocytes are classified based on their ligands and receptors, efficiency in phagocytosis, antigen degradation and antigen presentation, all of which will be discussed in more detail.

Phagocytic ligands can be classified as opsonins and non-opsonic ligands, and there are specific receptors for each class. Internalization of endogenous ligands such as lipopolysaccharides (LPS) of bacteria and phosphatidylserine (PS) in apoptotic cells are referred to as non-opsonic ligands that can be recognized by CD14/TLR4/MD2 receptor complex, scavenger receptors such as scavenger receptor A (SR-A), macrophage receptor with collagenous structure (MARCO) and CD36 (Bergmann, Lang et al. 2009) and also the C-type lectin receptors such as Dectin-1, which binds β-glucan on the cell wall of certain bacteria and fungi (Drummond and Brown 2011). Alternatively, phagocytosis of host-derived proteins (opsonin ligands), like C3b and IgG are

recognized by complement receptor 3 (CR3) and FcγRs respectively (Yefenof 2000, Goodridge, Underhill et al. 2012).

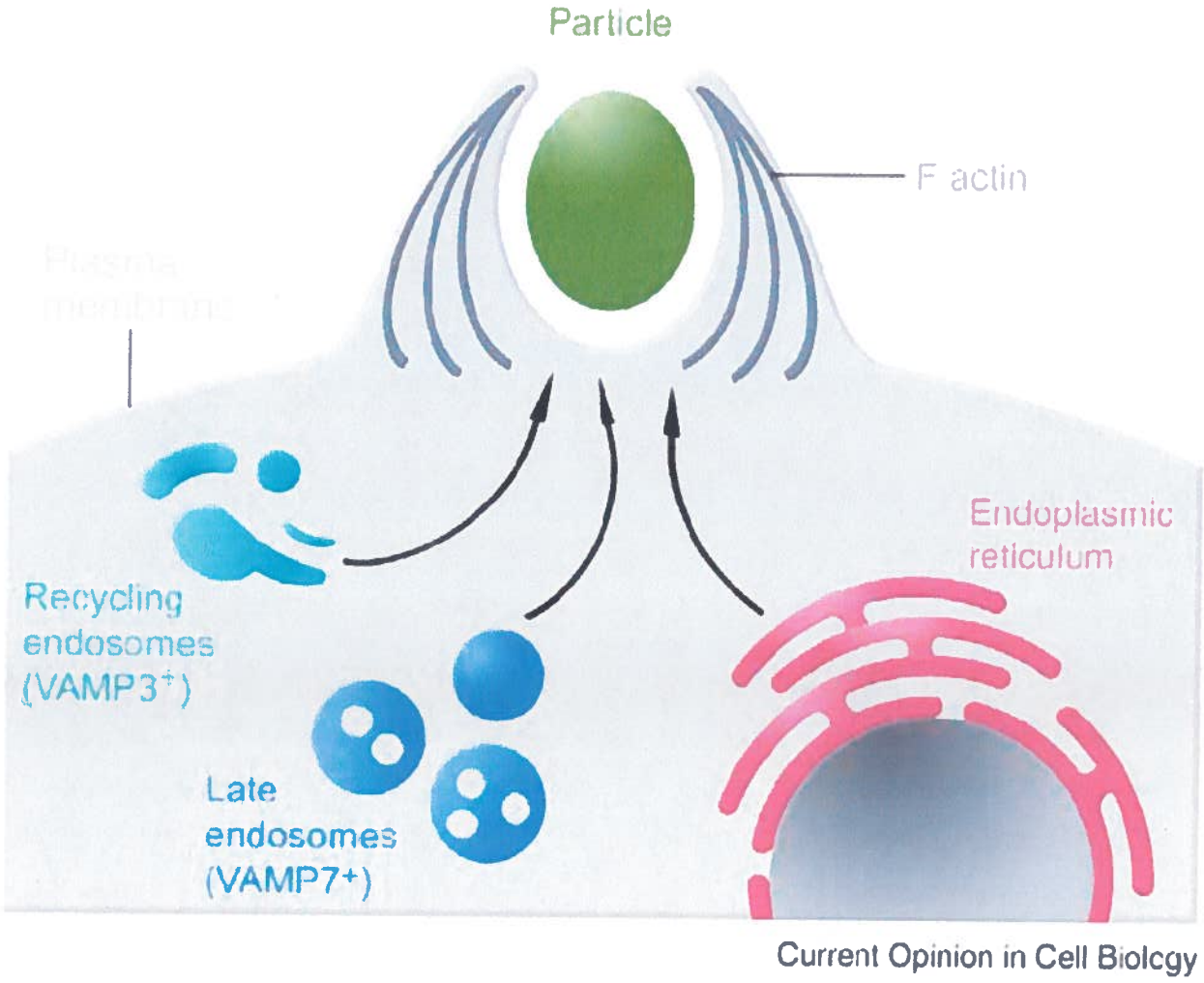



**Figure 1) Phagocytosis**. Different intracellular compartments such as recycling endosomes, late endosomal compartments, and the endoplasmic reticulum are involved during phagocytosis. Polymerization of F-actin at the phagocytic cup is also observed (Niedergang and Chavrier 2004).

## 1. 1. FcγR-mediated phagocytosis

Three types of Fcγ receptors, namely FcγR I, II, and III mediate phagocytosis. FcγRII is present in human but not mouse cells. IgG-coated particles bind to Immunoreceptor Tyrosine Activation Motif (ITAM) receptors and this triggers phosphorylation of the γ chain of ITAM domains through the Src family of tyrosine kinases (Figure 2). Downstream of the initial tyrosine phosphorylation, there are two different modes of activation. In the first pathway, one or several guanine nucleotide exchange factors (GEFs) are activated, which in turn activate the small GTPases Rac and Cdc42 by replacing GDP with GTP. Wiscott-Aldrich Syndrome protein (WASP) is the responsible effector protein in cytoskeletal remodeling, which is activated upon activation of Rac and Cdc42 proteins. Consequently, activated WASP directly binds to a complex of actin-binding proteins (Arp2/3). The Arp2/3 complex nucleates actin filaments resulting in polymerization of actin at the phagocytic cup. Since phagocytosis is a dynamic event, the signal should be inactivated and this happens through depolymerization of actin around the phagocytic cup, making actin available for another new cycle of polymerization. Depolymerization of actin happens through Cdc42 and Rac inactivation via hydrolysis of GTP to GDP (Deschamps, Echard et al. 2013).

The second pathway is activation of one or more PI3-kinases which results in production of phosphatidylinositol 3,4,5 triphosphate. Inhibition of PI 3-kinase inhibits closure of the phagocytic cup (Ghosh, Bose et al. 2013).

## 1.2. Complement receptor type 3-mediated phagocytosis

There are similarities and differences between FcγR- and CR-mediated phagocytosis, but C3R- and C4R-mediated phagocytosis are not as well characterized. In contrast to the FcγR pathway, which is characterized by extension of lamellipodia around IgG-opsonized targets, C3-opsonized particles enter the cell without extensions of cytoskletal elements. It has been shown that activation of Rho GTPase mediates phagocytosis via complement receptors, but as opposed to FcγR-dependent phagocytosis, Cdc42 and Rac activation is not required. This explains why the FcγR pathway, and not the C3R pathway, is accompanied by extension of lamellipodia around IgG-opsonized targets. Another difference between FcγR and CR-mediated phagocytosis is that the latter does not require tyrosine kinase activation (Canton and Kima 2012). Nonetheless, both modes of phagocytosis require protein kinase C activation.

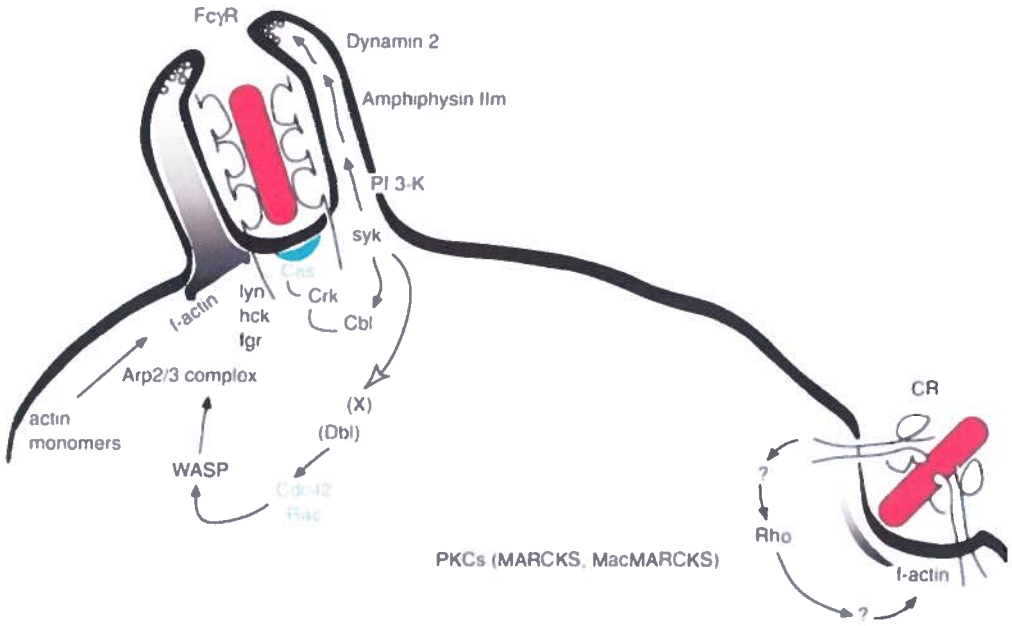



**Figure 2) Fcγ receptor and complement receptor pathway**. Upon activation of FcγR pathway, a tyrosine kinase such as Lyn, Hck and Fgr, phosphorylates the γ chain of ITAM receptor, which in turn can activate Cdc42 and Rac proteins. WASP activates through activation of Cdc42 and Rac and at the end, WASP activates Arp2/3 protein to polymerase actin filament at the phagocytic cup. Syk also phosphorylates Cbl as an adaptor protein. Cbl interacts with p85 subunit of phosphatidylinositol 3-kinase and with Crk. Activated PI 3-kinase is essential for the fusion of vesicles with the plasma membrane and supply membrane for phagocytosis. Also, activated PI 3-kinase is required to recruit amphiphysin IIm and dynamin 2 to phagosome. Cbl interacts with Crk, which in turn interacts with Cas at phagocytic cups. CR-mediated pathway is Rho-GTPase-dependent but independent of Rac and Cdc42. Both pathways are mediated by activation of protein kinase C (PKC), which regulates F-actin formation (Ernst 2000)

## 1.3. Phagocytes

Phagocytosis is extremely complex, and this complexity is in part because of the diversity of receptors capable of stimulating phagocytosis, and in part due to the capacity of a variety of microbes to influence their fate as they are internalized. A large number of mammalian cell types are capable of phagocytosis, but their phagocytic efficiency is different. Rabinovitch divided phagocytic cells into three different categories: non-professional phagocytes, paraprofessional phagocytes and professional phagocytes (Rabinovitch 1995). Epithelial cells and fibroblasts are considered as "non-professional phagocytes". Certain specialized cells such as dendritic cells, which are well-known for antigen presentation, can also take up certain particles as well as microorganisms; dendritic cells are deemed as "paraprofessional phagocytes". Polymorphonuclear cells (PMN) and monocytes, which arise from bone marrow precursors, are in the category of professional phagocytes. PMN have a short term life; they can differentiate and survive for one or two days, but monocytes and macrophages can live for days, weeks, months or even years. The role of professional phagocytes is not restricted to phagocytosis of foreign particles, pathogens, apoptotic, or defective host cells; they also secrete important molecules including cytotoxic oxygen and nitrogen radicals, enzymes, and cytokines which have bactericidal and inflammatory roles. At the high levels, both cytotoxic oxygen and nitrogen radicals, damage organelles particularly mitochondria while at low levels, they play a role as signaling molecules. Cytokines are released in two different ways; in some cells through secretory granules while in other cells via constitutive secretory pathways (Stanley and Lacy 2010). Some cytokines such as IL-1β are released through a non-canonical secretory pathway which does not pass through traditional organelles such as the ER and the Golgi complex. It has

been shown that IL-1β is released from exosomes which contains caspase-1 and other inflammasome components (Qu, Franchi et al. 2007).

To highlight the role of receptors, it has been shown that the phagocytic capacity of "non-professional" phagocytes such as Chinese hamster ovary or COS cells (CV-1 (simian) in origin and carrying the SV40 genetic material), is significantly increased by the heterologous expression of specialized phagocytic receptors such as FcγRs that are endogenous in "professional phagocytes" (Indik, Park et al. 1995, Caron and Hall 1998). The number of receptors and their variations bring the unique abilities for professional phagocytes to engulf a variety of particles. Different receptors can recognize conserved motifs on pathogens that are not found on higher eukaryotes. This is why these receptors are conserved evolutionarily. Janeway named these receptors "pattern-recognition receptors" (PRRs), and their targets "pathogen-associated molecular patterns" (PAMPs) (Janeway 1992).

### 1.3.1. Pathogen survival in phagocytes

In general, pathogen survival post-phagocytosis depends on specific strategies to avoid being eradicated by their host cells. Some pathogens change their PAMPs to enter stealthily into phagocytes and by using this strategy they are no longer recognized by PRRs. Another strategy is to undergo chemical changes like altering their LPS (e.g. *Yersinia spp*) or modify their flagella (e.g. *Campylobacter jejuni*) to avoid recognition by TLR4 and TLR5, respectively. More specifically, TLR5 normally recognizes an evolutionary conserved site on bacterial flagellin while some pathogens such as *Campylobacter jejuni* alters flagellin molecules thereby they are not recognized by TLR5. (Andersen-Nissen, Smith et al. 2005, Montminy, Khan et al. 2006). Regarding *Mycobacteria*, they remodel their phagosome to survive by excluding some specific

markers from late endosomes and lysosomes such as Rab7, v-ATPase, LAMP-1 and also, PI(3)P-binding proteins EEA1 and Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) (Kagan, Stein et al. 2004). In the case of the v-ATPase, it has been shown that *Mycobacterium tuberculosis* protein tyrosine phosphatase (PtpA) binds to the macrophage vacuolar-$H^+$-ATPase complex and excludes the v-ATPase from infected phagosomes (Joshi, Sacks et al. 1998). Some other pathogens like *Listeria* can pierce phagosomes, through their virulence factors including listeriolysin O, phospholipase A and B. Once they are out of phagosomes, they rapidly divide in cytoplasm of host cells (Zhang, Zhang et al. 2003). There is another group of pathogens like *Coxiella* that are resistant to the antimicrobial environment of mature phagosomes and can survive in the acidic lumen of phagosomes. *Coxiella* has several acid survival systems which are called acid resistance. These additional systems help *Coxiella* to function in a very acidic environment (Lin, Lee et al. 1995, Ernst 2000).

### 1.3.2. Phagosome maturation

After internalisation of particles through their own specific receptors, nascent phagosomes undergo a maturation process in which they sequentially interact with early endosomes, late endosomes and finally lysosomes to form phagolysosomes (Figure 3). Consequently, early phagosomes carry early endosomal markers like Rab5 or early endosome antigen 1 (EEA1) which they lose throughout the maturation process to gain late enodosomal and lysosomal markers such as Rab7 (late endosome), lysobisphosphatidic acid (or Bis [monoacylglycerol] phosphate is synthesized from phosphatidylglycerol in the late endosomal system and it stimulates the enzymes involved in the degradation of glycosylceramides), mannose-6-phosphate receptor (lysosomal marker) and lysosomal-associated membrane proteins

(LAMPs) in an acidic pH lumen (Fratti, Backer et al. 2001). There are different hypothesis about phagosome maturation. Some groups like Grinstein and his clolleagues believe that the ER is not involved in phagosome maturation (Touret, Paroutis et al. 2005) but some others including Desjardins and his colleagues suggested the "kiss-and-run" model whereby phagosomes undergo only transient and partial fusion with endocytic organelles including ER without complete mixing of two compartments (Desjardins 1995); as a result, only specific and selected membranes and luminal contents transfer between the phagosome and the endosomal compartment. In this regard, Rab5 plays a key role. It has been shown that Rab5 restricts the complete fusion between phagosomes and endosomes. Further studies show that in mutant active form of Rab5 (Rab5(Q79L)) fusion between phagosomes and endosomes happens without limitation. Consequently the generated giant phagosome acquires LAMP1 and proceeds for maturation but it lacks the microbicidal conditions to kill intracellular parasites (Derre and Isberg 2004).

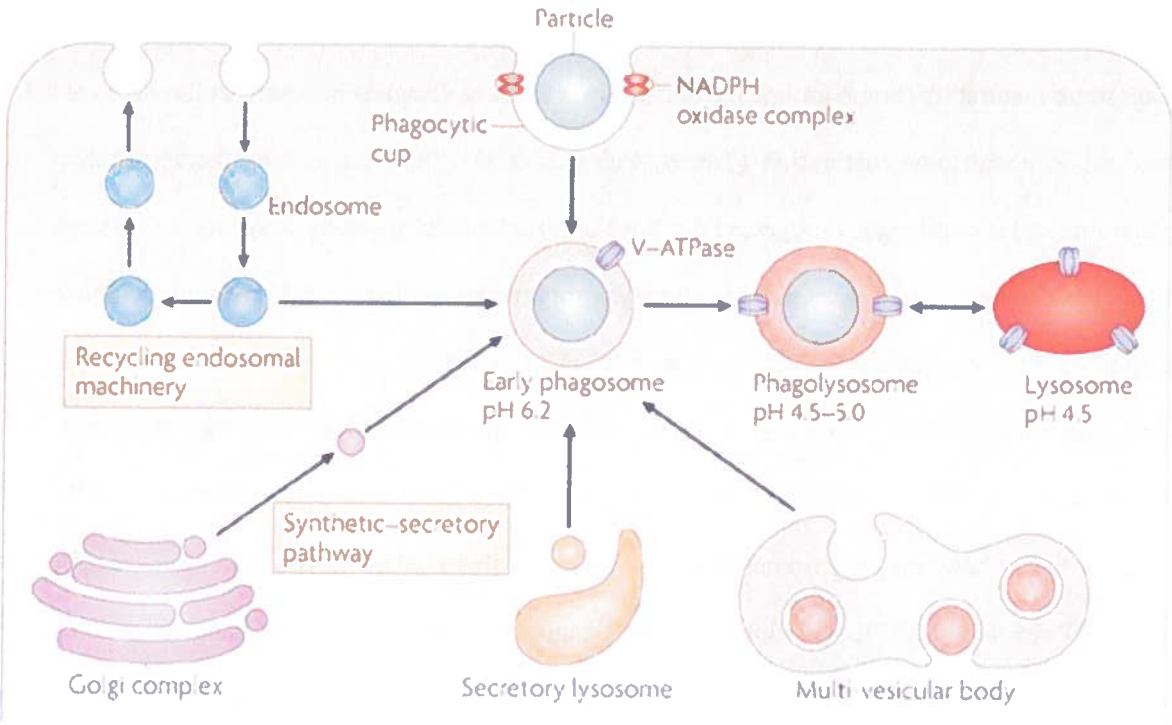



**Figure 3) Phagosome maturation.** During phagosome maturation, there are transient interactions among different endosomal compartments such as endoplasmic reticulum, recycling endosome, lysosome, Golgi complex, secretory lysosome and multivesicular body. Some proteins like NADPH oxidase can be recruited prior to phagosomal closing. Once the phagosome is closed, it acidifies through lysosomal enzymes and also via the recruitment of V-ATPase, which pumps protons into lumen (Russell, Vanderven et al. 2009)

## 1.4. Involvement of SNAREs in phagolysosome biogenesis

SNAREs (Soluble-*N*-ethylmaleimide-sensitive-factor accessory-protein (SNAP) receptor) play central roles in intracellular trafficking and membrane fusion (Ungar and Hughson 2003). SNAREs are a super family of 38 currently known members in mammals. They all share the SNARE motif (also referred to as H3 domain), which is a ~60-amino-acid α-helical domain that is important for SNARE-SNARE protein interaction.

The two different SNAREs, v-SNAREs and t-SNAREs, are located on donor and target membranes, respectively. There is an alternative structure-based terminology for these proteins, wherein v-SNAREs are called R-SNAREs (because of the central functional arginine in their SNARE motif), and t-SNAREs are referred to as Q-SNAREs because of the active glutamine in their SNARE motif (Fasshauer, Sutton et al. 1998). The positively charged guanidino group of the arginine (R) residue interacts with the carboxyl group of glutamine (Q) residues.

Q-SNAREs can be further classified as Qa (syntaxin- 1, 2, 3, 4, 5, 7, 11, 13, 16, 17 and 18), Qb (Vti1a [vesicle transport through interaction with the t-SNARE homologue 1a], GS27 (Golgi SNARE of 27 kDa), GS28), Qc (syntaxin- 6, 8, 10, GS15, BET1), Qb,c (SNAP23, SNAP25, SNAP29 and SNAP47). There is an addition unclassified SNARE (D12, Sec20, Sec22a, Sec22c). R-SNARE (VAMP1 [vesicle-associated membrane protein 1], VAMP2, 3, 4, 5, 7, 8, ERS24 [Sec22b] and YKT6) (Stow, Manderson et al. 2006).

R-SNAREs are single transmembrane proteins that contribute one SNARE motif to the *trans*-SNARE complex (Figure 4). The *trans*-SNARE complex is a four-α-helical bundle that is formed by intertwining a single R-SNARE helix with three helices (Habc) of the Q-SNARE

complex (Stow, Manderson et al. 2006). At the beginning, Q-SNARE motif is blocked through Sec1 as a regulator. GTPase Rab dissociates Sec1 protein so that the Q-SNARE motif is available to bind to R-SNARE. This transient form of *trans*-SNARE complex causes the vesicle and target membrane to come close together. Furthermore, crystallographic studies show that assembled transient *trans*-SNARE complexes have the required force to fuse lipid bilayers (Chen and Scheller 2001). In the following step, transient *cis*-SNARE complexes are formed in the same membrane. At this point, the vesicle membrane is in continuity with the target membrane. After the fusion of the two membranes, SNARE complexes are rapidly disassembled and they are ready to be re-used again for subsequent membrane fusion events. This disassembly of *cis*-SNARE complexes is mediated by a soluble complex containing the ubiquitous cytoplasmic ATPase NSF and α-SNAP (Figure 4) (Alpadi, Kulkarni et al. 2012, Parisotto, Malsam et al. 2012).

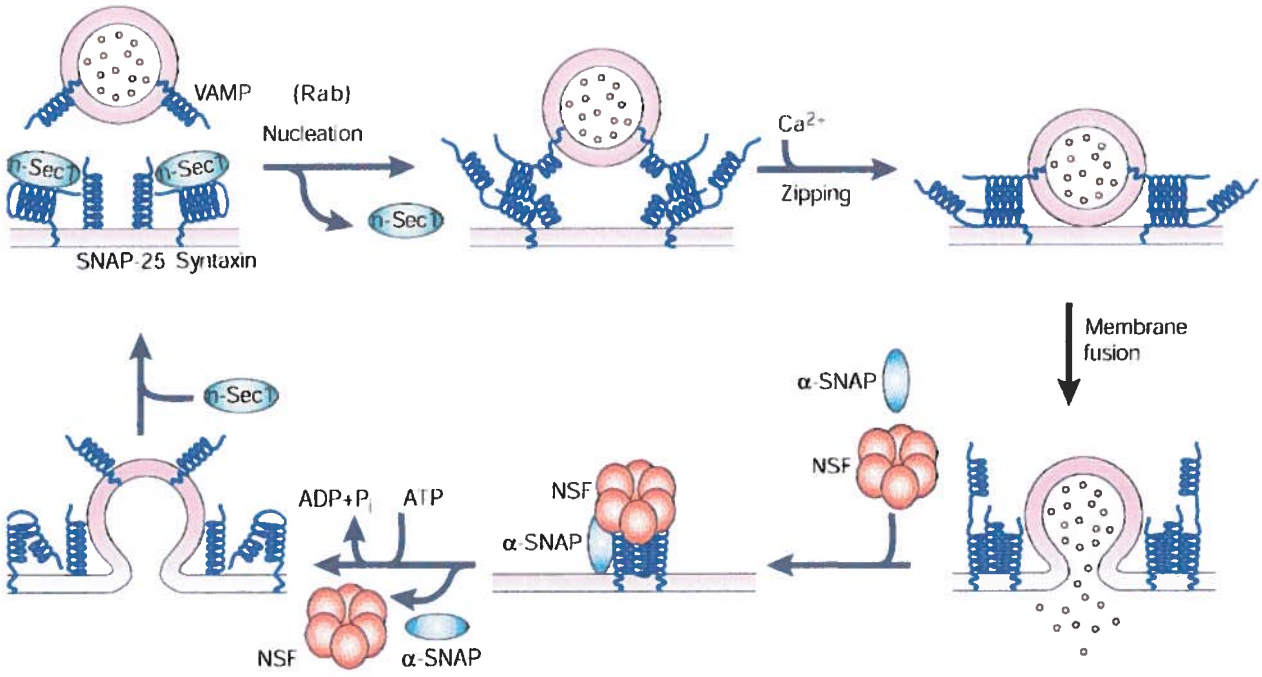



**Figure 4) *Trans*-SNARE complex and role of NSF and α-SNAP.** To form a *Trans*-SNARE complex one alpha helix on a vesicle membrane and three alpha helixes on a target membrane interact and make a four alpha helix bundle and bring the acceptor and donor membranes so tight and close together and fusion happens. To release the involved SNAREs and initiate another cycle of binding and fusion, some ATPase proteins, including α-SNAP and NSF, by hydrolysing ATP disassemble the SNARE complex. (Chen and Scheller 2001)

### 1.4.1. SNARE Regulation

Post-translational modifications of SNARE proteins and binding of regulatory proteins are two suggested regulatory mechanisms to modulate *trans*-SNARE complex formation. Phosphorylation is one means of regulation. In some immune cells such as mast cells, phosphorylation of Q-SNAREs can turn them on or off. In resting mast cells, syntaxin 4 is inactive through phosphorylation by Rab3D-associated kinase. On the contrary, SNAP23 in activated mast cells is activated through phosphorylation to mediate mast cell degranulation (Pombo, Martin-Verdeaux et al. 2001, Hepp, Puri et al. 2005). Complexin II (is a small cytoplasmic protein, which is a modulator for synaptic vesicle release) binds to the SNARE complex of syntaxin 3, SNAP23 and VAMP2 or VAMP8 to regulate degranulation in mast cells (Tadokoro, Nakanishi et al. 2010). Another regulator of SNARE activity is the Sec/Munc-like protein family. They can bind to N-terminal domains of syntaxins and regulate the *trans*-SNARE complex either positively or negatively. Sec/Munc-like protein family can make SNAREs available for *trans*-SNARE complex. For instance, in macrophages Munc 18 binds to syntaxin 4 in non-raft membrane domains and then by moving into cholesterol-rich lipid rafts, syntaxin 4 is separated from Munc 18 and participates in complex assembly for phagocytosis or cytokine secretion (Kay, Murray et al. 2006). The Sec/Munc-like protein family can also regulate the stability of some SNARE proteins to cycles from *trans*- to *cis*-SNARE complexes (Rizo and Sudhof 2002).

### 1.4.2. SNAREs distribution

SNAREs are expressed specifically in different cell types and they are selectively distributed on endosomal compartments and membranes. These selective SNARE combinations could be a powerful approach for mapping intracellular pathways and manipulating both trafficking steps and cellular responses (Figure 5). In macrophages, internalization of particles is dictated by sequential membrane-fusion events that lead to phagolysosome formation. During the whole phagosome maturation process, multiple SNAREs are involved and as noted earlier, they are specific for certain vesicles in each step of phagosome maturation.

VAMP3 regulates fusion of the recycling endosome membrane with the plasma membrane upon FcγR-dependent internalization; this happens at newly-formed phagocytic cups (Allen, Yang et al. 2002). Another example is involvement of syntaxin 18 and D12 during phagocytosis of large (3 μm), but not small (0.8 μm) IgG-coated particles. During phagocytosis of large particles, and to compensate for plasma membrane, some endosomal compartments like the ER are involved, and R-SNARE Sec22b regulates this interaction (Becker, Volchuk et al. 2005, Hatsuzawa, Tamura et al. 2006).

SNAP29 is a SNARE protein regulator in mast cells and plays role in the phagocytosis of *E. coli*. Wesolowski and his colleagues showed for the first time the bactericidal role for SNAP29 in mast cells, in which they found that both phagocytosis and killing of *E. coli* significantly increase upon overexpressing SNAP29 (Wesolowski, Caldwell et al. 2012).

According to another study, SNAP23 can regulate phagocytosis in macrophages. Further studies by the same research team showed that SNAP23 has a role in phagosome maturation by regulating the recruitment of NADPH oxidase complex and vacuolar-type $H^+$-ATPase to phagosomes (Sakurai, Hashimoto et al. 2012).

The roles of SNAREs in the immune system are not limited to phagocytosis and phagosome maturation. For instance, VAMP3 on recycling endosomes and syntaxin 4/SNAP23 at the cell surface can also play critical roles in cell adhesion, spreading and migration of macrophages to infected tissues (Veale, Offenhauser et al. 2011).

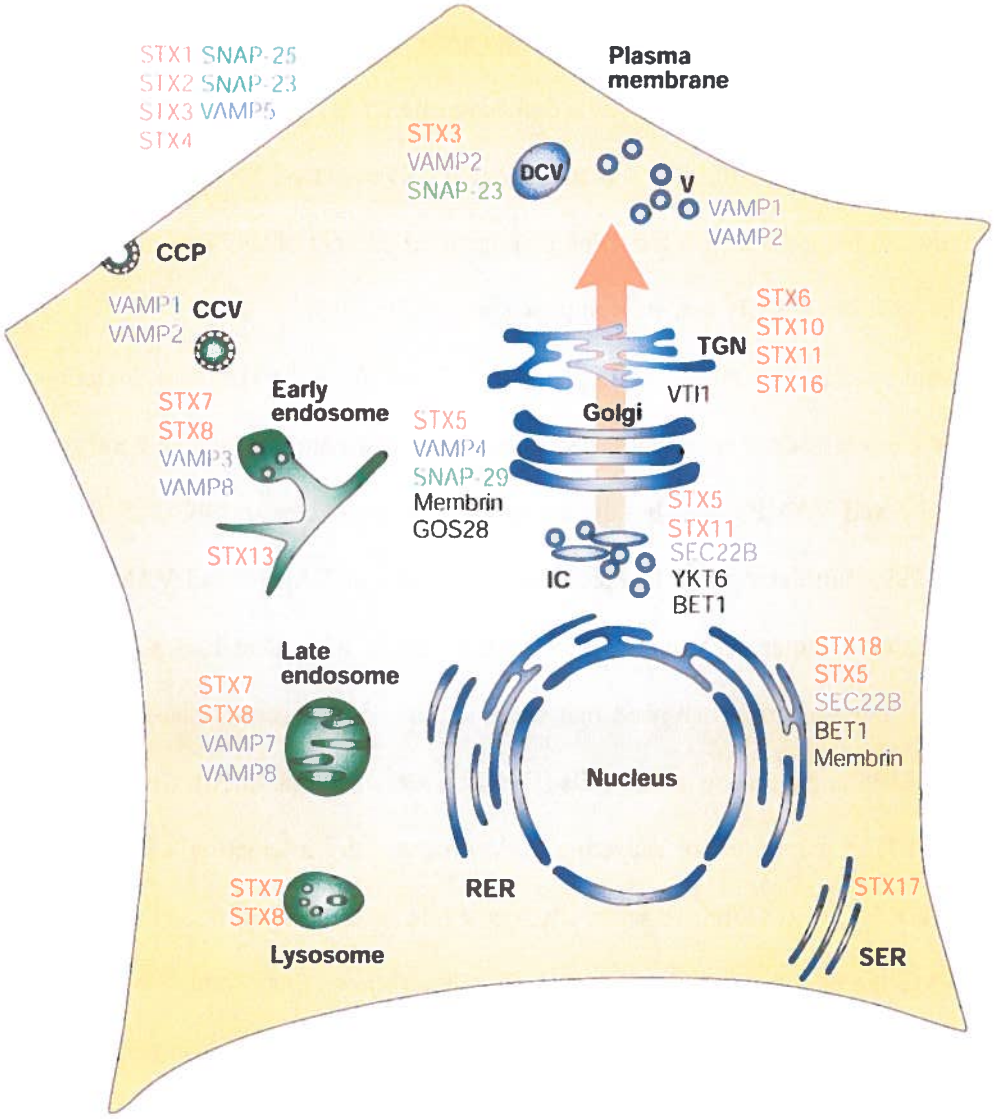



**Figure 5) Distribution of SNAREs on different endosomal compartments**. CCP (clathrin-coated pit), CCV (clathrin-coated vesicles), DCV (dense core vesicles), IC (intermediate compartment), RER (rough endoplasmic reticulum), SER (smooth endoplasmic reticulum), V (vesicle), TGN (*trans*-Golgi network) (Chen and Scheller 2001).

### 1.4.3. SNAREs and infectious pathogens

There are several diseases which are linked to defects in specific SNAREs such as early-onset myocardial infarction that is caused by a defective mutant of VAMP8 (Shiffman, Rowland et al. 2006). In addition, according to the sequential involvement of SNAREs in phagosome maturation, they can be good targets for some pathogens to prevent phagolysosome biogenesis, hence inhibit bactericidal activity and antigen presentation (Figure 6).

There are several studies that show the degradation of specific SNAREs after infection. For instance active zinc-dependent endopeptidase of the clostridial neurotoxin specifically cleaves VAMP1, VAMP2 and VAMP3, and botulinum toxin serotype A cleaves SNAP25 (Pellizzari, Rossetto et al. 1999). Similarly, there is a proteolytic cleavage of VAMP2 and VAMP8 through a zinc-metalloprotease (antarease) extracted from the venom of a Brazilian scorpion *Tityus serrulatus* (TSV). Further studies showed that the fraction *v* of the venom causes cleavage of VAMP2 and VAMP8 in pancreatic acinar cells (Fletcher, Fletcher et al. 2010). Cellubrevin (also known as VAMP3) is a marker for recycling endosome and by interacting with syntaxin 6, which is a marker for *trans*-Golgi network, it plays a role in trafficking from the *trans*-Golgi network (TGN) to the endocytic pathway. Also, it has been shown that VAMP3 plays a role in phagosomal maturation and it is targeted by *M. tuberculosis* . It arrests phagosome maturation by disrupting trafficking pathways and consequently it can survive in macrophages (Fratti, Chua et al. 2002).

Interestingly, some pathogens that enter host cells through membranes use molecular components that contain heptad repeats. These repeats usually consist of hydrophobic residues and they have a similar structure and function to the SNARE motifs in SNAREs proteins. These SNARE-like proteins are used by microorganisms to promote or block membrane fusion

depending on their particular survival strategy (Wesolowski and Paumet 2010). Intracellular microorganisms such as *Mycobacterium*, *Salmonella*, *Chlamydia* or *Legionella* block unfavourable fusion events but on the other hand create new profitable fusion pathways to survive and replicate in their host cells (Figure 7). As a result, pathogen-containing vacuoles become unique organelles with characteristics of late endosomes/lysosomes (*Salmonella*), early endosomes (*Mycobacterium*), ER (*Legionella*) or without any prominent features (*Chlamydia* and *Toxoplasma*) (Kumar and Valdivia 2009).

In conclusion, SNAREs play critical roles in vesicle trafficking in the immune system and this explains why they are good targets for different pathogens. The rest of this section specifically describes some important SNAREs proteins.

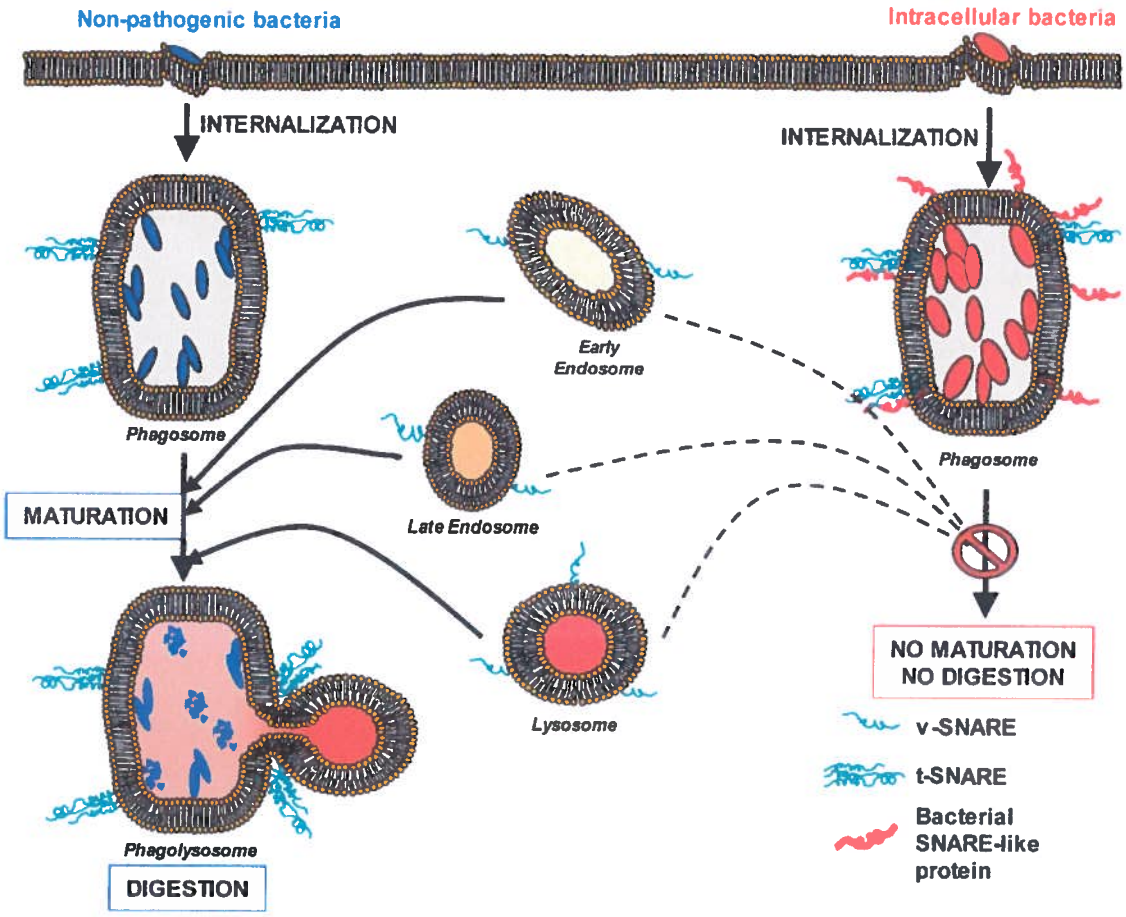



**Figure 6) Some pathogens selectively target SNAREs to prevent fusion and phagosome maturation**. The figure shows that virulent pathogens inhibit phagosome maturation and replicate in phagosomes. Phagosomes containing avirulent microbes proceed for phagosome maturation (Wesolowski and Paumet 2010).

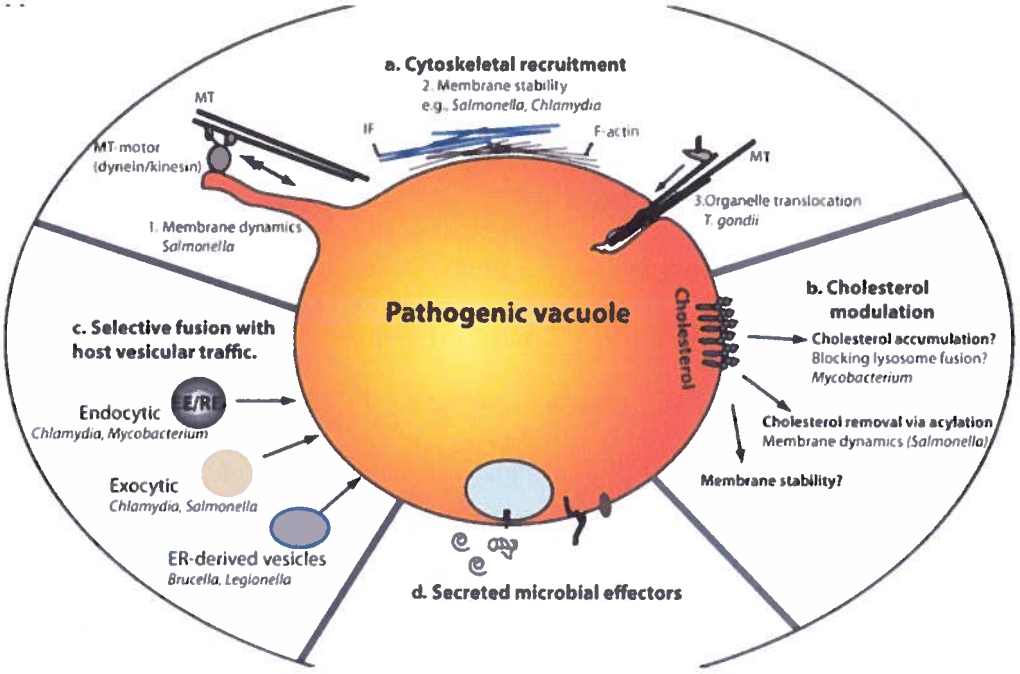



**Figure 7) Pathogenic vacuoles escape host immue system.** a) *Chlamydia* and *Salmonella* modulate the host cytoskeleton, which may change the structural ability and membrane dynamics of a pathogen-containing vacuole b) Modulation of vacuolar membrane lipids. c) Selective fusion with endosomal compartments in host cells. d) Pathogen regulates the characteristic of their vacuoles by secreting microbial effectors. (Kumar and Valdivia 2009)

### 1.4.4. R-SNARE (VAMP8)

Advani and his colleagues in 1998, found seven new mammalian SNARE proteins including VAMP8 (Endobrevin) (Advani, Bae et al. 1998). It shows 32%, 33% and 31% similarity in amino acid sequence with VAMP-1, -2 and -3 respectively (Wong, Zhang et al. 1998). Further studies on VAMP8 showed that this R-SNARE protein is located on late endosomes, the *trans*-Golgi network, coated pits and plasma membrane in addition to early endosomal compartments (Antonin, Holroyd et al. 2000). VAMP8 interaction with syntaxin 7, syntaxin 8 and Vti1b (vesicle transport through interaction with the t-SNARE homologue 1b) mediates fusion of early and late endosomes. Noteworthy, VAMP7 with the same complex of syntaxin 7 and syntaxin 8 and Vti 1b mediates the fusion of late endosomes and lysosomes (Pryor, Mullock et al. 2004). It has been shown that, VAMP8 and syntaxin 2 play a role in cytokinesis as well (Low, Li et al. 2003). Aside from positive regulatory roles, VAMP8 also can be a negative regulator of phagocytosis in dendritic cells (Ho, Cai et al. 2008). However, the negative regulatory role of VAMP8 in dendritic cells is controversial. The positive or negative role of VAMP8 in phagocytosis depends on the pathogens and specific involved receptors and consequently, initiating of different phagocytic pathways, can change.

As mentioned earlier, VAMP2 and VAMP3 are cleaved by botulinal neurotoxins and they have a consensus cleavage site "LERDQKLSE"; interestingly, VAMP8 does not contain this conserved toxin cleavage site (Wong, Zhang et al. 1998), and it is one of the differences between VAMP8 and VAMP2 and 3.

The role of VAMP8 is not limited to phagocytosis and phagosome maturation. It has been shown that VAMP8 in combination with VAMP7 and Vti1b regulate fusion of autophagosomes with

lysosomes. Confocal microscopy results show the co-localization of VAMP7, VAMP8 and Vti 1b with the LC3 protein in xenophagosomes. Autophagy is an intracellular degradation system for unnecessary or dysfunctional cells. Antimicrobial autophagy is called xenophagy and related phagosomes are xenophagosomes that degrade the intracellular bacteria after fusing with lysosome (Furuta, Fujita et al. 2010).

### 1.4.5. R-SNARE (Sec22b)

As noted earlier, each endosomal compartment has specific individual SNAREs or SNARE complexes that define their functions. It has been also shown that the ER is a membrane donor during phagocytosis. The ER membrane contains SNAREs, including syntaxin 18 (Qa), BNIP1 (Qb), p31(D12) (Qc) and Sec22b (R), which usually interact with other accessory proteins such as Sly1 (Suppressor of loss of Yeast Rab GTPase (YPT1) protein 1), ZW10 (spindle check point protein) and RINT-1 (DNA repair protein (RAD50)-interacting protein 1). Accessory proteins may help and control the formation of ER SNARE core complexes. Further studies highlight the role of Sec22b as an ER-SNARE and show that ER with its protein complex (Sec22b with syntaxin 18, P31 and BNIP1) plays a role in phagocytosis and post-Golgi trafficking (Hatsuzawa, Hirose et al. 2000). Interestingly, interaction between syntaxin 18 and BNIP-1 and p31 is weak but syntaxin 18 and Sec22b interact specifically and after binding together due to the conformational changes in their SNARE motifs, they create high affinity binding sites to interact with two the other proteins (p31 and BNIP-1) and make a stable and functional complex (Aoki, Kojima et al. 2008).

Although there is a positive correlation between syntaxin 18 and phagocytosis, Sec22b negatively regulates phagocytosis in macrophages (Hatsuzawa, Hashimoto et al. 2009). In this

study, the authors showed that overexpression of Sec22b inhibits phagocytosis but knock down of Sec22b increases phagocytosis in a macrophage cell line (J774 cells). Domain analysis showed that the R-SNARE motif of Sec22b is involved in phagocytosis inhibition. This motif selectively binds to syntaxin 18 and hinders its role as positive regulator of phagocytosis.

Another protein complex partner of Sec22b in the ER is syntaxin 5 (Qa), membrin (Qb) and Bet1 (Qc). It has been shown that Sec22b does not bind to any of these individual SNAREs; it is always found as a three-protein complex (Xu, Joglekar et al. 2000).

Studies on the biogenesis of vacuoles containing *Legionella pneumophila* (an intracellular bacterial pathogen) showed that Sec22b is recruited to phagosomes harbouring wild type of *L. pneumophila,* while phagosomes containing an avirulent mutant form (Arasaki and Roy 2010) do not recruit Sec22b. In addition to Sec22b, which is an ER-localized SNARE, some plasma membrane SNAREs such as syntaxin 2, syntaxin 3, syntaxin 4 and SNAP23 are recuited in vacuoles containing *L. pneumophila.* These can be dissociated upon interaction with the ATPases NSF and α-SNAP.

Sec22b localizes to the ER-Golgi intermediate compartment (ERGIC) and pairs to the plasma membrane SNARE syntaxin 4, which is present in phagosomes. A study on the intracellular parasite *Toxoplasma gondii* parasite showed that by deleting Sec22b in infected dendritic cells, recruitment of ER-resident proteins to phagosomes and vacuoles containing parasites is impaired. Also, it has been shown that Sec22b regulates antigen cross-presentation in dendritic cells and it explains the severe impairment in cross-presentation in cells lacking Sec22b. Cells lacking Sec22b show increased lysosomal recruitment and consequently, more proteolytic activity (Wesolowski and Paumet 2010).

Altogether, knowing more about ER-associated SNAREs can help us better understand phagosome remodeling after infection.

### 1.4.6. R-SNARE (VAMP3)

During phagosome maturation, the recycling endosome interacts with the phagosome. VAMP3 is a marker for recycling vesicles and it is shown that during phagocytosis and phagosome maturation, plays an important role (Bajno, Peng et al. 2000). However, Allen and colleagues pointed out to the fact that VAMP3 is not necessary for phagosome maturation and also, phagocytosis of opsonized and non-opsonized particles late time points (1 hour after phagocytosis). In that study, Zymosan was the only affected particle in phagocytosis but only at early time points (5 min to 15 min) (Allen, Yang et al. 2002). Since different endosomal compartments are involved in the formation of an active membrane complex during phagocytosis and the mechanism is not clearly defined, describing the role of each SNAREs individually in phagocytosis is difficult and somehow inaccurate. In addition, it is possible that VAMP-2, -3 and -8 play redundant roles in phagocytosis.

### 1.4.7. Q-SNARE (syntaxin 5)

Syntaxin 5 is a ubiquitously expressed single-pass type IV membrane protein. It is a protein marker for ER, ERGIC and *cis*-Golgi cisternae of the early secretory pathway. In addition to the regulatory function of syntaxin 5 in coat protein (COP) II vesicle transport , it is also capable to bind to different proteins such as presenilin (multipass transmembrane proteins that function as a part of gamma secretase intramembrane protease complex) and the very low density lipoprotein (VLDL)- receptor ( a type I transmembrane glycoprotein) (Wagner,

Dieckmann et al. 2013). There is an interaction between syntaxin 5 and Sec22b in dendritic cells. Syntaxin 5, unlike Sec22b, is required for delivery of MHC class I molecules and their trafficking to the cell surface; therefore, syntaxin 5 knock down causes reduced MHC class I molecules at the cell surface and reduced the presentation of the SIINFEKL model peptide (which is routinely used in measurement of cross-presentation) to associated OVA-specific TCR (Cebrian, Visentin et al. 2011).

### 1.4.8.Q-SNARE (syntaxin 4)

The t-SNARE syntaxin 4 is involved in the exocytic pathway like two other SNAREs, including syntaxin 2 and syntaxin 6. Functional studies on syntaxin 4 show that this SNARE is located at the cell surface of macrophages and is in a complex with SNAP23/Munc 18c. This protein complex regulates membrane trafficking and also TNF secretion upon LPS induction (Pagan, Wylie et al. 2003).

## 2. *Leishmania*

*Leishmania,* is a parasitic protozoa and causes Leishmaniasis. It was named after the pathologist William Boog Leishman in 1903. *Leishmania* currently affects 12 million people in 98 countries; in the WHO website (http://www.who.int/leishmaniasis/leishmaniasis_maps/en/) the distribution of this parasitic disease all over the world can be appreciated. Different types of leishmaniasis show distinct epidemiological characterization. The global prevalence shows 2 million new cases per year (0.5 million of visceral leishmanaisis and 1.5 million of cutaneous leishmaniasis) and there is more than 350 million people at risk (Wang, Cui et al. 2012)

The invertebrate hosts or vectors, phlebotomine sand flies, have two important genera: *Phlebotomus* of the "Old World", which are divided into 12 different subgenera, and *Lutzomyia* of the "New World", which are divided into 25 subgenera. Both sand flies and *Leishmania* vary in their geographical distribution (Killick-Kendrick 1999). *Leishmania* are successful parasites that infect different orders of mammals as potential reservoirs for Leishmaniasis (Figure 8). These include rodents, canids, edentates, marsupials, procyonids, primitive ungulates and primates.

*Leishmania* parasites are exposed to two different extra- and intracellular environments. In the extracellular stage, they live in the sand fly gut (Phlebotomine), and during intracellular stage, they reside in vertebrate host cells. Therefore, the parasites have two different morphological forms, promastigotes and amastigotes, in invertebrate and vertebrate hosts, respectively. Promastigotes are flagellated and motile, whereas amastigotes lack flagella.

Leishmaniasis is transmitted by the bite of a female sand fly that transmits metacyclic promastigotes to a vertebrate host. There are several stages for parasites to differentiate from amastigotes to highly active metacyclic promastigotes in the invertebrate host, as follows (Sacks and Kamhawi 2001).

1) Infected bloodmeal, which contains amastigotes, is transferred to the abdominal midgut. Usually transfromation from amastigotes to promastigotes happens between 12 to 18 hours. Promastigotes at this stage are called procyclics and they are short, ovoid and slightly motile. Between 18 to 24 hours, promastigotes multiply fast and form rosettes whith their flagella directed toward the center. Later on, during 36 to 60 hours, promastigotes transform to a long, slender and highly motile forms, which are called nectomonad.

2) Nectomonads are packed in the anterior portion of the abdominal midgut while most of them are attached to epithelial cell through their flagella.

3) The anterior migration of promastigotes to the thoracic midgut and stomodeal valve is completed by day 7. At this stage, promastigotes are termed haptomonads which are transformed to metacyclic promastigotes that are short, slender, highly active and with a long flagellum, at least twice longer than the cell body. Metacyclic promastigotes are never seen in division.

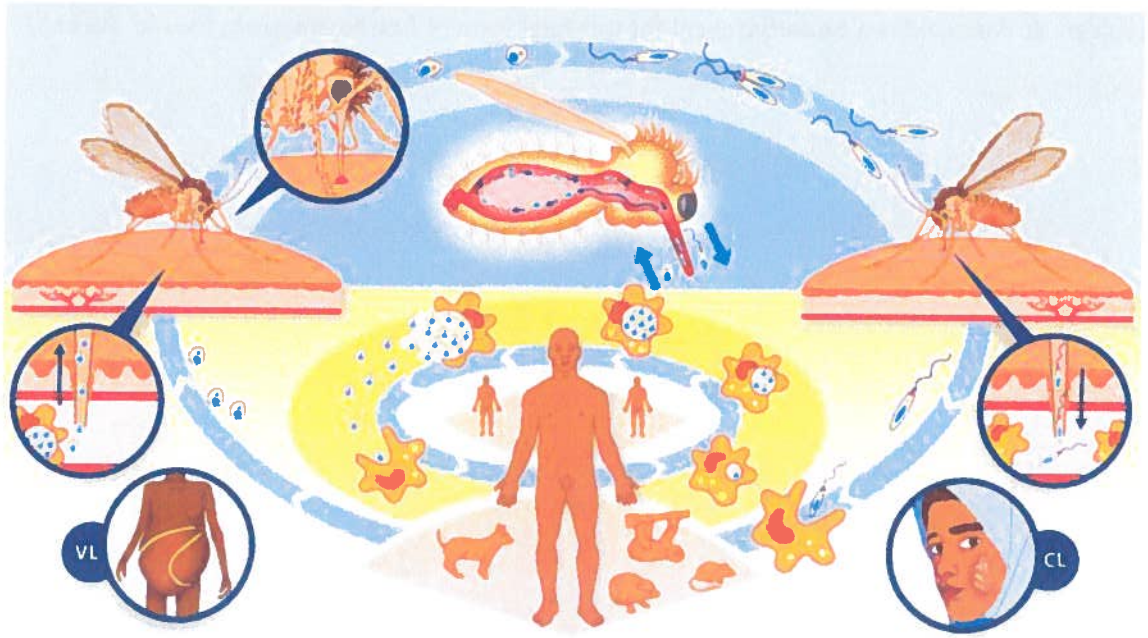
Mainly metacyclic promastigotes migrate into esophagus, pharynx and proboscis and during blood feeding are transferred to the vertebrate host. They are engulfed by macrophages wherein they transform to amastigotes. The harsh environment of the macrophage phagolysosome compartment which harbours amastigotes is acidic and it is different from phagosomes containing promastigotes. Under these milder conditions, promastigotes morphologically and physiologically changes and transform to amastigotes. Environmental signals including elevated temperature and acidification are crucial in trigging differentiation to amastigotes. In this stage, only some specific genes express which are different from the ones express in promastigotes form (Chow, Cloutier et al. 2011).

Amastigotes multiply in macrophages but depending on the different species of *Leishmania*, they target different organs. There are three different forms of Leishmaniasis (Matte, Mallégol et al. 2009):

1) Cutaneous Leishmaniasis (Baghdad ulcer, Delhi boil) causes skin lessions and is caused by the most common speies in the "Old World" such as *L. major, L. tropica* and *L. aethiopica* and by *L. mexicana* in the new world.

2) Mucocutaneous Leishmaniasis (Espondia or Uta) is characterized by lesions that metastasize in mucous membrane. It is caused by *L. braziliensis braziliensis*.

3) Visceral Leishmaniasis (Kala-azar) is characterized by fever, anemia, and swelling of the liver and spleen. *L. donovani* is a causative agent for this fatal form of Leishmaniasis in the old world.




**(MCL)**

**Figure 8. *Leishmania* life cycle**. The innfectious form of *Leishmania* (metacyclic promastigote form) is transmited by bite of the sand fly Phlebotomine to the vertebrate host. The promastigote form transforms to the amastigote form and depending of the species of *Leishmania*, they cause visceral leishmaniasis (VL), cutaneous leishmaniasis (CL) or mucocutaneous leishmaniasis (MCL) (Laurenti and Moreira 2011)

## 2.1. Virulence factors in *Leishmania*

Although there is no proof that *Leishmania* spp. produce toxins, *Leishmania* produces pathogenicity factors that allow these parasites to establish intracellular infection in macrophages. Some of the virulence factors in *Leishmania* are as follows: Secreted acid phosphatases (sAP), Glycophosphatidylinositol (GPI), Glycoinositolphospholipid (GIPL), Lipophosphoglycan (LPG), Phosphoglycan (PG), Proteophosphoglycans (PPGs), Glycoprotein 63 (GP63) and Cysteine protease (CPs) (Figure 9) (Chang and McGwire 2002). Details on LPG and GP63 will be presented in sections 2.3 and 2.5, respectively, as the major *Leishmania* virulence factors. In addition, there is evidence that phosphorylation plays critical roles in adaptation of the parasite to the environment of both the vertebrate and the invertebrate host. This can be explained by the large number of 179 protein kinases in the genome of *L. major* (Wiese 2007). Several studies show the role of mitogen-activated protein (MAP) kinases in controlling flagellum lenght in promastigote form of *Leishmania* and as mentioned earlier in metacyclic form of promastigote (the most infectious form), flagella is two times longer than body (Rotureau, Morales et al. 2009). In another study, it has been shown that a *Leishmania* protein-tyrosine phosphatase, LPTP1, that is necessary for amastigote survival (Nascimento, Zhang et al. 2006).

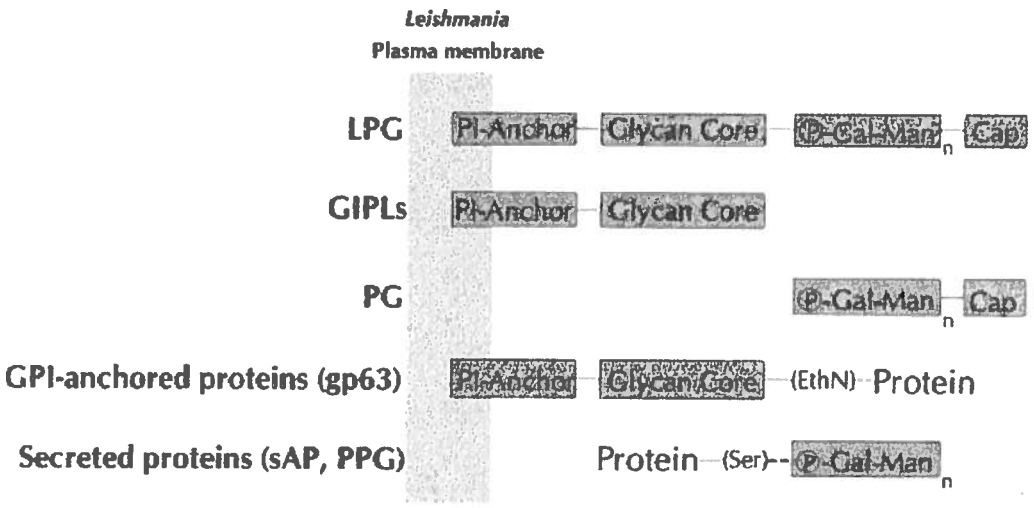



**Figure 9) Glycoconjugates as virulence factors in *Leishmania*.** These molecules are either attached to the cell surface through glycosylphosphatidylinositol (GPI) lipid anchors, including the lipophosphoglycan (LPG) and the glycosylinositol phospholipids (GIPL) or they are secreted like secreted acid phosphatase (sAP), extracellular phosphoglycan (PG) and proteophosphoglycan (PPG). EthN, phosphoethanolamine (Descoteaux and Turco 1999).

## 2.2. Entry of *Leishmania* into macrophages

Uptake of *Leishmania* promastigotes by macrophages is mediated through their surface receptor molecules. Among the *Leishmania* virulence factors, GP63 and LPG can bind and interact with the complement units C3b and C3bi on macrophages and consequently bind to CR1 and CR3 (Mac-1) receptors for entry to macrophages. It has been shown that although LPG is involved in this pathway, it is not indispensable for binding or internalization of promastigotes (Descoteaux, Matlashewski et al. 1992). Therefore, other promastigote surface molecules must also play this role but they have not been identified. The involved receptors for entry of promastigotes do not limit only to CR1 and CR3 (Mac-1) but other receptors such as the mannose-fucose receptor (MFR), TLR 2, TLR 9, the fibronectin receptor and receptors for advanced glycoconjugates are involved as well (Mosser and Rosenthal 1993, Martinez-Salazar, Berzunza-Cruz et al. 2008).

After binding promastigotes on the surface of macrophages, some proteins like actin and Rho-family GTPases play a role in *Leishmania* promastigote internalization (Lodge and Descoteaux 2005). After activation of Rho-family GTPases, several effector proteins interact with them and mediate the formation of F-actin filaments around newly formed phagosomes (Etienne-Manneville and Hall 2002). As noted earlier (in phagocytosis section), in Fcγ-receptor mediated phagocytosis, Rac-1 and Cdc42, are recruited to the phagocytic cup while they are phosphorylated. Sequentially, Wiscott-Aldrich syndrome protein (WASP) interacts with phosphorylated Cdc42 and activates the Arp2/3 complex to polymerize actin around the phagosome. Later, adaptor proteins, including Nck, Vasodilator-stimulated phosphoprotein

(VASP) and molecules needed in phosphoinositide signaling become involved as well (Lodge and Descoteaux 2008).

The internalization of amastigotes is not as well understood as that of promastigotes. It has been shown that amastigotes enter macrophages through a Rac-1 and Arf6-dependent process and there is a colocalization of F-actin, paxillin and talin to phagocytic cups (Lodge and Descoteaux 2006). Amastigote forms of some parasites including *L. amazonensis* make phosphatidylserine (PS) on their surface to infect host cells and promote alternative macrophage activation. It has been shown that phosphatidylserine on the surface of amastigotes down regulates dendritic cell activation and antigen presentation (Wanderley, Thorpe et al. 2013). Internalization of *Leishmania* amastigotes to macrophages is mediated by Fc-γR and phosphatidylserine (PS) receptors which induce transforming growth factor beta (TGF-β) and IL-10 production. This decreases classical macrophage activation and enhances parasite survival (Kane and Mosser 2000).

## 2.3. Lipophosphoglycan

LPG is the main molecule of cell surface of *Leishmania* promastigotes. It has approximately 5 million copies per cell and it covers the whole cell surface including the flagellum. This molecule is strongly down-regulated or absent in the amastigotes (Turco and Sacks 1991). LPG is synthesized by all *Leishmania* species and it is necessary for survival in both vector and host (Novozhilova and Bovin 2010). It consists of four different parts as follows: a lipid anchor (1-*O*-alkyl-2-*lyso*-phosphatidyl [*myo*] inositol), a glycan core, a chain of repetitive units (Galβ1,4Manα1-$PO_4$), which is identical for all *Leishmania*, and an oligosaccharide cap. In *L. donovani* metacyclics the number of oligosaccharide-phosphate units increases and causes the

elongation of LPG. Also, bacause of the conformational changes, the availability of oligosaccharide cap decreases. The functional consequences of these structural changes are helping the transmission of *Leishmania* from sand fly to vertebrate host and the survival and replication of the parasite in host cells (Sacks, Pimenta et al. 1995).

Depending on the side chain nature and structure, three types of LPG are known. LPG type 1, has no side substituent (*L. donovani*). Type 2, is glycosylated at the galactose position C3 in repetitive units (*L. mexicana, L. major, L. tropica*). Type 3 of LPG, is specific to *L.aethiopica*. It has 35% of repetitive units that are manosylated at position C2 of mannose (McConville, Schnur et al. 1995).

LPG has different functions, including to allow *Leishmania* binding to stomach epithelium cells of the sand fly (Gantt, Goldman et al. 2001). In addition, LPG makes a barrier around *Leishmania* and protects it against oxidative damage. LPG also regulates nitiric oxide (NO) synthesis by decreasing inducible NO synthase (iNOS) expression in infected macrophages (Winberg, Rasmusson et al. 2007). Probably LPG has a gene silencer to inhibit IL-12 secretion by macrophage (Assis, Ibraim et al. 2012) and, last but not least, LPG causes delay in phagosome maturation (Desjardins and Descoteaux 1997). It has been shown that *L. major lpg*1$^{-/-}$, which only lacks LPG, were susceptible to human complement system, lost the ability to inhibit transient phagolysosomal fusion and were sensitive to oxidant reagents (Spath, Garraway et al. 2003).

## 2.4. Delay in phagosome biogenesis after infection by *Leishmania*

*Leishmania* amastigotes multiply in phagosomes that fuse with lysosomes. This acidic environment with pH of (~ 4.7 - 5.2) is optimal for amastigote activities and it is caused by the

vacuolar-type $H^+$-ATPase (Vinet, Fukuda et al. 2009). Different protein markers for late endosomes and lysosomes like Rab7, LAMP1 and LAMP2 are present on vacuoles containing amastigotes. In addition, some molecules of the ER such as calnexin and the membrane fusion regulator Sec22b are recruited to vacuoles containing amastigotes as well (Ndjamen, Kang et al. 2010). There is no assembly for NADPH oxidase complex on the membrane of parasitophorous vacuole containing amastigotes, because amastigotes fail to induce phosphorylation of cytosolic $p47^{phox}$, a key factor for NADPH oxidase activation during phagocytosis (Figure 10) (Lodge and Descoteaux 2008). Another change in parasitophorous vacuoles containing amastigotes is the disruption of lipid microdomains present within the phagosome membrane (Lodge, Diallo et al. 2006).

In contrast to amastigotes, phagosomes containing promastigotes poorly interact with late endosomes and lysosomes and this explains delayed recruitment of LAMP1 and probably, an impaired recruitment of Rab7 (Lodge and Descoteaux 2008). Periphagosomal F-actin accumulation is induced in phagosome harbouring promastigotes. An actin barrier possibly interferes with the recruitment of signal transducers and vesicle trafficking to the forming phagolysosome (Lodge and Descoteaux 2005). However, F-actin accumulation is not observed with all *Leishmania* species. There is a rapid dissociation of F-actin around phagosomes harbouring *L. amazonensis* metacyclic promastigotes (Wilson, Huynh et al. 2008), which could be explaind by the formation of the communal vacuole containing *L. amazonensis* but further studies are required to conform this hypothesis. Similar to amastigotes, promastigotes disrupt lipid microdomains, like flotillin, in the phagosomal membrane. These membrane microdomains play an important role in different cell functional events such as by disruption of these structures in phagosomes, promastigotes can reside safely in macrophages (Simons and Vaz 2004).

Exclusion of Synaptotagmin V, a regulator of phagocytosis and phagosome maturation, is an example of microdomain disorganization after infection with promastigotes (Vinet, Fukuda et al. 2008). One of the functional consequences of the inhibition of phagosome maturation by promastigotes is disabling the NADPH oxidase assembly at the membrane of phagosomes. NADPH oxidase produces reactive species oxygen (ROS) as an important antimicrobial factor (Lodge and Descoteaux 2006). Interestingly, recruitment of NADPH oxidase to the phagosome membrane reduces the proteolytic activity of the phagosome in dendritic cells. NADPH oxidase uses pumped protons into the lumen of the phagosome in dendritic cells and by doing so keeps the lumen basic with less proteolytic activity to proceed for antigen presentation by dendritic cells.

Phagosomes in macrophages, unlike dendritic cells, are acidic. It is well known that most lysosomal hydrolyses are optimally active at acidic pH and this is a key factor for killing pathogens including *Leishmania* in the phagosome of macrophages (Moradin and Descoteaux 2012, Rybicka, Balce et al. 2012). Thus, some pathogens inhibit phagosome acidification in macrophages to alter the phagosome to a non-hostile environment to survive and multiply. For example *L. donovani* excludes v-ATPase from phagosomes after 24 h of phagocytosis and inhibits phagosome acidification (Vinet, Fukuda et al. 2009).

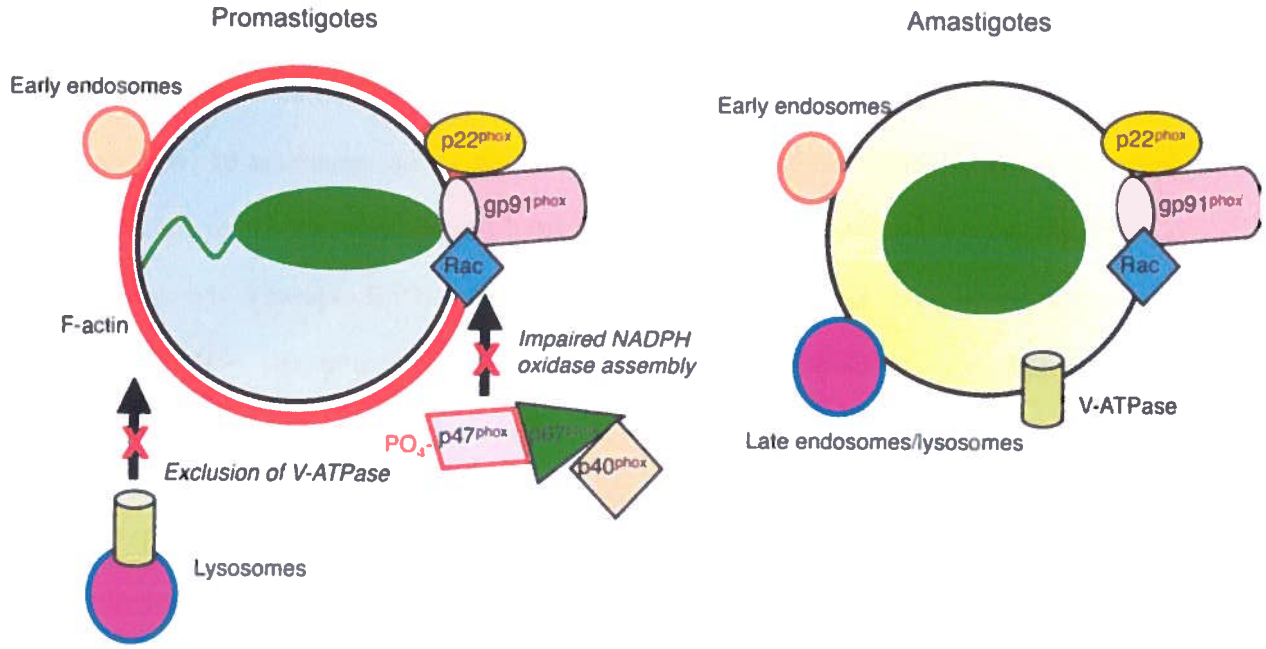



**Figure 10) Difference in phagosome remodeling in promastigote- and amastigote-containing parasitophorous vacuoles.** F-actin accumulation is observed in phagosomes harbouring promastigotes but not amastigotes. In contrast to amastigote-phagosome, the ones containing promastigotes interact poorly with late endosomes/lysosomes. In addition, V-ATPase is excluded from phagosomes containing promastigotes but is recruited to amastigote-phagosome (Moradin and Descoteaux 2012).

## 2.5. GP63

GP63 was discovered in the 1980s and it is a major surface antigen of the promastigote form of *Leishmania* ($5x10^5$ molecules per cell). There are several names for this virulence factor, including major surface glycoprotein (MSG), promastigote surface protease (PSP) and Leishmanolysin (Yao, Donelson et al. 2003). GP63 gene sequencing of several *Leishmania* spp. show the same sequence motif for zinc-binding (HExxHxxGxxH) which it has been shown that they mediate internalization of *Leishmania* into macrophages (Puentes, Guzman et al. 1999). GP63 and other major cell surface components such as lipophosphoglycan (LPG) and glycoinositolphospholipids (GIPL) have important roles in establishing an infection in macrophages after injection of a small number (<1000) of promastigotes by sand flies (Schlagenhauf, Etges et al. 1998). In contrast to promastigotes, amastigotes express low levels of GP63 that might still be enough to compensate for the absence of LPG. In the amstigote forms, the remaining GP63 molecules are not packed with the otherwise highly abundant LPG, making GP63 more likely to disrupt the host immune response and promote amastigote survival (Olivier, Atayde et al. 2012).

### 2.5.1. Structure of GP63

GP63 from *L. major* has been crystallized (Figure 11). The predicted protein sequence for GP63 contains signal peptide (KDEL) to target the protein to the ER during synthesis; it also contains an immature pro-peptide that is removed during the maturation process. In *L. major,* the conserved cysteine amino acid in the pro-peptide form of GP63 binds to a zinc ion at the active

site of the enzyme and inhibits enzyme activity before maturation, hence protecting the cell from self-destruction by active GP63 (Macdonald, Morrison et al. 1995). It has been also shown that some zinc chelators like 1,10-phenanthroline reduce the release of *L. amazonensis* GP63 from the cell surface, which suggests that GP63 release is dependent on autoproteolysis (McGwire, O'Connell et al. 2002). Crystal structure of mature GP63 shows three different domains; N-terminal, central, and C-terminal domains (Schlagenhauf, Etges et al. 1998). The N-terminal domain has a similar folding structure to the catalytic part of other zinc proteinases with the following sequence motif: HExxHxxGxxH. The zinc-coordinated histidine plays a highlight role in GP63 function as a zinc metalloprotease and also in its stability. Indeed, substitution of any of the three histidine residues in the motif cited above leads to intracellular degradation of GP63 (McGwire and Chang 1996). The difference between GP63 and other zinc proteinases is the insertion of 62-amino acids between glycine and third histidine residue. The spacing between the three histidines stabilizes energy to the zinc coordination and contributes to the conformation of an active structure (Vallee and Auld 1990). In addition, it may also be required to facilitate the conformation of discontinuous receptor binding sites. All GP63s from different *Leishmania* species, except *L. guyanensis*, have 18 cysteine residues that are involved in forming 9 intramolecular disulfide bridges (2 disulfide bonds in N-terminal, 6 bonds in C-terminal and 1 in central domain). The C-terminal domain contains a site for glycosylphosphatidylinositol (GPI) anchor addition. There is an abundance of different GP63 isoforms which are located either internally whitin the parasite, anchored to the surface membrane, or secreted (Yao, Donelson et al. 2007). Ellis et al. found that glycosylation is necessary for secretion of non-GPI anchored GP63 (Ellis, Sharma et al. 2002). It has been also shown that both forms of GP63 are associated with lipid rafts (Figure 12). Other studies show that GP63 can be secreted in exosomes. There is

a recent study that shows a direct delivery of *Leishmania* molecules into macrophages through exosomes (Silverman, Clos et al. 2010). Exososmes are small vesicles (40-100 nm) and they are released from different kinds of eukaryotic cells including parasites. Comparative quantitative proteomics identified 329 proteins in *Leishmania* exosomes, which are delivered to host cells through an exosome-based secretion system (Zhao, Yang et al. 2009). Fusion of exosomes with the macrophage plasma membrane hence delivers GP63 to the host cell cytoplasm but the exact molecular mechanism is not clear yet (Descoteaux, Moradin et al. 2013, Ghosh, Bose et al. 2013). However, there are different suggested mechanisms: 1) GPI-GP63 interacts with macrophages through lipid rafts or a flipping protein. 2) GPI-GP63 and non-GPI-GP63 can be transported to the cytoplasm via transporter proteins. It is possible that cleavage of phospholipase mediates the release of some proteins from the cell membrane. 3) Lipid raft-dependent endocytosis followed by retrograde trafficking. 4) GP63 can also be released into the cytoplasm of host cells through microvesicles or exosomes containg GPI-GP63 (Gomez and Olivier 2010).

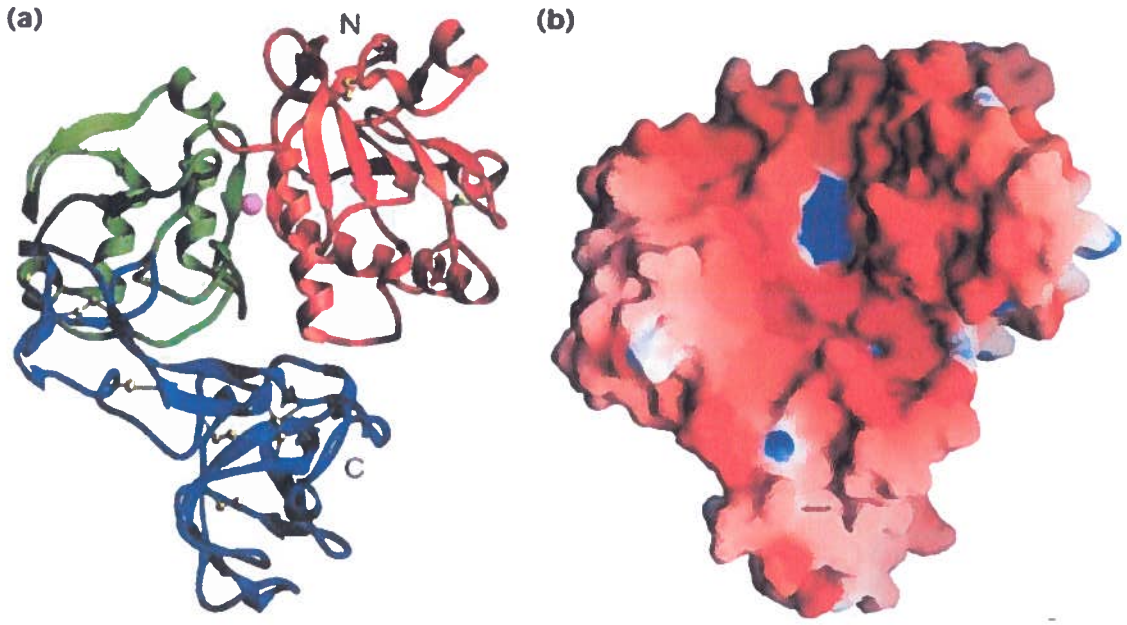



**Figure 11)** ***Leishmania major*** **GP63 domain structure.** a) N-terminal domain in red, central domain in green and C-terminal domain in blue. Disulfide bonds in yellow and the active site zinc atom is represented as a magenta sphere. b) Surface representation of the molecules with negative charge shown in red and positive region in blue (Schlagenhauf, Etges et al. 1998)

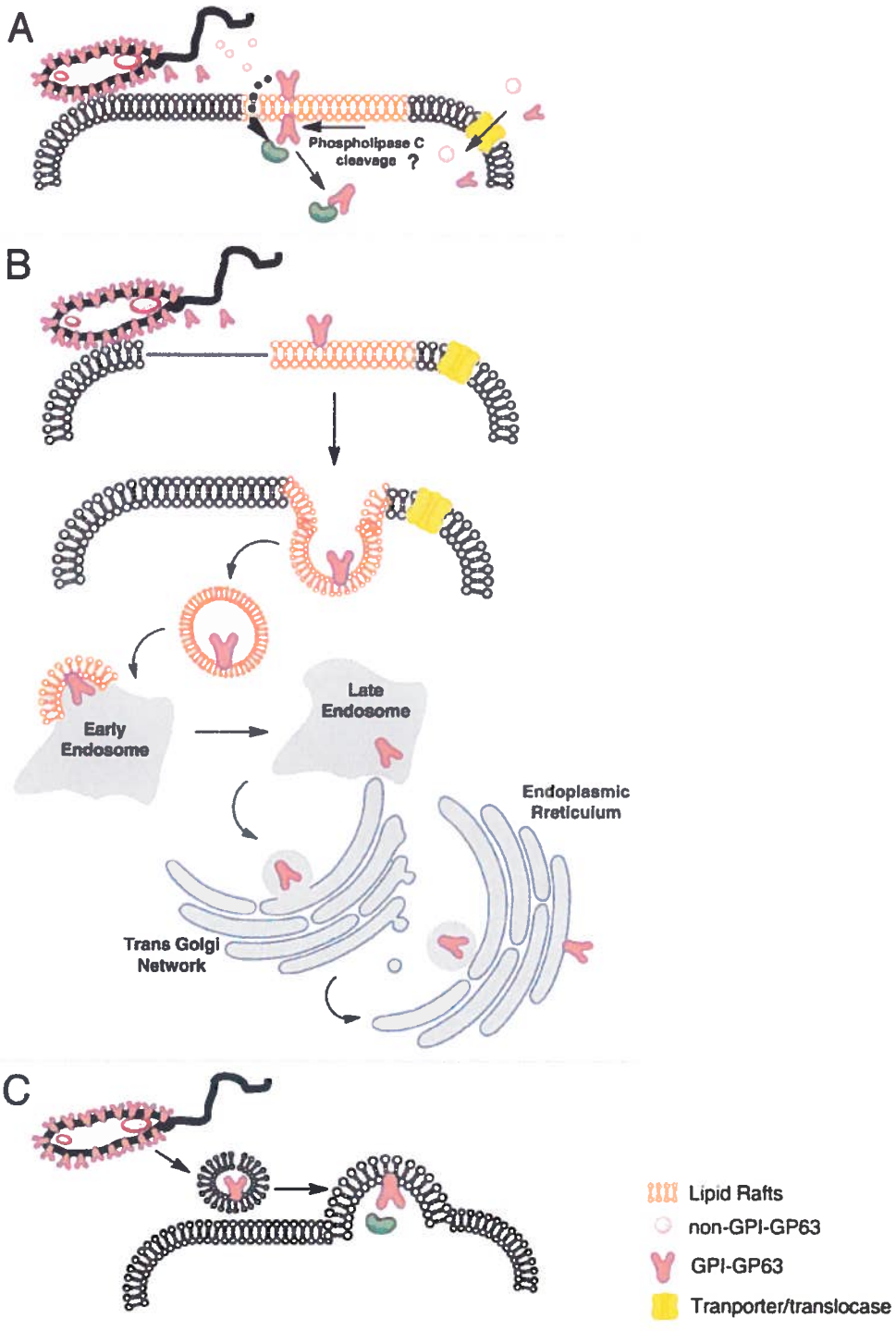

A
Phospholipase C
cleavage ?
B
Early
Endosome
Late
Endosome
Endoplasmic
Rreticulum
Trans Golgi
Network
C
Lipid Rafts
non-GPI-GP63
GPI-GP63
Tranporter/translocase

**Figure 12) Putative mechanisms of access of GP63 to the cytoplasm of macrophages.** (A) There are different possible ways for GP63 molecules to enter the host cytoplasm including through lipid rafts, protein flipping and protein transporters; (B) Lipid raft-dependent endocytosis that releases GP63 from the original vesicle and it is followed by retrograde trafficking from *trans* Golgi/ER network (C) GP63 can be released to the cytoplasm of host cells by membrane-membrane fusion between vesicles in the form of exosomes and host cells (Gomez and Olivier 2010).

### 2.5.2. Regulation of GP63 expression

RNA polymerase II facilitates gene expression at the level of transcription in higher eukaryotic cells, but trypanosomatid protozoa do not have this conventional regulatory system. Alternatively, they use a post-transcriptional mechanism to modulate gene expression. Studies in *L. major*, *L. amazonensis* and *L. chagasi* show the same post-transcriptional regulatory systems (Yao, Donelson et al. 2003). Several *Leishmania* species have different types of *gp63* genes that are developmentally regulated in different life cycle stages of the parasite, such as amastigotes, procyclic and metacyclic promastigotes (Joshi, Sacks et al. 1998).

There are distinct gene classes for *gp63* and the expression of their mRNA switches during *Leishmania* development; however, the total abundance of *GP63* mRNA remains relatively constant. The protein expression of GP63 increases 14- to 16-times during the transformation of promastiges from logarithmic to stationary phase. This augmentation of GP63 protein levels does not correlate with the steady amount of *GP63* mRNA and further studies are required to explain this phenomenon (Yao, Leidal et al. 2002, Hsiao, Yao et al. 2008).

### 2.5.3. Function of GP63

The amastigotes and promastigotes express different isoforms of GP63, which are different biochemically and functionally. There is a wide range of *in vitro* substrates for GP63, including casein, gelatin, albumin, haemoglobin, and fibrinogen (Yao, Donelson et al. 2003). The pH optimum varies among *Leishmania* species and it is substrate dependent (Tzinia and Soteriadou 1991). For instance, *L. major* GP63 is active at neutral to alkaline pH, whereas *L.*

*mexicana* GP63 is most active at pH ~4. Studies of GP63 substrate specificity show that this enzyme is an endopeptidase with no exopeptidase activity that can hydrolyze a range of peptides. Identification of 11 cleavage sites shows that there is no conserved cleavage motif for GP63 substrates: P1↓P'1-P'2-P'3, where the arrow shows the site of cleavage, P1 is a polar amino acid, P1' is an hydrophobic amino acid, and P2' and P3' are basic amino acids. A tyrosine residue is also usually present at P1 site (Bouvier, Schneider et al. 1990), but *L. mexicana* GP63 preferentially cleaves at a serine or threonine site (Ip, Orn et al. 1990).

GP63 serves different functions in different parasite life stages (Figure 13). It has been shown that in *L. donovani*, GP63 regulates multiplication and also plays a role in the morphology of these parasites (Pandey, Chakraborti et al. 2004) It can bind to the C3 protein of the complement system and convert it to the inactive form C3bi, in this way promastigotes can escape complement-mediated lysis. GP63 also acts as an opsonin for host complement system and facilitates the attachment of *Leishmania* to macrophages. CR3 is a receptor for C3bi and can bind to promastigotes. CR1 is a receptor for both C3b and C3bi and binds to *L. major* promastigotes as well. Altogether, by acting like an opsonin, GP63 facilitates promastigote phagocytosis by host macrophages (Ueno and Wilson 2012). Serum-resistant promastigotes contain membrane-associated proteins that block the lytic activity of the complement on red blood cell membranes. These putative inhibitors impair C9, but not C3 binding to complement-activating complexes and it shows that blocking happens at the late step of complement cascade. In addition, it is suggested that sensitivity or resistancy to complement lysis depends on the amount of inhibitory protein expression in different stages of promastigotes (Nunes, Almeida-Campos et al. 1997). GP63 has been detected in amastigotes of all *Leishmania* species so far but the exact role of GP63 is not clarified yet (Yao, Donelson et al. 2003). One of the suggested

roles for GP63 in amastigotes is protecting intracellular amastigotes within macrophage phagolysosomes. It has been shown that attenuated *L. mexicana amazonensis* with 20- to 50-fold reduction in GP63 expression cannot survive inside macrophage phagolysosomes like the wild type ones. Also, intracellular *Leishmania* amastigotes were eradicated by inhibiting GP63 activity through 1, 10 phenantroline. In support of this idea, it has been shown that when attenuated cells or proteins are coated by active GP63, while they are entrapped in liposomes, they can be protected from phagolysosome degradation (Seay, Heard et al. 1996). Another role of GP63 related to its protease activity is cleavage of CD4 molecules and reduction T cells responses (Hey, Theander et al. 1994). In a classical antigen processing pathway, MHC class II molecules are recognized by $CD4^+$ T cells; thus, cleavage of CD4 molecules by GP63 causes inhibition of T cell responses. T cell epitopes presented by MHC class I molecules are typically peptides between 8 - 11 amino acids in length. It has been shown that GP63 cleaves intracellular peptides in a way that they cannot be loaded on MHC class I molecules; thereby preventing antigen presentation on MHC class I molecules. (Garcia, Graham et al. 1997). Recently, further studies showed that in a non-canonical pathway in which exogenous antigens are loaded on MHC class I and recognized by $CD8^+$ T cells, the failure of exogenous antigen presentation by MHC class I is due to the degradation of some SNAREs proteins by GP63. Consequently, recruitment of some subunits of NADPH to phagosomes, which is SNARE dependent, fails. Altogether, by changing oxidation and acidification in phagosomes *Leishmania* affects antigen processing and presentation on MHC class I molecules (Matheoud, Moradin et al.2013).

GP63 alters host macrophage signaling pathway by degrading different key proteins. Here are some examples:

1) Myristoylated alanine-rich C kinase substrate (MARKS) and MARKS-related proteins (MRP) are substrates for PKC in macrophages. These two proteins (MARKS and MRP) increase significantly in macrophages after stimulation by LPS and cytokines (Aderem 1992). Further studies showed that MARKS and MRP are cleaved upon infection with *L. major* and the cleavage is GP63-dependent (Corradin, Ransijn et al. 2002).

2) PKC is an important serine/threonine kinase involved in signal transduction. There are several studies that show that PKC is affected by LPG on the surface of *Leishmania* promastigotes but also, it has been shown that *L. donovani* amastigotes, which do not contain LPG, inhibit PKC-dependent signalling pathway (Shadab and Ali 2011). Thus, inhibition of PKC might be at least in part explained by the effect of GP63 but further investigations are necessary to clarify this issue (Olivier, Atayde et al. 2012).

3) Janus kinase/signal transducer and activator of transcription (JAK2/STAT1) pathway is one of the important signalling pathways which is altered after infection with *Leishmania.* This pathway is responsible for producing different antimicrobial factors such as NO and that is why it is altered in *Leishmania*-infected cells. The protein tyrosine phosphatase (PTP) SHP-1 is a negative regulator of this pathway, which inactivates JAK2 and it is activated through *Leishmania* GP63. In addition to SHP-1, GP63 activates two more protein tyrosine phosphatases PTP1B and TCPTP by cleaving their C' terminal domain (Gomez, Contreras et al. 2009, Shio, Hassani et al. 2012).

4) There are several transcription factors such as NF-κB and activated protein-1(AP-1), which are cleaved by *Leishmania* GP63. Normally, NF-κB p65RelA interacts with p50 to mediate normal macrophage functions but after cleavage of NF-κB p65RelA through GP63, p35RelA is

released and it interacts with NF- κB p50. This protein complex binds to DNA and stimulates the secretion of specific chemokines (MIP-2/CXCL2, MCP-1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4) in infected macrophages with *Leishmania* instead of proceeding with normal macrophage activities (Gregory, Godbout et al. 2008). In addition to NF-κB, AP-1 is an important transcription factor and it plays a role in IFN γ-induced NO production (Olivier, Gregory et al. 2005). It has two forms of homodimers and heterodimers. Homodimers form from Jun family members (c-Jun, JunB and Jun D) while heterodimers form from Jun and Fos family members (c-Fos, FosB, Fra 1 and Fra 2) (Ding, Pan et al. 2013). Further studies on AP-1 showed that its different subunits (c-Fos and c-Jun) are degraded by *Leishmania* GP63 and consequently inactivate AP-1 (Contreras, Gomez et al. 2010).

5) In addition to the transcription system, the macrophage translation system is altered through GP63 as well. Normally, the formation of the eukaryotic translation initiation factor 4F (eIF4F) is suppressed by the translational repressor eIF4E-binding protein 1 (4E-BP1). The eIF4F complex is formed by the cap-binding subunit, eIF4E; the RNA helicase, eIF4A; and the scafolding protein, eIF4G. The activity of 4E-BP1 is controlled by its phosphorylation through mTOR (mammalian/ mechanistic target of rapamycin), a serine/threonine kinase. When 4E-BP1 is hyper-phosphorylated through mTOR, it dissociates from eIF4E so that active eIF4F can initiate cap-dependent translation. Further studies by Jaramillo and colleagues showed that GP63 cleaves mTOR after infection with *Leishmania* (Jaramillo, Gomez et al. 2011). mTOR is a key regulator of the innate immune response including the synthesis of cytokines, such as type I IFN, which are important for host defence against Leishmaniasis (Weinstein, Finn et al. 2000, Bogdan, Mattner et al. 2004).

In addition to macrophages, GP63 migrates to the extracellular matrix and degrades collagen IV and fibronectin (McGwire, Chang et al. 2003). Interestingly, recent studies showed that GP63 targets Dicer1, which is a pre-miRNA (precursor microRNA) processor that inhibits miRNP (microRNA ribonucleoprotein complex) formation in infected hepatic cells (Descoteaux, Moradin et al. 2013, Ghosh, Bose et al. 2013). miR-122 expression is specific to the liver and is a regulator of fatty-acid metabolism. Restoration of miR-122 in infected mouse liver increases serum cholesterol and reduces liver parasite burden. In addition, proliferation, receptor expression and IFN-γ release by natural killer (NK) cells are affected by GP63 although the mechanism is unknown (Lieke, Nylen et al. 2008). Since the functional studies on the role of GP63 was done on *L. major* Δ*gp63* and WT, so they are specific.

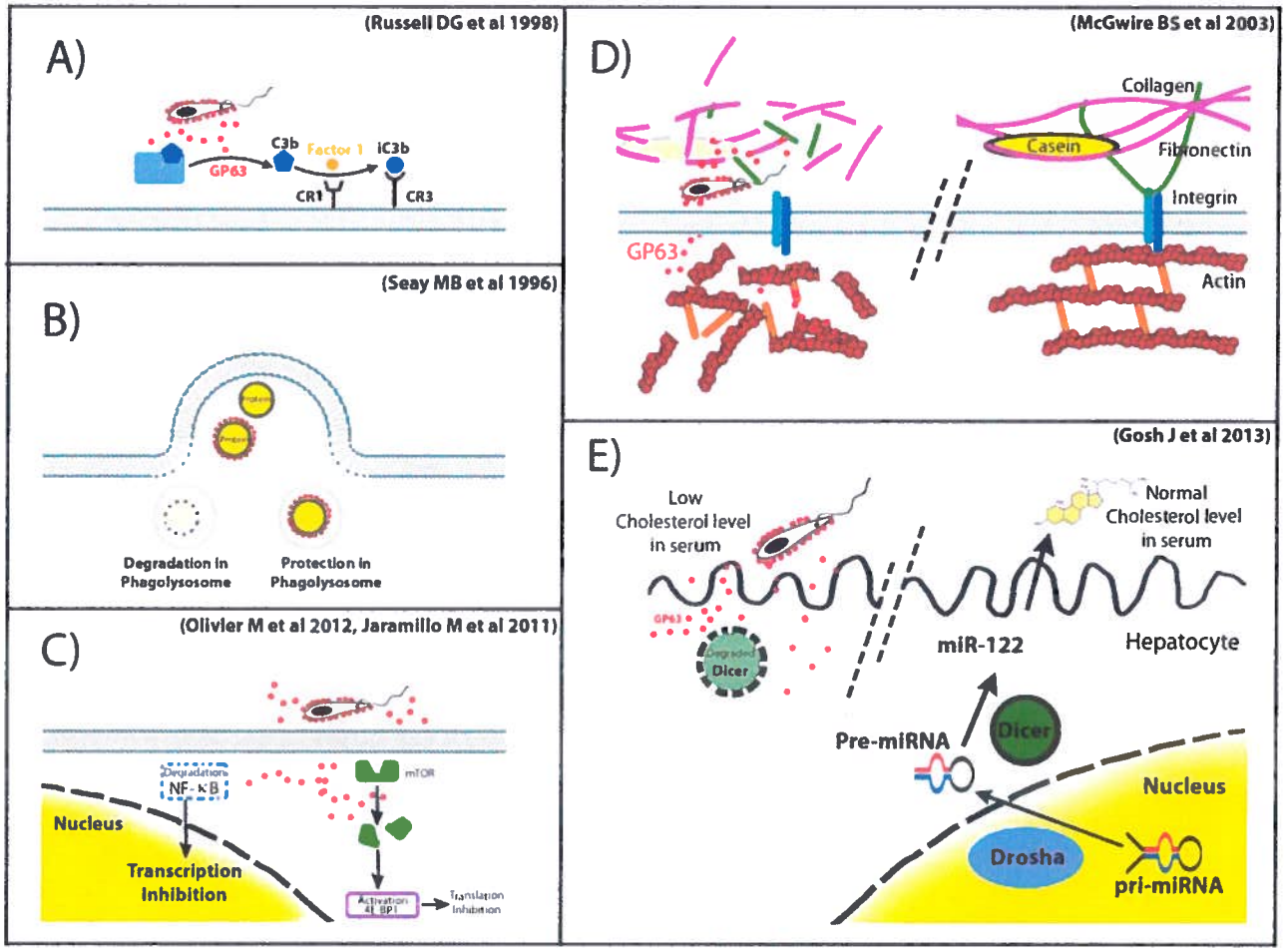

A)
(Russell DG et al 1998)
C3b
Factor 1
iC3b
GP63
CR1
CR3
B)
(Seay MB et al 1996)
Degradation in Phagolysosome
Protection in Phagolysosome
C)
(Olivier M et al 2012, Jaramillo M et al 2011)
NF-κB
mTOR
Nucleus
Transcription Inhibition
Translation Inhibition
D)
(McGwire BS et al 2003)
Collagen
Casein
Fibronectin
Integrin
GP63
Actin
E)
(Gosh J et al 2013)
Low Cholesterol level in serum
Normal Cholesterol level in serum
GP63
Dicer
miR-122
Hepatocyte
Pre-miRNA
Dicer
Nucleus
Drosha
pri-miRNA

**Figure 13) Different functions of GP63.** (A) GP63 can use lipid raft microdomains and receptors (such as third complement receptor CR3) to enter the host cells. C3b is a target for GP63 and it is degraded to iC3b, which in turn binds to CR3. (B) Proteins entrapped in liposomes, which are covered by GP63 are protected from phagolysosomal degradation while the non-protected ones are sensitive to phagolysosome degradation. (C) GP63 alters cell signalling by selectively cleaving transcription factor regulators such as NF-κB and also, translation factor regulators, including mTOR. (D) Extracellular matrix proteins, fibronectin and collagen are degraded by GP63. (E) GP63 degrades Dicer, which in turn causes hypocholesterolemia.

# 3. NADPH Oxidase (NOX2)

As noted earlier, after infection with *Leishmania*, there is no assembly of NADPH oxidase (NOX2) belongs to NOX family on the phagosome membrane. There are different members in NOX family including 1, 2, 3, 4. Here, there is a description of NOX2, both functionally and structurally, to better understand the functional consequences of the enzyme disassembly after infection with *Leishmania*. NOX2 is an enzyme by following reaction formula: it catalyzes oxidation of NADPH with simultaneous reduction of oxygen to superoxide (Pohanka 2013).

$$NADPH + 2O_2 \longrightarrow NADP^+ + H^+ + 2O_2^-$$

$$2O_2^- + 2H^+ \longrightarrow O_2 + H_2O_2 \text{ (Babior 1999)}$$

NOX2 is expressed during inflammatory responses and provides antibacterial agents to kill invading pathogens (Lee and Yang 2012). This enzyme is responsible for the major percentage of superoxide ($O^-_2$) produced by professional phagocytes (e.g. macrophages, neutrophils, monocytes and eosinophils). These phagocytic cells can produce NOX2 at a certain stage of their development. Macrophages play a key role in the digestion and clearance of antigens; thus, they have a more acidic lumen with highly proteolytic activity due to a high ROS production. Since the main role of dendritic cells is antigen processing to be presented by MHC molecules, preserving epitopes is necessary. Therefore, an acidic lumen within a phagosome with high proteolytic activity, such as that of the macrophage, is not suitable. In dendritic cells, NOX2 leads to the production of low levels of ROS that consume protons in the phagosomal lumen and

inhibit acidification (pH~7.5) (Ribeiro-de-Jesus, Almeida et al. 1998, Hepp, Puri et al. 2005). If ROS generation is arrested in dendritic cells, the pH in the phagosome decreases and it acidifies very fast (Russell 2007).

Mice deficient for some NOX2 subunits are susceptible to infections such as *Salmonella typhimurium* (Sakurai, Hashimoto et al. 2012). Some pathogens have developed strategies to interfere with NOX2 activity such as preventing NOX2 assembly on the phagosome or reducing the steady-state levels of NOX2 components. A novel mechanism discovered in *Helicobater pylori* is that these bacteria can misdirect the assembly of functional NOX2 complex. In this case, NOX2 complex assembles in the plasma membrane instead of in the phagosome membrane so that generated ROS are released into the extracellular space instead of in the phagosome lumen (Allen, Beecher et al. 2005). Recently, a study on *Mycobacterium tuberclosis* showed a novel role for NOX2 complex. Normaly TNF binds to TNF receptor 1 (TNF-R1) and subsequently activates JNK to enter apoptosis. High levels of ROS inactivate MAP kinase phosphatases which in turn prolong the activation of JNK activity and apoptosis. The authors showed that NOX2 helps host macrophages to remove intracellular bacteria by increasing the levels of ROS in the phagosome and subsequently induce host cell apoptosis while in infected macrophages with *M. tuberclosis*, the levels of NOX2 decreased (host defense mechanism is inhibited by *M. tuberclosis* ) (Goldszmid, Coppens et al. 2009, Miller, Velmurugan et al. 2010).

ROS has bactericidal functions but also, when they are released to the extracellular space, they can compromise the function and viability of adjacent cells. For instance, T cells and other lymphocytes are sensitive to ROS and they rapidly inactivate and undergo apoptosis (Martner, Aurelius et al. 2011). Since effector T cells are constantly exposed to the oxidative media of

infected or inflamed tissues, there should be a strategy applied by T cells to escape inactivation by ROS. It has been shown that, molecules carrying reduced sulfhydryl groups (thiols) neutralize ROS and by doing so, protect different cell types from oxidative stress (Yan, Garg et al. 2009). The exact role of thiols in T cell survival under oxidative stress conditions is not clear yet, but is already known that thiols promote T cell functions. Also, it was shown that dendritic cells release thiols and activate thiol expression on adjacent cells including NK cells and T cells. A recent study showed that after interaction between antigen (Ag)-specific T cells and dendritic cells, T cells neutralize exogenous oxygen radicals and consequently they can be resistant to ROS-induced apoptosis. Dendritic cells provide Ag-specific T cells with reduced sulfhydryl groups (thiols) after presentation of viral and bacterial Ags. Altogether, these data unveil a novel aspect of dendritic cell/T cell cross-talk of relevance for preservation of specific immunity in inflammed or infected tissue (Campbell-Valois, Trost et al. 2012).

## 3.1. Structure of NOX2

Phagocyte NOX2 consists of an integral membrane heterodimer which is called cytochrom b558 and it is composed of a catalytic core, the enzyme gp91$^{phox}$ and also p22 $^{phox}$. The cytosolic part is formed by p67$^{phox}$, p47$^{phox}$ and p40$^{phox}$ in interaction with a low molecular weight G protein Rac1 or Rac2 (depends on the phagocyte cell type), which altogether make a quite large active protein complex (Figure 14). Rac1 is expressed ubiquitously while Rac2 is restricted to haematopoietic cells. In resting conditions, Rac proteins are associated with GDI (guanine nucleotide dissociation inhibitor), which is a 55 kDa protein while the active forms bind to GTP. The conversion between the active and inactive states is regulated by GEFs(guanine-nucleotide-exchange factors) and GAPs (GTPase-activating proteins), which

control the release of GDP and binding to GTP and also, the hydrolysis of GTP, respectively (Groemping and Rittinger 2005).

### 3.1.1. $p91^{phox}$

$gp91^{phox}$ (also called the β-subunit of the cytochrome) is an important subunit in the NOX2 protein complex. There are electron carrying components in the NOX2 such as flavine adenine dinucleotide (FAD) and two hemes which are associated with the cytosolic and membrane parts of the complex, respectively. The N-terminal part forms six transmembrane α-helices. Helices 3 and 5 contain two conserved histidines that bind to two hemes. The C-terminal cytoplasmic domain contains the binding sites for FAD and NADPH. Altogether, $gp91^{phox}$ contains all co-factors required for electron transfer reactions (Groemping and Rittinger 2005).

### 3.1.2. $p67^{phox}$

This subunit is an accessory protein and although it is required for the function of NOX2, its exact function is not clear yet. $p67^{phox}$ has two SH3 domains (Src homology 3), one located in the center and the other one at the C-terminal. Further studies showed that $p67^{phox}$ catalyzes the transfer of electrons from NADPH to electron acceptor dyes, which suggests that $p67^{phox}$ might play a role in transferring electrons from NADPH to oxygen in the cell; however, this should be confirmed in presence of $O_2$ instead of dye (Dang, Babior et al. 1999).

### 3.1.3. $p47^{phox}$

It recognizes phosphatidylinositol 3,4 bisphosphate and thereby contributes to membrane anchoring. $p47^{phox}$ consists of two SH3 domains that are involved in protein-protein interactions; depending on resting or active states, some of these proteins are targeted by phosphorylation. $p47^{phox}$ is the phosphorylated unit of the NADPH oxidase and there are 11 known phosphorylation sites for this subunit (Groemping and Rittinger 2005). As noted earlier,

amastigotes evade the phosphorylation of $p47^{phox}$ during phagocytosis so that NADPH assembly does not occur (Lodge and Descoteaux 2006, Moradin and Descoteaux 2012).

### 3.1.4. $p22^{phox}$

It is located in the membrane of phagocytes and interacts with $gp91^{phox}$ and Rac. $p22^{phox}$ has a cytosolic tail (Dinauer, Pierce et al. 1990) and it binds to phosphorylated $p47^{phox}$ to assemble the active oxidase.

### 3.1.5. $p40^{phox}$

It is a protein in NOX2 without clear function and it seems not to be essential for the oxidase activity of the enzyme. It is in the protein complex with $p47^{phox}$ and $p67^{phox}$ but further studies are required to elucidate its real function.

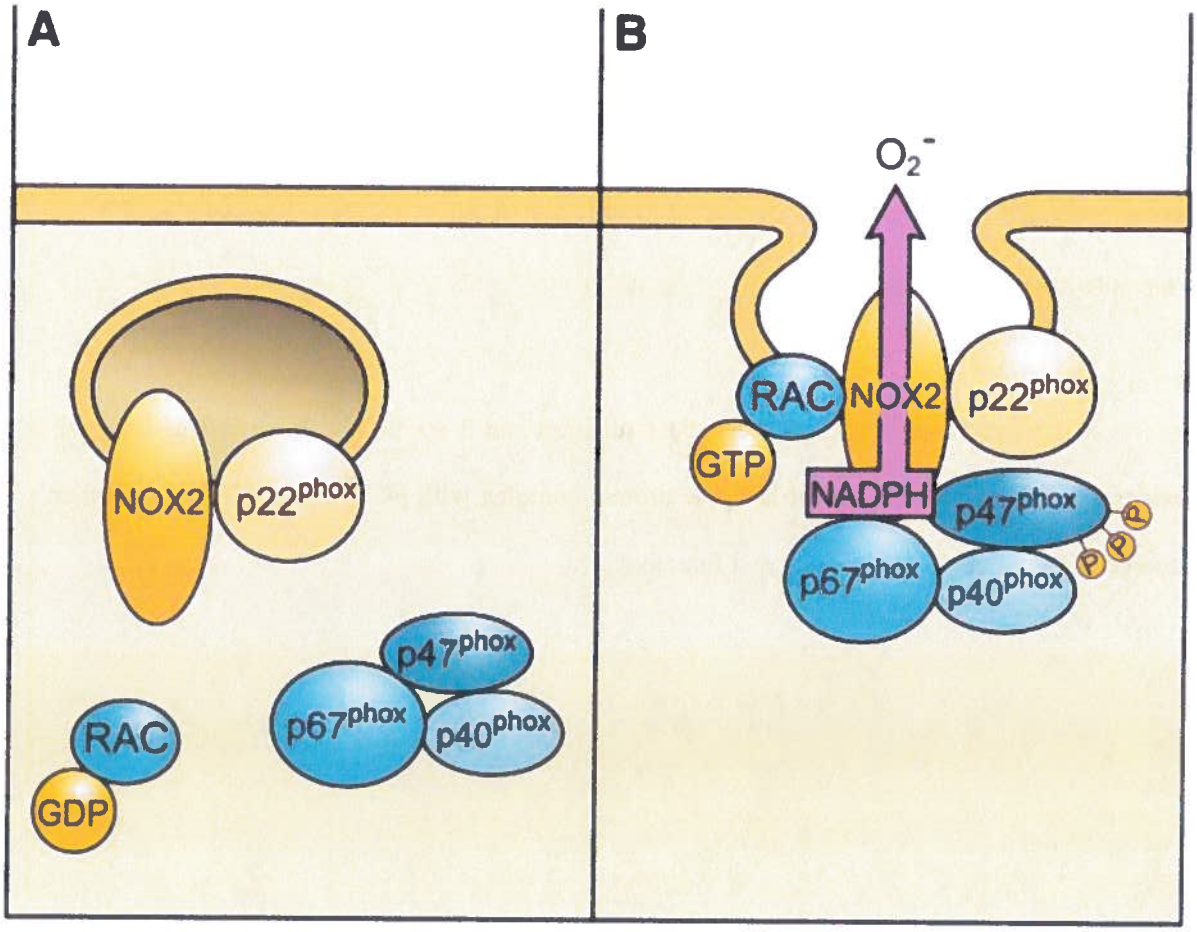



**Figure 14) Assembly of NOX2 on the phagosome membrane.** In resting cells, gp91$^{phox}$ and p22$^{phox}$ are found in the membrane of intracellular vesicles. They are in close interaction and co-stabilize each other. Upon activation, cytosolic p47$^{phox}$ is phosphorylated and its conformational structure changes, which in turn leads to its interaction with p22$^{phox}$. The movement of p47$^{phox}$ brings other cytoplasmic subunits(p67$^{phox}$ and p40$^{phox}$) together to form an active NOX2 enzyme (Bedard and Krause 2007).

# 4. Cross-presentation

Rock and colleagues were the first to describe cross-presentation in cultured splenocytes in 1990 (Rock, Gamble et al. 1990). Antigen presenting cells (APCs) are critical cells of the immune system, which uptake antigen particles, process them and present them to antigen-specific T lymphocytes in the context of MHC or related proteins. Phagocytosis plays a crucial role in up-taking foreign antigens and presenting them on MHC class II molecules. Recent studies show that even though MHC class I molecules are normally loaded with peptides derived from cytosolic proteolysis, exogenous antigens can be presented on MHC class I as well. This non conventional pathway is called cross-presentation pathway (Mantegazza, Magalhaes et al. 2013).

## 4.1.Presentation of antigens through MHC class I and MHC class II

Endogenous antigens in the cytoplasm, nucleus and mitochondria of most cell types are presented on the context of MHC class I molecules in the classical pathway. Such antigens go through cytosolic proteolysis and are cleaved into peptides. Later on peptides translocate into the ER where they can encounter and assemble with newly synthesized MHC class I molecules (Savina, Peres et al. 2009). Cross-presentation has a different pathway in which exogenous antigens or soluble proteins are assembled on MHC class I in non-canonical pathway. The process of cross-presentation is restricted to dendritic cells *in vivo* while it can be observed in other cell types in culture as well (Rock, Gamble et al. 1990).

MHC class II molecule expression is limited to professional APCs such as, dendritic cells, macrophages and B lymphocytes and also certain activated epithelial cells. Before the sources of

cross-presented antigens were identified, phagosomes were thought to be responsible for providing antigens only for loading on MHC class II. Later on, it was shown that there is a sequential presentation of exogenous antigens by MHC class I and MHC class II respectively (Burgdorf, Scholz et al. 2008). According to this study exogenous antigens (phagocytosed antigens) can load on both MHC class I and class II molecules. The mechanism is not clear yet but the authors suggested that in the less acidic lumen of early phagosome, antigens preferably proceed to load on MHC class I molecules (Savina, Jancic et al. 2006) while later on in acidic phagosomes with increased proteolytic activity, they are processed to be presented by MHC class II molecules. Altogether, they proposed that phagocytosed antigens do not load only on MHC class II but also they load on MHC class I.

## 4.2. Molecular mechanism of MHC class II and cellular pathway of exogenous antigen presentation by MHC class II

MHC class II molecules have two integral membrane chains, α and β. In the ER, α and β chains associate with Ii, which is called invariant chain or CD74, and leave the ER. Blocking the peptide binding site with Ii prevents the binding of premature peptides on the MHC class II molecules (Cresswell 1996). Within late endosomal compartments, which in professional phagocytes are called MIIC (MHC class II-containing compartments), Ii is degraded by Class II-associated invariant chain peptide (CLIP) so that the peptide binding site is available in MHC class II molecules. Meanwhile internalized particles are degraded by proteases in the late endosomal compartment and they become ready to load on available binding sites of MHC class II molecules. HLA-DM, which is present in MIIC, mediates peptide exchange by dissociating CLIP and by doing so, the MHC class II binding pocket is free and available for a new cycle of

binding to new peptides (Xu, Wu et al. 2013). Finally, MHC class II : peptide complexes are exposed at the cell surface and they are ready to be recognized by Ag-specific $CD4^+$ T cells.

## 4.3. Molecular mechanism of MHC class I and cellular pathway of endogenous antigen presentation by MHC class I

MHC class I molecules are assembled in the ER and they are composed of two chains, the polymorphic α chain and the non-polymorphic β2 microglobulin (β2m) light chain. There is a N-linked oligosaccharide on the α chain, which inhibits the exit of immature MHC class I molecules from the ER. In addition, the assembly of MHC class I molecules is complete if only the chains bind to peptides in the peptide binding site of the molecule. The peptides that can bind to MHC class I molecules are between eight to nine amino acids long. The peptides enter ER through ATP-dependent TAP1/2 transporters. In the ER, they are trimmed at their N-terminal by the ER protease called ERAAPs (ER aminopeptidase associated with antigen processing). Transfer of trimmed peptides from TAP onto folding MHC class I is mediated by chaperons (Tapasin and calreticulin) (Peaper and Cresswell 2008). Once the assembly of chains and trimmed peptides is completed, the whole protein complex exits the ER through the classical pathway and is exposed on plasma membrane to be recognized by $CD8^+$ cytotoxic T lymphocytes (CTL).

## 4.4. Cross-presentation by MHC class I

Although some viruses can infect dendritic cells and load on MHC class I molecules by the classical pathway, in the rest of cases such as bacteria, parasites and viruses that do not infect dendritic cells, exogenous antigens are not processed and expressed by dendritic cells

themselves. In a significant number of these cases, it has been shown that dendritic cells can phagocytose antigens from pathogens and cross-present peptides derived from these antigens on their MHC class I molecules.

Here are two main models for the cross-presentation pathway. One of them is referred to as "vacuolar" pathway and the other is "cytosolic" pathway (Figure 15):

In the TAP and proteasome-dependent cytosolic pathway, antigens in the phagosomes enter to the cytosol via an Endoplasmic-Reticulum-Associated protein Degradation (ERAD)-associated retrotranslocon. Later on, antigens are ubiquitylated by E3 ubiquitin ligases in order to be targeted and degraded by the proteasome. The generated peptides from targeted antigens can either be transferred into the phagosome by TAP and loaded on MHC class I in the phagosome or they can proceed through classical TAP-dependent MHC class I antigen presentation in the ER (Mantegazza, Magalhaes et al. 2013).

The Endoplasmic-Reticulum-Golgi Intermediate Compartment (ERGIC) is a vesicle which carries TAP, ERAD and MHC class I molecules and transfers them from the ER to the phagosomes and play a role in phagosomal loading pathway. This interaction between ER/ERGIC is important for some pathogens like *Legionella*, *Brucella* and *Toxoplasma* because they actively target ER/phagosome pathway to survive (Cebrian, Visentin et al. 2011).

The TAP- and proteasome-independent "vacuolar" pathway is simpler than the other one. In this pathway antigens are phagocytosed and degraded in phagosomes through cathepsins, which are proteases mostly active at low pH. Generated peptides are loaded on MHC class I molecules within the phagosome (Mantegazza, Magalhaes et al. 2013).

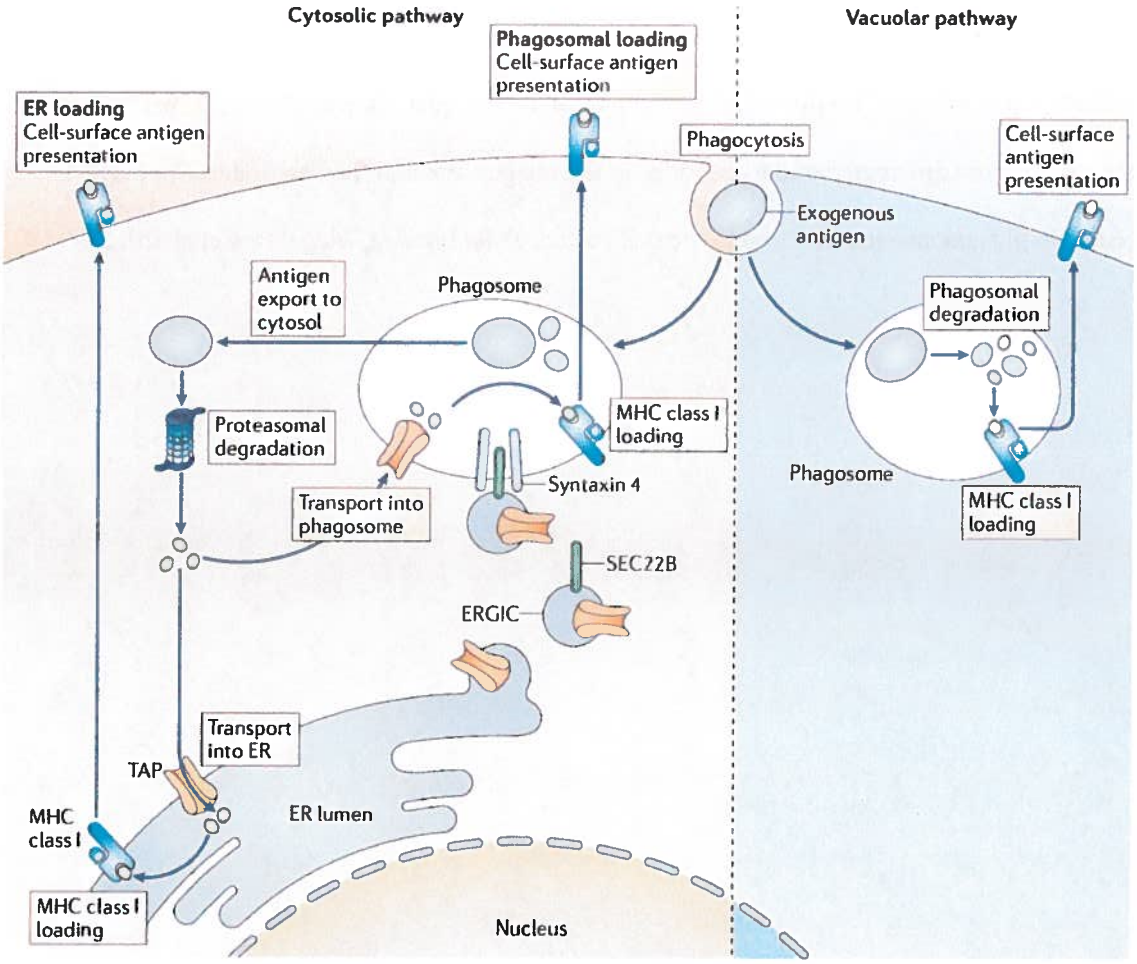

Cytosolic pathway
Vacuolar pathway
Phagosomal loading
Cell-surface antigen
presentation
ER loading
Cell-surface antigen
presentation
Phagocytosis
Cell-surface
antigen
presentation
Exogenous
antigen
Antigen
export to
cytosol
Phagosome
Phagosomal
degradation
Proteasomal
degradation
MHC class I
loading
Syntaxin 4
Phagosome
Transport into
phagosome
MHC class I
loading
SEC22B
ERGIC
Transport
into ER
TAP
ER lumen
MHC
class I
MHC class I
loading
Nucleus

**Figure 15) Two different intracellular pathways for cross-presentation.** Cytosolic pathway; After phagocytosis of exogenous antigens, they are processed by the proteasome. The processed antigens are loaded on MHC class I molecules in the ER or re-imported into the phagosome to be loaded on MHC class I molecules residing in phagosomes. Sec22b in interaction with syntaxin 4 on phagosomes, mediate the recruitment of TAP to phagosomes. Vacuolar pathway; exogenous antigens are degraded into peptides in the phagosome and then are loaded on MHC I molecules in phagosome and exposed to the cell surface (Mantegazza, Magalhaes et al. 2013).

### 4.4.1. Cross-presentation regulation

Cross-presentation requires conserved potential MHC class I binding epitopes. Hence protein degradation should not be high in dendritic cell phagosomes in order to protect the epitopes.

There are several factors that have effects on the efficiency of cross-presentation. In dendritic cells, NOX2 resides in secretory lysosomes that fuse with newly formed phagosomes under the control of Rab27A as a regulatory protein. Rab27A induces the fusion of "inhibitory lysosome-related organelles" to phagosomes. Phagosomes in Rab27A knock-down dendritic cells have a low level of NOX2 with a very acidic lumen and high antigen degradation activity. In these knock-down cells cross-presentation is impaired (Jancic, Savina et al. 2007). Mutation in *rab27a* causes Griscelli's syndrome in human and corresponding mouse model called ashen mice (*Rab27a*$^{ash}$). This syndrome is an autosomal recessive disorder characterized by albinism and immunodeficiency that usually causes death at early age (Sanal, Kucukali et al. 1993).

It has been shown that NOX2 regulates the phagosomal pH in dendritic cells and in this regard, there is a genetic disease (chronic granulomatous), which is caused by defect in the *nox2* gene; the patients have high phagosome acidification and impaired cross-presentation (Figure 16) (Hepp, Puri et al. 2005). Recently, it has been shown that phagosomal protease activity in dendritic cells can be regulated by NOX2 but is pH-independent. In this pathway NOX2 oxidazes the proteases directly. Also, they showed that there is an incomplete activation of V-

ATPase in dendritic cells, which keeps the lumen alkaline with low proteolytic activity to proceed with cross-presentation (Huynh and Grinstein 2007, Rybicka, Balce et al. 2012).

Particle size but not antigen load influences the cross-presentation pathway. It has been shown that, cross-presentation of small particles (0.8 μm) occurs through the phagosome-to-cytosol pathway, while for big particles (3μm) it involves the vacuolar pathway and that explains why small particles (0.8μm) are sensitive to proteasome inhibition and block the exit of MHC class I from ER whereas larger particles are resistant to proteasome inhibition. (Bethani, Werner et al. 2009).

Another factor that influences positively cross-presentation is targeting antigens to the receptors that are not delivered to lysosomes (lysosome is acidic and cleaves the antigens into small peptides so epitopes cannot be recognized by MHC class I anymore). Mannose receptor, DEC205, DNGR-1 or CD40L are categorized in this class of receptors (Ernst 2000).

Sec22b is another regulator of the cross-presentation pathway. Sec22b is an ERGIC protein and interacts with syntaxin 4 on the phagosome. Deletion of Sec22b inhibits the recruitment of ER-resident proteins to phagosomes where phagosomal loading of cross-presentation is impaired. It has been shown that siRNA-mediated knock down of Sec22b increases antigen degradation and inhibits cross-presentation (Cebrian, Visentin et al. 2011).

Also, it has been shown that in cross-presentation of viral antigens, gamma-interferon-inducible lysosomal thiolreductase (GILT), which is expressed in endosomal/lysosomal compartments, plays a critical role in cross-presentation (Cebrian, Visentin et al. 2011).

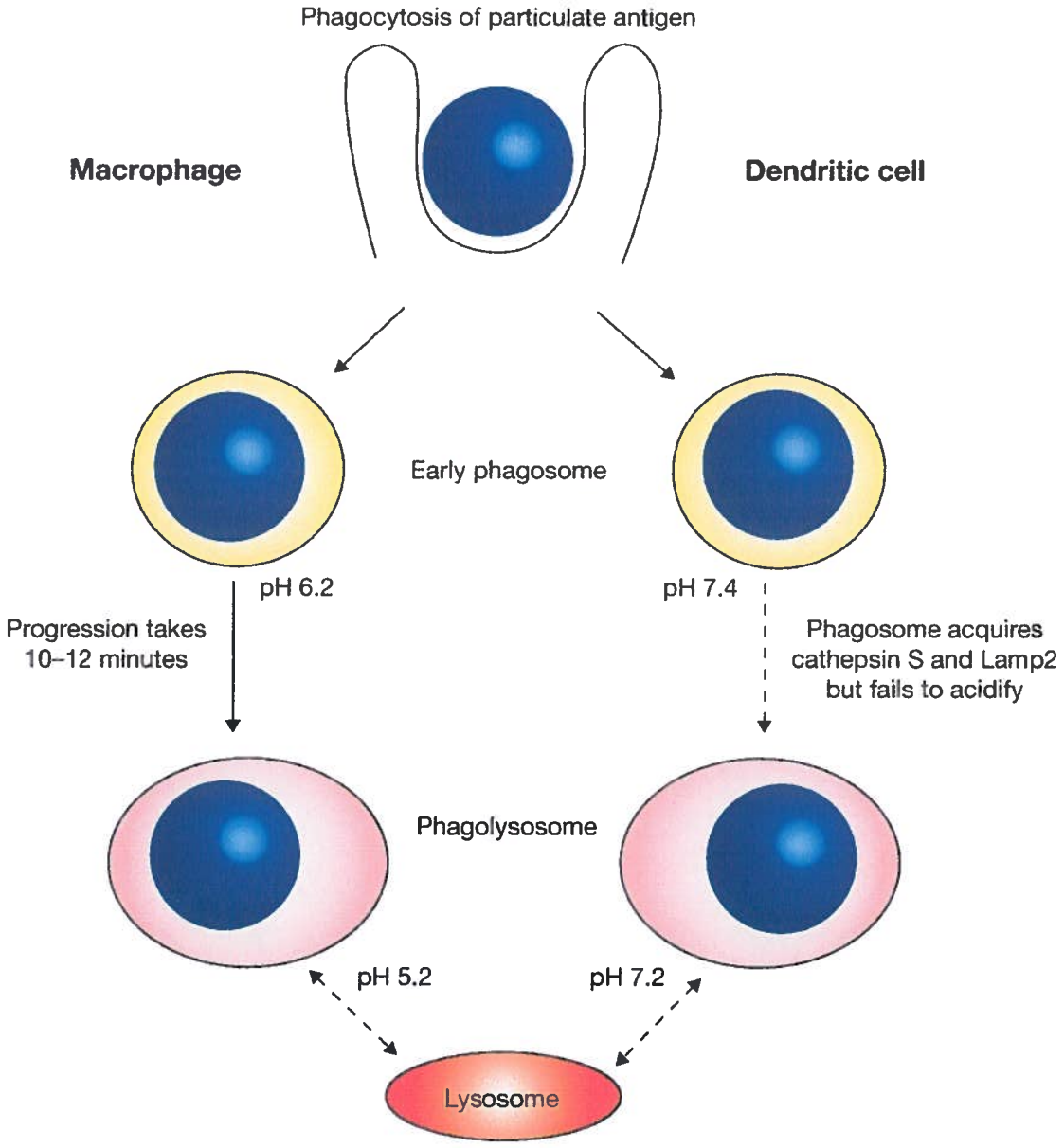

Phagocytosis of particulate antigen
Macrophage
Dendritic cell
Early phagosome
pH 6.2
pH 7.4
Progression takes
10–12 minutes
Phagosome acquires
cathepsin S and Lamp2
but fails to acidify
Phagolysosome
pH 5.2
pH 7.2
Lysosome

**Figure 16) Different phagosomal pH in dendritic cells and macrophages.** In macrophages, phagosomes acidify rapidly (in 10-12 min) and reach 5.2 while in dendritic cells, the pH is around 7.2 (Russell 2007).

## 4.5. Role of innate signaling on antigen presentation

As discussed earlier, phagosomes by receiving signals through phagosome receptors initiate actin and membrane remodeling to form phagosomes. Similarly, phagosome maturation and MHC class I/II processing and antigen presentation are also affected by signaling pathways. Some pathogens like *M. tuberclosis* interfere with intracellular signals and thereby block phagosome maturation and antigen presentation (Halle, Gomez et al. 2009).

Pattern recognition receptors (PRRs) play role in antigen presentation. PRRs include TLRs, Nod-like receptors (NLR) and C-type lectin receptors such as mannose receptor, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) and dectin-1. C-type lectin receptors recognize branched oligosaccharides on the surface of pathogens and tumor cells (Zhang, Czabotar et al. 2012). Also, phagocytic receptors for complement and immunoglobulin Fc regions, are not categorized as PRRs but they can detect and kill the pathogens through the involvement of opsonized microbes and cross-talk with PRRs (Rittirsch, Flierl et al. 2009). Signaling via PRRs stimulate dendritic cell maturation and in parallel modulate phagosome maturation and antigen presentation. The precise mechanisms that link innate cell signalling with phagosome maturation and antigen presentation are not clear yet (Mantegazza, Magalhaes et al. 2013).

# CHAPTER 2

# (Publications)

## Publication No.1

## *Leishmania* evades host immunity by inhibiting antigen cross-presentation through direct cleavage of the SNARE VAMP8

# *Leishmania* evades host immunity by inhibiting antigen cross-presentation through direct cleavage of the SNARE VAMP8

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*Running title: Leishmania* GP63 inhibits cross-presentation

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# SUMMARY

During phagocytosis, microorganisms are taken up by immune cells into phagosomes. Through membrane trafficking events mediated by SNARE proteins, phagosomes fuse with lysosomes, generating degradative phagolysosomes. Phagolysosomes contribute to host immunity by linking microbial killing within these organelles with antigen processing for presentation on MHC class I or II molecules to T cells. We show that the intracellular parasite *Leishmania* evades immune recognition by inhibiting phagolysosome biogenesis. The *Leishmania* cell surface metalloprotease GP63 cleaves a subset of SNAREs, including VAMP8. GP63-mediated VAMP8 inactivation or *Vamp8* disruption prevents the NADPH oxidase complex from assembling on phagosomes, thus altering their pH and degradative properties. Consequently, the presentation of exogenous *Leishmania* antigens on MHC class I molecules, also known as cross-presentation, is inhibited, resulting in reduced T cell activation. These findings indicate that *Leishmania* subverts immune recognition by altering phagosome function and highlight the importance of VAMP8 in phagosome biogenesis and antigen cross-presentation.


# HIGHLIGHTS

- The *Leishmania* metalloprotease GP63 cleaves phagosomal SNAREs
- Cleavage of VAMP8 enables *Leishmania* promastigotes to inhibit cross-presentation
- VAMP8 controls the recruitment of $gp91^{phox}$ to phagosomes
- VAMP8 controls the proteolytic activity of phagosomes

# INTRODUCTION

Phagocytosis, the process by which cells of the immune system such as macrophages and dendritic cells engulf microorganisms at sites of infection, leads to the formation of phagosomes where microbes are killed and processed for antigen presentation (Boulais, Trost et al. 2010, Botelho and Grinstein 2011, Joffre, Segura et al. 2012). Because phagosomes play a key role in both innate and adaptive immunity, the functional properties of these organelles and the molecular mechanisms regulating their interactions with pathogens has been the attention of a large number of studies in the last 25 years. These studies have highlighted the diversity of molecular strategies used by a variety of microorganisms to interfere with the maturation of phagosomes into phagolysosomes, a process referred to as phagolysosome biogenesis (Flannagan, Cosio et al. 2009, Alix, Mukherjee et al. 2011). Phagolysosome biogenesis is essential for the acquisition of the microbicidal properties required for the killing of microbes (Desjardins, Houde et al. 2005, Jutras and Desjardins 2005). This process is driven by complex sets of membrane trafficking events involving soluble *N*-ethylmaleimide-sensitive factor attachment receptors (SNAREs) proteins-regulated sequential fusion events between phagosomes and intracellular organelles including early endosomes, late endosomes and lysosomes (Stow, Manderson et al. 2006). These interactions allow the coordinated transfer of sets of hydrolases, as well as the acquisition of the oxidative machinery, involved in the killing of microorganisms and the processing of some of their proteins for antigen presentation on MHC class II molecules. Furthermore, the interaction between phagosomes and sub-regions of the endoplasmic reticulum plays a key role in the contribution of this organelle to cross-presentation,

the process by which exogenous microbial antigens are presented on MHC class I molecules (Jutras and Desjardins 2005, Campbell-Valois, Trost et al. 2012).

The protozoan *Leishmania* parasitizes phagocytic cells and causes a spectrum of human diseases known as leishmaniases. Once inoculated by sand fly vectors into mammals, promastigotes are taken up by macrophages. We previously reported that one mechanism used by promastigotes to evade the microbicidal consequences of phagocytosis is the inhibition of phagolysosome biogenesis (Desjardins and Descoteaux 1997). Hence, promastigotes are internalized in phagosomes that poorly interact with late endosomes and lysosomes and display a delayed recruitment of LAMP-1 (Scianimanico, Desrosiers et al. 1999, Dermine, Scianimanico et al. 2000, Spath, Garraway et al. 2003). Lipophosphoglycan (LPG), the major promastigote surface glycolipid, is responsible for this inhibition (Desjardins and Descoteaux 1997, Lodge and Descoteaux 2008). LPG inserts itself in the phagosome membrane where it destabilizes lipid microdomains (Dermine, Goyette et al. 2005), thereby impairing processes required for the generation of a microbicidal compartment within macrophages. One consequence of LPG-mediated microdomain disorganization is the exclusion of the membrane fusion regulator Syt V from the phagosome early after infection. This in turn abrogates recruitment of the V-ATPase and impedes phagosome acidification (Vinet, Fukuda et al. 2009). Targeting of the phagosome fusion machinery thus represents an efficient way for *Leishmania* promastigotes to create an intracellular niche favorable to the establishment of infection.

These findings led us to seek further insight into the impact of *Leishmania* promastigotes on the host cell fusion machinery that controls phagosome maturation and function. Here, we provide

evidence that *Leishmania* promastigotes impair phagosome maturation and antigen cross-presentation through the proteolytic cleavage of SNAREs.

# RESULTS

## *Leishmania* Promastigotes Exclude SNAREs from Phagosomes

Infection with the intracellular pathogen *Leishmania* is initiated when promastigotes are engulfed by phagocytic cells at the site of inoculation. The main survival strategy of this parasite is then to inhibit phagolysosome biogenesis. An important aspect of the molecular mechanisms enabling this inhibition relies on the exclusion of synaptotagmin V (Syt V), a key regulator of membrane fusion, from the phagosomal membrane (Vinet, Fukuda et al. 2009). In addition to Syt V, the phagosome proteome contains several Soluble NSF Attachment Protein Receptors (SNAREs) (Boulais, Trost et al. 2010). Hence, it is conceivable that multiple aspects of phagolysosome biogenesis, crucial for the acquisition of the microbicidal properties of this organelle and its ability to properly process proteins for antigen presentation, are regulated by series of membrane fusion regulators such as SNAREs. To determine whether *Leishmania* impairs the recruitment of other SNAREs, we infected bone marrow-derived macrophages (BMM) with *L. donovani* promastigotes and assessed the intracellular localization of VAMP3 and VAMP8, two SNAREs known to be present on phagosomes (Murray, Kay et al. 2005, Kay, Murray et al. 2006, Furuta, Fujita et al. 2010). Immunofluorescence analyses at the confocal microscope show that both VAMP3 and VAMP8 were present on zymosan-containing phagosomes at 2 h and 6 h after the initiation of phagocytosis (Fig. 1A). In contrast, these two SNAREs were absent from the majority of phagosomes containing wild-type (WT) *L. donovani* promastigotes (Fig. 1A). Because the virulence glycolipid LPG is the only known *Leishmania* factor responsible for phagosome remodeling (Desjardins and Descoteaux 1997, Scianimanico,

Desrosiers et al. 1999, Dermine, Duclos et al. 2001, Lodge, Diallo et al. 2006, Vinet, Fukuda et al. 2009), we tested whether this molecule was involved in the exclusion of VAMP3 and VAMP8 from *Leishmania*-containing phagosomes. Our results show that VAMP3 and VAMP8 are still excluded from phagosomes containing the LPG-defective Δ*lpg2 L. donovani* mutant, indicating that LPG is not responsible for the phagosomal exclusion of both SNAREs (Fig. 1A). This finding pointed towards the existence of an unsuspected mechanism used by *Leishmania* promastigotes to remodel their phagosomes. Intriguingly, we observed that VAMP3 and VAMP8 were not only excluded from phagosomes but that the intensity of the labeling for these proteins was also reduced throughout the cell in BMM infected with *L. donovani* promastigotes compared to BMM that had internalized Zym, or to uninfected BMM (Fig. 1A). Western blotting analyses confirmed that the amount of both proteins was much lower in infected cells. Furthermore, the appearance of a second band of lower molecular mass suggested that VAMP8 was actually cleaved in infected cells (Fig. 1B). Thus, the cleavage of key proteins involved in membrane trafficking may be part of a strategy used by *Leishmania* promastigotes to remodel their intracellular niche.

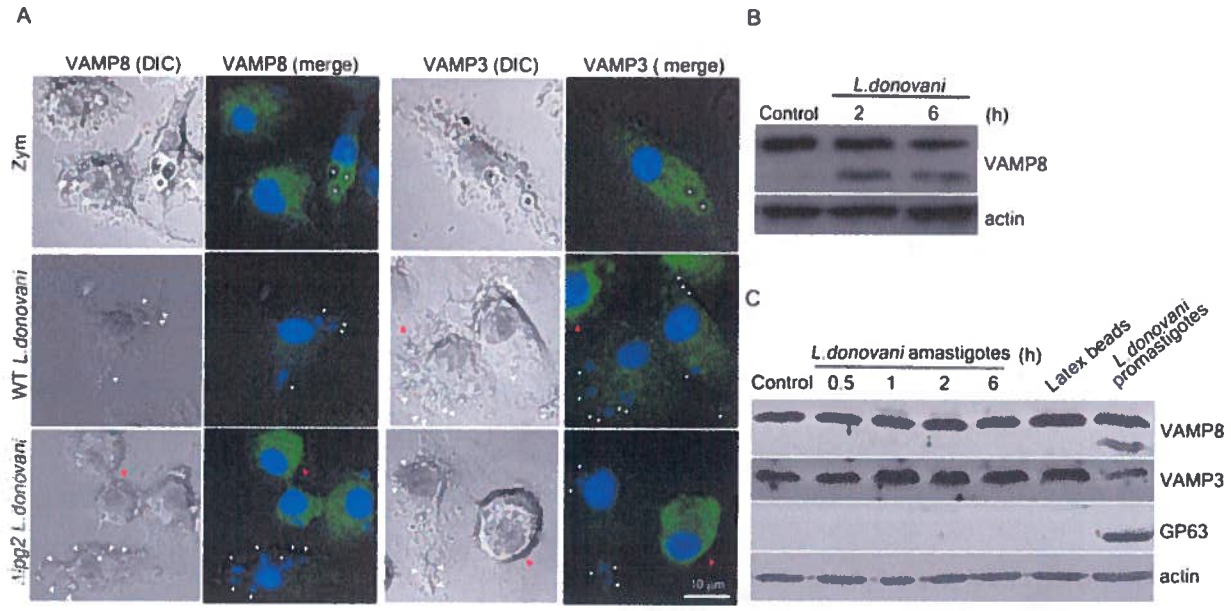



**Figure 1. *Leishmania* promastigotes target host cell SNAREs.** (**A**) Confocal microscopy images of BMM 2 h after internalization of either zymosan, wild-type (WT), or Δ*lpg2 L. donovani* promastigotes. VAMP3 and VAMP8 are in green, nuclei are in blue. Asterisks show phagosomes containing zymosan and white arrowheads show phagosomes containing *L. donovani*. Red arrowheads show uninfected cells. (**B**) VAMP8 and actin in lysates of BMM infected with *L. donovani* promastigotes for the indicated time points. (**C**) VAMP8, VAMP3, GP63, and actin in lysates of BMM infected with *L. donovani* amastigotes for the indicated time points. Controls consisted of BMM infected for 2 h with *L. donovani* promastigotes or with latex beads. Similar results were obtained in three independent experiments. See also Figure S1.

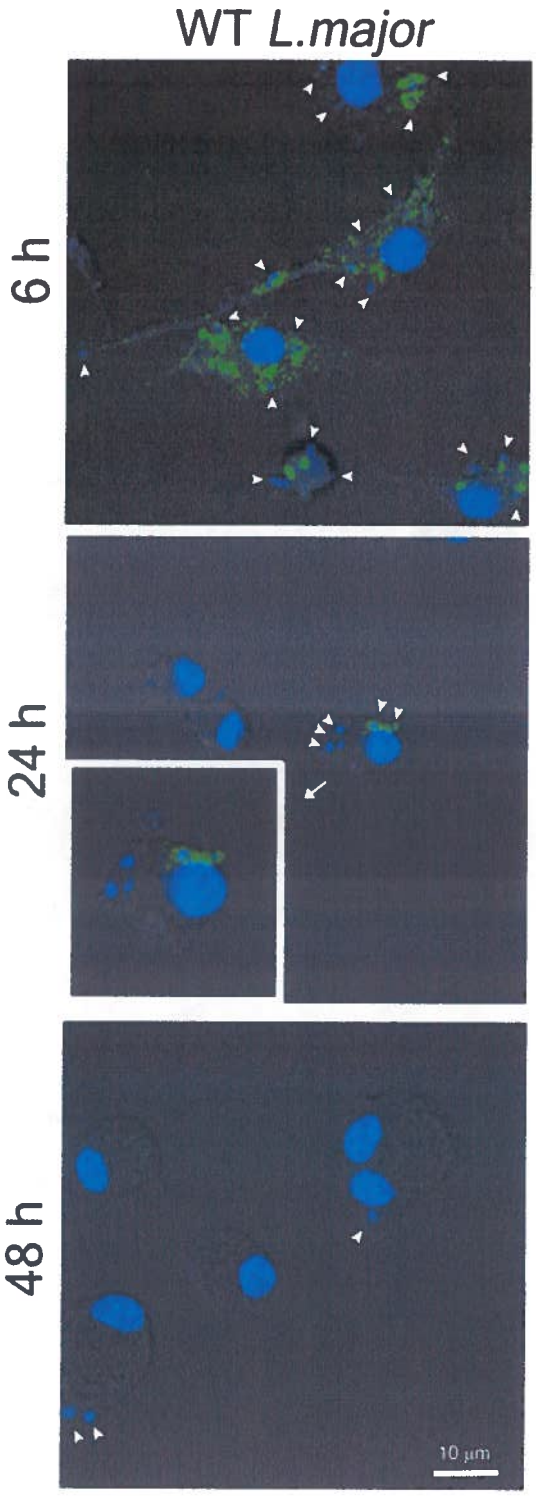

WT L.major
6 h
24 h
48 h
10 µm

**Figure S1. GP63 is present during the early phase of infection in BMM.** Representative confocal immunofluorescence images of showing the presence of GP63 in BMM 6, 24, and 48 h after internalization of WT *L. major* promastigotes. GP63 is in green, nuclei are in blue. White arrowheads show the parasites.

## GP63 Mediates the Cleavage of SNAREs

The cleavage of VAMP8 in *Leishmania*-infected BMM is reminiscent of that of SNAREs catalyzed by the zinc-dependent metalloproteases botulinium neurotoxins and antearase from scorpion venom (Fletcher, Fletcher et al. 2010, Montal 2010). We therefore investigated whether a *Leishmania* protease is involved in VAMP8 cleavage. The *Leishmania* genome encodes several proteases (Ivens, Peacock et al. 2005) including the surface GPI-anchored GP63, a zinc-dependent metalloprotease expressed at high levels in promastigotes (Etges, Bouvier et al. 1986). GP63 is a virulence factor that contributes to the pathogenesis of *Leishmania* by cleaving molecules controlling a variety of host defense mechanisms (Corradin, Ransijn et al. 1999, Joshi, Kelly et al. 2002, Gomez, Contreras et al. 2009, Halle, Gomez et al. 2009, Jaramillo, Gomez et al. 2011). During the differentiation of promastigotes into amastigotes, GP63 is down-regulated (Fig. S1). Interestingly, we did not observe cleavage of either VAMP8 or VAMP3 in BMM infected with *L. donovani* amastigotes and this corresponds to the absence of GP63 (Fig. 1C). These findings lent support to the possibility that GP63 was responsible for the cleavage of VAMP8 and VAMP3 in BMM infected with promastigotes. The availability of a Δ*gp63* mutant in *L. major* allowed us to directly test this hypothesis (Joshi, Kelly et al. 2002). We first showed that similar to *L. donovani*, infection with *L. major* promastigotes resulted in a cleavage of VAMP8 that was detectable as early as 30 min post-infection (Fig. 2A). Remarkably, VAMP8 remained intact in cells infected with the Δ*gp63* mutant (Fig. 2B). The ability to cleave VAMP8 was regained when cells were infected with the add-back mutant (Δ*gp63*+*gp63 L. major*) promastigotes. VAMP3 was also degraded in a GP63-dependent manner (Fig. 2B). By

incubating purified glutathione S-transferase (GST)-tagged VAMP8 with *L. major* promastigotes, we showed that GP63 directly cleaves VAMP8 (Fig. 2C). Collectively, these results identify GP63 as the parasite molecule responsible for the cleavage of host SNAREs during infection (Fig. 2 and Fig. S2). Using confocal microscopy, we confirmed that phagosomal exclusion of VAMP8 (Fig. 2D and 2E) and VAMP3 (not shown) is GP63-dependent. Our results thus far highlight a pathogenesis mechanism used by *Leishmania* parasites to control biogenesis of the parasitophorous vacuole, whereby GP63 targets SNAREs that regulate vesicular trafficking to and from phagosomes.

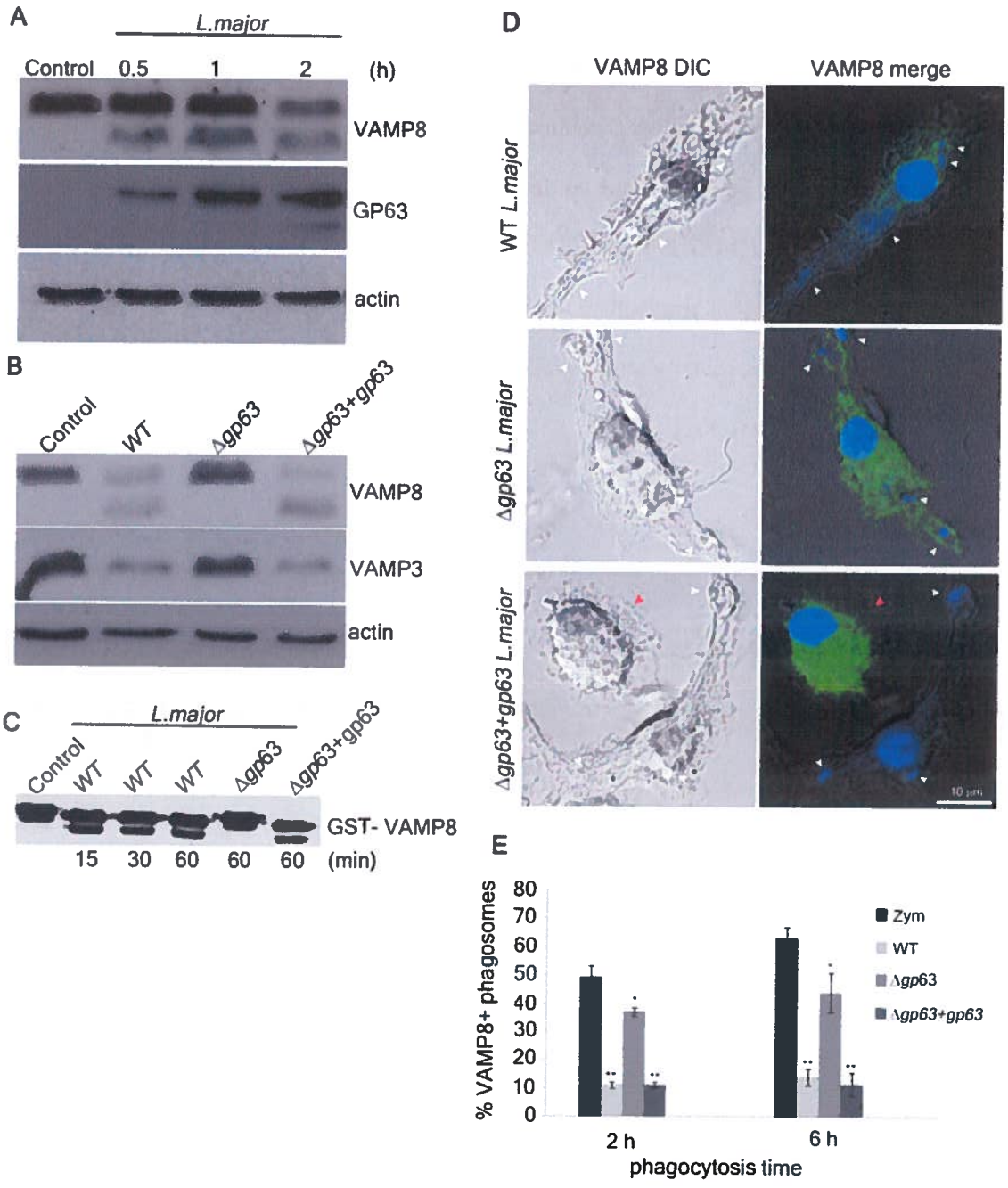

A
L.major
Control 0.5 1 2 (h)
VAMP8
GP63
actin
B
Control
WT
Δgp63
Δgp63+gp63
VAMP8
VAMP3
actin
C
L.major
Control
WT
WT
WT
Δgp63
Δgp63+gp63
GST- VAMP8
15 30 60 60 60 (min)
D
VAMP8 DIC
VAMP8 merge
WT L.major
Δgp63 L.major
Δgp63+gp63 L.major
E
% VAMP8+ phagosomes
80
70
60
50
40
30
20
10
0
Zym
WT
Δgp63
Δgp63+gp63
2 h
6 h
phagocytosis time

**Figure 2. GP63 mediates SNARE cleavage in infected BMM.** (**A**) Kinetics of VAMP8 cleavage by *L. major* and GP63 accumulation in lysates of BMM was assessed by immunoblot analysis. (**B**) VAMP8 and VAMP3 in lysates from BMM infected for 2 hr with WT, Δ*gp63*, or Δ*gp63*+*gp63 L. major* promastigotes. (**C**) GST-VAMP8 was incubated with *L. major* promastigotes and cleavage was assessed by immunoblot analysis. (**D**) Confocal microscopy images of BMM infected for 2 hr with WT, Δ*gp63*, or Δ*gp63*+*gp63 L. major* promastigotes. VAMP8 is in green, nuclei are in blue. White arrowheads show phagosomes containing *L. major*. Red arrowheads show uninfected cells. (**E**) Quantification of VAMP8-positive phagosomes at 2 and 6 hr after infection. Data are presented as the mean ± SD of three independent experiments. *, $p \leq 0.05$; **, $p \leq 0.005$. See also Figure S2.

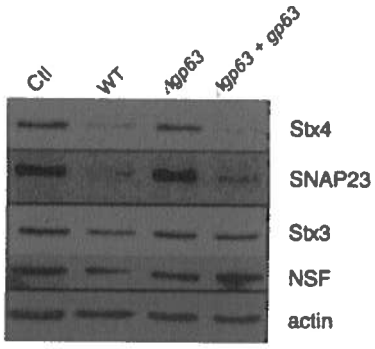



**Figure S2. *Leishmania* promastigotes selectively cleave additional SNAREs.** Lysates from BMM infected for 2 hr with WT, Δ*gp63*, or Δ*gp63*+*gp63 L. major* promastigotes were subjected to immunoblots analysis for the indicated SNAREs. Data are representative of two independent experiments.

## *Leishmania* Impairs Antigen Cross-presentation Through the Action of GP63

In addition to the killing of the parasite, the processing of *Leishmania* proteins for antigen presentation on both MHC class II molecules and MHC class I molecules during cross-presentation is a highly regulated process that requires molecular properties acquired during phagolysosome biogenesis (Jutras and Desjardins 2005). Although the precise molecular mechanisms involved in the processing of *Leishmania* proteins for cross-presentation are still poorly understood, it is assumed that parasite proteins are proteolytically cleaved in maturing phagosomes, generating peptides that can either be directly loaded on MHC class I molecules in phagosomes or translocated to the cytoplasm for further processing by the proteasome (Bertholet, Debrabant et al. 2005, Bertholet, Goldszmid et al. 2006). Hence, trafficking events involved in phagolysosome biogenesis are likely to play key roles in cross-presentation during *Leishmania* infection. In that context, we elected to study whether the GP63-dependent cleavage of SNAREs observed during infection alters key immune processes such as cross-presentation in BMM and BMDC (bone marrow-derived dendritic cells).

To test this hypothesis in the context of *Leishmania* infection, we produced WT *L. major* and Δ*gp63 L. major* expressing ovalbumin (*L. major*-OVA) to measure the cross-presentation of the OVA antigen on MHC class I molecules. BMM and BMDC were infected with promastigotes before the addition of OVA-specific $CD8^+$ OT-I T cells and cross-presentation was assessed through the expression of the early T cell activation marker CD69. Negative

controls consisted of *L. major* promastigotes not expressing OVA. Our results showed that cross-presentation was significantly higher in BMM and BMDC infected with Δ*gp63 L. major*-OVA promastigotes compared to cells infected with WT parasites (Fig. 3A, 3B), demonstrating that GP63 endows these parasites with the ability to alter cross-presentation. A key question that arose at this point was whether the action of GP63 was restricted to phagosomes containing parasites, or if the whole cellular machinery of cross-presentation was altered. We addressed this issue by showing that VAMP8 present on latex bead-containing phagosomes formed in cells infected with WT and Δ*gp63*+*gp63 L. major* promastigotes was also cleaved (Fig. 3C). This result allowed us to test whether the cleavage of VAMP8 by GP63 is sufficient to alter cross-presentation by measuring the presentation of the SIINFEKL peptide following the internalization of ovalbumin-coated latex beads in infected cells. Indeed, cross-presentation from OVA-coated beads was greatly diminished in BMM and BMDC infected with WT promastigotes compared to uninfected cells or cells infected with the Δ*gp63* mutant (Fig. 3D). Infection with the GP63 add-back parasites (Δ*gp63* + *gp63*) restored the ability of the Δ*gp63* mutant to inhibit cross-presentation, highlighting the global effect of GP63 on this immune process. Interestingly, the non-pathogenic parasite *L. tarentolae*, in which GP63 is inactive (Campbell, Kurath et al. 1992), was unable to cleave VAMP8 and to inhibit cross-presentation (Fig. 3B, D). The inhibition of cross-presentation by *L. major* promastigotes was not caused by a decreased MHC class I expression as shown by SIINFEKL peptide loading control experiment (Fig 3D), or by a defect in phagocytosis (Fig. S3). Furthermore, infection with *Leishmania* promastigotes had no noticeable effect on the phagosomal recruitment of molecules involved in the antigenic peptide loading on MHC class I (Fig. 3E). Importantly, amastigotes, in which GP63 is down-modulated (Fig. 1C), did not inhibit cross-presentation (Fig. 3F). Collectively,

these results show that a microbial molecule, GP63, directly impairs the ability of infected cells to crosspresent exogenous peptides on MHC class I molecules. These results also support the hypothesis that *Leishmania* promastigotes transiently alter antigen cross-presentation by the degradation of a restricted set of molecules including SNAREs. Thus far, our study also indicates that the well-established latex bead system can be used to decipher the molecular mechanisms enabling GP63 to cleave VAMPs, and the potential role played by these proteins in the regulation of cross-presentation.

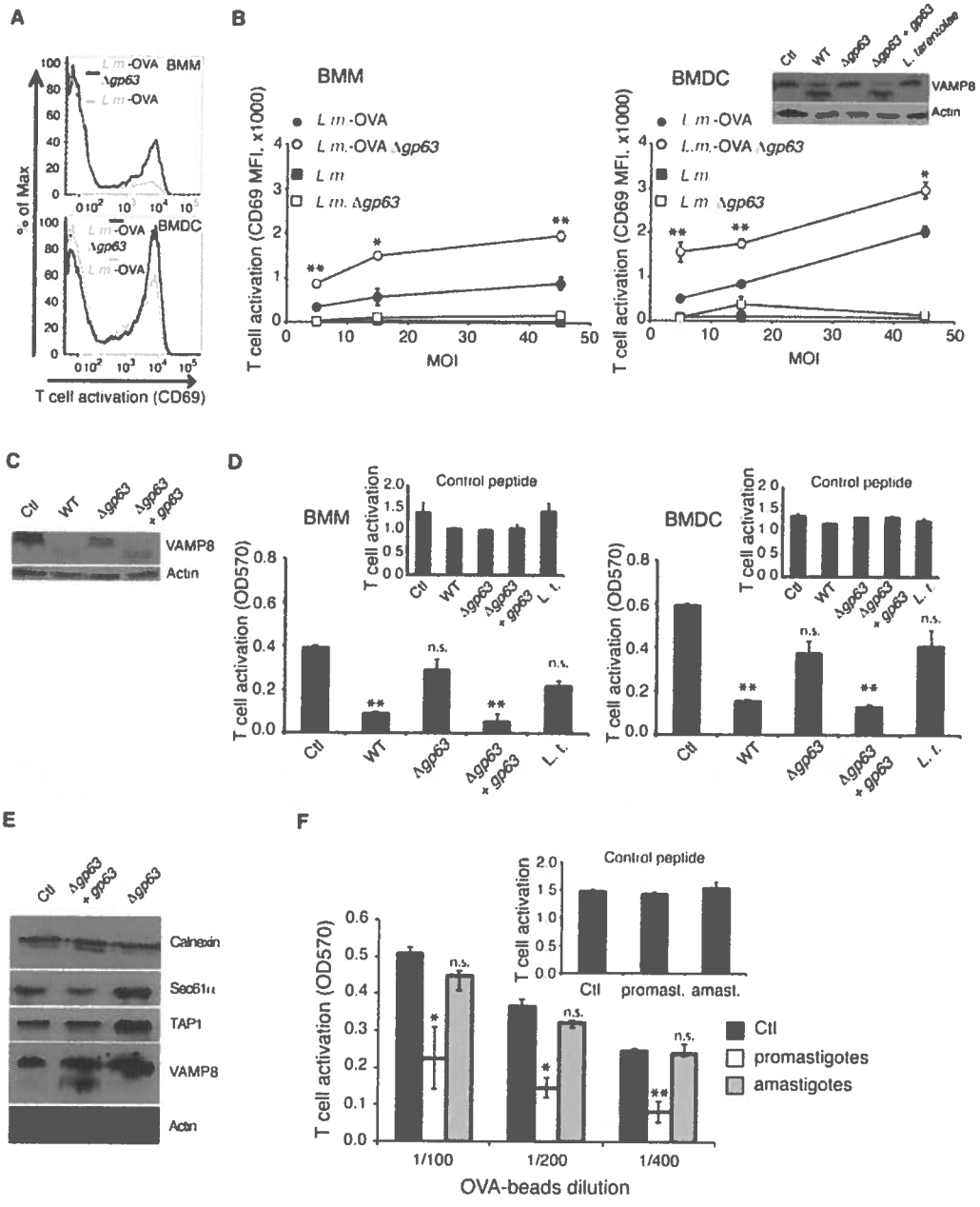

A
B
C
D
E
F
BMM
BMDC
L m-OVA
L m-OVA Δgp63
L m
L m Δgp63
% of Max
T cell activation (CD69)
T cell activation (CD69 MFI, x1000)
MOI
Ctl
WT
Δgp63
Δgp63 + gp63
L. tarentolae
VAMP8
Actin
T cell activation (OD570)
Control peptide
T cell activation
n.s.
Calnexin
Sec61α
TAP1
promast.
amast.
promastigotes
amastigotes
1/100
1/200
1/400
OVA-beads dilution

**Figure 3. *Leishmania* promastigotes inhibit cross-presentation in a GP63-dependent manner.** (**A**) Representative FACS histograms of CD8 OT-I T cells CD69 expression after culture with BMM or BMDC infected with WT or Δ*gp63 L. major*-OVA promastigotes. (**B**) Cells infected with WT or Δ*gp63 L. major*-OVA promastigotes or with nontransfected parasites were incubated with CD8 OT-I T cells and cross-presentation was assessed by CD69 expression. Right upper-panel shows VAMP8 and actin in noninfected BMDC or infected with WT, Δ*gp63*, Δ*gp63*+*gp63 L. major* or *L. tarentolae* promastigotes. Ctl, control (**C**) VAMP8 in latex bead-containing phagosomes isolated from noninfected cells (Ctl) or infected with WT, Δ*gp63* or Δ*gp63*+*gp63* promastigotes. (**D**) Cells were infected with WT, Δ*gp63*, Δ*gp63*+*gp63 L. major* or *L. tarentolae* promastigotes or left untreated (Ctl). OVA-coated beads or SIINFEKL peptide (assessment of surface MHC class I expression, upper graph) were added and cross-presentation and MHC I expression were evaluated using the B3Z T cell hybridoma. (**E**) Immunoblot analysis of molecules involved in the MHC class I machinery (calnexin, Sec61α, TAP1) and VAMP8 in non infected cells (Ctl) or cells infected with Δ*gp63* or Δ*gp63*+*gp63 L. major* promastigotes. (**F**) BMDCs were infected with WT *L. donovani* promastigotes (promast.) or amastigotes (amast.), or left untreated (Ctl). OVA-coated beads or SIINFEKL peptide (assessment of surface MHC class I expression, upper graph) was then added. Cross-presentation and MHC class I expression were evaluated using B3Z T cell hybridoma. Data are presented as the mean ± SEM of one experiment representative of at least two independent experiments. *, $p \leq 0.05$; **, $p \leq 0.01$. See also Figure S3.

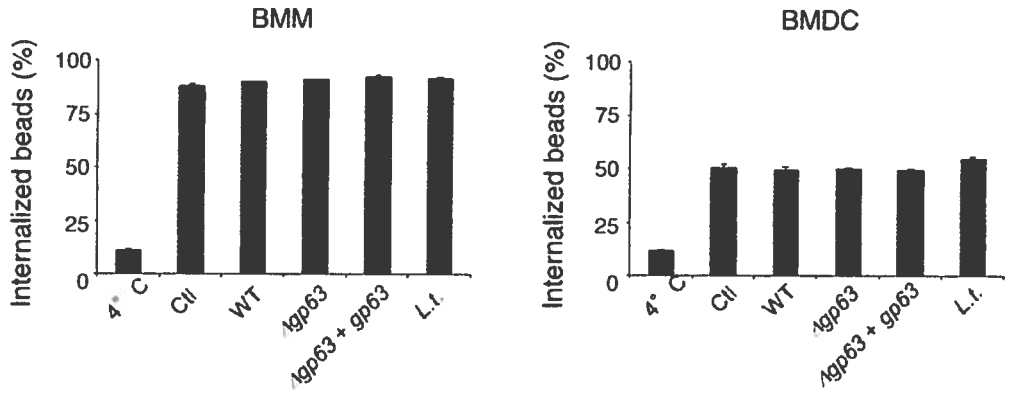



**Figure S3. Phagocytosis is not affected in infected cells.** BMM or BMDC were infected with WT, Δ*gp63*, Δ*gp63*+*gp63 L. major* or *L. tarentolae* promastigotes or left untreated (Ctl). They were cultured 1 h in the presence of 1.9 μm fluorescent latex beads. Phagocytosis was assessed by FACS and surface non internalized beads were excluded. Data are presented as the mean ± SEM of one experiment representative of two independent experiments.

## VAMP8 is required for Antigen Cross-presentation

Thus far, our results suggest that the cleavage of SNAREs by GP63 might be responsible for the alteration of cross-presentation in *Leishmania*-infected cells. Whereas VAMP3 regulates cytokine secretion at the phagocytic cup (Murray, Kay et al. 2005), little is known about the role of VAMP8 in phagosome function. However, VAMP8 is present on late endosomes where it controls homotypic fusion between these organelles (Pryor, Mullock et al. 2004). Considering the importance of the regulation of fusion events during phagolysosome biogenesis, including phagosome-late endosome fusion (Desjardins, Nzala et al. 1997), we investigated whether VAMP8 is required for cross-presentation. To this end, we used BMM and BMDC from VAMP8 null mice (Wang, Ng et al. 2004). Using our OVA-coated bead assay, we observed a decrease of around 50% in the level of cross-presentation in *Vamp8*$^{-/-}$ BMDC and BMM compared to WT cells (Fig. 4A). This inhibition was not the consequence of a reduced MHC class I expression (Fig. 4A, inset), or due to a defect in phagocytosis (Fig. S4A) in *Vamp8*$^{-/-}$ cells. In addition, the absence of VAMP8 did not significantly affect the recruitment of the machinery involved in the antigenic peptide loading on MHC class I to phagosomes (Fig. S4B). To determine whether the effect of GP63-mediated cleavage of VAMP8 on cross-presentation was specific, we assessed the impact of GP63 and VAMP8 on MHC class II-restricted antigen presentation in BMDC. Interestingly, we found that this process was also inhibited by GP63 (Fig. S4C). However, in contrast to cross-presentation, MHC class II-restricted antigen presentation was normal or even slightly superior in the absence of VAMP8 (Fig. S4D). These

results strongly support the proposal that VAMP8 is one of the key molecules targeted by *Leishmania* to specifically alter cross-presentation.

Our data indicate that the role of VAMP8 in cross-presentation within phagosomes can be tested either in uninfected *Vamp8*$^{-/-}$ BMDC and BMM, or/and in *Leishmania*-infected WT BMDC and BMM. Accordingly, we hypothesized that the phagosome functional properties observed in *Vamp8*$^{-/-}$ cells should be similar to those observed in *Vamp8*$^{+/+}$ cells infected with WT *L. major* promastigotes (a condition that degrades VAMP8). Based on this assumption, we first compared phagosomal acidification (a hallmark of phagosome maturation). As shown in Figure 4B, the phagosomal pH was significantly lower in *Vamp8*$^{-/-}$ BMDC at all time points evaluated after phagocytosis compared to *Vamp8*$^{+/+}$ BMDC. Similarly, the phagosomal pH was significantly lower in WT *L. major*-infected *Vamp8*$^{+/+}$ BMDC compared to uninfected BMDC or to BMDC infected with the Δ*gp63 L. major* mutant (Fig. 4B). From these results, we concluded that the presence of VAMP8 is required to maintain a near neutral pH in BMDC phagosomes.

The pH in the phagosome lumen influences directly the proteolytic activity and antigen processing within this organelle (Delamarre, Pack et al. 2005). We therefore determined whether the observed decrease in antigen cross-presentation in *Leishmania*-infected WT BMDC and in *Vamp8*$^{-/-}$ BMDC was accompanied by a defect in phagosomal proteolytic activity. Degradation of the general protease substrate DQ-ovalbumin was significantly increased in *Vamp8*$^{-/-}$ BMDC as well as in *Vamp8*$^{+/+}$ BMDC infected with WT *L. major*, when compared to uninfected and to Δ*gp63 L. major*-infected *Vamp8*$^{+/+}$ BMDC (Fig. 4C). The processing of antigens for cross-presentation in phagosomes is also tightly controlled by the oxidation level generated through the NADPH oxidase activity. Conflicting reports either link the control of the proteolytic activity

to a direct effect of the NADPH oxidase on the phagosome pH level (Savina, Jancic et al. 2006), or through the redox modulation of local cysteine cathepsins (Rybicka, Balce et al. 2011). Remarkably, treatment with the NADPH oxidase complex inhibitor DPI reversed the phagosomal proteolytic activity in *Vamp8*$^{+/+}$ BMDC to the levels observed in *Vamp8*$^{-/-}$ BMDC (Fig. 4C). Treatment of Δ*gp63 L. major*-infected cells with DPI also led to the levels of phagosomal proteolytic activity observed in WT *L. major*-infected cells. These observations suggest that phagosomal oxidative activity is impaired in *Vamp8*$^{-/-}$ BMDC and in WT *L. major*-infected cells, which may be responsible for the increased antigen degradation in those cells.

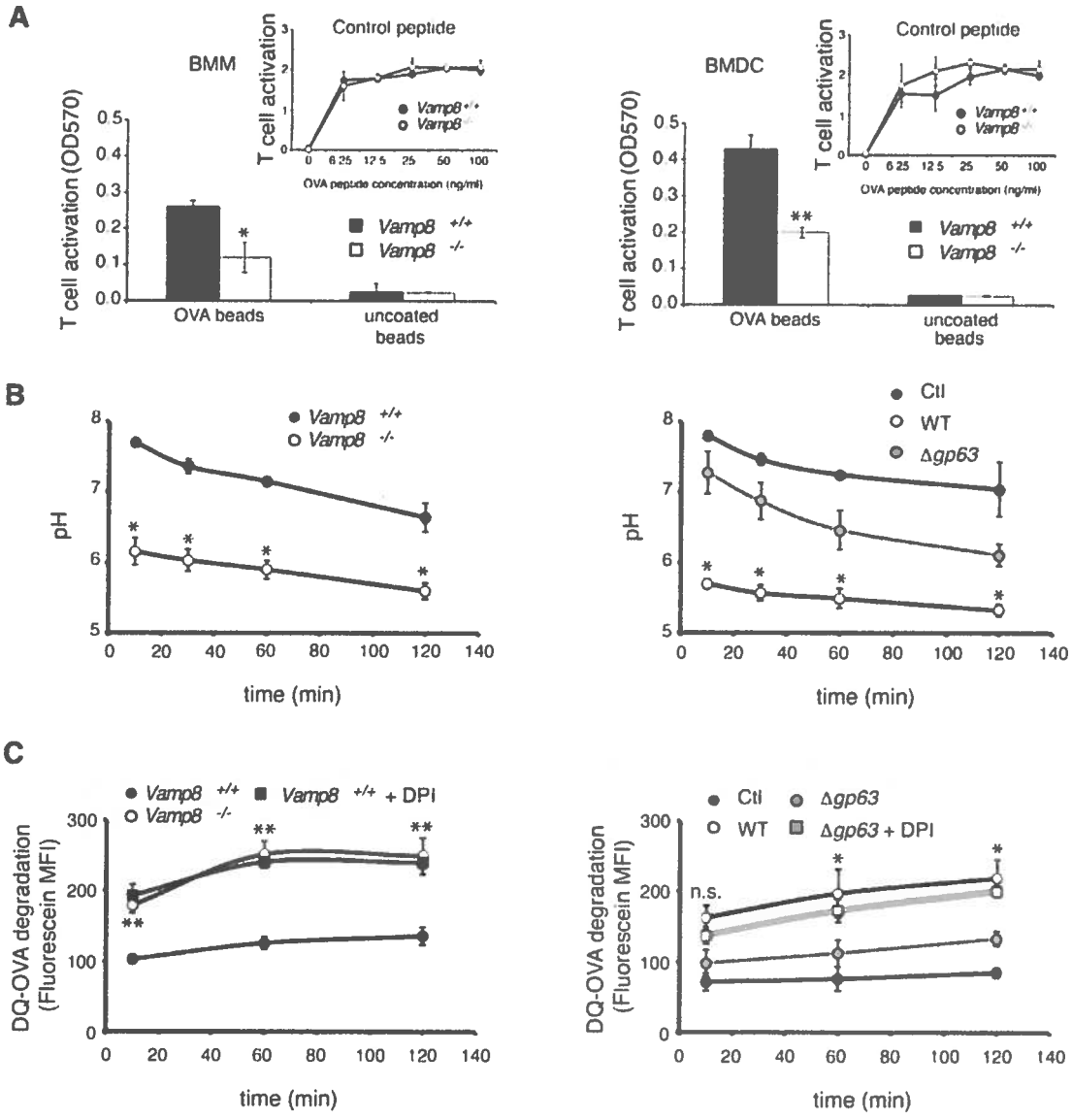
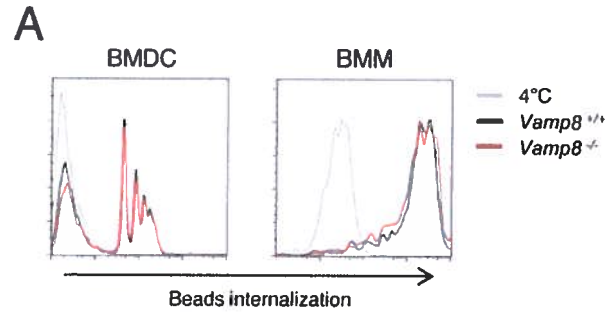
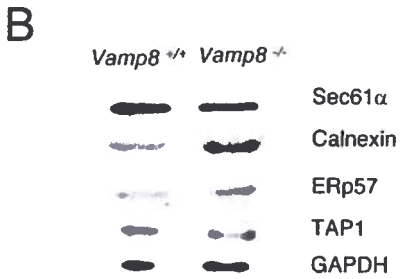
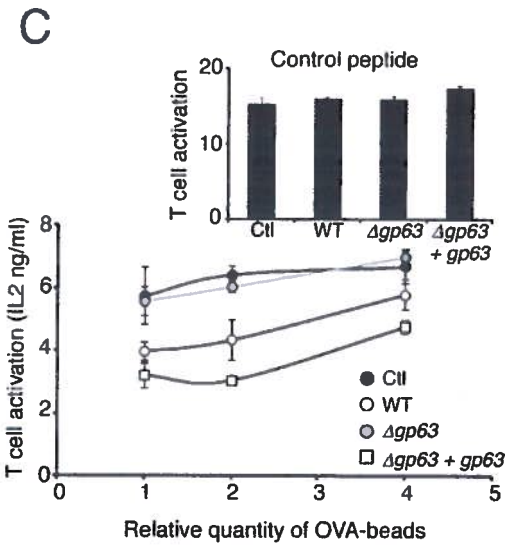

A
BMM
Control peptide
T cell activation
Vamp8 +/+
Vamp8 -/-
OVA peptide concentration (ng/ml)
T cell activation (OD570)
OVA beads
uncoated beads
BMDC
B
pH
time (min)
Ctl
WT
Δgp63
C
DQ-OVA degradation (Fluorescein MFI)
Vamp8 +/+ + DPI
Δgp63 + DPI
n.s.

**Figure 4. VAMP8 regulates antigen cross-presentation through the control of phagosome acidification and antigen degradation.** (**A**) *Vamp8*$^{+/+}$ and *Vamp8*$^{-/-}$ BMMs or BMDCs were fed with OVA-coated beads or SIINFEKL peptide (upper graph). Cross-presentation and MHC class I expression were evaluated using B3Z T cell hybridoma. (**B**) *Vamp8*$^{+/+}$ and *Vamp8*$^{-/-}$ BMDCs (left panel) and *Vamp8*$^{+/+}$ BMDCs infected with WT or *Δgp63 L. major* promastigotes (right panel) were fed with FITC-coated beads to evaluate phagosomal pH. (**C**) *Vamp8*$^{+/+}$ and *Vamp8*$^{-/-}$ BMDCs (left panel) and *Vamp8*$^{+/+}$ BMDCs infected with WT or *Δgp63* promastigotes (right panel) were fed with OVA-DQ-coated beads to assess phagosomal proteolysis. Data are presented as the mean ± SEM of one experiment representative of at least three independent experiments. *, $p \leq 0.05$; **, $p \leq 0.01$. See also Figure S4.


A
BMDC
BMM
4°C
Vamp8 +/+
Vamp8 -/-
Beads internalization


B
Vamp8 +/+
Vamp8 -/-
Sec61α
Calnexin
ERp57
TAP1
GAPDH


C
Control peptide
T cell activation
Ctl
WT
Δgp63
Δgp63 + gp63
T cell activation (IL2 ng/ml)
Relative quantity of OVA-beads

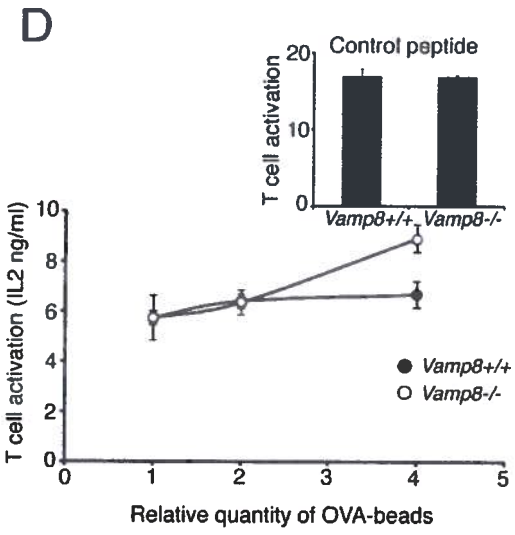

D
Control peptide
T cell activation
Vamp8+/+
Vamp8-/-
T cell activation (IL2 ng/ml)
Relative quantity of OVA-beads

**Figure S4. Impact of VAMP8 on phagocytosis, MHC class I machinery, and MHC class II presentation. (A)** Phagocytosis is not affected in *Vamp8*$^{-/-}$ cells. *Vamp8*$^{+/+}$ and *Vamp8*$^{-/-}$ BMM or BMDC were culture 1 h in the presence of 1.9 µm fluorescent latex beads. Phagocytosis was assessed by FACS and non-internalized beads were excluded. **(B)** MHC class I machinery recruitment is not affected in *Vamp8*$^{-/-}$ BMDC. Immunoblot analysis of molecules involved in the MHC class I machinery (Calnexin, Sec61, TAP1, ERp57) in *Vamp8*$^{+/+}$ and *Vamp8*$^{-/-}$ cells. **(C)** MHC class II presentation is altered after *Leishmania* infection through the action of GP63. BMDC were infected with WT, Δ*gp63*, Δ*gp63+gp63 L. major* promastigotes or left untreated (Ctl). OVA-coated beads or $OVA_{323-339}$ peptide (for assessment of surface MHC class II expression, upper graphics) were then added. MHC class II presentation and expression were evaluated using BO97 T cell hybridoma. **(D)** MHC class II presentation is not altered in *Vamp8*$^{-/-}$ cells. *Vamp8*$^{+/+}$ and *Vamp8*$^{-/-}$ BMDC were fed with OVA-coated beads or $OVA_{323-339}$ peptide (upper graphics). MHC class II presentation and expression were evaluated using BO97 T cell hybridoma. Data are presented as the mean ± SEM of one experiment representative of two independent experiments.

## VAMP8 Regulates Phagosomal Oxidative Activity

To verify the possible involvement of VAMP8 in the regulation of phagosomal oxidative activity, BMDC that internalized DHR-coated beads were analyzed by FACS for ROS production. The phagosomal oxidative activity observed in *Vamp8*$^{+/+}$ BMDC was inhibited by DPI, indicating that it was predominantly generated by the NADPH oxidase complex. In contrast, phagosomal oxidative activity was defective in *Vamp8*$^{-/-}$ BMDC and was not further reduced by DPI (Fig. 5A). Similar to *Vamp8*$^{-/-}$ BMDC, phagosomal oxidative activity was impaired in *Vamp8*$^{+/+}$ BMDC infected with WT *L. major* promastigotes in a GP63-dependent manner (Fig. 5A). We concluded that VAMP8 is required for phagosomal oxidative activity and that its degradation by GP63 may be responsible for the alteration of oxidative activity in WT *L. major*-infected BMDC.

To further understand the mechanisms possibly linking VAMP8 to the phagosomal oxidative activity, we assessed whether this SNARE participates in the recruitment of the NADPH oxidase complex subunits to phagosomes. A key component of this complex, gp91$^{phox}$, is found in late endosomes called "inhibitory lysosome-related organelles" in reference to the ability of these organelles to regulate phagosomal proteolytic activity after NADPH complex recruitment (Savina, Jancic et al. 2006). VAMP8 also localizes to late endosomes (Pryor, Mullock et al. 2004). Immunofluorescence analyses indicate that VAMP8 and gp91$^{phox}$ indeed partially co-localize in BMDC (Fig. 5B) and that in the absence of VAMP8, the recruitment of gp91$^{phox}$ to phagosomes was strongly inhibited (Fig. 5C). To analyze further the impact of VAMP8 on the recruitment of components of the NADPH oxidase to phagosomes, we purified phagosomes from BMDC at different time points after the initiation of phagocytosis. Consistent with the

results obtained by confocal microscopy (Fig. 5C), we observed an inhibition of gp91$^{phox}$ recruitment in *Vamp8*$^{-/-}$ phagosome extracts, whereas recruitment of the others subunits was not altered (Fig. 5D). These results indicate that VAMP8 is required for the recruitment of gp91$^{phox}$ to phagosomes, a process that plays a key role in the regulation of antigen processing for cross-presentation. In agreement with these observations, DPI inhibited cross-presentation in *Vamp8*$^{+/+}$ BMDC as well as in cells infected with Δ*gp63 L. major* promastigotes (Fig. 5E). In contrast, DPI treatment did not further inhibit cross-presentation in either *Vamp8*$^{-/-}$ or gp91$^{phox}$ KD BMDC and WT *L. major*-infected *Vamp8*$^{+/+}$ BMDC (Fig. 5E), consistent with the fact that phagosome oxidation is already impaired in these cells (Fig. 5A). To test whether our results obtained with OVA-coated beads were also relevant in *Leishmania* phagosomes, we infected BMDC with WT or Δ*gp63 L. major*-OVA promastigotes and measured OT-I T cells activation. Figures S5A and S5B show that DPI inhibited cross-presentation of *Leishmania*-derived OVA in BMDC infected with Δ*gp63 L. major*-OVA promastigotes, and no further inhibition was observed in WT *L. major*-OVA promastigotes infected cells. Furthermore, compared to normal BMDC, *Vamp8*$^{-/-}$ BMDC infected with Δ*gp63 L. major*-OVA promastigotes displayed a strong defect for the activation of OT-I T cells, and no further inhibition of cross-presentation was observed when we added DPI (Fig. S5C and S5D). Collectively, our results indicate that cleavage of VAMP8 enables *Leishmania* promastigotes to inhibit cross-presentation by impairing the recruitment of gp91$^{phox}$ to phagosomes, a key step for the control of phagosomal proteolysis. Consistently, absence of VAMP8 resulted in an increase susceptibility to *L. major* infection in a mouse model of cutaneous leishmaniasis (Fig. S5F).

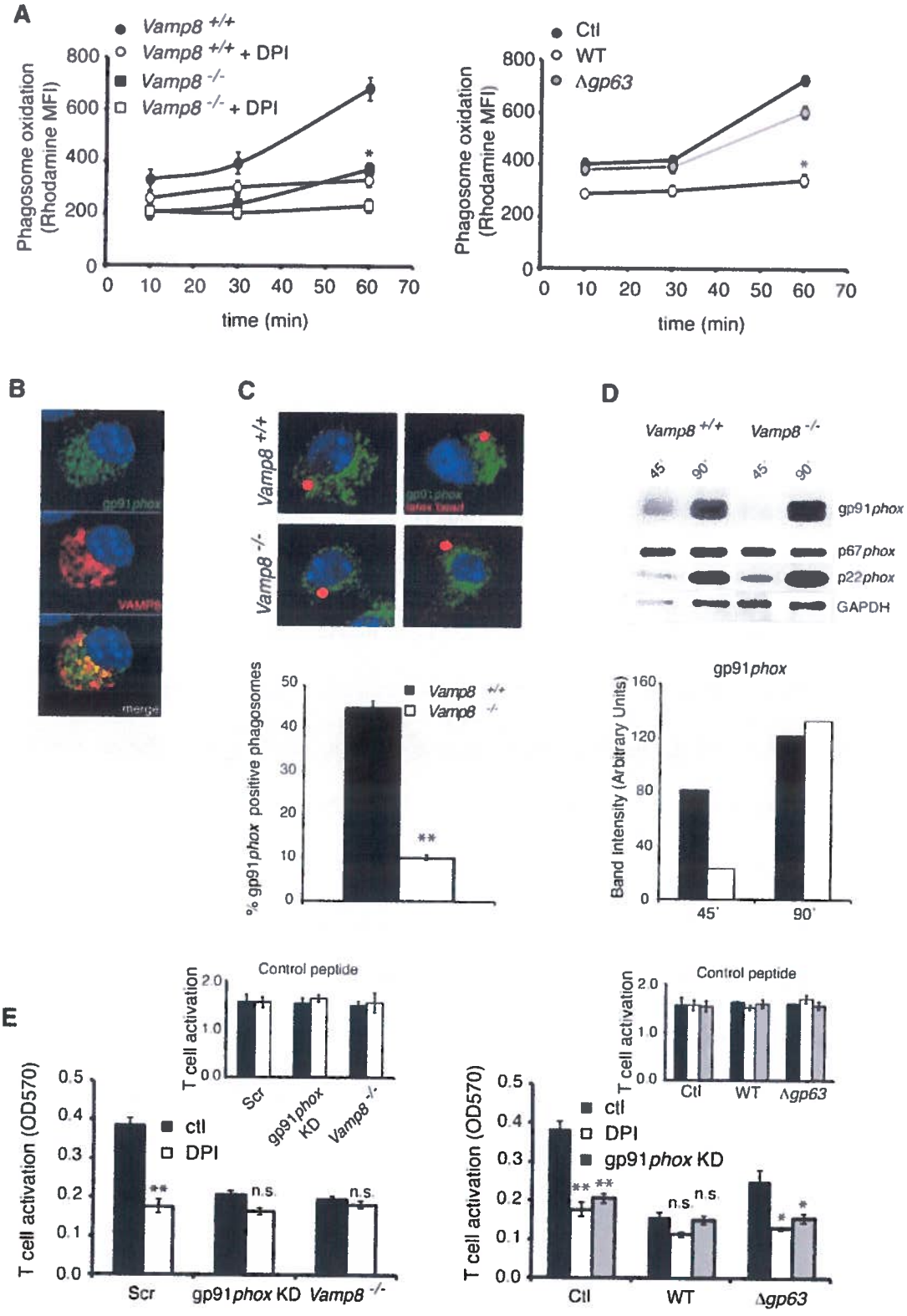

A
Vamp8 +/+
Vamp8 +/+ + DPI
Vamp8 -/-
Vamp8 -/- + DPI
Phagosome oxidation (Rhodamine MFI)
time (min)
Ctl
WT
Δgp63
B
gp91phox
VAMP8
merge
C
Vamp8 +/+
Vamp8 -/-
% gp91phox positive phagosomes
D
Vamp8 +/+
Vamp8 -/-
45'
90'
gp91phox
p67phox
p22phox
GAPDH
Band Intensity (Arbitrary Units)
E
Control peptide
T cell activation
Scr
gp91phox KD
Vamp8 -/-
ctl
DPI
T cell activation (OD570)
n.s.
Ctl
WT
Δgp63
gp91phox KD

**Figure 5. gp91$^{phox}$ is inefficiently recruited to the phagosome in *Vamp8*$^{-/-}$ cells.** (**A**) *Vamp8*$^{+/+}$ and *Vamp8*$^{-/-}$ cells (left panel) or *Vamp8*$^{+/+}$ cells infected with WT or *Δgp63 L. major* promastigotes (right panel) were fed with DHR-coated beads and assayed for phagosome oxidation. (**B**) gp91$^{phox}$ (green) and VAMP8 (red) partly colocalize in BMDC. (**C**) Cells were fed with fluorescent latex beads (red) and gp91$^{phox}$ (green) recruitment to the phagosome was evaluated after 45 min. The lower histogram shows the quantification of gp91$^{phox}$ recruitment to phagosomes. (**D**) The presence of NADPH oxidase subunits in purified latex bead phagosome extracts (45 min and 90 min) was assessed by immunoblots. The lower histogram shows the densitometry quantification of the gp91$^{phox}$ immunoblots. (**E**) BMDCs expressing shRNA-targeting gp91$^{phox}$ (gp91$^{phox}$ KD), nontargeting scramble (Scr) control or *Vamp8*$^{-/-}$ BMDCs (left histogram) and gp91$^{phox}$ KD or Scr BMDCs infected or not with WT or *Δgp63* promastigotes (right histogram) were fed with OVA-coated beads in the presence or not of DPI. Cross-presentation or SIINFEKL peptide (assessment of surface MHC class I expression, upper graph) was evaluated using B3Z T cell hybridoma. Data are presented as the mean ± SEM of one experiment representative of at least two independent experiments. *, $p \leq 0.05$; **, $p \leq 0.01$. See also Figure S5.

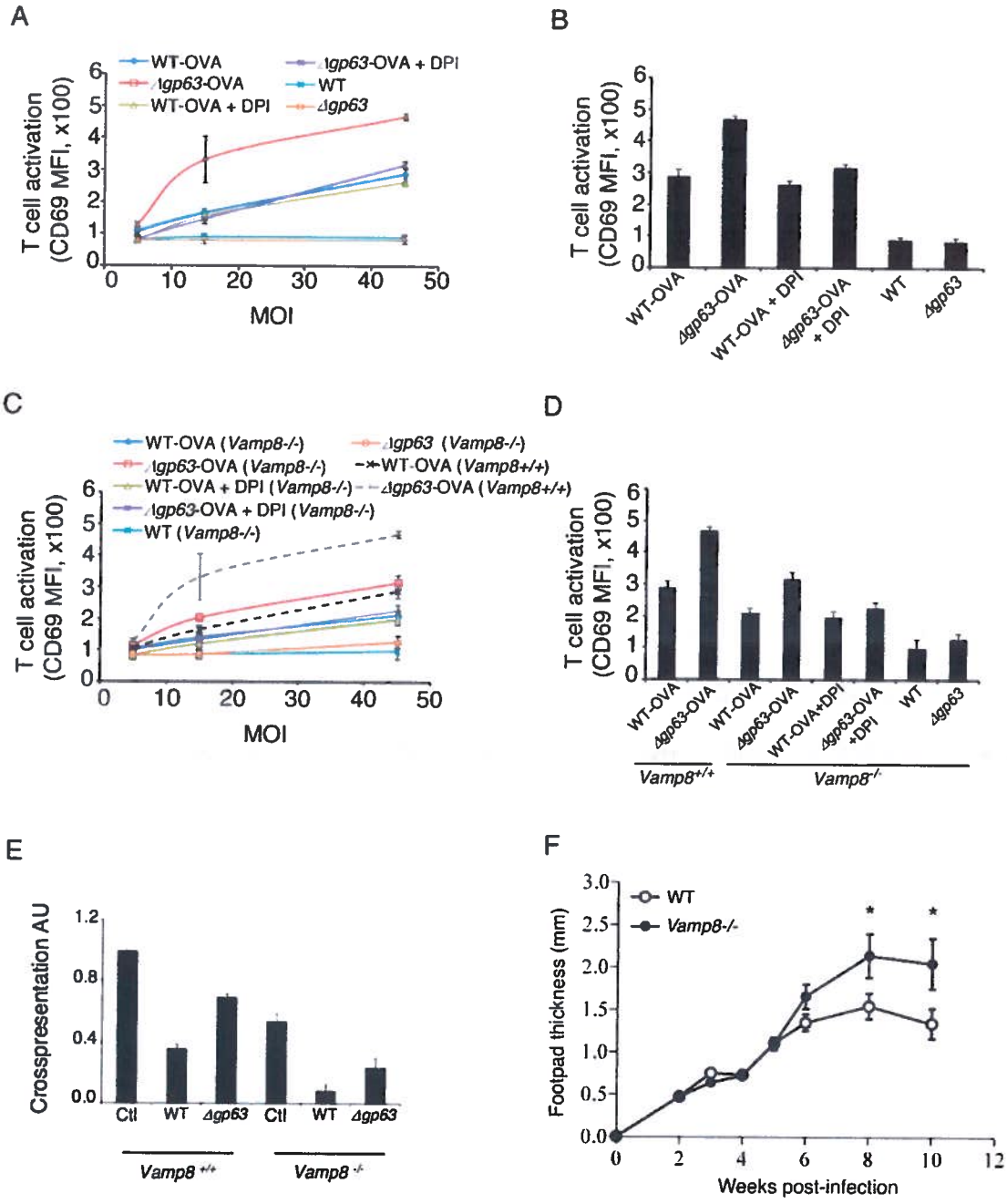

A
WT-OVA
Δgp63-OVA
WT-OVA + DPI
Δgp63-OVA + DPI
WT
Δgp63
T cell activation (CD69 MFI, x100)
MOI
B
T cell activation (CD69 MFI, x100)
WT-OVA
Δgp63-OVA
WT-OVA + DPI
Δgp63-OVA + DPI
WT
Δgp63
C
WT-OVA (Vamp8-/-)
Δgp63 (Vamp8-/-)
Δgp63-OVA (Vamp8-/-)
WT-OVA (Vamp8+/+)
WT-OVA + DPI (Vamp8-/-)
Δgp63-OVA (Vamp8+/+)
Δgp63-OVA + DPI (Vamp8-/-)
WT (Vamp8-/-)
T cell activation (CD69 MFI, x100)
MOI
D
T cell activation (CD69 MFI, x100)
WT-OVA
Δgp63-OVA
WT-OVA
Δgp63-OVA
WT-OVA+DPI
Δgp63-OVA +DPI
WT
Δgp63
Vamp8+/+
Vamp8-/-
E
Crosspresentation AU
Ctl
WT
Δgp63
Ctl
WT
Δgp63
Vamp8 +/+
Vamp8 -/-
F
WT
Vamp8-/-
Footpad thickness (mm)
Weeks post-infection

**Figure S5. *Leishmania* further impairs phagosome oxidation and cross-presentation in the absence of VAMP8, and causes more important lesions in VAMP8-null mice.** (**A**) *Vamp8*$^{+/+}$ BMDC were infected with WT-OVA or Δ*gp63*-OVA *L. major* promastigotes or with non-transfected parasites, in the presence or not of DPI. CD8 OT-I T cells were then added and cross-presentation was evaluated by OT-I T cells CD69 expression. (**B**) Representative histogram of the highest MOI in panel (A). (**C**) *Vamp8*$^{+/+}$ or *Vamp8*$^{-/-}$ BMDC were infected with WT-OVA or Δ*gp63*-OVA *L. major* promastigotes or with non-transfected parasites, in the presence or not of DPI. CD8 OT-I T cells were then added and cross-presentation was evaluated by OT-I T cells CD69 expression. (**D**) Representative histogram of the highest MOI in panel (C). (**E**) *Leishmania* further suppresses antigen cross-presentation in *Vamp8*$^{-/-}$ BMDC. *Vamp8*$^{+/+}$ and *Vamp8*$^{-/-}$ cells were infected with WT or Δ*gp63 L. major* promastigotes or left untreated (Ctl). OVA coated beads were then added. Cross-presentation was evaluated using B3Z T cell hybridoma (left panel). Results were normalized using untreated *Vamp8*$^{+/+}$ control cells and are presented as the mean ± SEM of one experiment representative of two independent experiments. (**F**) Absence of VAMP8 increases susceptibility to *Leishmania* in a mouse model of cutaneous leishmaniasis. WT and *Vamp8*$^{-/-}$ mice were injected in the hind footpad with *L. major* promastigotes and disease progression was monitored over 10 weeks by measuring changes in footpad thickness. N= 11 for WT mice and 9 for *Vamp8*$^{-/-}$ mice. *, $p \leq 0.01$. Data are presented as the mean ± SEM of one experiment representative of two independent experiments.

# DISCUSSION

In the present study, we unraveled a strategy used by *Leishmania* parasites to evade immune recognition by inhibiting cross-presentation, a process involved in the development of a protective immune response during leishmaniasis (Muller, Pedrazzini et al. 1991, Da-Cruz, Conceicao-Silva et al. 1994, Belkaid, Von Stebut et al. 2002, Uzonna, Joyce et al. 2004, Stager and Rafati 2012). We found that cross-presentation is inhibited by GP63 and is inhibited in the absence of VAMP8, supporting the proposal that cleavage of this SNARE enables *Leishmania* promastigotes to inhibit this important immune process. A recent publication showed that Sec22b, a SNARE associated with the endoplasmic reticulum and localized to the phagosome membrane, is also required for cross-presentation (Cebrian, Visentin et al. 2011). Accordingly, it appears that the molecular mechanisms involved in cross-presentation are finely regulated by a series of SNAREs that can be directly targeted by intracellular pathogens to evade immune recognition. The findings that bacteria such as *Chlamydia* and *Legionella* encode proteins displaying SNARE-like motifs that interfere with the function of endocytic SNAREs (Paumet, Wesolowski et al. 2009, Wesolowski and Paumet 2010), highlight the significance of targeting this type of molecules to manipulate the host response.

The processing of exogenous antigens for cross-presentation within phagosomes is a highly complex process that requires limited proteolytic activities regulated in part by the intraphagosomal pH, and the levels of hydrolytic enzymes (Delamarre, Pack et al. 2005) (Trombetta, Ebersold et al. 2003). Recent reports indicated that phagosomal proteolysis, and potentially pH, are regulated by the activity of the NADPH oxidase complex (Savina, Jancic et al. 2006) (Ribicka 2011). In the absence of VAMP8, the pH within the phagosome lumen was decreased, while the proteolytic activity was increased. Furthermore, we observed a severe

impairment of the oxidative activity, together with an inhibition of the recruitment of gp91$^{phox}$ to phagosomes, supporting the link between oxidation and the regulation of proteolytic activities. Interestingly, our results indicate that VAMP8 is required for the early recruitment of gp91$^{phox}$ to phagosomes, which is crucial for the establishment of the conditions required for the processing of exogenous antigens for cross-presentation. Recent findings indicated that SNAP-23 regulates the recruitment of gp91$^{phox}$ to phagosomes (Sakurai, Hashimoto et al. 2012). Since VAMP8 and SNAP-23 can form SNARE complexes (Wang, Ng et al. 2004), it is possible that both act in concert to control the association of gp91$^{phox}$ to phagosomes. Remarkably, the impairment of the phagosomal properties in cells lacking VAMP8 also occurred in a GP63-dependent way in *Vamp8*$^{+/+}$ cells infected with *Leishmania* promastigotes, supporting the concept that the cleavage of this SNARE is a key aspect of the strategy used by the parasite to subvert the immune response. In support to this proposal, we found that absence of VAMP8 resulted in an increased susceptibility to *L. major* infection in a mouse model of cutaneous leishmaniasis (Fig. S5F). Future studies will be aimed at elucidating the impact of VAMP8 on the immune response during *in vivo* infection. Although VAMP8 clearly plays a role in cross-presentation, other molecules targeted by *Leishmania* may also be involved in this process. Hence, we observed that cross-presentation is further decreased when *Vamp8*$^{-/-}$ BMDC are infected with *L. major* promastigotes (Fig. S5E). Whether this is related to the cleavage of SNAP-23 or syntaxin-4 (Fig. S2) remains to be investigated.

We discovered that an intracellular pathogen can directly target and cleave components of the membrane fusion machinery of its host cells to inhibit antigen presentation. These findings thus significantly contribute to our understanding of the mechanisms associated with immune evasion during *Leishmania* infection. Our data clearly identified the metalloprotease GP63 as the parasite

molecule responsible for the cleavage of VAMP8. Our observations that GP63-mediated cleavage of VAMP8 (and other SNAREs) and the consequential inhibition of cross-presentation are specific to the promastigote stage, indicate that these events are limited to the early phase of the establishment of *Leishmania* infection. Sustained cleavage of SNAREs in parasitized cells would most likely be deleterious for host cells and for the parasites, since the membrane fusion machinery must remain operational to allow for the maintenance of amastigote-harboring phagosomes and parasite replication. The mechanism by which GP63 accesses and degrades host cell substrates remains to be elucidated. The recent discovery that GP63 is a constituent of exosomes released by *Leishmania* provides a potential mechanism for the export of this molecule across the phagosome membrane (Silverman, Clos et al. 2010). Furthermore, previous studies indicated that GP63 is released from the parasite surface, exits the phagosome, and colocalizes with lipid microdomains on some of the macrophage membranes (Gomez, Contreras et al. 2009). Remarkably, the finding that sets of SNAREs associate to various degrees with lipid microdomains (Gil, Soler-Jover et al. 2005) may facilitate their encounter with GP63. Further studies will address the relative contribution of other SNAREs in the fine regulation of the membrane trafficking events regulating both the innate and adaptive immune response in antigen presenting cells.

# EXPERIMENTAL PROCEDURES

## Animals and cells

BALB/c, 129/SvJ, VAMP8 null (Wang, Ng et al. 2004) and *Rag*$^{-/-}$ OT-I T-cell receptor transgenic mice (specific for the $K^b$-restricted OVA257-264 epitope) were maintained in our animal facility. Animals were handled in strict accordance with good animal practice as defined by the Canadian Council on Animal care and animal work was approved by the Comité institutionel de protection des animaux of INRS-Institut Armand-Frappier (protocol 0811-09). BMM or BMDC were obtained by growing bone marrow cells from female mice for 7 days in Dulbecco Modified Eagle Medium with L-glutamine (Life Technologies) for BMM, or RPMI-1640 for BMDCs, supplemented with 10% heat-inactivated FBS (Hyclone, Logan, UT), 10 mM Hepes (pH 7.4) and antibiotics (complete medium) in the presence of 15% (v/v) L929 cell-conditioned medium as a source of CSF-1 for BMM, or in the presence of 20 ng/ml GM-CSF for BMDCs. Cells were made quiescent by culturing them in the absence of CSF-1 or GM-CSF for 18 hr prior to use. RAW 264.7 mouse macrophages were cultured in complete medium in a 37°C incubator with 5% $CO_2$. The β-galactosidase–inducible OVA–specific $CD8^+$ T cell hybridoma B3Z (provided by W. Heath, University of Melbourne) was maintained in RPMI-1640 medium supplemented with 5% (vol/vol) FCS, glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 mg/ml), G418 (0.5 mg/ml) and hygromycin B (100 mg/ml). The $OVA_{323-339}$-specific, $I\text{–}A^b$–restricted BO97.10 hybridoma (a kind gift from P Marrack) was maintained in Kappler Marrack Complete Tumor Medium.

## Parasites

*Leishmania* promastigotes (WT and Δ*lpg2 L. donovani* LV9; WT, Δ*gp63*, and Δ*gp63*+*gp63 L. major* Seidman clone A2; *L. tarentolae*) were grown at 26°C in M199 medium supplemented with 20% heat inactivated FBS, 100 μM hypoxanthine, 40 mM HEPES, 5 μM hemin, 3 μM biopterin, 1 μM biotin and antibiotics (*Leishmania* medium). *L. donovani* amastigotes were recovered from the spleen of hamsters infected for 6 to 8 weeks. *L. major* promastigotes expressing a secreted form of OVA (*L. major*-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 OVA protein (139 to 386) containing both MHC class I $OVA_{257-264}$- and class II $OVA_{323-339}$-restricted epitopes (Bertholet, Debrabant et al. 2005)(kindly provided by Alain Debrabant, FDA). Transfected parasites were grown in *Leishmania* medium supplemented with 50 μg/ml G418. For infections, promastigotes were used in the late stationary phase of growth (Vinet, Fukuda et al. 2009).

## Reagents and Antibodies

Rabbit antibodies to VAMP8 and SNAP-23 were from Synaptic Systems. Rabbit antibodies to Syntaxin 3, VAMP3, Calnexin, and ERp57, and the mouse monoclonal to Syntaxin 4 were from AbCam. Mouse monoclonal to β-actin and rabbit anti-OVA were from Sigma-Aldrich. Mouse monoclonal antibodies to GP63 were kindly provided by W. Robert McMaster (University of British Columbia). Goat antibodies to TAP1 were from Santa Cruz Biotechnology. Rabbit antibodies to Sec61 were from Affinity BioReagents. Mouse monoclonal

antibody to NSF was a kind gift from Sidney Whiteheart (University of Kentucky). Mouse monoclonal antibodies to gp91$^{phox}$, CD8α APC-H7, Vα2 PerCP, CD3 (conjugated to PE-Cy7), and CD69 (conjugated to APC) were from BD Biosciences. Purified OVA (grade VI, 99% pure), DPI (diphenylene iodonium), CPRG (chlorophenol red-β-D-galactopyranoside) and PMA (Phorbol Myristate Acetate) were from Sigma. The SIINFEKL peptide was from Anaspec.

## Phagocytosis Assays

Complement opsonisation of *Leishmania* promastigotes with BALB/c mouse serum was done as described (Vinet, Fukuda et al. 2009). Cells were incubated with zymosan or parasites at a particle-to-cell ratio of 15:1 for different time points. For FACS experiments, cells were incubated 30 min with 1.9 μm fluorescent latex beads, previously coated with OVA. Cells were then washed in PBS, 5 mM EDTA to eliminate non-internalized beads. Cells were then cultured in complete medium for various times of chase and then labelled with an anti-OVA antibody. Phagocytosis was evaluated using FACSCalibur flow cytometer, and noninternalized beads (OVA-positive cells) were excluded.

## Microscopy and Immunofluorescence

Cells were fixed, permeabilized and fixed as described (Vinet, Fukuda et al. 2009). Phagosomal recruitment of VAMP8, VAMP3, and gp91$^{phox}$ was quantified by immunofluorescence microscopy using an oil immersion Axio Observer Z1 microscope equipped with a Zeiss LSM780 confocal system. Results are based on at least 300 cells in each independent experiment (100 cells per each cover slip) and each experiment was repeated twice. For gp91$^{phox}$ staining, cells were visualized with a wide-field microscope (DeltaVision Elite

Inverted Microscope) equipped with a Coolsnap HQ2 camera using a 60X/1.42 NA oil objective. Images were acquired and deconvoluted using SoftWoRx Software (Applied Precision).

## Phagosome Preparation

Cells were incubated for 15 min with 3.19 μm polysterene magnetic particles, then chased for different periods of time and disrupted in homogeneization buffer. Magnetic phagosomes were removed from the postnuclear supernatant using a magnet, washed 3 times in cold PBS, and lysed. After 10 min at 4°C, magnetic beads phagosomes were removed by centrifugation. Phagosomes were also prepared using latex beads (Estapor) and discontinuous sucrose gradients as previously described (Desjardins, Celis et al. 1994).

## Western Blot Analyses

Phagosomal and total cell lysates proteins were separated on 12% and 15% SDS-PAGE, transferred onto Hybond-ECL membranes (Amersham Biosciences) and immunodetection was achieved by chemiluminescence (Amersham Biosciences).

## *In vitro* Cleavage Assay

Reactions were performed in 200 μl binding buffer (PBS 1X, $ZnCl_2$ 1 mM) with 1 μg of purified protein. GST-tagged VAMP8 was incubated with 30 X $10^6$ *L. major* promastigotes for various time points at room temperature (Gomez, Contreras et al. 2009), and subjected to Western blot analyses.

## Design of shRNA and knock-down strategy

Specific shRNA targeting the gp91$^{phox}$ and nontargeting scramble control were designed based on the sequences presented on the Sigma MISSION-RNA website. Annealed forward and reverse hairpins oligonucleotides were cloned into a modified pLKO.1-TRC1.5 vector where the puromycin resistance gene was replaced with mAmetrine. Lentiviral particles were made by co-transfecting in HEK293T cells the shRNA-containing pLKO-mAM vector along with pMD2-VSVG, pMDLg/pREE, and pRSV-REV. Viral supernatants were used to transduce BMDC (day 2 of differentiation) and 4 days after lentivirus transduction, cells were sorted according to mAmetrine fluorescence. Validation of the knock down was done by RT-qPCR and western blot analyses.

## Antigen Presentation Assay

BMMs or BMDCs were infected for 6 hr with WT or *Δgp63 L. major*-OVA promastigotes, or with nontransfected promastigotes. Cells were then washed and fixed for 5 min at 23°C with 1% (wt/vol) paraformadehyde, followed by three washes in complete medium containing 0.1 M of glycin. Purified OT-I T cells (using EasySep Mouse CD8+ T cells Enrichment Kit, Stem Cell) were then added for 16 hr and T cell activation was assayed by assessing CD69 expression within the $CD3^+CD8\alpha^+V\alpha2^+$ population. Activation was measured with a LSRFortessa flow cytometer

For OVA latex bead assays, at 2 or 6 hr postinfection, $10^5$ BMM or BMDC were incubated with uncoated or OVA-coated 0.8 μm latex beads for 1 hr of pulse, in the presence or not of DPI. Cells were incubated for a 3 hr chase, fixed with 1% (wt/vol) paraformadehyde, and washed in complete medium containing 0.1 M glycin. For MHC class I and class II expression control, cells received the SIINFEKL peptide or the $OVA_{323-339}$ peptide respectively, 30 min before the end of the chase. Cells were then cultured for 12 hr at 37°C together with $10^5$ B3Z or BO97.10 cells for analysis of T cell activation. B3Z cells, which expresses β-galactosidase upon specific recognition of the $OVA_{257-264}$ (SIINFEKL)-H-$2K^b$ complex, were washed in PBS and lysed (0.125 M Tris base, 0.01 M cyclohexane diaminotetraacetic acid, 50% (vol/vol) glycerol, 0.025% (vol/vol) Triton X-100 and 3 mM dithiothreitol, pH 7.8). A β-galactosidase substrate buffer (1 mM $MgSO_4$ x7 $H_2O$, 10 mM KCl, 0.39 M $NaH_2PO_4$ $xH_2O$, 0.6 M $Na_2HPO_4$ x7 $H_2O$, 100 mM 2-mercaptoethanol and 0.15 mM CPRG, pH 7.8) was added for 2–4 h at 37°C. Cleavage of CPRG was quantified in a spectrophotometer as absorbance at 570 nm, reflecting T cell activation after cross-presentation. Production of IL-2 by BO97.10 was measured by ELISA (Biolegend).

## Measurement of Phagosomal pH and Oxidation

Phagosome pH and oxidation were measured as described (Savina, Jancic et al. 2006). Polybeads amino microspheres (3 μm; Polysciences) were covalently coupled with FITC (Sigma) for measuring pH, or with DHR (Dihydrorhodamine, Invitrogen) for the measuring oxidation. In both cases, beads were also coupled to FluoProbes 647 (pH insensitive and oxidation insensitive, Interchim). Cells were pulsed with the beads for 20 min, washed and further incubated for the indicated times before FACS analyses, using a gating FCS/SSC

selective for cells that have phagocytosed one latex bead. For oxidation experiments, PMA (1 μg/ml) was added after phagocytosis. For DPI assays, cells were incubated in the presence of 10 μM DPI for 30 min before and during the 20 min pulse. The ratio of the mean fluorescence intensity (MFI) emission between the two dyes was determined. For pH measurement, values were compared with a standard curve obtained by resuspending the cells that had phagocytosed beads at a fixed pH (ranging from pH 4 to 8).

## Measurement of OVA Phagosomal Degradation

Cells were incubated with 3 μm polybeads coupled to DQ OVA and FluoProbes 647 (Molecular Probes) for 20 min at 37 °C and washed three times with PBS. At the indicated time points, OVA degradation was evaluated by flow cytometry with the fluorescein MFI measure normalized by the FluoProbes 647 MFI values. For DPI assay, cells were incubated in the presence of 10 μM DPI for 30 min before and during the 20 min pulse.

## In Vivo *Leishmania* Infections

Mice were infected by injecting *L. major* stationary-phase parasites (5 X $10^6$ in 50 μl) into the right hind footpad of 6- to 8-week-old animals. Footpad swelling was measured weekly for up to 10 weeks after infection using an electronic caliper as described (Gomez et al., 2009).

## Statistical Analysis

An unpaired T-Test was performed using GraphPad software to asses whether the differences between control and infected groups were significant or not. P values $< 0.05$ were considered significant.

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## Publication No.2

## *Leishmania* inhibits the recruitment of ER-SNARE Sec22b on the phagosome membrane

# *Leishmania* inhibits the recruitment of ER-SNARE Sec22b on the phagosome membrane

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# Summary

Soluble *N*-ethylmaleimide-sensitive-factor attachment protein receptor (SNARE) proteins mediate vesicle fusion and play an important role in macrophages during phagocytosis of microbes. The endoplasmic reticulum-associated SNARE, Sec22b, was recently shown to regulate antigen cross-presentation. We observed a significantly reduced Sec22b recruitment to the phagosomes containing *Leishmania*. Using mutants defective in either LPG or GP63, we found that this inhibtion was GP63-dependent. We did not observe cleavage of Sec22b, suggesting that the inhibition of Sec22b recruitment to *Leishmania*-phagosomes is indirect, probably due to the cleavage of a cognate SNARE required for the recruitment of Sec22b to the phagosome. Knocked down of Sec22b interacting proteins, syntaxin 4, syntaxin 5 and SNAP23, which are cleaved by GP63 and are involved in microbicidal activity of phagosomes, had no effect on the recruitment of Sec22b to phagosomes. Identification of the involved protein in fusion with Sec22b that is targeted by *Leishmania*, might uncover a novel mechanism employed by this parasite to evade recognition by the immune system, through the degradation of key regulators of vesicular trafficking and impairement of cross-presentation.

# Introduction

Many intracellular pathogens evolved to alter membrane fusion between intracellular compartments while residing in the phagosome of host cells; by this means, they impair phagosome maturation and antigen presentation. Altering antigen presentation pathways is a key factor for intracellular pathogens such as *Leishmania* to escape the immune system and replicate successfully in phagocytes. *Leishmania* is the causative agent of leishmaniasis with a wide spectrum of human diseases ranging from a confined cutaneous form to fatal visceral leishmaniasis. Different virulence factors have been characterized for this intracellular parasite but the ones that modulate phagosome maturation are lipophosphoglycan (LPG) and the cell surface metalloprotease GP63 (Moradin and Descoteaux 2012, Olivier, Atayde et al. 2012). Different studies show that GP63, an abundant surface glycoprotein in promastigotes, cleaves several regulators of cell transcription and translation to alter cell signaling pathways, thereby helping the parasite to escape the immune responses (Contreras, Gomez et al. 2010, Jaramillo, Gomez et al. 2011). More recently, it has been shown that VAMP8 (endobrevin) is cleaved directly by GP63, and since VAMP8 is a key regulator in cross-presentation, then antigen presentation is altered upon infection with *Leishmania* in both macrophages and dendritic cells (Matheoud, Moradin et al. 2013).

The SNAREs (soluble NSF attachment receptors, where NSF stands for *N*-ethylmaleimide-sensitive factor) were first reported in 1993 and since then, many studies have been published on the roles of this large protein family. SNAREs are distributed selectively on different endosomal compartments with different functions. They are key players in the immune system as they

regulate membrane fusion and protein trafficking (Stow, Manderson et al. 2006). There are two different categories of SNAREs: R-SNAREs and Q-SNAREs, which are located on vesicle and target membranes, respectively. VAMP8 is a R-SNARE with a widespread distribution in intracellular compartments (late endosomes, the *trans*-Golgi network, coated pits and plasma membrane, and early endosomal compartments) (Pryor, Mullock et al. 2004). VAMP8 controls the assembly of NADPH oxidase on phagosomes where it regulates pH and antigen degradation. Consequently, VAMP8 plays a key role in cross-presentation in antigen-presenting cells (Matheoud, Moradin et al. 2013). The ER-SNAREs Sec22b is a negative regulator of phagocytosis (Hatsuzawa, Hashimoto et al. 2009). Further studies on Sec22b showed that it is a critical regulator of cross-presentation in dendritic cells through ER/phagosome fusion pathway (Cebrian, Visentin et al. 2011).

Antigens are processed and presented by antigen presenting cells in the context of MHC I and MHC II molecules to trigger $CD8^+$ and $CD4^+$ responses, respectively. In the classical pathway, endogenous antigens induce a $CD8^+$ response and exogenous antigens initiate a $CD4^+$ T cell response. Alternatively, some exogenous antigens are loaded on MHC I molecules through a"cross-presentation" pathway and initiate the $CD8^+$ cytotoxic T cell response (Mantegazza, Magalhaes et al. 2013). Dendritic cells have specific characteristics that enhance their efficiency in cross-presentation such as a less acidic pH in their phagosomes in comparison with macrophage phagosomes. It has been shown that production of reactive oxygen species (ROS) by NADPH oxidase and also, incomplete activation of the V-ATPase inhibit further acidification in dendritic cells and consequently, epitopes remain intact (i.e. they keep their recognition sites) and they are not degraded into small pieces and can be loaded on MHC I molecules (Russell 2007).

In this study, we show that *Leishmania* excludes Sec22b from phagosomes harbouring this intracellular parasite. We also show that the exclusion of Sec22b is indirectly dependent on GP63. Further analyses on the protein complex formed by Sec22b with other SNAREs indicate that syntaxin 2 and syntaxin 5 are degraded through GP63 but interestingly, Sec22b itself is not degraded.

# Results

## GP63-mediated exclusion of Sec22b from the phagosome

In our previous work, we showed that cross-presentation is inhibited by *Leishmania* through the cleavage of VAMP8 (Matheoud, Moradin et al. 2013). In addition, we confirm that this mechanism is GP63-dependent. To better understanding the mechanisms by which *Leishmania* escapes the immune response, we studied the ER-SNARE protein called Sec22b. This protein also has a role in regulating cross-presentation (Cebrian, Visentin et al. 2011). In order to investigate the effect of *Leishmania* on Sec22b, we first analysed the distribution of Sec22b in infected primary bone marrow derived macrophages (BMMs) from BALB/c mice. As can be observed in Figures 1-A and 1-B, BMMs were infected with *L. major* (WT, Δ*gp63*, Δ*gp63+gp63*) for 2 and 6 hours. Confocal microscopy analysis showed that Sec22b was recruited to phagosomes containing zymosan and *L.major* Δ*gp63*, but not *L.major* Δ*gp63+gp63* and WT parasites. The absence of Sec22b recruitment to phagosomes containing *L.major* Δ*gp63+gp63* and WT parasites could be due to its cleavage through the GP63 metalloprotease.

Next, BMMs were infected with *L. major* (WT, Δ*gp63*, Δ*gp63+gp63*) for the same times as before (2 and 6 hours). Non-infected BMMs were used as control. BMMs were lysed with a lysis buffer containing 1mM of 1, 10 phenanthroline (an inhibitor of zinc metallopeptidases) and total proteins were separated on 15% SDS-PAGE acrylamide gel. Interestingly, there was no cleavage or degradation of Sec22b upon infection by *Leishmania*. In this experiment, cleavage of the R-SNARE VAMP8 was used as a positive control for GP63 function (Figure 1-C).

In another experiment, RAW 264.7, a cell line of mouse macrophages, were infected with *L. major* (WT, Δ*gp63*, Δ*gp63*+*gp63*) for 2 hours; non-internalized parasites were washed away. The experiment was followed by adding 3μm magnetic beads and phagosomes were prepared as described before (Arango Duque, Fukuda et al. 2013) . Macrophages were lysed with a 1, 10 phenanthroline-containing lysis buffer and subjected to Western blot analyses. As expected, there was no Sec22b cleavage in phagosomes from infected macrophages, similar to phagosomes from non-infected cells. The VAMP8 protein was used as a positive control for GP63 cleavage (Figure 1-D). Thus, we concluded that although Sec22b was excluded from phagosomes harbouring *L. major* (WT and Δ*gp63*+*gp63*), there was no degradation of Sec22b. We hypothesize that the effect of GP63 on Sec22b is indirect and that there might be an interacting protein with Sec22b that is degraded by GP63. This would impede the recruitment of Sec22b to phagosomes containing *Leishmania*. In support of this notion, a study on the p38 protein (mitogen-activated protein kinase) shows that the effect of GP63 on this protein is indirect and due to the cleavage of upstream protein TAB1 (TGF-beta activated kinase 1) (Halle, Gomez et al. 2009).

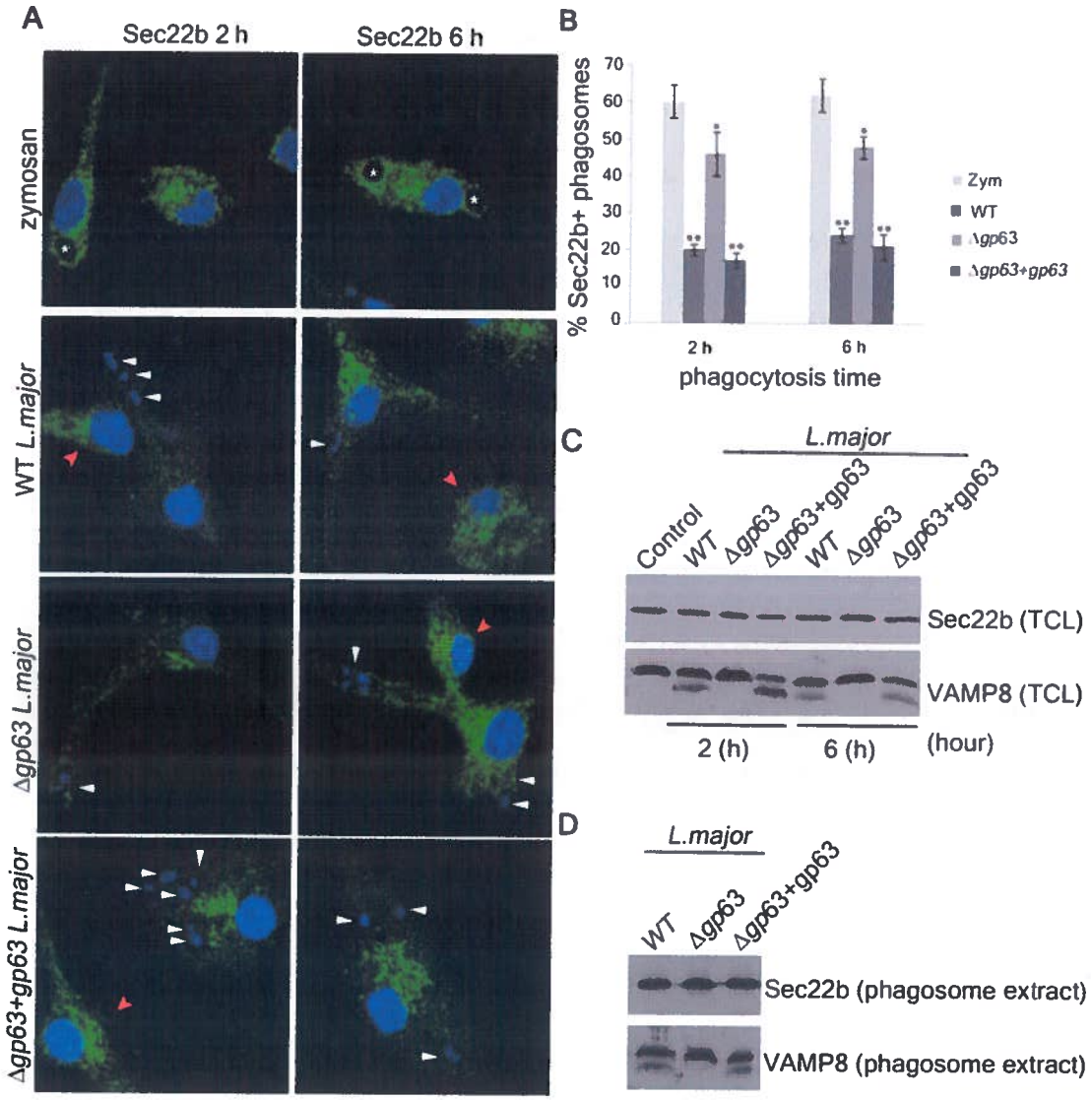
A
Sec22b 2 h
Sec22b 6 h
zymosan
WT L.major
Δgp63 L.major
Δgp63+gp63 L.major
B
% Sec22b+ phagosomes
70
60
50
40
30
20
10
0
2 h
6 h
phagocytosis time
Zym
WT
Δgp63
Δgp63+gp63
C
L.major
Control
WT
Δgp63
Δgp63+gp63
WT
Δgp63
Δgp63+gp63
Sec22b (TCL)
VAMP8 (TCL)
2 (h)
6 (h)
(hour)
D
L.major
WT
Δgp63
Δgp63+gp63
Sec22b (phagosome extract)
VAMP8 (phagosome extract)

**Figure 1. Sec22b is not degraded but it is excluded from phagosomes containing *L. major* WT and Δ*gp63*+Δ*gp63* via GP63. (A)** Representative confocal immunofluorescence images of BMMs 2 h and 6 h after internalisation of zymosan, *L. major* (WT, Δ*gp63*, Δ*gp63*+Δ*gp63*). Sec22b is shown in green and nuclei are in blue. Asterisks denote the zymosan, white arrowheads indicate *Leishmania* nucleus and red arrowheads show non-infected cells. **(B)** Quantification of Sec22b recruitment to the phagosome at 2 and 6 h after internalization of zymosan, *L.major* (WT, Δ*gp63*, Δ*gp63*+Δ*gp63*). Results are representatives of three independent experiments (100 cells per each experiment). *, $p \leq 0.05$; **, $p \leq 0.005$. **(C)** Immunoblot analysis of Sec22b and VAMP8 was done in total BMM lysates after infection with *L.major* (WT, Δ*gp63*, Δ*gp63*+Δ*gp63*) at 2 and 6 h. Here, the control is total cell lysates of non-infected BMM. **(D)** Immunoblot analysis of Sec22b and VAMP8 of phagosomes isolated from infected RAW264.7 macrophages; cells were infected for 2 h followed by 1 h magnetic bead internalization.

## Decreased Sec22b recruitment to the phagosome is not LPG-dependent

It has been shown that *Leishmania* through lipophosphoglycan (LPG) excludes V-ATPase from the phagosome membrane by impairing the recruitment of synaptotagmin V (Vinet, Fukuda et al. 2009). To find out whether LPG has a role in excluding Sec22b from the phagosome, macrophages were infected by *L. donovani* WT and also Δ*lpg2* (which lacks LPG completely) for different time points (2 and 6 hours). Immunofluorescence assays showed that Sec22b was significantly less recruited to phagosomes containing zymosan in infected BMMs with *L.donovani* (WT and Δ*lpg2*) in contrast to non-infected macrophages (Figure 2-A, -B). These results showed that in contrast to the role of LPG in many alterations of phagolysosome biogenesis (Moradin and Descoteaux 2012), the decrease of Sec22b recruited to *Leishmania*-harbouring phagosomes is LPG-independent. Interestingly, the decrease in Sec22b recruitment was not specific for only infected phagosomes; we found that absence of Sec22b in infected BMMs is not limited to phagosomes harboring *L. donovani* (WT and Δ*lpg2*) but it is also observed in phagosomes harboring zymosan in infected BMMs.

To confirm the decreased recruitment of Sec22b in zymosan-containing phagosomes of infected BMMs, cells were infected with *L. major* (WT, Δ*gp63*, Δ*gp63*+*gp63*) for 2 or 6 hours and at the end of each specific time point, zymosan was added for 1 hour. As confocal microscopy analyses show, recruitment of Sec22b to the phagosomes containing zymosan in BMMs infected with *L. major* (WT and Δ*gp63*+*gp63*) decreases significantly but not in the case of *L. major* Δ*gp63* (Figure 3-A, -B). These results further confirm that Sec22b is excluded from the phagosome through the GP63 metalloprotease only in infected macrophages. Absence of Sec22b

is not limited to infected phagosomes but also phagosomes with zymosan in infected BMM macrophages show less recruitment of Sec22b.

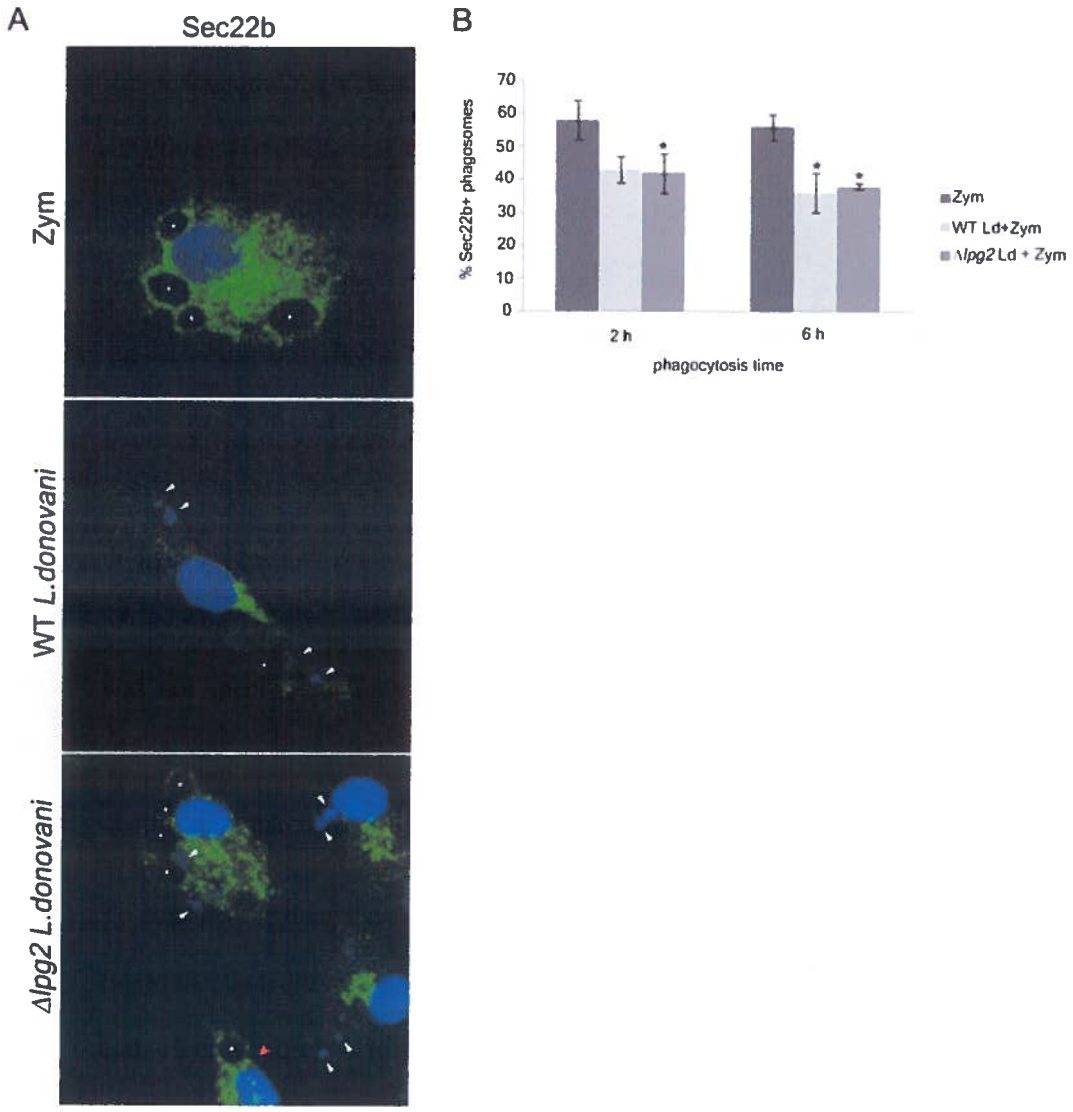
A
Sec22b
Zym
WT L.donovani
Δlpg2 L.donovani
B
% Sec22b+ phagosomes
70
60
50
40
30
20
10
0
2 h
6 h
phagocytosis time
Zym
WT Ld+Zym
Δlpg2 Ld + Zym

**Figure 2. Exclusion of Sec22b from phagosomes containing *Leishmania* is LPG-independent. (A)** Confocal immunofluorescent images of a 1 h zymosan treatment alone or of a 2 h infection with *L. donovani* (WT, Δ*lpg2*) followed by 1 h internalization of zymosan. Sec22b is in green and nuclei are in blue. Asterisks show zymosan in phagosomes. White arrowheads point at the nucleus of parasites. **(B)** Quantification of Sec22b recruitment around zymosan in infected macrophages with *L. donovani* (WT, Δ*lpg2*). The graph shows decreased recruitment of Sec22b around zymosan in both *L. donovani* wild-type and Δ*lpg2*-infected BMMs, meaning that exclusion of Sec22b is not through LPG. Results here are representative of three independent experiments. *, $p \leq 0.05$

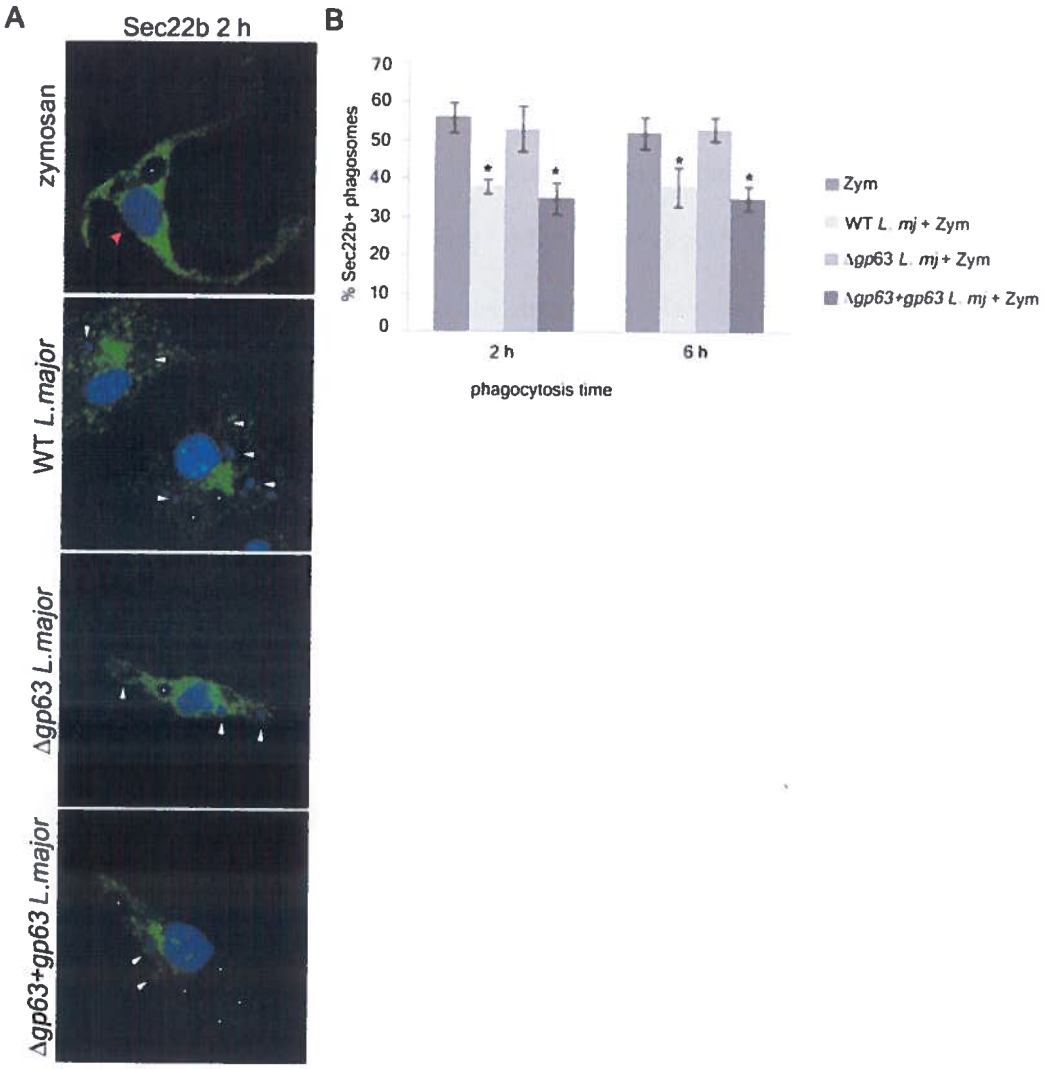
A
Sec22b 2 h
zymosan
WT L.major
Δgp63 L.major
Δgp63+gp63 L.major
B
% Sec22b+ phagosomes
70
60
50
40
30
20
10
0
2 h
6 h
phagocytosis time
Zym
WT L. mj + Zym
\gp63 L. mj + Zym
\gp63+gp63 L. mj + Zym

**Figure 3. GP63-mediated decrease in Sec22b recruitment from phagosomes containing zymosan in infected BMMs. (A)** Confocal immunofluorescence images after 2 h infection with *L. major* (WT, Δ*gp63*, Δ*gp63*+Δ*gp63*) followed by 1 h internalization with zymosan. Zymosan alone was used as a control. Sec22b is in green and nuclei are in blue. Asterisks show zymosan in the phagosomes. White arrow heads are pointed at the nucleus of parasites. **(B)** Quantification of Sec22b recruitment around zymosan in infected BMMs with *L. major* (WT, Δ*gp63*, Δ*gp63*+Δ*gp63*). The graph shows decreased recruitment of Sec22b around zymosan in both *L. major* (WT and Δ*gp63*+Δ*gp63*)-infected macrophages but not for *L. major* Δ*gp63*, which means that exclusion of Sec22b is modulated by GP63. Results are representative of three independent experiments. *, $p \leq 0.05$

## Degradation of Sec22b-interacting proteins by GP63

It has been shown that syntaxin 2, syntaxin 3, syntaxin 4 and SNAP23 localize to vacuoles harbouring *Legionella pneumophila* and interact with Sec22b (Arasaki and Roy 2010). There are other studies that show the interaction between syntaxin 5 and Sec22b (Xu, Joglekar et al. 2000). In addition, it is well known that syntaxin 5 localizes on the parasitophorous vacuole membrane of phagosomes containing *Leishmania* (Canton, Ndjamen et al. 2012). Our hypothesis is that GP63 might inactivate one of the Sec22b-interacting proteins and that is why decided to study syntaxin 2, syntaxin 3, syntaxin 4 and SNAP23. In our previous work, we observed the degradation of Syntaxin 4 and SNAP23 through GP63, but not the cleavage of syntaxin 3 (Matheoud, Moradin et al. 2013). In this study we show the degradation of syntaxin 2 (degradation is not as strong as what we observed with other SNAREs) and syntaxin 5 in BMMs through GP63 (Figure 4-A). To further investigate the functional consequences for degradation of these SNAREs by *Leishmania* and also to find the correlation between the degradation of these SNAREs with the exclusion of Sec22b from phagosomes containing *Leishmania*, we knocked down some of these SNAREs and proceeded with functional experiments.

## Sec22b recruitment is not altered after knockdown of interacting SNAREs

Small interfering RNA (siRNA) was used to deplete syntaxin 4, syntaxin 5 and also SNAP23. RAW 264.7 cells were incubated with ON-TARGETplus SMARTpool siRNA for 48 hours. Lysed knock down cells were monitored by Western blot to verify the efficiency of the knock down assay (Figure 4-B). The recruitment of Sec22b to the phagosomes containing zymosan was

followed with the same kinetics as when macrophages were infected with *Leishmania*, 2 and 6 hours after knockdown with siRNA. Confocal microscopy revealed that absence of either syntaxin 4, SNAP 23 or syntaxin 5 had no effect on the recruitment of Sec22b to the phagosome (Figure 4C).

VAMP8 is a widespread R-SNARE in cells in which it plays a role in membrane fusion and protein trafficking. It also has been shown that VAMP8 is degraded by *Leishmania* to inhibit NADPH oxidase assembly to the phagosome and impair cross-presentation (Matheoud, Moradin et al. 2013). Considering the common role for both VAMP8 and Sec22b in cross-presentation, we hypotesized that in the absence of VAMP8, Sec22b function is altered as well. To further analyse the exclusion mechanism of Sec22b from the phagosome, we isolated BMMs from $VAMP8^{-/-}$ and $VAMP8^{+/+}$ mice and compared the Sec22b recruitment around phagosomes containing zymosan. Sec22b recruitment was not altered in the absence of VAMP8 (Figure 4-D).

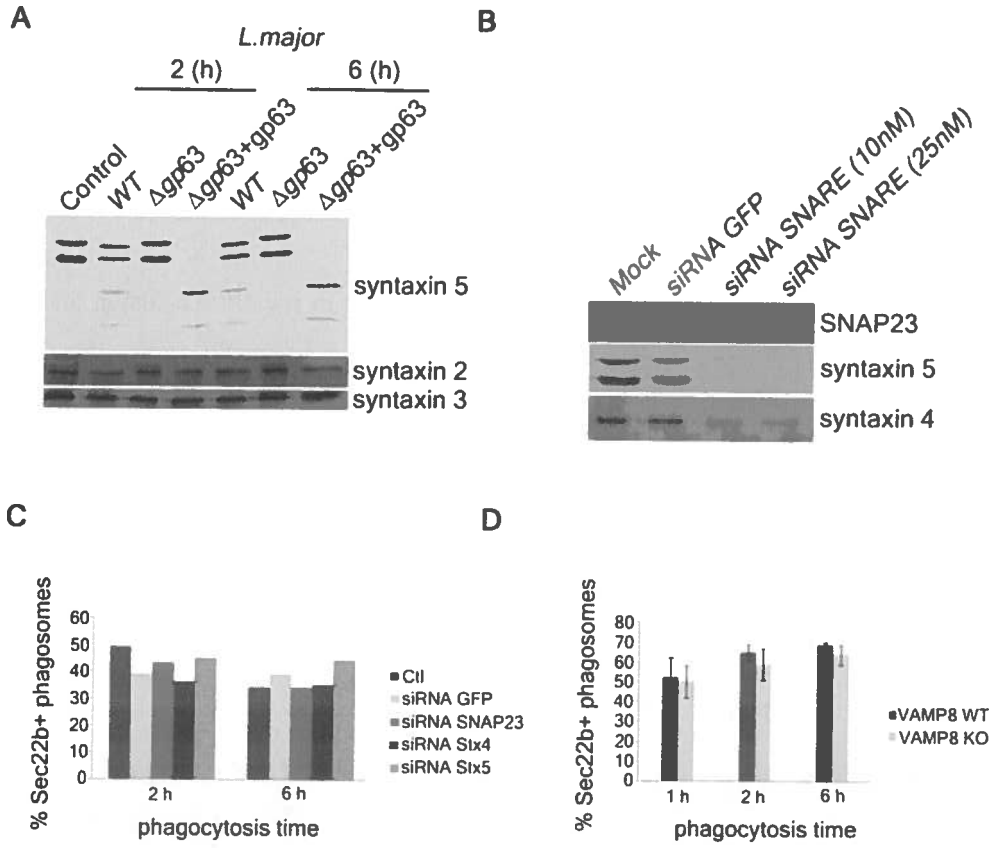
A
L.major
2 (h)
6 (h)
Control
WT
Δgp63
Δgp63+gp63
WT
Δgp63
Δgp63+gp63
syntaxin 5
syntaxin 2
syntaxin 3
B
Mock
siRNA GFP
siRNA SNARE (10nM)
siRNA SNARE (25nM)
SNAP23
syntaxin 5
syntaxin 4
C
% Sec22b+ phagosomes
60
50
40
30
20
10
0
2 h
6 h
phagocytosis time
Ctl
siRNA GFP
siRNA SNAP23
siRNA Stx4
siRNA Stx5
D
% Sec22b+ phagosomes
80
70
60
50
40
30
20
10
0
1 h
2 h
6 h
phagocytosis time
VAMP8 WT
VAMP8 KO

**Figure 4. Sec22b-interacting proteins (syntaxin 4, -5 and SNAP23), although degraded by *Leishmania* through GP63, are not required for Sec22b recruitment to phagosomes containing zymosan.** **(A)** Immunoblot analysis of syntaxin 5, syntaxin 2 and syntaxin 3 in total cell lysates of infected BMMs with *L. major* (WT, Δ*gp63*, Δ*gp63*+Δ*gp63*) at 2 and 6 h post infection. Lysis buffer contains 1, 10 phenanthroline. As can be observed, syntaxin 3 is not degraded by GP63 at any time point. **(B)** Immunoblot analysis on total cell lysates of RAW264.7 cells after knock down SNAP23, syntaxin 5 and -4 by siRNA. For both siRNA concentrations (10 nM and 25 nM), gene knock down is efficient. Mock sample received only lipofetamine (RNAiMAX reagent) and siRNA GFP is a negative control to verify the specificity of the various siRNAs employed. **(C)** Quantification of Sec22b phagosome recruitment around zymosan at 2 and 6 h post-phagocytosis. RAW264.7 cells were knocked-down for SNAP23, syntaxin 4 and syntaxin 5 separately. Here, we counted 100 cells per experiment. Control bars are RAW264.7 cells with 2 and 6 h of internalization with zymosan without any lipofectamine or siRNA treatment. **(D)** Quantification of Sec22b phagosome recruitment around zymosan in BMMs from VAMP8$^{+/+}$ and VAMP8$^{-/-}$ mice. The results are representative of counting 300 cells (100 cell per cover slip).

## Significantly different Sec22b recruitment pattern in RAW 264.7 and BMMs

Since siRNA knock down assays were performed in RAW 264.7 macrophage but not in primary BMMs, we decided to monitor the Sec22b recruitment to phagosomes containing zymosan and *L. major* Δ*gp63* during 2 and 6 hours of phagocytosis in knock down RAW 264.7 cells for syntaxin 5 and also siRNA GFP as a control. Confocal microscopy analyses show significant decrease in Sec22b recruitment to the phagosomes containing *L. major* Δ*gp63* in both transiently knock down syntaxin 5 and also GFP (Figure 5-A). This result revealed that recruitment of Sec22b is not similar to what is observed in BMMs. The next question was whether Sec22b recruitment in RAW 264.7 cells infected with *L. major* WT and Δ*gp63*+Δ*gp63* is the same as with *L. major* Δ*gp63*.

To address this issue, infection was done with the same kinetics (2 and 6 hours) with *L. major* (WT, Δ*gp63*, Δ*gp63*+*gp63*). Similar to what we observed in BMMs, exclusion of Sec22b from the membrane of phagosomes harbouring *L. major* WT and Δ*gp63*+Δ*gp63* is less than *L. major* Δ*gp63* but in contrast to BMMs, exclusion of Sec22b from the phagosomes containing *L. major* Δ*gp63* is dramatically reduced as compared to cells treated with zymosan alone (Figure 5-B). We conclude that althought both RAW264.7 and BMMs are macrophages, they do not behave completely similar; thereby, further studies concerning the exclusion of Sec22b should be done on either RAW264.7 or BMMs separately and interpret the mechanism independently as well. It is clear that results obtained using primary BMMs are biologically more important than those obtained in macrophage cell lines such as RAW264.7.

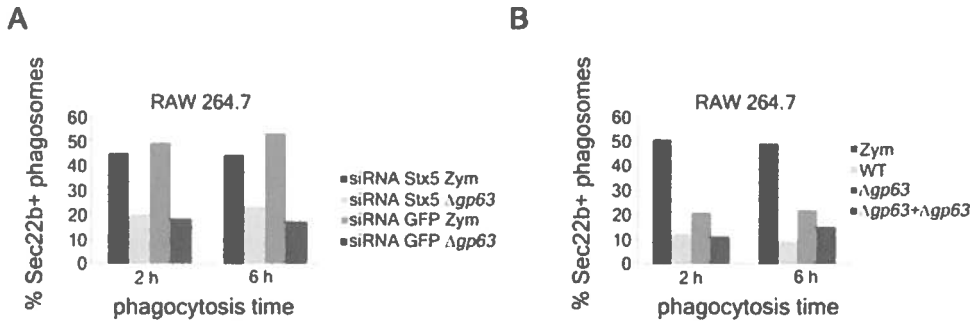


**Figure 5. Significantly less recruitment of Sec22b to phagosomes harbouring *L. major* Δ*gp63* in RAW264.7 cells compared to zymosan. (A)** Quantification of Sec22b recruitment to the phagosomes containing either zymosan or *L. major* Δ*gp63* after 2 and 6 h of phagocytosis. RAW264.7 cells were knocked-down for syntaxin 5 or GFP, as a control. Here we counted 100 cells per experiment. **(B)** Quantification of Sec22b recruitment to the phagosomes containing either zymosan or *L.major* (WT, Δ*gp63*, Δ*gp63*+Δ*gp63*) at 2 and 6 h of phagocytosis. Here we counted 100 cells per experiment. Recruitment of Sec22b to the phagosomes containing *L. major* (WT and Δ*gp63*+Δ*gp63*) is less than in those harbouring *L. major* Δ*gp63*. In contrast to what we observed in BMMs, recruitment of Sec22b to the phagosomes containing *L. major* Δ*gp63* was dramatically less than in those containing zymosan.

## Syntaxin 4, syntaxin 5 and SNAP23 play a bactericidal role in macrophages

Sec22b regulates antigen cross-presentation by interacting with syntaxin 4 on the phagosome membrane (Cebrian, Visentin et al. 2011). Also, it has been shown that SNAP23 is a critical protein in the recruitment of NADPH oxidase complex and V-ATPase to the phagosomes (Sakurai, Hashimoto et al. 2012). Considering the role of these Sec22b-interacting proteins in phagosome acidification and maturation, we decided to investigate their bactericidal effect.

In order to define the bactericidal ability of syntaxin 4, syntaxin 5 and SNAP23, we knocked-down each protein in RAW 264.7 macrophages (which are more efficient cells for conducing siRNA than primary BMMs) individually. Then, knocked down RAW 264.7 macrophages were infected with *E.coli* DH1α, as described before (Steele-Mortimer, Meresse et al. 1999)

In the absence of syntaxin 4, there is more phagocytosis while the bactericidal effect decreases (Figure 6-A). This result shows a bactericidal role for syntaxin 4 and could be one of the reasons why it is one of the proteins targeted by *Leishmania*. It is possible that upon infection with *Leishmania* and targeting syntaxin 4, fusion of the vesicle compartment to the plasma membrane, where syntaxin 4 is located, will be impaired. In addition, it has been shown that syntaxin 4 plays a role in TNF secretion (Stow, Manderson et al. 2006). Thus, if secretion of TNF is inhibited by *Leishmania*, at least in part, via syntaxin 4 degradation, this could favor parasite survival and cause a delay in the resolution of the infection.

Bacterial infection with *E.coli* DH1α was also conducted on RAW 264.7 cells knocked down in SNAP23. The results show that there is less phagocytosis of bacteria in comparison with cells

knocked down in GFP as control and the bactericidal effect was inhibited (Figure 6-B). There is a recent study on SNAP23 which explains that this protein regulates phagosome formation and maturation in macrophages (Sakurai, Hashimoto et al. 2012). Also, it has already been shown that SNAP23 in interaction with syntaxin 4 is located on the plasma membrane and plays a role in TNF secretion (Stow, Manderson et al. 2006). Therefore, it might be possible that cleaving SNAP23 is another strategy applied by *Leishmania* to cause a delay in phagosome maturation through GP63.

RAW264.7 cells knocked down in syntaxin 5 showed less bactericidal effect as compared to control ones (siRNA GFP bar), but there was no effect in phagocytosis (Figure 6-C).

In conlusion, all the Sec22b-interacting proteins studied here, play a role in microbicidal activity of macrphages. The exact mechanism for each protein is not clear yet and further investigations are required.

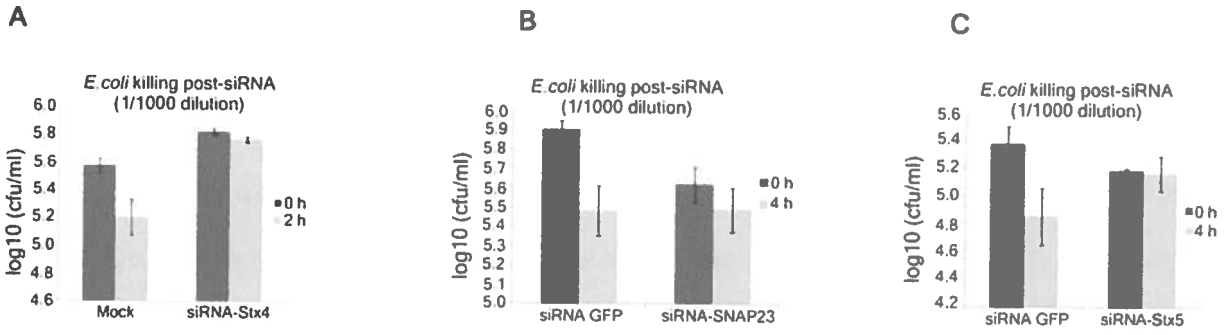


**Figure 6. Syntaxin 4, -5 and SNAP23 play bactericidal roles in RAW264.7 cells. (A)** Quantification of colonies 1/1000 dilution at 0 and 2 h post-infection. Syntaxin 4 knockdown RAW264.7 cells were infected with *E.coli* (the ratio of infection was 20:1), then Gentamicin was added and after a 20-min incubation, cells were lysed (this is referred to as 0 time point). Mock is a control sample which only received Lipofectamine. These results are representative of three independent experiments and each experiment was done in triplicate. **(B, C)** Quantifications were done on knockdown RAW264.7 for SNAP23 and syntaxin 5, respectively. siRNA GFP is a control and post-infection times were 0 and 4 h.

# Discussion

It is well established that *Leishmania*, through modifications of phagolysososme biogenesis, make their phagosomes a propicious environment to replicate. Among the different virulence factors in *Leishmania*, LPG plays critical roles in promastigotes and interferes with phagosome maturation (Moradin and Descoteaux 2012). Recently, in addition to LPG, there are several studies on GP63 as well, a metalloprotease primarily expressed in promastigotes. These studies show that GP63 alters the host cell signalling pathways and impairs antigen presentation. Consequently, *Leishmania* can escape the immune response safely and survive (Olivier, Atayde et al. 2012, Matheoud, Moradin et al. 2013). Increasing evidence confirms the temporary fusion of the ER to the phagosome during phagosome maturation (Goldszmid, Coppens et al. 2009). It was also shown that more than 90% of *Leishmania* parasitophorous vacuoles (LPV) containing either *L. donovani* or *L. mexicana* display resident ER membrane molecules at a very early time after their formation (Canton, Ndjamen et al. 2012). Altogether, these results led us to investigate the effect of *Leishmania* on ER-SNARE Sec22b as a regulator of phagosome maturation and also of cross-presentation (Cebrian, Visentin et al. 2011). Our results show that exclusion of Sec22b from phagosomes harbouring *Leishmania* happens through GP63 and not LPG. However, we show that exclusion of this ER-SNARE from the infected phagosomes is not due to its cleavage or degradation by GP63. We suggest that it is probably mediated by cleavage or degradation of a cognate protein in Sec22b complex such as syntaxin 2, -3, -4, -5 or SNAP23 (Arasaki and Roy 2010, Canton, Ndjamen et al. 2012). There was no cleavage of syntaxin 3 protein after infection with *Leishmania*; however, syntaxin 2, 4, 5 and SNAP23 were degraded in a GP63-dependent manner. Confocal microscopy analyses showed no effect on Sec22b recruitment to zymosan-containing phagosomes in the absence of syntaxin 4, -5 or SNAP23

individually. Thus, we hypothesize that by knocking down all the three SNAREs simultaneously, exclusion of Sec22b might be observed. Our explanation is that, normally after infection by *Leishmania*, syntaxin 4, -5 and SNAP23 are degraded in the infected phagosomes. Hence, degradation of several components of the protein complex might cause the exclusion of Sec22b. It is possible that the absence of only one subunit will be compensated by other subunits so Sec22b recruitment would still be observed. It has been also shown that only 10% of SNARE levels are sufficient for SNARE-SNARE interaction (Bethani, Werner et al. 2009), so by knocking down proteins via siRNA, there might be still enough residual SNARE levels to maintain their functions in the cell. In support of this notion, in another study it is indicated that simultaneously blocking of VAMP2, -3 and -8 completely inhibits insulin-stimulated GLUT4 insertion into the plasma membrane while re-expression of only one of these SNAREs rescues the inhibition (Bethani, Werner et al. 2009, Zhao, Yang et al. 2009). Furthermore, there is a study which shows that recruitment of Sec22b to the vacuole containing virulent *L. pneumophila* is required but not for the avirulent form of these bacteria. In that study, it is shown that recruitment of Sec22b to the vacuole containing *L. pneumophila* encounters a delay with triple knock down cells for syntaxin 2, -3 and -4 while single knock down of these proteins does not cause any delay in Sec22b recruitment. These observations, have been explained in part by the fact that mammalian cells overexpress some SNAREs to ensure that main cellular processes are not perturbed (Arasaki and Roy 2010) This also explains why pathogens target several SNAREs in a protein complex and not only one.

Besides these explanations, it is also possible that some other cognate proteins such as syntaxin 18, p31 or BNIP1 (Aoki, Kojima et al. 2008), which are all Q-SNAREs and in a protein complex with Sec22b, are involved in Sec22b recruitment to the phagosome. There is a proteomic

analysis by Desjardins and his colleagues which shows recruitment of syntaxin 18 to the phagosomes (Campbell-Valois, Trost et al. 2012), suggesting that it can be another target protein for *Leishmania*. Since syntaxin 18 interacts with Sec22b, it is possible that it is involved in recruitment of Sec22b to the phagosome. Further analyses on the functions of syntaxin 18, p31 or BNIP1 and their interactions with Sec22b might shed some light on the mechanism of impaired Sec22b recruitment to the phagosomes of *Leishmania*-infected cells.

Furthermore, it has been shown that Rab1 is an important effector protein for recruitment of Sec22b to the *Legionella*-containing vesicle (Derre and Isberg 2004, Kagan, Stein et al. 2004). It has been shown that Rab1 recruitment to the *Legionella*-containing vesicle mediates the transport and fusion of ER-derived vesicles containing Sec22b. Inhibiting Rab1 function impairs the recruitment of Sec22b to the *Legionella*-containing vesicle. Therefore, investigating the interaction of Rab1 with Sec22b and its role on the impairement of Sec22b recruitment to the *Leishmania*-containing phagosomes could be an interesting approach.

Another point to take into account is the differences in Sec22b recruitment in RAW264.7 macrophages compared to primary BMMs. We showed that syntaxin 4, syntaxin 5 and SNAP23 are cleaved by *Leishmania* and it explains why we chose *E. coli* as a bacterial model to investigate their microbicidal effect. In cells infected with *Leishmania*, there is degradation of all above-cited proteins, which mimicks a triple siRNA knock-down. Althought we are aware of the fact that RAW264.7 cells do not behave well after treating them with transfection buffer for siRNA and infection with *Leishmania,* it still would be interesting to monitor the survival of *L. donovani* WT at 6, 24 and 48 h post-infection in knock-down RAW264.7 for SNAP23 and

syntaxin 4. It has already shown that by deleting syntaxin 5, replication of *Leishmania* amastigotes in the parasitophorous vacuole is interrupted (Canton, Ndjamen et al. 2012).

What then is the functional consequence of SNARE degradation by *Leishmania*? Our study showed that the microbicidal effect of macrophages after knockdown of syntaxin 4, -5 and SNAP23 decreases significantly. Syntaxin 4 and SNAP23 are described as SNAREs involved in TNF secretion, which is one of the most important cytokines secreted by macrophages to kill *Leishmania* (Kuroda, Nishio et al. 2008). Thus, it is possible that through SNARE cleavage, *Leishmania* reduces TNF secretion and thereby inhibits nitric oxide production in macrophages (Frankova and Zidek 1998). There is a recent study showing that SNAP23 is a regulator of phagosome maturation. Indeed, this SNARE protein has a critical role in the recruitment of V-ATPase to phagosome membranes (Sakurai, Hashimoto et al. 2012). Consequently, it is possible that *Leishmania* targets SNAP23 to interfere with phagosome maturation and acidification and by doing so decrease the microbicidal effect of the macrophages.

It has been shown that syntaxin 5 is present on LPVs and plays a role in *Leishmania* replication (Canton, Ndjamen et al. 2012). Furthermore, to confirm the role of syntaxin 5 in LPV development, Canton and his colleague used Retro-2 as a small molecule that has a role in the redistribution of syntaxin 5 (Canton and Kima 2012). They showed that targeting syntaxin 5 by Retro-2 blocks LPV development in infected cells. Considering all these studies on syntaxin 5 and its role in LPV formation, the question that remains to be answered is why *Leishmania* degrades syntaxin 5 despite being acquired in LPVs and being seemingly necessary for parasite survival? Further studies are required to answer this question. It is possible that the role of individual SNAREs in some cellular processes show contrasting results in part because many

physiological processes can be insensitive to disruption of single SNARE molecules. On the other hand, redundancy of some individual SNAREs has evolved to buffer some vital cellular processes by compensation with other proteins.

# Material and Methods

## Macrophages

All animals were handled in strict accordance with good animal practice as defined by the Canadian Council on Animal care and all animal work was approved by the Comité institutional de protection des animaux of INRS-Institut Armand-Frappier (protocol 0508-01). BMM were obtained by growing bone marrow cells from female BALB/c mice at 37° C in 5% $CO_2$ for 7 days in Dulbecco Modified Eagle Medium with L-glutamine (Life Technologies) supplemented with 10% heat-inactivated FBS (Hyclone, Logan, UT), 10mM Hepes (pH 7.4) and antibiotics (complete medium) in the presence of 15% (v/v) L929 cell-conditioned medium as a source of colony-stimulating factor (CSF)-1 (Vinet, Fukuda et al. 2009). BMM were made quiescent by culturing them in the absence of CSF-1 for 18 h prior to use. RAW 264.7 mouse macrophages were cultured in complete medium in a 37° C incubator with 5% $CO_2$. The VAMP8 knockout mice with a genetic background of 129 SVJ have been described previously (Wang, Shi et al. 2007) and they were kindly provided by Dr. Wang (Institute of Molecular and Cell Biology, Singapore). The VAMP8 knockout mice were kept in the National Centre for Experimental Biology of INRS-Institut Armand-Frappier (protocol 1004-03). PCR method was employed to genotype tail with primers specific for wild type and mutant alleles (Wang, Ng et al. 2004). The BMM were obtained from knockout mice by growing bone marrow cells as described previously for BALB/c mice.

## Parasites

Promastigotes of *L.major* WT, Δ*gp63*, and Δ*gp63*+Δ*gp63* (Joshi, Kelly et al. 2002), *L. donovani* LV9, Δ*lpg2* (which does not synthesize repeating Galβ1,4Manα1-$PO_4$ units) (Descoteaux, Luo et al. 1995), were grown at 26°C in M199 medium supplemented with 20% heat inactivated FBS, 100 μM Hypoxanthine, 40mM HEPES, 5 μM hemin, 3 μM biopterin, 1 μM biotin and antibiotics. The *L. major* Δ*gp63*+Δ*gp63* and *L. donovani* Δ*lpg2 parasites were* grown in the presence of 50 μg/ml G418 and 300 μg/ml Hygromycin B, respectively. For infections, promastigotes were used in the late stationary phase of growth.

## Reagents and antibodies

Rabbit polyclonal antibodies to VAMP8, Sec22b, syntaxin 4, syntaxin 5, syntaxin 2, syntaxin 3 and SNAP23, were purchased from SYSY (Synaptic Systems). Another Sec22b antibody was mouse monoclonal antibody for Novus Biological and another rabbit polyclonal antibodies specific for Syntaxin 3, were purchased from AbCam.

## Phagocytosis assays

Complement opsonisation of *Leishmania* promastigotes with BALB/c mouse serum was done as described before (Lodge and Descoteaux 2005), macrophages were incubated with parasites at the a particle to cell ratio of 15:1 for different kinetic time points. In the case of infectivity for more than 2 hours, excess particles were removed by two washes with DMEM after two hours of infection and cells were transferred again to 37° C for the indicated time points before processing for microscopy and western blot.

## Phagosome isolation

Adherent macrophages ($30X10^6$) per 150X20mm tissue culture dish were incubated overnight at 37° C in 5% $CO_2$. The day after, cells were infected with *L. major* (WT, Δ*gp63*, Δ*gp63*+Δ*gp63*) for 2 hours and then polystyrene magnetic beads of 3 μm diameter (Spherotech) were added for 1 h. Phagosome purification was performed as previously described (Vinet and Descoteaux 2009, Arango Duque, Fukuda et al. 2013).

## Microscopy and immunofluorescence

Macrophages were fixed, permeabilized using 0.1% Triton X-100 and nonspecific surface FcγR binding were blocked using 1% BSA, 2% normal goat serum, 6% skimmed milk and 50% inactivated FBS (Vinet, Fukuda et al. 2008). Recruitment of different proteins such as Sec22b were quantified by immunofluorescence microscopy. Results are based on at least 300 cells in each independent experiment (100 cells per each cover slip) and each experiment repeated two times. For recruitment experiments, cells were labelled with the appropriate combinations of primary and secondary Abs. Detailed analysis of protein recruitment and distribution was performed essentially as described before (Lodge and Descoteaux 2005). Confocal immunofluorescence analysis was done by using two different kinds of microscope, an oil immersion Nikon Eclipse E800 microscope equipped with a Bio-Rad radiance 2000 confocal imaging system (Bio-Rad) and also, Zeiss LSM780 with Axio observer Z1 installed. All the images were taken by plan-Apochromat objective (63X, 1.4 oil DIC) and aquired by the Zen 2011 software. Statistical analysis was performed by using GraphPad software to calculate unpaired *t*-test to test whether the differences between control and infected groups were significant.

## Western blot

Cells and purified phagosomes were lysed in ice-cold lysis buffer (20 mM Tris-HCl (pH 7.5) and 1% Nonidet P-40) containing protease and phosphatase inhibitors. Phagosomal and total cell lysates proteins were separated on 15% SDS-PAGE, transferred onto Hybond-ECL membranes (Amersham Biosciences) and immunodetection was achieved by chemiluminescence (Amersham Biosciences).

## RNA interference

RNA duplexes targeting syntaxin 5, syntaxin 4 and SNAP23 were purchased from Thermo Scientific as ON-TARGET plus SMART pools. The transfection of RNA duplexes into RAW264.7 cells was conducted using Lipofectamin RNAiMAX Reagent from Invitrogen. The final concentration of RNA duplexes was 25nM. Knockdown efficiency was determined after 48 h post-transfection by immunoblot and also bactericidal effect experiment was done after 48 h as well. For infection studies, cells were infected with *L. major* (WT, Δ*gp63*, Δ*gp63*+Δ*gp63*) promastigotes opsonized with BALB/c mouse serum.

## Bacterial infection

Bacteria *E.Coli* DH1α were grown in Luria-Bertani (LB) broth without antibiotics for 16 h at 37°C and then subcultured at a dilution of 1:20 in fresh LB medium without antibiotics until $OD_{600}$ ~ 0.3. RAW264.7 were seeded into 24 well plates and before adding bacteria, DMEM medium was changed with no antibiotic DMEM and 10% inactivated FCS. The ratio of infection *E. coli*: macrophage was 20:1. The bacterial inoculum was prepared by pelleting at 1000xg in a

microcentrifuge for 2 min and resuspended in PBS1X and then added to the cells. The infection was carried out at 37°C in 5% $CO_2$ for 20 min. Later on, non internalized bacteria were removed by washing 4 times with PBS1X. Thereafter, 5µg/ml of Gentamicin was added for 20 min at 37° C in 5% $CO_2$. For time 0 h, cells were lysed by 100µl Triton X-100 (1%) for 1 min. Lysates were diluted and plated in agar (Steele-Mortimer, Meresse et al. 1999).

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# CHAPTER 3

# Discussion

# 1. VAMP8 and cross-presentation

## 1.1. Phagosome maturation

Uptake of external particles is a critical process for eukaryotic cells and occurs via four key pathways: macropinocytosis, phagocytosis, clathrin-mediated endocytosis (CME) and caveolae-mediated endocytosis (Blander and Medzhitov 2006). Among these pathways, phagocytosis is the most essential endocytic mechanism in host defense to eliminate pathogens, hence unleashing an inflammatory response to alert the immune system about the presence of microbes. Each pathogen binds to different receptors on the surface of phagocytes and activates different signalling pathways. In general, there is actin polymerization at the phagocytic cup, which is under the control of effector proteins such as the GTPases Rho, Rac and Cdc42 (Hall and Nobes 2000). With time, early phagosomes mature by acquiring new components and sequentially fusing with early endosomes, late endosomes and lysosomes. Finally, mature phagosomes – except those of dendritic cells – attain an acidic pH of ~ 5.5 to degrade and kill internalized microorganisms (Janssen, Tabeta et al. 2006). The main role of macrophages is to kill and completely degrade internalized pathogens, and therefore require an acidic pH in their phagosomes to accomplish this task. Dendritic cells, on the other hand, act as APCs, and keep a less acidic pH within their phagosomes to avoid complete degradation of antigen epitopes. These epitopes are consequently presented via MHC class I and II complexes (Russell 2007).

## 1.2. SNAREs are targeted by pathogens to alter phagosome maturation

SNAREs play an essential role during phagocytosis and they selectively fuse with the phagosome membrane during the phagosome maturation process. SNAREs constitute a big

family of proteins with selective distribution in different endosomal compartments. There are 25 members in *Saccharomyces cerevisiae*, 36 members in *Homo sapiens* and 27 in *Leishmania* (Besteiro, Coombs et al. 2006). Remarkably, syntaxin 4 and SNAP23 (plasma membrane), VAMP3 and syntaxin 13 (recycling endosome), Sec22b, syntaxin 18 and D12 (ER), VAMP8 (late endosome), VAMP7 and syntaxin 7 (late endosome and lysosomes) are SNAREs involved in phagosome fusion and maturation (Sakurai, Hashimoto et al. 2012). Syntaxin 11 is another SNARE that is located on the plasma membrane, late endosomes and lysosomes in macrophages. It binds to Vti1b to regulate trafficking steps between late endosomes, lysosomes and plasma membrane in macrophages; it plays a role as a negative regulator for phagocytosis of apoptotic cells and IgG opsonized red blood cells (Zhang, Ma et al. 2008, Offenhauser, Lei et al. 2011).

Pathogens alter the phagosome maturation process through different mechanisms such as recruitment, exclusion, degradation and cleavage some specific SNAREs. Sequentially, pathogens make of phagosomes a safe compartment to survive and replicate. Here are some examples that illustrate the role of SNAREs in pathogen surviving in host cells.

Several intracellular pathogens like *Salmonella*, avoid interacting with lysosomes but they usually recruit lysosome-associated membrane protein 1 (LAMP1). The exact role of LAMP1 is not clear yet but recently it has been shown that recruitment of LAMP1 to the phagosome is mediated by SipC-Syntaxin 6 interaction. It is possible that *Salmonella* uses syntaxin 6 on *Salmonella*-containing phagosomes (SCP) to recruit LAMP1 by fusing to LAMP1-containing Golgi-derived vesicles, in order to stabilize their niche in macrophages (Madan, Rastogi et al.

2012). Another study shows that VAMP7 is required for biogenesis of *Coxiella* replicative vacuoles (Campoy, Mansilla et al. 2012).

*L. pneumophila* alters fusion mechanism in host cells by inducing Sec22b localization to the plasma membrane, whereby it recruits syntaxin proteins on the vacuole. DrrA is a Rab1-targeting effector protein that stimulates this noncanonical SNARE fusion in pathogen-containing vacuoles, and helps *L. pneumophila* survive (Arasaki, Toomre et al. 2012).

*Chlamydia* is an intracellular bacterium that replicates in a modified compartment called inclusion. It has been shown that syntaxin 6 is recruited to the chlamydial inclusion membrane. More specifically, *Chlamydia* alters the host membrane trafficking pathway to survive and replicate and syntaxin 6 has a critical role in the development of inclusions (Pokrovskaya, Szwedo et al. 2012).

It has been shown that IncA and IcmG/DotF are two SNARE-like proteins that are expressed by *Chlamydia* and *Legionella,* respectively, and they inhibit the function of endocytic SNAREs machinery in host cells; it means that SNARE-mediated fusion during phagosome maturation is inhibited (Paumet, Wesolowski et al. 2009).

Another study shows that VAMP3 is a target for *Mycobacterium tuberculosis* to inhibit phagosome maturation, and it is degraded in *M. tuberculosis* phagosome compartment (MPC). There are two different mechanisms to explain the absence of VAMP3 on MPC; first, proteolytic activity of *M. tuberculosis* (it produces two putative metalloproteases and contains other secreted hydrolases); the second one is through host cytosolic enzymes that degrade VAMP3 (Fratti, Chua et al. 2002).

In our study, we show that *Leishmania* parasites also selectively target key SNAREs in phagosomes to interfere with phagosome maturation and antigen presentation. This is a novel strategy by *Leishmania* to escape immune recognition. *Leishmania* GP63 is a metalloprotease that is expressed in high levels in promastigotes, and is responsible for cleaving VAMP8, syntaxin 4, SNAP23 and VAMP3 in *Leishmania*-infected cells.

## 1.3. GP63 as a virulence factor

Cleavage and degradation of some SNAREs such as SNAP25, VAMP2 and syntaxin 1 is mediated by the metalloproteases clostridial neurotoxins (CNT) (Proux-Gillardeaux and Galli 2008). It has been also shown that VAMP2 and VAMP8 are cleaved through the metalloprotease, antarease from the Brazilian scorpion *Tityus serrulatus* (Fletcher, Fletcher et al. 2010). Altogether, these observations drew our attention to analyse the effect of GP63 on host SNARE expression given its main role as a metalloprotease and virulence factor in *Leishmania*. There are several reports of cleavage and degradation of specific proteins by GP63 such as: a) mTOR, whose degradation stalls translation in macrophages, b) protein tyrosine phosphatases, which when cleaved stimulate phosphatase activity thereby altering cell signalling, c) TAB-1, whose cleavage inactivates p38 mitogen-activated protein kinase (Gomez, Contreras et al. 2009, Halle, Gomez et al. 2009, Jaramillo, Gomez et al. 2011).

The mechanism by which GP63 accesses and degrades host cell substrates remains unclear. However, there is a study showing that exosomes, which are released by *Leishmania* and contain GP63, could be a potential mechanism for this molecule to be exported across the phagosome membrane (Silverman, Clos et al. 2010). Further studies showed that GP63 colocalizes with lipid microdomains on some macrophage membranes (e.g. perinuclear membranes) (Gomez,

Contreras et al. 2009). On the other hand, some SNAREs associate with lipid microdomains (Gil, Soler-Jover et al. 2005), which could facilitate their interaction with GP63.

Our work shows for the first time that GP63 targets different SNAREs in phagosomes containing *Leishmania*. The VAMP8 protein contains four distinct domains: vesicular, transmembrane, SNARE motif and cytoplasmic domain. Based on mass spectrometry results, the cleavage site for VAMP8 is located at the beginning of the SNARE motif which is in the cytoplasmic region. The mechanism of how GP63 cleaves VAMP8 is not clear yet and further studies are required to shed light on this matter.

As can be seen in figure S1 of publication 1, GP63 redistributes itself in the cytoplasm of the host cell, but it is not known specifically in which organelle it resides. There are different mechanisms that could explain how GP63 cleaves VAMP8. For instance, GP63 and VAMP8 could be located on different vesicles that, upon coming close to each other would allow the cytoplasmic part of VAMP8 on one vesicle interacting with GP63 on the other vesicle and cleavage would occur. It is also possible that through interaction of intra-vesicular compartments – one containing GP63 and the other VAMP8 – two vesicles fuse so that both VAMP8 and GP63 are located in the same vesicle and VAMP8 would become cleaved upon interacting with GP63. Another way of cleavage could happen through free GP63 in the cytoplasm. Finally, invagination of the plasma membrane containing GP63 (originating from exosomes) could give rise to vesicles containing GP63. These vesicles could then traffic in the cell and find their way to VAMP8-containing organelles.

Cleavage of SNAREs by GP63 alters phagosome remodeling and converts the phagosome into a suitable compartment where parasites survive, replicate and spread the infection. We found out

that cleavage of VAMP8 significantly inhibits antigen cross-presentation. Furthermore, we confirmed the role of VAMP8 in cross-presentation by using VAMP8$^{-/-}$ mice.

## 1.4. Regulation of cross-presentation

Based on our results on the regulation of cross-presentation by VAMP8, and also on the role of Sec22b – another SNARE at the phagosome membrane – on cross-presentation (Cebrian, Visentin et al. 2011), it appears that molecular mechanisms involved in cross-presentation are regulated by a series of SNARE molecules. The involvement of these SNAREs in the regulation of cross-presentation makes them attractive targets for pathogenic attack.

The processing of exogenous antigens for cross-presentation is a complex mechanism that is regulated by several pathways. *In vitro* experiments showed that cross-presented OVA antigens are reduced by macrophages and dendritic cells after infection with *Leishmania*; which means that antigen processing happens in both kinds of phagocytes. The exact mechanism for OVA processing is not clear yet and further studies are required to define whether OVA cross-presentation after infection with *Leishmania* happens through the cytosolic or the vacuolar pathway. Consequently, although macrophages are not professional phagocytes for antigen cross-presentation and dendritic cells not efficient at phagocytosis, it is possible that in *in vivo* *Leishmania* infections, both kinds of cells are involved in *Leishmania* phagocytosis and both cross-present *Leishmania* antigens. To address this unclear mechanism, more studies are required to show that *in vivo* cross-presentation of *Leishmania* antigens follows the same mechanism as *in vitro* for OVA and also whether this is mainly done by macrophages or by dendritic cells. In addition, the intracellular pathways of cross-presentation in human dendritic cells are poorly investigated; however, recent studies on human blood dendritic cell antigen 3

show clear phylogenetic homology with mouse $CD8^+$ dendritic cells. Also, human lamina propria $CD103^+$ dendritic cells have similar functions to mouse lamina propria $CD103^+$ dendritic cells (Joffre, Segura et al. 2012). In summary, there is little known about subpopulations of human dendritic cells and almost nothing about their functions. Nonetheless, because of existing similarities between mouse and human dendritic cells, it is plausible to consider that there is an inhibition of cross-presentation after infection with *Leishmania* in human dendritic cells through a similar molecular mechanism. Further studies are required to confirm this hypothesis.

As noted earlier, one of the key factors in dendritic cell phagosome function is pH, which should be alkaline to inhibit cleavage of antigen epitopes and present these on MHC class I molecules. Recent reports indicate that the NOX2 complex regulates pH and phagosomal proteolysis (Rybicka, Balce et al. 2012). In our study, we show that in the absence of VAMP8, the pH in the phagosome lumen was decreased while the proteolytic activity was increased. Furthermore, in the absence of VAMP8, severe impairment of oxidative activity through inhibition of $gp91^{phox}$ recruitment to phagosomes was observed, supporting the link between oxidation and proteolytic activity. It has been shown that the NOX2 produces ROS and ensues in a less acidic pH in the phagosomal lumen in dendritic cells. In contrast to normal phagosomes, absence of NOX2 increases phagosome acidification and sequentially increases antigen degradation and impairs cross-presentation in dendritic cells (Savina, Jancic et al. 2006). In regards to our results for colocalization of VAMP8 with $gp91^{phox}$, we strongly suggest that VAMP8 is a key regulator protein that mediates the fusion between vesicles bearing $gp91^{phox}$, also called "inhibitory lysosome-related organelles", and phagosomes. Furthermore, the critical role of SNAP23 in the functional recruitment of NADPH oxidase complex to the phagosomes has been shown (Sakurai, Hashimoto et al. 2012). Based on the fact that VAMP8 and SNAP23 are cognate proteins and

make a protein complex (Wang, Ng et al. 2004), it is possible that both of these two SNARE participate in gp91$^{phox}$ recruitment to phagosomes. In addition to the key role of VAMP8 in cross-presentation, it is possible that other molecules targeted by *Leishmania* may also be involved in this process. Our observations on further decrease in cross-presentation after infection of VAMP8$^{-/-}$ BMDC with *L. major* promastigotes support this hypothesis. Accordingly, SNAP23 and syntaxin 4 are good candidates to continue the investigation on the inhibition of cross-presentation after infection of VAMP8$^{-/-}$ BMDC infected with *Leishmania*.

# 2. Exclusion of Sec22b from phagosomes harbouring *Leishmania*

## 2.1. Phagosome remodeling through LPG

The impact of lipophosphoglycan (LPG), the major surface glycolipid in *Leishmania* promastigotes, on phagosome remodeling is significant (Moradin and Descoteaux 2012). Inhibition of phagolysosome biogenesis through LPG helps *Leishmania* to survive and replicate safely in phagosomes. In spite of the accumulation of some proteins like actin around phagosomes, LPG is also responsible for interfering with recruitment of some proteins such as synaptotagmin V, protein kinase C-α and V-ATPase (Holm, Tejle et al. 2001, Vinet, Fukuda et al. 2009). In this study we showed the exclusion of Sec22b from *Leishmania*-containing phagosomes, albeit not due to an LPG-, but to a GP63-dependent pathway.

## 2.2. Cognate proteins for Sec22b

Increasing evidence confirms the direct fusion of the ER itself (not ER-derived vesicles) to phagosomes during maturation. It has been shown that Sec22b is one the ER protein markers

that regulates cross-presentation in dendritic cells (Guermonprez, Saveanu et al. 2003, Goldszmid, Coppens et al. 2009, Cebrian, Visentin et al. 2011). Our work has shown GP63-mediated exclusion of Sec22b from phagosomes containing *Leishmania* which might inhibit cross-presentation and it would be another novel strategy used by *Leishmania* to decrease antigen presentation and escape from the immune response. Concerning the fact that Sec22b is neither degraded, nor cleaved by GP63, other Sec22b-interacting proteins might be responsible for the exclusion of Sec22b from the phagosome.

Direct membrane fusion between ER and the plasma or phagosomal membrane requires different protein complexes. In *L. pneumophila*-containing- vacuoles, Sec22b interacts with syntaxin 2, -3, -4 and SNAP23. Further analysis has confirmed the functional interaction among these SNAREs, since addition of NSF and α-SNAP dissociates Sec22b from the other SNAREs (Arasaki and Roy 2010). Phagosomes purified from J774 macrophages showed the interactions among syntaxin 18, BNIP1, p31 and Sec22b. Further structural analyses show that interaction of syntaxin 18 and Sec22b causes conformational changes and creates high-affinity binding sites for the two other proteins (p31 and BNIP1) in the complex (Hatsuzawa, Tamura et al. 2006, Aoki, Kojima et al. 2008). A proteomic study also shows the recruitment of syntaxin 18 to the phagosome (Campbell-Valois, Trost et al. 2012). In another study, it has been shown that purified recombinant syntaxin 5, membrin and rbet1 assemble to create a high affinity binding site for Sec22b. This quaternary complex is observed in NRK cells (normal rat kidney cells) (Xu, Joglekar et al. 2000). Rab1 is a small guanosine triphosphatase (GTPase) that plays an important role in fusion of ER-derived vesicles and it has been shown that Rab1 is involved in Sec22b recruitment. Concerning Sec22b and Rab1, both of them play an important role in converting *L. pneumophila* containing vacuoles (LCV) into supporting organelles for *L.*

*pneumophila* replication (Kagan, Stein et al. 2004). ER markers including Sec22b, calnexin, as well as LAMP1 as a marker for late endosomes and lysosomes are recruited to *Leishmania* parasitophorous vesicles (LPV) containing amastigotes (Ndjamen, Kang et al. 2010).

Among all the proteins interacting with Sec22b, we studied selectively syntaxin 2, -3, -4, -5 and SNAP23 for degradation via GP63 after infection with *Leishmania* promastigotes. Immunoblot analyses showed the cleavage of syntaxin 2, -4, -5 and SNAP23; syntaxin 3 was not cleaved. Consequently, degraded SNAREs were potential candidates to mediate the exclusion of Sec22b from phagosomes harbouring *Leishmania*. However, after individually knocking-down syntaxin 4, -5 and SNAP23, no effect on Sec22b recruitment to zymosan-containing phagosomes was observed. This phenomenon can be explained in two different ways: first, there are probably some other proteins involved in the Sec22b protein complex that become altered upon infection with *Leishmania*. These cognate proteins can be degraded or cleaved by GP63, or their recruitment to the phagosomes might be impaired. Consequently, this could explain the indirect effect of *Leishmania* GP63 on Sec22b exclusion from the phagosome. Second, it is possible that absence of only one protein through siRNA knock-down can be compensated by increased expression of other proteins; this would explain why recruitment of Sec22b does not change under these experimental conditions. To support this hypothesis, there is a study showing that individual knock-down of syntaxin 2, -3 and -4 did not change Sec22b recruitment while triple knock-down cells showed a delay in Sec22b recruitment (Arasaki and Roy 2010). In this regard, there is another report that demonstrates that only 10% expression of SNAREs is sufficient to have proper SNARE-SNARE interactions in the cell (Bethani, Werner et al. 2009), and since efficiency of siRNA knock-down is never 100%, it is possible that low levels of syntaxin 4, -5 or SNAP23 expression are enough to mediate Sec22b recruitment to the phagosome. These

observations can be explained in part by the fact that usually in mammalian cells some SNAREs are over expressed to guarantee that main cellular process proceed without delay. Likewise, this might be a reason why pathogens target more than one SNARE in host cells. Regarding Sec22b, it is possible that since Sec22b is a regulator of cross-presentation, which is a key function in phagocytic cells, cognate proteins in the Sec22b complex are overexpressed to ensure that cross-presentation does not fail easily. Consequently, by knocking-down these genes individually there might still be a certain amount of cognate proteins in the Sec22b complex to mediate the recruitment of Sec22b to the phagosomes.

## 2.3. Microbicidal activity of Stx5, Stx4 and SNAP23

The next question we addressed was about the functional consequences of the cleavage of each individual SNARE after infection with *Leishmania*. Our study showed a bactericidal role for syntaxin 4, -5 and SNAP23 individually. It is possible that by triple knock-down of syntaxin 4, -5 and SNAP23, the bactericidal ability of macrophages becomes dramatically reduced and this could explain why *Leishmania* degrades these three SNAREs simultaneously.

Despite the presence of different defense mechanisms in macrophages, *Leishmania* appears to survive quite well within macrophage phagolysosomes and this implies evolved applied strategies by *Leishmania* to efficiently escape macrophage microbicidal effector mechanisms. Regarding the bactericidal role of SNAP23 and syntaxin 4, they are both involved in TNF secretion (Uriarte, Rane et al. 2011). TNF is a proinflammatory cytokine produced by activated macrophages and also many other cell types such as natural killer cells (Wilhelm, Ritter et al. 2001, Allenbach, Launois et al. 2008). SNAP23 is a critical SNARE in phagosome maturation and modulates recruitment of V-ATPase to phagosome membranes. It is possible that

*Leishmania* targets SNAP23 to inhibit phagosome acidification and microbicidal activity of macrophages (Sakurai, Hashimoto et al. 2012). Finally, syntaxin 5 was the last SNARE we studied here, which is found on LPVs and acts as a regulator of *Leishmania* amastigote replication and LPV development (Canton and Kima 2012). We used *E.coli* to show the bactericidal effect of syntaxin 5, but strikingly, syntaxin 5 seems to promote *Leishmania* replication in LPVs after 48 h infection. Further studies to explain why *Leishmania* promastigotes cleave syntaxin 5, while being a regulator of *Leishmania* amastigotes replication, need to be done. It is possible that Sec22b in interaction with syntaxin 5 regulates cross-presentation and *Leishmania* promastigotes could down-regulate cross-presentation temporarily at early time points of infection by cleaving syntaxin 5. Consequently, *Leishmania* promastigotes would have time to escape from the immune system and differentiate to amastigotes. In addition, it is possible that although syntaxin 5 is acquired by LPVs, its cleavage through GP63 is needed for the overall survival strategy of the parasite. Altogether, it is likely that the role of individual SNAREs in some cellular processes show differing results in part because many physiological processes are insensitive to disruption of single SNARE molecules. On the other hand, redundancy of SNARE function could have evolved to buffer vital cellular processes whereby failure or absence of one SNARE is functionally compensated with another SNARE.

# CHAPTER 4

## Résumé en français

# 1. Introduction

## 1.1. La phagocytose

La phagocytose est le mécanisme permettant l'internalisation de particules de grande taille, de microorganismes et de débris cellulaires par les cellules phagocytaires telles que les macrophages, les cellules dendritiques et les neutrophiles. La phagocytose est initiée par différents récepteurs situés en surface des cellules et impliqués dans diverses voies de signalisation à travers différents mécanismes moléculaires. La polymérisation de la F-actine, à l'origine de la formation de pseudopodes au niveau des coupes phagocytiques, est suivie séquentiellement de la fusion des membranes et du transfert de protéines à travers les compartiments endosomaux, suivant le processus appelé maturation du phagosome (Deschamps, Echard et al. 2013). Pendant la maturation du phagosome, chaque compartiment individuel possède une composition unique aux niveaux de sa membrane et de son lumen. Le pH luminal est un des facteurs cruciaux permettant de déterminer la fonction des phagosomes chez différentes cellules phagocytaires. Le rôle des macrophages étant d'éliminer les particules étrangères, le pH du phagosome est très acide, contrairement au pH interne des cellules dendritiques qui est moins acide afin de garder intacts les épitopes antigéniques pour pouvoir les présenter aux cellules T (Russell, Vanderven et al. 2009). Durant la maturation du phagosome, plusieurs changements s'opèrent au niveau de la fusion des membranes et du trafic des protéines. Certains pathogènes parviennent à échapper à la réponse immunitaire en portant atteinte à la maturation du phagosome.

## 1.2. Les SNAREs et leur rôle dans la biogénèse du phagolysosome

Le NSF (*N*-ethylmaleimide sensitive factor), en collaboration avec une protéine adaptatrice appelée SNAP (NSF attachment protein), joue un rôle dans de nombreuses voies de trafic intracellulaire. Les SNAREs (SNAP receptors) constituent une super-famille de 38 membres identifiés à ce jour chez les mammifères. Ils sont distribués de manière sélective dans différents compartiments endosomaux et sont séparés en deux catégories différentes : les v-SNAREs et les t-SNAREs. Les v-SNAREs (également appelés R-SNAREs) sont situés sur des vésicules membranaires, tandis que les t-SNAREs (également appelés Q-SNAREs) sont situés sur les membranes cibles. Au cours de la fusion membranaire, un complexe *trans*-SNARE est formé de façon à rapprocher les deux membranes. Ce complexe est formé d'une simple hélice R-SNARE et de trois hélices Q-SNARE. Ensuite, des complexes transitoires *cis*-SNAREs sont formés sur la même membrane. En d'autres termes, la membrane de la vésicule fusionne avec la membrane cible. A la suite de cette fusion, les complexes SNARE sont rapidement désassemblés pour être rapidement disponibles pour un prochain cycle de fusion membranaire. Le démantèlement de ces complexes *cis*-SNARE est effectué par un complexe soluble formé de l'ATPase cytoplasmique ubiquitaire de NSF et de l' α-SNAP (Alpadi, Kulkarni et al. 2012).

### 1.2.1. Le R-SNARE VAMP8

La protéine VAMP8 (aussi appelée endobrévine) a été découverte en 1998 et est située dans les endosomes tardifs, le réseau *trans*-Golgi, les « coated pits , plasma membrane » ainsi que dans les compartiments endosomaux (Antonin, Holroyd et al. 2000). Le rôle de VAMP8 ne se limite pas à la phagocytose et la maturation des phagosomes. Il a en effet été démontré que la syntaxine 17 interagit avec SNAP29 et la protéine VAMP8 lysosomale pour effectuer la fusion entre les autophagosomes et le lysosome (Itakura and Mizushima 2013).

### 1.2.2. Le R-SNARE Sec22b

Il a été démontré que le réticulum endoplasmique (RE) constitue un donneur de membrane lors de la phagocytose (Desjardins 2003). Sec22b est un des marqueurs protéiques du RE. Il interagit avec la syntaxine 18, P31 et BNIP1 pour permettre la phagocytose et le trafic post-Golgi (Aoki, Kojima et al. 2008). Sec22b régule négativement la phagocytose dans les macrophages (Hatsuzawa, Hashimoto et al. 2009). La syntaxine 5, la membrine et Bet1 sont des molécules apparentées à Sec22b, qui peut uniquement se lier à elles en hétérocomplexes trimériques et non individuellement ou en complexe binaire (Xu, Joglekar et al. 2000). Récemment, il a également été démontré que Sec22b régule la cross-présentation des antigènes dans les cellules dendritiques (Wesolowski and Paumet 2010).

### 1.2.3. Le R-SNARE VAMP3

VAMP3 est un marqueur de vésicules de recyclage et joue un rôle important dans la phagocytose dans les macrophages (Bajno, Peng et al. 2000). Il est possible que VAMP2, -3 et -8 jouent des rôles redondants dans la phagocytose, la présence de l'une permettant de compenser l'absence d'une autre (Allen, Yang et al. 2002).

### 1.2.4. Le Q-SNARE syntaxine 5

Une interaction a lieu entre la syntaxine 5 et Sec22b dans les cellules dentritiques. Contrairement à Sec22b, la syntaxine 5 est nécessaire à la livraison de molécules MHC de classe I et à son acheminement vers la surface de la cellule (Cebrian, Visentin et al. 2011).

### 1.2.5. Le Q-SNARE syntaxine 4

Des études fonctionnelles menées sur la syntaxine 4 ont montré que cette protéine est située à la surface des macrophages et qu'elle forme un complexe avec SNAP23/Munc 18c. La syntaxine 4 régule le trafic membranaire et la sécrétion de TNF suite à une induction par le LPS (Pagan, Wylie et al. 2003).

### 1.2.6. Les SNAREs et les pathogènes

Si l'on considère l'implication séquentielle des SNAREs dans la maturation du phagosome, ceux-ci pourraient constituer de bonnes cibles pour permettre à certains pathogènes d'empêcher la biogénèse du phagolysosome, l'activité bactéricide et la présentation d'antigènes.

Voici plusieurs études ayant démontré le clivage de SNAREs spécifiques après infection. Une endopeptidase dépendante du zinc de la neurotoxine clostridiale est capable de cliver les protéines VAMP1, -2 et-3 (VAMP = vesicle-associated membrane protein), tandis que la toxine botulique du sérotype A clive SNAP25 (Pellizzari, Rossetto et al. 1999). Une autre metalloprotéase dépendante du zinc (l'anteréase), extraite du venin du scorpion brésilien *Tityus serrulatus*, clive VAMP2 et VAMP8 (Fletcher, Fletcher et al. 2010). L'activité protéolytique de *Mycobacterium tuberculosis* vise VAMP3, interférant ainsi avec la maturation du phagosome de façon à survivre dans les macrophages (Fratti, Chua et al. 2002). Des pathogènes intracellulaires tels que *Mycobacterium*, *Salmonella*, *Chlamydia* ou *Legionella* bloquent certaines étapes de fusion défavorables mais d'un autre côté créent de nouvelles voies de fusion favorables à leur survie et à leur réplication à l'intérieur des cellules hôtes.

## 1.3. *Leishmania*

*Leishmania* est un parasite protozoaire responsable de la leishmaniose. Il se transmet suite à une morsure par une mouche phlébotome femelle (des genres *Lutzomyia* et *Phlebotomus*). Ces parasites sont exposés aux environnements extra- et intracellulaires. Au stade extracellulaire, ils vivent dans les intestins des mouches phlébotomes (phlébotomine). Au stade intracellulaire, ils vivent dans les cellules de vertébrés. Par conséquent, les parasites présentent deux stades morphologiques distincts (promastigote et amastigote), retrouvées chez leurs hôtes invertébré et vertébré, respectivement. Les promastigotes sont flagellés tandis que les amastigotes ne le sont pas (Sacks and Kamhawi 2001).

Les amastigotes se multiplient dans les macrophages mais en fonction des espèces de *Leishmania*, ils peuvent cibler différents organes. Il existe trois formes différentes de Leishmaniose (Matte, Mallégol et al. 2009):

1) La leishmaniose cutanée (ou ulcère de Baghdad, bouton de Delhi) est responsable de lésions cutanées et est provoquée par l'espèce la plus commune du monde ancien comme *L. major*, *L. tropica*, ainsi que *L. aethiopica* et *L. mexicana* dans le nouveau monde.

2. la leishmaniose mucocutanée (Espondia ou Uta) se caractérise par des lésions formant des métastases dans la membrane muqueuse. Elle est causée par *L. braziliensis braziliensis*.

3. La leishmaniose viscérale (Kala-Azar) se manifeste par de la fièvre, de l'anémie et le gonflement du foie et de la rate. *L. donovani* est responsable de cette forme fatale de leishmaniose dans l' "ancien monde".

### 1.3.1. Les facteurs de virulence de *Leishmania*

*Leishmania* ne produit pas de toxines mais plusieurs facteurs de virulence lui permettant d'établir l'infection dans les macrophages, comme le glycophosphatidylinositol (GPI), le glycoinositolphospholipide (GIPL), le lipophosphoglycan (LPG), le phosphoglycan (PG), le proteophosphoglycan (PPGs), la glycoprotéine 63 (GP63) et les protéases à cystéine (CPs) (Chang and McGwire 2002).

### 1.3.2. Le lipophosphoglycan (LPG)

Le LPG est le principal constituant de la surface cellulaire des *Leishmania* promastigotes. On en compte approximativement 5 millions de copies par cellule, qui en recouvrent toute la surface, y compris le flagelle. Cette molécule est fortement régulée à la baisse, voire absente, chez les amastigotes (Jecna, Dostalova et al. 2013). Le LPG est constitué de quatre parties distinctes : 1) une ancre lipidique de type 1-*O*-alkyl-2-*lyso*-phosphatidyl (*myo*) inositol ; 2) un noyau glycan ; 3) un enchaînement d'unités répétitives Galβ1,4Manα1-PO4 identique chez tous les *Leishmania* et 4) une structure cap oligosaccharidique (Descoteaux, Avila et al. 2002).

#### 1.3.2.1. Retard de la maturation du phagosome provoqué par le LPG

Les amastigotes de *Leishmania* se multiplient dans les macrophages fusionnant avec les lysosomes. Cet environnement acide (pH ~4.7 - 5.2) est optimal pour l'activité des amastigotes et est causé par l'ATP-ase H+ de type vacuolaire (Vinet, Fukuda et al. 2009). Différents marqueurs protéiques d'endosomes tardifs et de lysosomes tels que Rab7, LAMP1et LAMP2, de même que la calnexine et Sec22b comme protéines résidant dans le RE, sont présentes dans les vacuoles

contenant les amastigotes (Ndjamen, Kang et al. 2010). Le complexe NADPH oxidase ne peut pas s'assembler sur la membrane des vacuoles parasitophores contenant les amastigotes, car les amastigotes ne sont pas capables d'induire la phosphorylation de la sous-unité $p47^{phox}$ cytosolique (Lodge and Descoteaux 2008). Les vacuoles parasitophores contenant les amastigotes présentent également une disruption des microdomaines lipidiques situés à l'intérieur de la membrane du phagosome (Lodge, Diallo et al. 2006).

Contrairement aux phagosomes contenant des amastigotes, les phagosomes contenant des promastigotes interagissent peu avec les endosomes tardifs et les lysosomes, ils présentent donc un retard dans le recrutement de LAMP1 et un défaut dans le recrutement de Rab7. L'accumulation de F-actine periphagosomale est induite dans le phagosome porteur de promastigotes pour interférer avec le recrutement de transducteurs de signaux et avec le trafic vésiculaire vers le phagolysosome en formation (Lodge and Descoteaux 2005). L'exclusion de la synaptotagmin V comme régulateur de la phagocytose, tout comme la maturation des phagosomes, sont des exemples de la désorganisation faisant suite à une infection par des promastigotes (Vinet, Fukuda et al. 2008). Il a aussi été démontré que les phagosomes contenant des promastigotes n'étaient pas capables d'assembler le NADPH oxidase à la membrane des phagosomes, ce qui empêcherait la formation d'espèces réactives de l'oxygène (ROS), qui sont d'importants agents antimicrobiens (Moradin and Descoteaux 2012). Le pH acide des phagosomes est essentiel pour la dégradation des pathogènes. L'inhibition de l'acidification des phagosomes est donc cruciale pour la survie et la multiplication de pathogènes tels que *L. donovani*, qui exclut la V-ATPase des phagosomes de façon à inhiber leur acidification (Vinet, Fukuda et al. 2009).

### 1.3.3. GP63

GP63 a été découverte dans les années 1980 et constitue un antigène de surface majeur pour la forme promastigote de *Leishmania*. On donne différents noms à ce facteur de virulence : major surface glycoprotein (MSG), promastigote surface protease (PSP) and Leishmanolysine (Yao, Donelson et al. 2003). Toutes les protéines GP63 possèdent le même motif de liaison au zinc (HExxHxxGxxH). On retrouve une quantité d'isoformes de GP63. La majorité est transmembranaire grâce à un GPI et une faible proportion est intracellulaire. Le reste est sécrété (Yao, Donelson et al. 2007). Il a été démontré que les formes sécrétées et transmembranaires sont associées à des microdomaines lipidiques. Le mise en évidence de 11 sites de clivage démontre qu'il n'y a pas de site de clivage conservé pour GP63, mais il se clive préférentiellement au peptide P1' (acide aminé hydrophobe)-P2' (acide aminé basique)-P3' (acide aminé basique) (Contreras, Gomez et al. 2010).

#### 1.3.3.1. Fonction de GP63

GP63 joue différents rôles selon le stade du parasite. Elle peut se lier à la protéine C3 du complément et la convertir en une forme inactive C3bi pour échapper à la lyse provoquée par le complément. GP63 agit comme une opsonine auprès du complément pour faciliter l'attachement de *Leishmania* aux macrophages (Ueno and Wilson 2012). GP63 a jusqu'à présent été détectée chez les amastigotes de toutes les espèces de *Leishmania*, cependant son rôle exact n'a pas encore été clarifié. Il a été suggéré que GP63 protègerait les amastigotes à l'intérieur des phagolysosomes des macrophages (Yao, Donelson et al. 2003). GP63 modifie la voie de signalisation des macrophages de l'hôte en dégradant différentes protéines clés impliquées dans

la signalisation cellulaire telles que SHP-1, PTP1B et TCPTP (Shio, Hassani et al. 2012). Plusieurs facteurs de transcription tels que NF-κB et la protéine-1 activée (AP-1) sont clivés par la protéine GP63 de *Leishmania* (Contreras, Gomez et al. 2010). De plus, la transcription et la traduction sont affectées par GP63. Il a été démontré que GP63 clive la cible mammifère de la rapamicine (mTOR) après infection par *Leishmania*. mTOR est une serine/threonine kinase qui active le suppresseur de facteur d'initiation eucaryote 4F (eIF4F) appelé $4^{E}$-BP1 (Jaramillo, Gomez et al. 2011). En plus des macrophages, GP63 migre vers la matrice extracellulaire et dégrade Dicer 1, un processeur de pré-ARNmi inhibant la formation de miRNP chez les cellules hépatiques infectées, causant ainsi une hypocholestérolémie (Descoteaux, Moradin et al. 2013, Ghosh, Bose et al. 2013).

## 1.4. La Cross-présentation

Les cellules présentatrices d'antigènes (CPAs) sont des cellules clés du système immunitaire impliquées dans l'acquisition des particules antigéniques, leur traitement et leur présentation aux lymphocytes T spécifiques au sein du complexe majeur d'histocompatibilité (CMH) ou des protéines associées. La phagocytose joue un rôle traditionnel dans l'acquisition des antigènes étrangers et leur présentation au CMH de classe II mais des études récentes démontrent que des antigènes exogènes peuvent être également présentés au CMH de classe I, alors que les molécules du CMH de classe I portent normalement des peptides dérivés de la protéolyse cytosolique. Cette voie non conventionnelle est appelée voie de cross-présentation (Mantegazza, Magalhaes et al. 2013).

Voici deux modèles de voie de cross-présentation. L'un est qualifié de « vésiculaire », l'autre de « cytosolique ».

Au sein de la voie cytosolique dépendante du protéasome et du TAP, les antigènes présents à l'intérieur des phagosomes sont acheminés vers le cytosol via le rétrotranslocon associé à la protéine de dégradation associée au RE (ERAD). Il a été démontré que la source de ce rétrotranslocon, de nouvelles molécules synthétisées par le CMH de classe I et du TAP est le compartiment intermédiaire Golgi-RE (ERGIC) fusionnant avec le phagosome. Cette interaction RE/ERGIC est importante pour certains pathogènes comme *Legionella*, *Brucella* et *Toxoplasma* car elle cible activement la voie RE/phagosome pour permettre leur survie. Ensuite, les antigènes sont ubiquitinés par les ligases à ubiquitine E3 de façon à être ciblées et dégradées par le protéasome. Les peptides générés par les antigènes ciblés peuvent soit être transférés au phagosome par TAP et chargés sur le CMH de classe I, soit poursuivre par la voie classique présentation d'antigène dans le RE par le CMH de classe I dépendant de TAP.

La voie TAP indépendante du protéasome est plus simple que l'autre voie. Dans cette voie, les antigènes phagocytés sont dégradés dans les phagosomes par les cathepsines, des protéases principalement actives à bas pH. Les peptides générés sont chargés sur les molécules du CMH de classe I à l'intérieur du phagosome.

### 1.4.1. La régulation de la cross-présentation

Récemment, il a été démontré que l'activité de la protéase phagosomale dans les cellules dendritiques pouvait être régulée par NOX2 de façon indépendante du pH. Dans cette voie, NOX2 oxyde directement les protéases (Rybicka, Balce et al. 2012). En effet, il y a activation

incomplète de la V-ATPase dans les cellules dendritiques suite au bas pH luminal, ce qui porte atteinte à la cross-présentation (Huynh and Grinstein 2007). En résumé, la dégradation des protéines ne devrait pas être importante dans les phagosomes des cellules dendritiques de façon à conserver les épitopes.

# 2. Premier article

## 2.1. Résultats

### 2.1.1. Les promastigotes de *Leishmania* excluent VAMP8 et VAMP3 des phagosomes

Après infection des macrophages dérivés de la moelle épinière (BMMs) par des promastigotes de *L. donovani*, la localisation intracellulaire de VAMP8 et VAMP3 a été déterminée par des analyses en immunofluorescence. Les résultats démontrent que VAMP3 et VAMP8 sont toutes deux absentes dans la majorité de phagosomes contenant les promastigotes sauvages de *L. donovani*. Nous avons démontré que cette exclusion passe par une voie indépendante du LPG. Des analyses par immunobuvardage de type Western ont confirmé le clivage de VAMP8 et VAMP3 dans les cellules infectées par *Leishmania*, nous en avons donc déduit que le clivage de protéines clés impliquées dans le trafic membranaire pourrait faire partie d'une stratégie utilisée par les promastigotes de *Leishmania* pour remodeler leur niche intracellulaire.

### 2.1.2. GP63 est responsable du clivage de VAMP3 et VAMP8

Le clivage de VAMP8 et VAMP3 dans les cellules infectées n'est pas sans rappeler l'activité de la métalloprotéase dépendante du zinc de *Clostridium botulinium* (Montal 2010). Nous nous sommes donc demandé si la protéine GP63 de *Leishmania* (également une métalloprotéase

dépendante du zinc) était responsable du clivage observé. Nous n'avons pas observé de clivage de VAMP8 ni de VAMP3 dans les BMM infectés avec des amastigotes de *L. donovani*, condition dans laquelle GP63 n'est pas détectée. Nous avons ensuite pu confirmer les résultats obtenus en n'observant pas de clivage de SNAREs particuliers après infection avec *L. major* Δ*gp63*. En résumé, ces résultats mettent en évidence un nouveau mécanisme grâce auquel les parasites *Leishmania* contrôlent la biogénèse de vacuoles parasitophores, dans lequel GP63 cible des SNAREs régulant le trafic vésiculaire depuis et vers les phagosomes.

### 2.1.3. *Leishmania* porte atteinte à la cross-présentation d'antigènes grâce à GP63

Les étapes du trafic impliquées dans la biogénèse du phagolysosome pourraient jouer un rôle clé dans la cross-présentation pendant une infection par *Leishmania*. Par conséquent, nous avons cherché à savoir si le clivage des SNAREs par GP63 observé durant l'infection pouvait altérer la cross-présentation.

Dans cette étude, la cross-présentation a été induite par l'expression de marqueurs d'activation des cellules T précoces par les cellules OT-I. L"inhibition de la cross-présentation a été observée après infection avec *L. major* de type sauvage et Δ*gp63*+*gp63* mais pas après infection avec le mutant Δ*gp63*. Nous avons confirmé que cette inhibition n'était pas due à une diminution de l'expression de molécules du CMH de classe I. De plus, les amastigotes, chez lesquels GP63 est sous-exprimée, n'inhibaient pas la cross-présentation. Jusqu'à présent, notre étude semble indiquer que cette inhibition de la cross-présentation est dépendante de la voie dépendante de GP63.

### 2.1.4. VAMP8 est nécessaire à la cross-présentation d'antigènes

Pour vérifier le rôle de VAMP8 dans la cross-présentation, nous avons utilisé des macrophages de moelle épinière (BMMs) et des cellules dendritiques de moelle épinière (BMDCs) de souris *Vamp8*$^{-/-}$ (Wang, Ng et al. 2004). En effectuant un test avec des billes recouvertes d'OVA, nous avons pu observer une diminution d'environ 50% du niveau de cross-présentation dans des BMDCs et BMMs *Vamp8*$^{-/-}$ par rapport aux niveaux observés chez les cellules sauvages. Il est intéressant de constater que contrairement à la cross-présentation, la présentation d'antigènes liée au CMH de classe II était normale voire même légèrement supérieure en absence de VAMP8. Ces résultats suggèrent fortement que VAMP8 est une des molécules régulatrices clés dans la cross-régulation ciblée par *Leishmania*.

### 2.1.5. VAMP8 régule l'activité oxydative du phagosome

Pour vérifier le rôle possible de VAMP8 dans la régulation de l'activité oxydative du phagosome, les BMDCs qui ayant internalisé des billes recouvertes de DHR ont été analysées par FACS pour déterminer leur production de ROS. Nous avons conclu que VAMP8 est nécessaire à l'activité oxydative du phagosome et que la dégradation par GP63 pourrait être responsable de l'altération de l'activité oxydative dans les BMDCs infectés avec *L. major* sauvage. Des études ultérieures ont démontré que VAMP8 est nécessaire pour le recrutement de gp91$^{phox}$ par les phagosomes, un processus jouant un rôle clé dans la régulation du traitement des antigènes pour la cross-présentation.

## 2.2. Discussion

Les pathogènes ont développé les stratégies suivantes afin d'éviter d'être éliminés par les phagosomes : prévenir la maturation du phagosome, résister à l'action microbicide des

phagolysosomes, échapper aux phagosomes du cytosol, altérer la maturation du phagosome grâce à la fusion avec d'autres organelles pour échapper à la caractérisation microbicide, incluant la présentation d'antigène. Nous démontrons ici que l'inhibition de la cross-présentation par *Leishmania* est contrôlée par GP63, une metalloprotéase fortement exprimée par la forme promastigote du parasite. Notre travail montre également que *Leishmania*, par l'intermédiaire de GP63, clive certaines molécules SNAREs endosomales, ce qui module directement la composition des phagosomes. Il semble que VAMP8 soit l'une des molécules SNAREs clivées par *Leishmania.* En l'absence de VAMP8, la cross-présentation est fortement inhibée, ce qui supporte l'hypothèse selon laquelle le clivage de VAMP8 serait le moyen grâce auquel les promastigotes de *Leishmania* inhiberaient la présentation d'antigène, une réponse immunitaire importante.

Récemment, il a été démontré que Sec22b, une molécule SNARE du RE localisée à la membrane du phagosome, est également nécessaire à la cross-présentation (Cebrian, Visentin et al. 2011). Il semble que les mécanismes moléculaires impliqués dans la cross-présentation soient régulés par une succession de molécules SNARE qui peuvent être directement ciblées par les pathogènes intracellulaires pour échapper à la reconnaissance par le système immunitaire.

Des éléments récents indiquent que la protéolyse phagosomale, et potentiellement le pH, sont régulés par le complexe NADPH oxydase (Rybicka, Balce et al. 2012). En l'absence de VAMP8, nous avons observé que le pH à l'intérieur de la lumière du phagosome se trouvait diminué, alors que l'activité protéolytique était augmentée. De plus, une atténuation sévère de l'activité oxydante a été observée, due à l'inhibition du recrutement de $gp91^{phox}$, ce qui confirme le lien entre oxydation et régulation de l'activité protéolytique. La colocalisation de VAMP8 et de

gp91$^{phox}$ dans les structures vésiculaires suggèrent fortement que cette molécule SNARE est impliquée dans l'accueil et la fusion des vésicules endocytotiques porteuses de gp91$^{phox}$. Comme VAMP8 et SNAP23 forment les complexes SNARE (Wang, Ng et al. 2004) et que SNAP23 régule le recrutement de gp91$^{phox}$, associée à la membrane « plasma » , vers le phagosome (Sakurai, Hashimoto et al. 2012), il est possible que VAMP8 et SNAP23 contrôlent conjointement l'association de gp91$^{phox}$ aux phagosomes.

En conclusion, nous reportons pour la première fois qu'un pathogène intracellulaire peut cibler et cliver des composants de la machinerie de fusion membranaire de sa cellule hôte pour inhiber la présentation d'antigène. Des études ultérieures permettront de déterminer la contribution relative d'autres molécules SNARE dans la régulation des étapes du trafic membranaire régulant les réponses immunitaires innée et acquise dans les cellules présentatrices d'antigène.

# 3. Deuxième article

## 3.1. Résultats

### 3.1.1. Exclusion de Sec22b du phagosome par GP63

En partant de nos travaux précédents démontrant que VAMP8 est ciblée par *Leishmania* et inhibe de façon significative la cross-présentation, et en prenant en considération le rôle de la protéine RE-SNARE Sec22b dans la cross-présentation (Cebrian, Visentin et al. 2011), nous avons décidé d'étudier le rôle de Sec22b après infection par *Leishmania.*

Nous avons utilisé *L. major* (sauvage, Δ*gp*63, Δ*gp*63+ *gp*63) et avons infecté des BMMs. L'absence de Sec22b dans les phagosomes contenant *L.major* Δ*gp*63+ *gp*63 et sauvage pourrait être due à son clivage par la métalloprotéase GP63. Un immunobuvardage de type Western dans

des lysats de cellules totales et des extraits de phagosomes ont démontré l'absence de clivage et de dégradation de Sec22b en conditions d'infection. Ensemble, ces résultats suggèrent que l'effet de GP63 sur la protéine Sec22b est indirect et qu'il pourrait y avoir une protéine intermédiaire agissant sur Sec22b qui serait dégradée par GP63 et qui affecterait le recrutement de Sec22b par les phagosomes contenant *Leishmania*. Une étude sur la protéine p38 (protéine kinase activée par le mitogène) a démontré que l'effet de GP63 sur cette protéine est indirect et qu'il est dû au clivage de la protéine agissant en aval, TAB1 (TGF-beta activated kinase 1) (Halle, Gomez et al. 2009).

### 3.1.2. Le recrutement atténué de Sec22b par les phagosomes n'est pas dépendant du LPG

Il a été démontré que *Leishmania*, par l'intermédiaire du LPG, était capable d'exclure la V-ATPase de la membrane du phagosome en empêchant le recrutement de la synaptotagmine V (Vinet, Fukuda et al. 2009). Nous avons infecté des BMMs avec *L. donovani* (sauvage et Δ*lpg*2) et analysé le recrutement de Seb22b dans les phagosomes infectés. Ces résultats démontrent que malgré le rôle du LPG dans beaucoup d'altérations de la biogénèse du phagolysosome (Moradin and Descoteaux 2012), l'absence de Sec22b dans les phagosomes contenant *Leishmania* n'est pas dépendante du LPG.

### 3.1.3. Dégradation de certaines protéines interagissant avec Sec22b par GP63

Il a été démontré que la syntaxine 2, la syntaxine 3, la syntaxine 4 et SNAP23, localisées au niveau des vacuoles porteuses de *L. pneumophila*, interagissaient avec Sec22b (Arasaki and Roy 2010). D'autres études montrent une interaction entre la syntaxine 5 et Sec22b (Xu, Joglekar et

al. 2000). Notre hypothèse est que GP63 pourrait inactiver l'une des protéines interagissant avec Sec22b pour affecter le recrutement par celle-ci. Nous avons démontré la dégradation des syntaxines 4, -5, -2 et de SNAP23 par GP63, et l'absence de dégradation de la syntaxine 3. Pour investiguer plus avant les conséquences fonctionnelles de la dégradation de ces SNAREs par *Leishmania* et déterminer la corrélation entre ces SNAREs avec l'exclusion de Sec22b des phagosomes porteurs de *Leishmania*, nous avons préparés des macrophages « Knock down » pour certains SNAREs et nous avons procédé à plusieurs expériences fonctionnelles supplémentaires.

### 3.1.4. Le recrutement n'est pas altéré en l'absence de SNAREs interagissant avec Sec22b

Des « Small interfering RNA » ( siRNA) ont été utilisés pour dépléter les syntaxines 4, 5 et SNAP23.

Le recrutement de Sec22b dans les phagosomes contenant du Zymosan a été réalisé dans des cellules knock down RAW 264.7. La microscopie confocale a révélé que l'absence des syntaxines 4 et 5 et de SNAP23 n'avait aucun effet sur le recrutement de Sec22b par les phagosomes. Pour analyser le mécanisme d'exclusion de Sec22b des phagosomes, nous avons utilisé les souris knockout VAMP8 et avons comparé le recrutement autour du zymosan dans les BMMs *Vamp8*$^{-/-}$ et *Vamp8*$^{+/+}$. Le recrutement de Sec22b n'est pas non plus affecté en l'absence de VAMP8.

### 3.1.5. La syntaxine 4, la syntaxine 5 and SNAP23 jouent un rôle bactéricide dans les macrophages

Pour caractériser le rôle bactéricide de la syntaxine 4, de la syntaxine 5 et de SNAP23, nous avons éliminé (knock down) individuellement chaque protéine dans des cellules RAW 264.7. Les cellules « knock down » RAW 2364.7 ont été infectées avec *E.coli* DH1α comme décrit précedemment (Steele-Mortimer, Meresse et al. 1999).

En l'absence des syntaxines 4 et 5 et de SNAP23, l'activité bactéricide est diminuée, ce qui prouve le rôle de ces SNAREs dans l'activité bactéricide des macrophages.

## 3.2. Discussion

Il a déjà été établi que *Leishmania* pouvait façonner le phagosome dans lequel il réside en modifiant la biogénèse du phagolysosome de façon à créer en environnement favorable à sa réplication. Un des facteurs de virulence connus chez *Leishmania*, le LPG, intervient particulièrement au stade promastigote pour interférer avec la maturation du phagosome (Moradin and Descoteaux 2012). Récemment, des études ont également été menées sur GP63, une metalloprotéase de surface des promastigotes. Ces études montrent que GP63 modifie la voie de signalisation cellulaire ou la présentation des antigènes ; par conséquent, *Leishmania* peut échapper sans risque à la réponse immunitaire et ainsi survivre (Olivier, Atayde et al. 2012). De plus en plus d'éléments tendent à confirmer l'établissement d'une fusion temporaire du RE avec le phagosome pendant sa maturation (Goldszmid, Coppens et al. 2009). Il a également été démontré que 90% des vacuoles parasitophores de *Leishmania* (LPV) contenant soit *L. donovani* soit *L. mexicana* présentent des molécules membranaires du RE résident sur les LPV directement après leur formation (Canton, Ndjamen et al. 2012). Ces résultats nous ont donc amenés à étudier l'effet de Leishmania sur le SNAREs du RE Sec22b, qui serait un régulateur de la maturation des phagosomes, ainsi que sur la cross-présentation (Cebrian, Visentin et al. 2011).

Nos résultats démontrent que l'exclusion de Sec22b des phagosomes porteurs de *Leishmania* est dépendante de GP63 mais pas du LPG. Cependant, nous avons également démontré que l'exclusion de ce SNARE relié au RE des phagosomes infectés n'était pas due au clivage ou à la dégradation par GP63. Nous suggérons que cette exclusion pourrait être contrôlée par le clivage ou la dégradation de protéines interagissant avec Sec22b comme les syntaxines 2, -3, -4 et -5 ou SNAP23 (Arasaki and Roy 2010, Canton, Ndjamen et al. 2012). Il n'y a pas de clivage de la syntaxine 3 après infection avec *Leishmania* mais les autres protéines citées sont bel et bien dégradées de manière dépendant de GP63. Des analyses par microscopie confocale ne montrent aucun effet de l'absence des syntaxines 4, -5 ou de SNAP23 prises séparément sur le recrutement de Sec22b. D'après ces résultats, nous suggérons qu'une élimination conjointe des trois SNAREs pourrait permettre d'observer l'exclusion de Sec22b. Cette hypothèse se base sur le fait qu'après infection par *Leishmania*, les syntaxines -4 et -5 et SNAP23 sont dégradées dans les phagosomes infectés. Il est possible que la dégradation de certaines sous-unités du complexe protéique soit à l'origine de l'exclusion de Sec22b. Il est également possible que seule une sous-unité soit capable de compenser l'absence des autres, permettant à Sec22b d'être toujours observée au niveau des macrophages infectés.

Outre toutes ces explications, il est également possible que d'autres protéines non investiguées telles que la syntaxine 18, p31 ou BNIP1 (Aoki, Kojima et al. 2008, Campbell-Valois, Trost et al. 2012), qui sont toutes des Q-SNAREs capables d'interagir avec Sec22b, soient impliquées dans son exclusion. Des analyses complémentaires sur d'autres SNAREs seraient donc nécessaires.

Quelles sont donc les effets fonctionnels de la dégradation de ces SNAREs par *Leishmania*? Notre étude montre que les effets bactéricides des macrophages après « knock down » des syntaxines 4, -5 et de SNAP23 diminuent fortement. La syntaxine 4 et SNAP23 sont décrites comme des types de SNAREs impliqués dans la sécrétion du TNF, qui est l'une des cytokines les plus importantes pour l'élimination de *Leishmania* par les macrophages (Kuroda, Nishio et al. 2008). Il est également possible que *Leishmania*, en réduisant la sécrétion du TNF par le clivage de certains SNAREs, réduise la production d'oxyde nitrique par les macrophages (Frankova and Zidek 1998). Une étude récente montre que SNAP23 est un régulateur de la maturation du phagosome. Cette protéine SNARE est connue pour jouer un rôle crucial dans le recrutement de la V-ATPase vers la membrane du phagosome (Sakurai, Hashimoto et al. 2012). Par conséquent, il est possible que *Leishmania* cible cette protéine pour interférer avec la maturation du phagosome et son acidification, de façon à diminuer son activité bactéricide.

Il a été démontré que la syntaxine 5 était présente sur les vacuoles parasitophores de *Leishmania* et qu'elle jouait un rôle dans la réplication de *Leishmania*. Par ailleurs, pour confirmer le rôle de la syntaxine 5 dans le développement des LPV, Canton et Kima ont utilisé Retro-2 comme petite molécule jouant un rôle dans la redistribution de la syntaxine 5 (Canton and Kima 2012). Ils ont ainsi démontré qu'en ciblant la syntaxine 5 par Retro-2, on bloque le développement des LPV dans les cellules infectées. Si l'on tient compte de toutes ces études menées sur la syntaxine 5 et son rôle dans la formation des LPV, une question reste sans réponse: pourquoi *Leishmania* degraderait la syntaxine 5 alors qu'elle est présente dans la vacuole parasitophore et qu'elle semble nécessaire à la survie dans les LPV? Des études complémentaires sont nécessaires pour pouvoir répondre à cette question. Il est possible que l'étude du rôle de SNAREs individuels dans certains mécanismes cellulaires aboutisse à des résultats mitigés, dus au fait que beaucoup de

processus physiologiques pourraient demeurer insensibles à la perte d'une simple molécule SNARE. En d'autres termes, la redondance de certains SNAREs individuels a pu évoluer de façon à pouvoir compenser la perte de certaines protéines et assurer les fonctions vitales.

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# Annex I

## *Leishmania* evades host immunity by inhibiting antigen cross-presentation through direct cleavage of the SNARE VAMP8

# Annex II

## *Leishmania* promastigotes: building a safe niche within macrophages

# Annex III

## *Leishmania* Dices Away Cholesterol for Survival

# Annex IV

# Alteration of the autophagic response by *Leishmania major* promastigotes

# Alteration of the autophagic response by *Leishmania major* promastigotes

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## ABSTRACT

The protozoan *Leishmania* parasitizes macrophages and is renowned for their ability to sabotage host-cell signaling pathways and antimicrobial processes. The mammalian target of rapamycin (mTOR) plays a pivotal role in the balanced coordination of multiple cellular processes, including protein synthesis, cytokine secretion and autophagy. In this study, we aimed to investigate the impact of *L. major* promastigotes on the mTOR signaling cascade and downstream autophagy-related events. Infection of bone marrow-derived macrophages with *L. major* promastigotes resulted in rapid proteolytic cleavage of mTOR and the upstream factor tuberous sclerosis complex-2 by the GPI-anchored, zinc-dependent metalloprotease GP63. Interestingly, although internalization of *L. major* promastigotes promoted LC3 lipidation, LC3 recruitment to the parasitophorous vacuole was blocked in a GP63-dependent manner. Western blotting and confocal microscopy experiments revealed that autophagic targeting of *L. major* parasites was impaired due to GP63-mediated cleavage of the R-SNARE vesicle-associated membrane protein 8. Indeed, in the absence of this SNARE, recruitment of LC3 to phagosomes containing GP63-deficient parasites was impaired. This study highlights a novel mechanism exploited by *L. major* to interfere with the autophagic response and provides a better understanding of *Leishmania* pathogenesis.

## AUTHOR SUMMARY

To establish an intracellular infection in macrophages, *Leishmania* parasites must evade a plethora of powerful antimicrobial responses, such as the production of reactive oxygen species and the activation of hydrolytic enzymes. Usually studied for its role in cellular homeostasis and survival, autophagy has recently been acknowledged as an important effector mechanism in the control and resolution of microbial infections. Here, we investigated whether the protozoan parasite *L. major* targets autophagy-related events. We showed that, upon *L. major* promastigote internalization, the autophagic marker LC3 becomes lipidated, indicative of autophagy induction. Yet, using promastigotes devoid of the metalloprotease GP63, an important virulence factor, we found that this molecule allows parasite evasion of autophagic targeting. Indeed, GP63 cleaves the membrane fusion mediator VAMP8, thereby preventing the recruitment of LC3 to phagosomes that contain *L. major* promastigotes. Our results highlight a novel mechanism used by *L. major* to modulate antimicrobial macrophage responses and provide a better understanding of *Leishmania* pathogenesis.

# Introduction

Leishmaniasis, a vector-borne disease that afflicts 12 million people worldwide, causes a wide spectrum of pathologies, ranging from self-healing ulcers to potentially fatal visceral infections. The etiological agents of leishmaniases, protozoa of the genus *Leishmania*, establish a persistent intracellular infection in the phagolysosome of host macrophages. To persist in this hostile environment, *Leishmania* parasites have evolved sophisticated mechanisms to sabotage macrophage antimicrobial functions and responses (Olivier, Gregory et al. 2005, Kima 2007, Lodge and Descoteaux 2008, Shadab and Ali 2011). Leishmanolysin, also referred to as GP63, is a glycophosphatidylinositol (GPI)-anchored, zinc-dependent metalloprotease highly expressed on the surface of *Leishmania* promastigotes (Yao, Donelson et al. 2003). This virulence factor plays a pivotal role in parasite evasion of host and macrophage immune responses (Yao 2010, Isnard, Shio et al. 2012). Upon promastigote internalization, GP63 is released from the parasite surface, a process thought to involve secretion of exosomes (Silverman and Reiner 2011), and inserts itself into lipid rafts, thereby rapidly gaining access to various intracellular compartments (Gomez, Contreras et al. 2009). Altough the precise mechanism remains to be elucidated, GP63 cleaves a number of macrophage function effectors, including plasma membrane receptors (Pelletier and Sato 2002), protein tyrosine phosphatases (Gomez, Contreras et al. 2009, Halle, Gomez et al. 2009), adaptor molecules (Halle, Gomez et al. 2009), transcription factors (Gregory, Godbout et al. 2008, Contreras, Gomez et al. 2010), and proteins involved in cytoskeletal rearrangement (Corradin, Ransijn et al. 1999).

Autophagy, or autophagocytosis, is a catabolic process by which cells degrade their own components, in a regulated manner, through the lysosomal machinery (Mizushima 2007, Ravikumar, Futter et al. 2009, Tanida 2011). In this process, structures known as isolation membranes or phagophores elongate and sequester cytosolic components into closed, double-membrane vacuoles called autophagosomes. Then, through stepwise fusion with endosomes and lysosomes, autophagosomes mature into acidic, highly degradative organelles, resulting in cargo breakdown. Constitutive autophagy serves in homeostasis and quality control by targeting long-lived or damaged organelles and aggregated or misfolded proteins for disposal. Inducible autophagy, on the other hand, acts as a cell-autonomous defense mechanism in response to environmental stress such as nutrient starvation, through bulk degradation of cytosolic material, and against microbial infections, through direct elimination of intracellular pathogens (Cemma and Brumell 2012).

Due to its wide spectrum of functions, aberrant regulation of autophagy contributes to numerous pathologies, including cancer, neurodegenerative disorder, chronic inflammation and infectious diseases (Sridhar, Botbol et al. 2012). Among the signals that coordinate the balance between proliferation and autophagy, mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that regulates transcription, translation, and metabolism in response to nutrients and growth factors. Under conditions of starvation, inactivation of mTOR results in autophagy, although the precise mechanism is not fully understood (Jung, Ro et al. 2010, Alers, Loffler et al. 2012). Autophagy is then orchestrated by soluble *N*-ethylmaleimide-sensitive factor attachment receptors (SNAREs). Assembly of Qa-, Qb-, Qc- and R-SNAREs into tetrameric coiled-coil structures is an essential step in vesicle fusion with target membranes, and therefore plays a significant role in autophagosome biogenesis and maturation (Ishihara, Hamasaki et al. 2001, Fader, Sanchez et al. 2009, Furuta, Fujita et al. 2010, Moreau, Ravikumar et al. 2011, Nair, Jotwani et al. 2011, Renna, Schaffner et al. 2011). SNAREs are also involved in autophagic elimination of pathogens: interactions between vesicle transport through interaction with t-SNAREs homolog 1b (Vti1b) and vesicle-associated membrane protein 8 (VAMP8) were recently found to be essential for antimicrobial autophagosome fusion with lysosomes (Furuta, Fujita et al. 2010).

Several pathogens have evolved strategies to prevent, circumvent, or even exploit autophagy to ensure intracellular survival (Deretic and Levine 2009, Orvedahl and Levine 2009, Levine, Mizushima et al. 2011, Yuk, Yoshimori et al. 2012). Reports providing a link between *Leishmania* infection and autophagy have so far been scarce. Promastigotes of *L. major* were recently found to cleave mTOR in a GP63-dependent manner, resulting in impaired host cell protein translation, weakened immune responses, and increased parasite replication (Jaramillo, Gomez et al. 2011). Given the role of mTOR in autophagy induction, the present study aimed to analyze the effect of *L. major* promastigotes on the autophagic response and the events that govern them. Our findings show that although infection leads to GP63-mediated inactivation of components of the Akt/mTOR axis, targeting of *L. major* promastigotes to autophagosomes is paradoxically inhibited in a GP63-dependent manner. Indeed, recruitment of the autophagic marker LC3 to the parasitophorous vacuole (PV) of *L. major* was blocked through a mechanism involving GP63-mediated cleavage of the endocytic R-SNARE VAMP8.

# Results

## L. major *GP63 cleaves key components of the Akt/mTOR pathway.*

The mTOR pathway is believed to play an important role in the autophagic response. To analyze the extent of disruption in this cascade in infected macrophages, we first investigated the effect of *L. major* promastigotes on proteins acting upstream and downstream of mTOR in BMM. As previously shown (Jaramillo, Gomez et al. 2011), infection resulted in rapid, time-dependent cleavage of mTOR, yielding multiple cleavage fragments (Fig. 1A). The tuberous sclerosis complex-2 (TSC-2) acts as a GTPase-activating protein (GAP) against Rheb and thereby acts a negative regulator of mTOR signalling (Inoki, Li et al. 2003, Tee, Manning et al. 2003). A significant reduction in TSC-2 protein levels was also detected upon infection with *L. major* promastigotes (Fig. 1A). As was previously described (Jaramillo, Gomez et al. 2011), the eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) levels were not altered in infected BMM, although phosphorylation of 4E-BP1 on residues Thr37/46 was impaired, likely as a result of mTOR cleavage (Fig. 1A).

To test the potential role of GP63 in the cleavage of TSC-2, as is the case for mTOR (Jaramillo, Gomez et al. 2011), we infected BMM with a *gp63*-deletion *L. major* mutant (Δ*gp63*). Δ*gp63* promastigotes failed to cleave TSC-2 and mTOR, in contrast to wild-type (WT) parasites or Δ*gp63* promastigotes rescued with the *gp63.1* gene (Δ*gp63*+*gp63*) (Fig. 1B). Altogether, these results indicate that infection with *L. major* promastigotes inflicts significant damage to the mTOR axis, through GP63-mediated cleavage of key proteins.

## L. major *promastigotes block LC3 recruitment to the PV in a GP63-dependent manner.*

Induction of the autophagic response is characterized by the lipidation of cytosolic LC3-I to form membrane bound LC3-II, which translocates to autophagosomes (Kabeya, Mizushima et al. 2000). To investigate whether disruption of the mTOR axis by *L. major* promastigotes leads to an autophagic response, we first analyzed by Western blotting the effect of *L. major* on LC3 lipidation in BMM. Both GP63-deficient and GP63-expressing parasites promoted conversion of LC3-I to LC3-II to a similar extent (Fig. 2A), indicating that *L. major* promastigotes induce a GP63-independent autophagic response. In addition, expression of the autophagic cargo receptor sequestasome (p62/SQSTM1) increased with time upon infection, in a GP63-independent (Fig. 2B) and cycloheximide-sensitive (data not shown) fashion. Next, to determine

whether the autophagic response induced upon infection targets the PV of *L. major* promastigotes, we examined LC3 intracellular distribution by confocal immunofluorescence microscopy. Despite conversion to its lipidated form, LC3 was detected on less than 10% of phagosomes containing WT *L. major* promastigotes (Fig. 2B,C). Remarkably, LC3 was present on a significantly higher percentage (~20%) of phagosomes containing Δ*gp63* parasites (Fig. 2B,C). As expected, phagosomes containing the Δ*gp63*+*gp63* parasites were similar to WT-phagosomes with respect to the presence of LC3. These results suggest that *L. major* promastigotes prevent phagosomal recruitment of LC3 through a GP63-dependent mechanism. These results also indicate that cleavage of mTOR by GP63 did not correlate with the induction of an autophagic response.

### *L. major* GP63 cleaves key SNAREs involved in autophagy.

Recent reports have begun to highlight the importance of SNARE-mediated membrane fusion in canonical autophagy (Ishihara, Hamasaki et al. 2001, Fader, Sanchez et al. 2009, Furuta, Fujita et al. 2010, Moreau, Ravikumar et al. 2011, Nair, Jotwani et al. 2011, Renna, Schaffner et al. 2011). Q-SNAREs Syntaxin 7, Syntaxin 8, and Vti1b, and R-SNAREs VAMP7 and VAMP8 are involved in the trafficking of phagocytosed microbes to the lysosome (Becken, Jeschke et al. 2010) and have also been attributed roles in autophagy (Atlashkin, Kreykenbohm et al. 2003, Furuta, Fujita et al. 2010, Moreau, Ravikumar et al. 2011). Interaction of Vti1b with VAMP7 orchestrates the maturation of Atg16L1-positive precursor vesicles into LC3-positive autophagosomes (Moreau, Ravikumar et al. 2011), while its interaction with VAMP8 mediates the fusion of autophagosomes with lysosomes (Furuta, Fujita et al. 2010). We previously showed that infection with *L. major* promastigotes results in a GP63-dependent proteolytic cleavage of VAMP8. This observation raised the possibility that *L. major* promastigotes prevented recruitment of LC3 to phagosomes through a GP63-mediated cleavage of SNAREs involved in the control of membrane fusion during autophagy. We thus assessed the impact o *L. major* infection on the levels and integrity of VAMP8, Vti1B, and VAMP7 in BMM. Western blotting analyses revealed that similar to VAMP8, Vti1B was cleaved in the presence o GP63 (Fig. 3). On the other hand, VAMP7 was insensitive to the proteolytic activity of GP63 (Fig. 3). Altogether, these findings suggest that *L. major* promastigotes may escape targeting to autophagosomes by disrupting SNARE complexes through proteolytic cleavage.

### *L. major* inhibits VAMP8 and Vti1b recruitment to the PV.

To determine the consequences of GP63-mediated proteolytic cleavage on VAMP8 and Vti1b functionality, we analyzed their recruitment to the phagosome upon infection. Although a significant number of phagosomes containing either zymosan or Δ*gp63 L. major* promastigotes

displayed VAMP8, this SNARE was absent from the vast majority of phagosomes containing either WT or GP63-rescued parasites (Fig. 4A). These observations suggest that exclusion of VAMP8 from PVs ensues from its cleavage by GP63, and provide a potential mechanism underlying GP63-dependent inhibition of LC3 recruitment to the PV. Akin to VAMP8, Vti1b was excluded from PVs, in contrast to phagosomes containing zymosan particles (Fig. 4B, C). Interestingly, however, inhibition of Vti1b recruitment to the PV was independent of GP63 and phosphoglycans (PGs), since *Δgp63 L. major* and the phosphoglycan-defective *Δlpg2 L. donovani* both displayed similarly low levels of Vti1b recruitment as their WT counterparts (Fig. 4B, C). These results show that *L. major* promastigotes interfere with key SNAREs involved in the autophagic response and suggest a role of particular importance for VAMP8 in *L. major* targeting to autophagosomes.

## *VAMP8 is essential for LC3 recruitment to the PV of* L. major.

To verify the hypothesis that GP63-mediated VAMP8 cleavage and exclusion from PVs is responsible for the impairment of LC3 recruitment to vacuoles containing promastigotes, we analyzed LC3 recruitment to PV in infected BMM derived from WT and VAMP8$^{-/-}$ 129 SvJ mice. As observed with BALB/c BMM (Fig. 2C,D), infection of WT 129 SvJ BMM with GP63-expressing promastigotes yielded a two-fold decrease in LC3 recruitment compared with GP63-deficient parasites (Fig. 5A,B). However, in *Vamp8*$^{-/-}$ BMM, LC3 failed to be recruited to PV containing *Δgp63* promastigotes, confirming that VAMP8 plays a role in LC3 recruitment to *L. major* promastigotes-harboring PV. The non-pathogenic *L. tarentolae* species, which do not cleave VAMP8, displayed LC3 recruitment to their PV to the same extent as GP63-deficient *L. major*, further corroborating these results (data not shown). In contrast, VAMP8 was not required for *L. major*- or rapamycin-induced LC3 conversion (Fig. 5C). Altogether, these results indicate that *L. major* prevents recruitment of LC3 to the PV through GP63-mediated cleavage of VAMP8.

# Discussion

Establishment of an intracellular infection by promastigotes of *Leishmania* species such as *L. donovani*, *L. chagasi* and *L. major* is characterized by a delay in phagolysosome maturation, most likely allowing time for differentiation into the mammalian stage amastigotes (Desjardins and Descoteaux 1997, Dermine, Scianimanico et al. 2000, Spath, Garraway et al. 2003, Rodriguez, Gaur Dixit et al. 2011). Inhibition of phagolysosome biogenesis is mostly achieved through lipophosphoglycan (LPG)-mediated disruption of lipid rafts (Winberg, Holm et al. 2009), causing the exclusion of antimicrobial effectors such as NADPH oxidase (Lodge, Diallo et al. 2006) and the vesicular proton pump v-ATPase (Vinet, Fukuda et al. 2009). Here, we provide the first evidence that *L. major* promastigotes escape the autophagic response, through a mechanism involving GP63-mediated proteolytic cleavage of SNAREs. Furthermore, our results revealed a role for VAMP8 in the recruitment of LC3 to phagosomes.

Autophagy is a specialized effector mechanism with connections to phagocytosis and innate immunity, through Fγc- and Dectin-1-mediated phagocytosis or recognition of pathogen- or damage-associated molecular patterns by Toll-like receptors (TLRs), NOD-like receptors, and RIG-I-like receptors (Sanjuan, Dillon et al. 2007, Delgado and Deretic 2009, Huang, Canadien et al. 2009, Deretic 2011, Ma, Becker et al. 2012). This interplay allows cells to monitor microbial invasion and contributes to autophagic macrophage activation (APMA), producing optimal conditions for autophagy-mediated pathogen recognition, capture and elimination, a process referred to as xenophagy. Our data show that phagocytosis of *L. major* promastigotes induces an autophagic response, as assessed by LC3-I to LC3-II conversion (Fig. 2A). This response, also seen *in vivo* upon *L. donovani* (Mitroulis, Kourtzelis et al. 2009) or *L. amazonensis* (Cyrino, Araujo et al. 2012) infection, may arise from the recognition of the parasite by TLRs such as TLR2, 3, 4 and 9 (Kropf, Freudenberg et al. 2004, Flandin, Chano et al. 2006, Gallego, Golenbock et al. 2011, Faria, Reis et al. 2012). Ligation of various TLRs has indeed been shown to promote autophagosome formation, LC3 conversion and LC3 association with phagosomes (Sanjuan, Dillon et al. 2007, Xu, Jagannath et al. 2007, Delgado, Elmaoued et al. 2008).

While an autophagic response is induced upon infection, our findings indicate that *L. major* promastigotes exploit the proteolytic activity of GP63 to block LC3 recruitment to phagosomes. The consequences of excluding LC3 from *Leishmania*-harboring phagosomes may be inferred from a recent study aimed at investigating the role of LC3 during Dectin-1-mediated phagocytosis in bone marrow-derived dendritic cells (Ma, Becker et al. 2012). Hence, Dectin-1-

triggered phagocytosis, reactive oxygen production, microbial killing, and cytokine production in response to β-glucan particles were normal in the absence of LC3. On the other hand, absence of LC3 reduced the ability of dendritic cells to present antigen in the context of MHCII and to activate CD4 T cells. This was the consequence of a reduced ability of phagosomes to acquire MHCII in the absence of LC3. Interestingly, the defect in antigen presentation observed in LC3-deficient dendritic cells is specific to MHCII (Ma, Becker et al. 2012). How LC3 influences MHCII recruitment to maturing phagosomes is not yet known. Similarly, whether exclusion of LC3 from *L. major*-harboring PV contributes to the ability of these parasites to evade the immune system (Shio, Hassani et al. 2012) is an issue that will deserve further investigation. Clearly, absence of VAMP8, and indirectly, inhibition of LC3 recruitment to *L. major*-containing phagosomes, did not significantly affect the survival of *L. major* promastigotes.

In the initial steps of xenophagy, which essentially remains a selective, cargo-dependent process, the identity of the molecular tags that earmark pathogens for autophagic degradation is still unclear. Autophagic capture of organelles and cytosolic proteins typically occurs through the recognition of ubiquitin tags by sequestasome (p62/SQSTM1)-like receptors (SLRs), which contain an LC3-interacting region that allows them to bring targeted cargoes into nascent autophagosomes (Kim, Hailey et al. 2008, Kirkin, McEwan et al. 2009, Johansen and Lamark 2011). SLRs are also involved in autophagic recognition of viruses, like Sindbis virus (Orvedahl, MacPherson et al. 2010), of bacteria ensconced in vacuoles, such as *Salmonella* (Thurston, Ryzhakov et al. 2009, Zheng, Shahnazari et al. 2009, Cemma, Kim et al. 2011, Wild, Farhan et al. 2011), and of bacteria that escape from vesicular compartments to establish a cytoplasmic lifestyle, namely *Shigella* (Dupont, Lacas-Gervais et al. 2009, Mostowy, Sancho-Shimizu et al. 2011), group A *Streptococcus* (Thurston, Ryzhakov et al. 2009) and ActA-deficient *Listeria* (Yoshikawa, Ogawa et al. 2009, Mostowy, Sancho-Shimizu et al. 2011). Interestingly, injury to the vesicular membrane seems to be a central event in subsequent bacterial targeting to autophagosomes. *Salmonella*-containing vacuoles (SCVs) that are damaged by the SPI-1 type III secretion system allow the inflow of ubiquitinated proteins and are engulfed by autophagosomes prior to bacterial escape to the cytoplasm (Birmingham, Smith et al. 2006, Zheng, Shahnazari et al. 2009). Similarly, the pore-forming toxin listeriolysin O, responsible for *Listeria* vacuole rupture, is indispensable for autophagy induction and LC3 conversion (Birmingham, Canadien et al. 2007, Py, Lipinski et al. 2007, Birmingham, Canadien et al. 2008, Meyer-Morse, Robbins et al. 2010). The membrane remnants generated by *Shigella*- and *Listeria*-induced vacuole rupture are even sufficient to promote LC3 recruitment, confirming that damaged membranes can act as triggering cues for autophagic degradation (Dupont, Lacas-Gervais et al. 2009, Meyer-Morse, Robbins et al. 2010). Some *Leishmania* species, such as *L. amazonensis* and *L. major*, express a pore-forming protein termed leishporin known to cause erythrocyte hemolysis and to damage the

membranes of nucleated cells, including macrophages, by forming small pores of 1.6 to more than 6.1 nm in diameter (Noronha, Ramalho-Pinto et al. 1994, Noronha, Ramalho-Pinto et al. 1996, Noronha, Cruz et al. 2000). Similarly, a study by Forestier *et al.* (Forestier, Machu et al. 2011) recently showed that, upon promastigote internalization, persistent flagellum activity sets off oscillatory parasite movements that generate localized wounds in the macrophage membrane. Whether leishporin- or oscillation-mediated damage to the parasitophorous vacuole membrane triggers its recognition by autophagosomes and whether this process is ubiquitin/p62-dependent will require further investigation. Although ubiquitinated proteins have been found on phagosomes (Houde, Bertholet et al. 2003, Lee, Kim et al. 2005), no data exists to confirm or infirm their association with *Leishmania* parasitophorous vacuoles. Yet, infection with *L. major* promastigotes promotes *de novo* p62 protein synthesis in a GP63-independent manner (Fig. 2B). Alternatively, recent data identified the lipid messenger diacylglycerol as another potential tag, aside from ubiquitin, linked to the targeting of a subset of SCVs to the autophagic system (Shahnazari, Yen et al. 2010).

Independently of the mechanisms through which macrophage invasion by *L. major* promastigotes set off an autophagic response, our results demonstrate that PV recognition by autophagosomes is prevented in a GP63-dependent manner, a process relying on proteolytic cleavage of SNAREs such as VAMP8. Recent advances have assigned a role to various SNAREs in autophagosome biogenesis, maturation and substrate clearance (Ishihara, Hamasaki et al. 2001, Fader, Sanchez et al. 2009, Furuta, Fujita et al. 2010, Moreau, Ravikumar et al. 2011, Nair, Jotwani et al. 2011, Renna, Schaffner et al. 2011). In yeast, the Q-SNARE Tlg2 and the R-SNAREs Sec22 and Ytk6 associate with Sso1/2 and Sec9 to organize Atg9-positive vesicles into tubulovesicular clusters (Nair, Jotwani et al. 2011). Other early secretory protein and coatmer proteins also seem to be important for yeast autophagosome biogenesis (Ishihara, Hamasaki et al. 2001, Hamasaki, Noda et al. 2003). In mammals, VAMP7 mediates the homotypic fusion of autophagosome precursors (Moreau, Ravikumar et al. 2011), whereas VAMP3 is involved in autophagosome fusion with multivesicular bodies, yielding an "amphisome" (Fader, Sanchez et al. 2009). Along with Vti1b, VAMP8 was recently shown to orchestrate fusion of antimicrobial autophagosomes with lysosomes (Furuta, Fujita et al. 2010). For its part, Syntaxin-5 promotes autophagic substrate clearance, presumably by ensuring appropriate lysosomal function (Renna, Schaffner et al. 2011). Yet, no role had ever been ascribed to SNAREs in the recognition of invading pathogens by autophagy. Typically, xenophagy results from *de novo* autophagosome formation around invading pathogens, whether they live free in the cytoplasm or secluded in a vesicle. Albeit slightly larger than their yeast counterparts (400-900 nm diameter), mammalian autophagosomes typically range from 0.5 to 1.5 μm in size. Enveloping the much larger *Leishmania* promastigotes (10-20 μm) therefore represents a daunting task. The data presented here clearly show that VAMP8 is essential for the recruitment of LC3 to phagosomes containing *L. major* promastigotes. The possibility that autophagosomes fuse with, rather than engulf, the

parasitophorous vacuole, as they do with other vesicles of the endocytic pathway is hypothesis that will deserve further attention.

Manipulating the host vesicle fusion machinery is an efficient strategy exploited by many pathogens to cause disease (Wesolowski and Paumet 2010). *Clostridium tetani* and *C. botulinum* neurotoxins cleave neuronal SNAREs such as synaptosomal-associated protein 25 (SNAP-25), VAMP1-3 and Syntaxin-1, thereby interfering with neurotransmitter release and causing paralysis (Sikorra, Henke et al. 2008, Chen and Barbieri 2011, Yamamoto, Ida et al. 2012). Likewise, the venom of the Brazilian scorpion *Tityus serrulatus* contains a metalloprotease termed antarease that is capable of cleaving VAMP2 and VAMP8 (Fletcher, Fletcher et al. 2010). There is growing evidence that the *Leishmania* metalloprotease GP63 rivals the clostridial neurotoxins by cleaving multiple SNAREs and Syts, namely VAMP8, Vti1b, VAMP3, SNAP-23 and Syt XI, thereby sabotaging key macrophage functions including as autophagy and antigen cross-presentation (unpublished data). Such observations warrant further studies on the mechanism and consequences of GP63-mediated SNARE cleavage, which may provide new avenues for the development of treatments against leishmaniasis.

# Materials and Methods

## *Macrophage culture*

Bone marrow-derived macrophages (BMM) were obtained from the femurs and tibias of 6- to 8-week-old female BALB/c (Charles River, St-Constant, Quebec, Canada) or 129 SvJ mice (WT and *Vamp8*$^{-/-}$, described elsewhere (Wang, Ng et al. 2004)) and differentiated as previously described (Descoteaux and Matlashewski 1989) in complete medium (Dulbecco"s Modified Eagle"s Medium with glutamine (Life Technologies, Burlington, ON, Canada), containing 10% heat-inactivated, fetal bovine serum (FBS) (PAA laboratories Inc., Etobicoke, ON, Canada), 10 mM HEPES pH 7.4 and penicillin-streptomycin), supplemented with 15% (v/v) 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 the absence of CSF-1 for 18 h prior to infection. Where indicated, BMM were treated with 10 μM rapamycin (Cayman Chemical Co., Ann Arbor, MI, USA), an inhibitor of mTOR, as a positive control for autophagy induction.

### Ethic statements

All animals were handled in strict accordance with good animal practice as defined by the Canadian Council on Animal Care, and all animal work was approved by the Comité Institutionnel de Protection des Animaux of the Institut National de la Recherche Scientifique-Institut Armand-Frappier (protocol 0811-09).

## *Parasites and infections*

Promastigotes of *L. major* NIH S (MHOM/SN/74/Seidman) clone A2, *L. major* GLC94 (MHOM/ TN/95/GLC94 zymodeme MON25), and *L. donovani* LV9 (MHOM/ET/67/HU3) were grown and maintained at 26°C in *Leishmania* medium (M199 medium supplemented with 10% heat-inactivated FBS, 100 μM hypoxanthine, 10 mM HEPES, 5 μM hemin, 3 μM biopterin, 1 μM biotin, and penicillin-streptomycin) by bi-weekly passage. The *L. major* NIH clone A2 isogenic mutants Δ*gp63* (knockout) and Δ*gp63*+*gp63* (add-back) have been previously described (Joshi, Kelly et al. 2002). The *L. donovani* LV9 isogenic mutant Δ*lpg2*, generated using the *LPG2* targeting construct previously described (Scianimanico, Desrosiers et al. 1999), does not synthesize repeating Galβ1,4Manα1-$PO_4$ units and therefore lacks lipophosphoglycan (LPG) and other PGs. Cultures of Δ*gp63*+*gp63* and Δ*lpg2* promastigotes were supplemented with 50 μg/ml G418 and 300 μg/ml hygromycin B, respectively. For BMM infections, promastigotes were used at the late stationary phase of growth. Complement opsonization of promastigotes and zymosan particles was performed prior to phagocytosis by incubating the particles in phosphate-buffered saline (PBS) containing 10% mouse serum for 30 min at 37°C. BMM were then incubated at 37°C with promastigotes (parasite-to-macrophage ratio of 20:1) or zymosan particles (ratio of 5:1) for the indicated times.

Infection levels were assessed by microscopic examination of infected cells upon Giemsa staining with the Hema 3 system (Fisher Scientific, Ottawa, ON, Canada).

## *Western blotting*

Adherent BMM were washed with ice-cold PBS containing 1 mM $Na_3VO_4$ and lysed in 50 mM Tris-HCl pH 8, 150 mM NaCl and 1% nonidet P-40, containing complete protease inhibitors (Roche Applied Science, Laval, QC, Canada) and phosphatase inhibitors (1 mM $Na_3VO_4$, 50 mM NaF, 1.5 mM EGTA and 10 mM $Na_4P_2O_7$). Occasionally, the zinc chelator 1,10-phenanthroline (Sigma-Aldrich, Oakville, ON, Canada)(Chaudhuri, Chaudhuri et al. 1989) was added to the lysis buffer, at a concentration of 2 mM, to confirm that the proteolytic cleavage performed by GP63 occurs during the infection process rather than during sample preparation (data not shown). Insoluble material was removed by centrifugation for 10 min at 4°C and protein concentrations were determined using the Pierce BCA protein assay kit (Pierce, Rockford, IL, USA). For LC3 conversion analysis, cells were lysed in RIPA buffer (50 mM Tris-HCl pH 8.0 150 mM NaCl, 0.1% SDS, 0.5% Na deoxycholate and 1% NP-40) containing protease and phosphatase inhibitors and whole cell extracts were briefly sonicated before insoluble material was removed by centrifugation for 10 min at 4°C. Samples were separated by SDS-PAGE on 6 to 15% polyacrylamide gels and then transferred to Hybond-LFP PVDF membranes (LC3) or Hybond-ECL nitrocellulose membranes from GE Healthcare Life Sciences (Baie d"Urfe, QC, Canada) using a Trans-Blot SD Semi-Dry Transfer Cell apparatus from BioRad (Mississauga, ON, Canada). Membranes were blocked with 5% BSA and incubated with the relevant antibodies. For immunodetection, horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG and the enhanced chemiluminescence (ECL) detection reagents from GE Healthcare Life Sciences were used. Rabbit polyclonal antibodies against TSC-2 and 4E-BP1 as well as rabbit monoclonal antibodies against mTOR (7C10) and phospho-4E-BP1 (Thr37/46; 236B4) were purchased from Cell Signalling Technology (Beverly, MA, USA). The rabbit polyclonal antibody against LC3B was from Novus Biologicals (Oakville, ON, Canada). Rabbit polyclonal antisera against VAMP8 and Vti1b were both from Synaptic Systems (Goettingen, Germany). Mouse monoclonal antibodies against p62 (2C11), VAMP7 (a36195) and β-actin (A5316) were purchased from Abnova Corporation (Walnut, CA, USA), Abcam (Cambridge, MA, USA) and Sigma-Aldrich, respectively.

## *Confocal immunofluorescence microscopy*

BMM were seeded in 24-well plates containing microscope coverslips (Fisher Scientific) and infected with *L. major* promastigotes or fed zymosan particles for the indicated times. Cells were washed with PBS, fixed with 2% formaldehyde for 10 min and then simultaneously blocked and permeabilized in 0.1% Triton X-100, 1% bovine serum albumin, 20% normal goat serum, 6% non-fat dry milk and 50% FBS for 20 min at room temperature. Cells were incubated for 1h at

room temperature with antibodies against LC3B (1:200), VAMP8 (1:100) or Vti1b (1:250). AlexaFluor 488-conjugated goat anti-rabbit IgG (1:500, Molecular Probes, OR, USA) was used for 30 min at room temperature, during which time macrophage and promastigote nuclei were stained with DRAQ5 (1:400, BioStatus Ltd., UK). Coverslips were washed three times with PBS between incubations and mounted on Fluoromount-G (SouthernBiotech, Birmingham, AL, USA). Analyses were performed using a Bio-Rad Radiance 2000 confocal imaging system (Bio-Rad Laboratories, Hercules, CA, USA) installed on an Eclipse E800 microscope. LC3, VAMP8 and Vti1b distribution was analyzed using an argon/krypton laser at 488 nm with a Plan Apo Nikon 60X (NA 1.4) oil immersion lens. DRAQ5 fluorescence was analyzed using a 638 nm diode laser at 650 nm long-pass with a Plan Apo Nikon 60x (NA 1.4) oil immersion lens. A minimum of 100 phagosomes per coverslip were examined for every experimental condition, each performed in triplicates. Images were acquired in the normal scanning mode with a Kalman filter of 3 to 6 using the LaserSharp software.

## *Statistical analysis*

The two-tailed, unpaired Student"s t test was performed to evaluate the significance of the differences observed. * $p < 0.001$.

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# Figure legends

**Figure 1.** *L. major* GP63 disrupts the mTOR pathway. Adherent BALB/c BMM were infected with *L. major* promastigotes (WT, Δ*gp63* or Δ*gp63*+*gp63*) for the indicated times (**A**) or for 1h (**B**). Whole cell extracts were separated by SDS-PAGE and analyzed by Western blotting using antibodies against mTOR, TSC-2, 4E-BP1 and phospho-4E-BP1, as described in *Materials and Methods*. Equal loading was verified with an antibody against β-actin. #, Cleavage fragment(s). *Ctl*, uninfected control.

**Figure 2.** *L. major* GP63 prevents parasite recognition by the autophagic response induced upon infection. Adherent BALB/c BMM were infected with *L. major* promastigotes (WT, Δ*gp63* or Δ*gp63*+*gp63*) for 1h (**A, C, D**) or for the indicated times (**B**). Whole-cell lysates were separated by SDS-PAGE and analyzed by Western blotting using antibodies specific for LC3B (**A**) and p62 (**B**), as described in *Materials and Methods*. Equal loading was verified with an antibody against β-actin. *Ctl*, uninfected control. (**C**) Infected cells were fixed and stained with the DNA marker DRAQ5 (blue) and with the anti-LC3B antibody (green) for immunofluorescence microscopy, as described in *Materials and Methods*. White arrows indicate parasite nuclei while red filled arrowheads point to LC3 recruitment. (**D**) LC3 recruitment to the phagosome was quantified for 300 phagosomes on triplicate coverslips. Data are presented as the mean ± standard error of the mean (SEM) of five independent experiments.

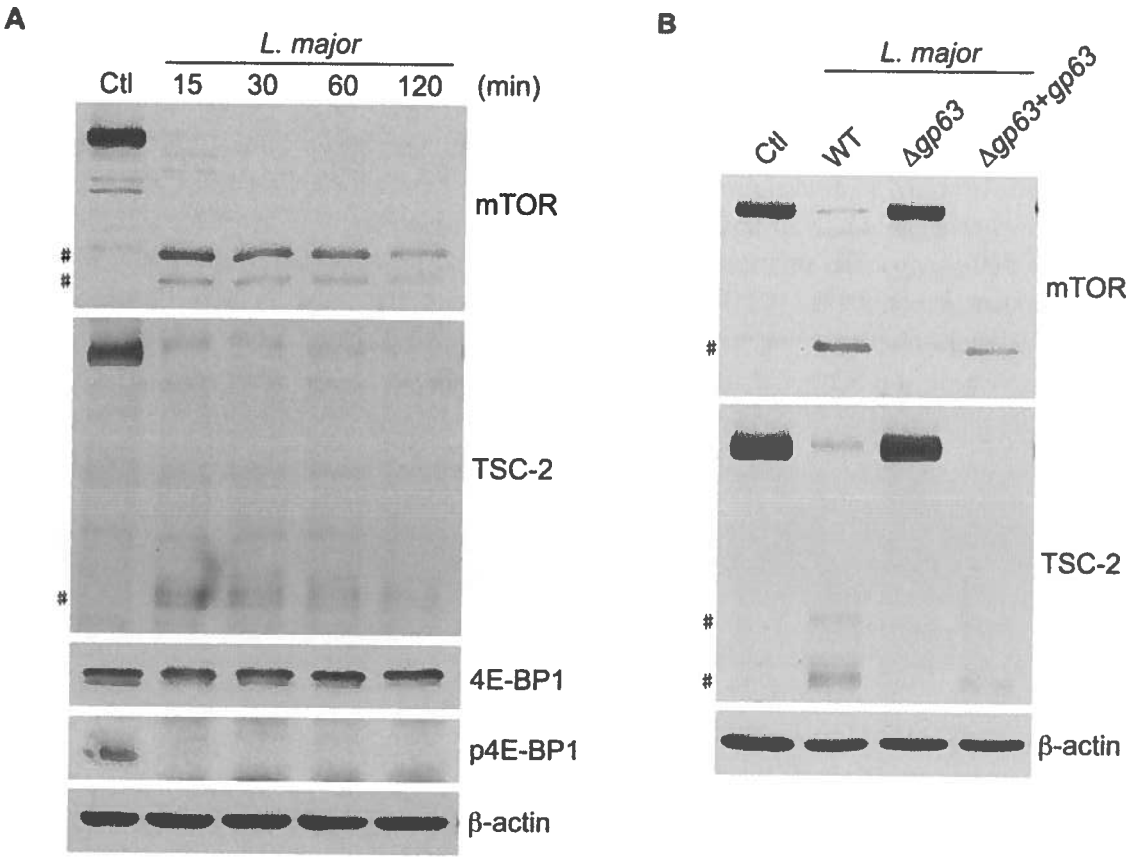
**Figure 3.** *L. major* infection leads to GP63-mediated proteolytic cleavage of SNAREs. Adherent BALB/c BMM were infected with *L. major* promastigotes (WT, Δ*gp63* or Δ*gp63*+*gp63*) for 1h. Whole-cell lysates were separated by SDS-PAGE and analyzed by Western blotting using antibodies against VAMP8, Vti1b and VAMP7, as described in *Materials and Methods*. Short and long radiographic exposures are shown for Vti1b to better visualize the cleavage fragments (#). Equal loading was verified with an antibody against β-actin. *Ctl*, uninfected control.

**Figure 4.** *L. major* inhibits VAMP8 and Vti1b recruitment to the PV. Adherent Balb/c BMM were fed zymosan particles or were infected with *L. major* promastigotes (WT, Δ*gp63* or Δ*gp63*+*gp63*). (**A**) VAMP8 recruitment to the phagosome was quantified for 300 phagosomes on triplicate coverslips. Data are presented as the mean ± SEM of an experiment representative of two independent experiments. (**B**) Infected cells were fixed and stained with the DNA marker

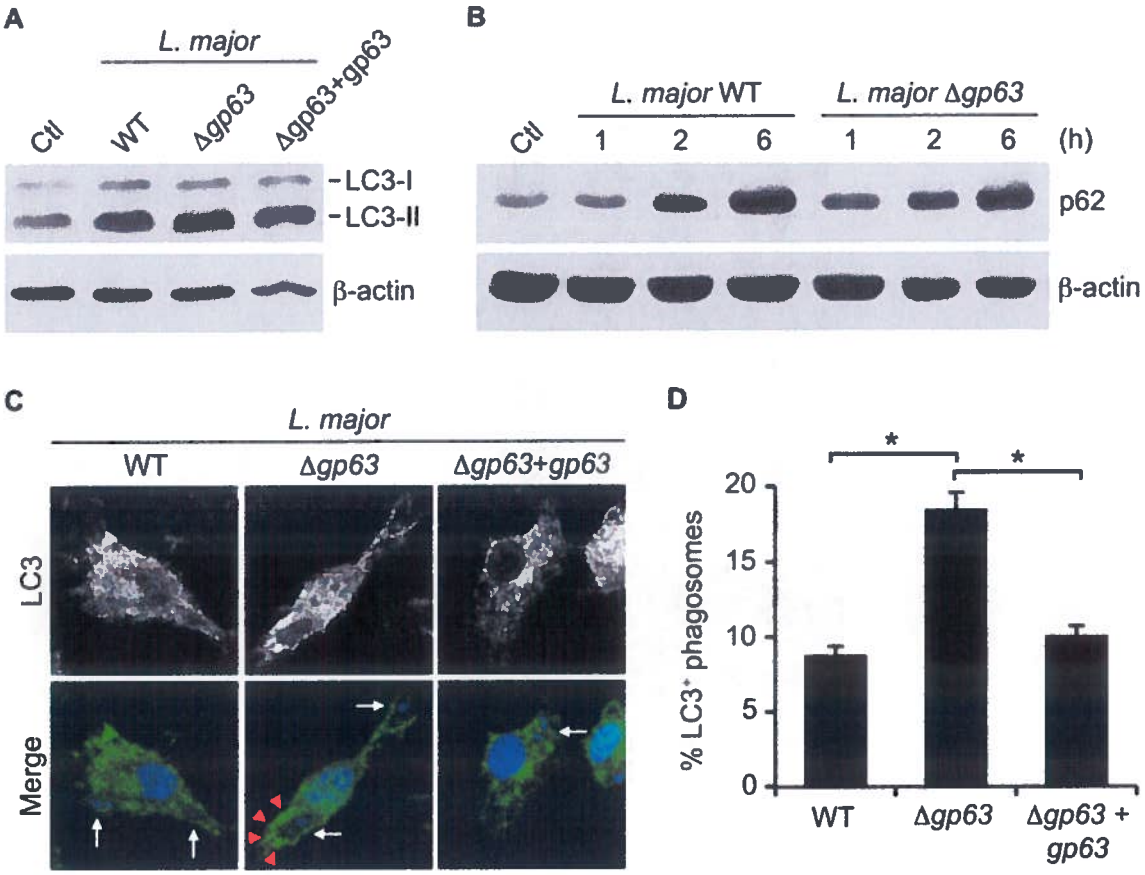
DRAQ5 (blue) and with the anti-Vti1b antibody (green) for immunofluorescence microscopy, as described in *Materials and Methods*. White arrows indicate parasite nuclei while red filled arrowheads point to VAMP8 recruitment. (**C**) Vti1b recruitment to the phagosome was quantified for 300 phagosomes on triplicate coverslips. Data are presented as the mean ± SEM of two independent experiments.

**Figure 5.** VAMP8 is essential for autophagic targeting of *L. major* PVs. Adherent 129 SvJ (WT or VAMP8$^{-/-}$) BMM were infected with *L. major* promastigotes (WT, Δ*gp63* or Δ*gp63*+*gp63*) (**A-C**) or treated with rapamycin (**C**) for 1h. (**A**) Infected cells were fixed and stained with the DNA marker DRAQ5 (blue) and with the anti-LC3B antibody (green) for immunofluorescence microscopy, as described in *Materials and Methods*. White arrows indicate parasite nuclei while red filled arrowheads point to VAMP8 recruitment. (**B**) LC3 recruitment to the phagosome was quantified for 300 phagosomes on triplicate coverslips. Data are presented as the mean ± SEM of three independent experiments. (**C**) Whole cell extracts were separated by SDS-PAGE and analyzed by Western blotting using an antibody specific for LC3B. Equal loading was verified with an antibody against β-actin. *Ctl*, uninfected, untreated control.

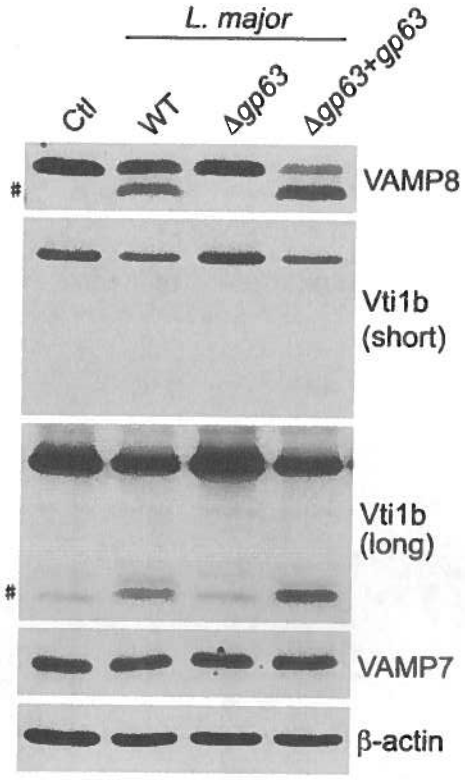
# Figure 1


A
L. major
Ctl
15
30
60
120
(min)
mTOR
TSC-2
4E-BP1
p4E-BP1
β-actin
B
L. major
Ctl
WT
Δgp63
Δgp63+gp63
mTOR
TSC-2
β-actin

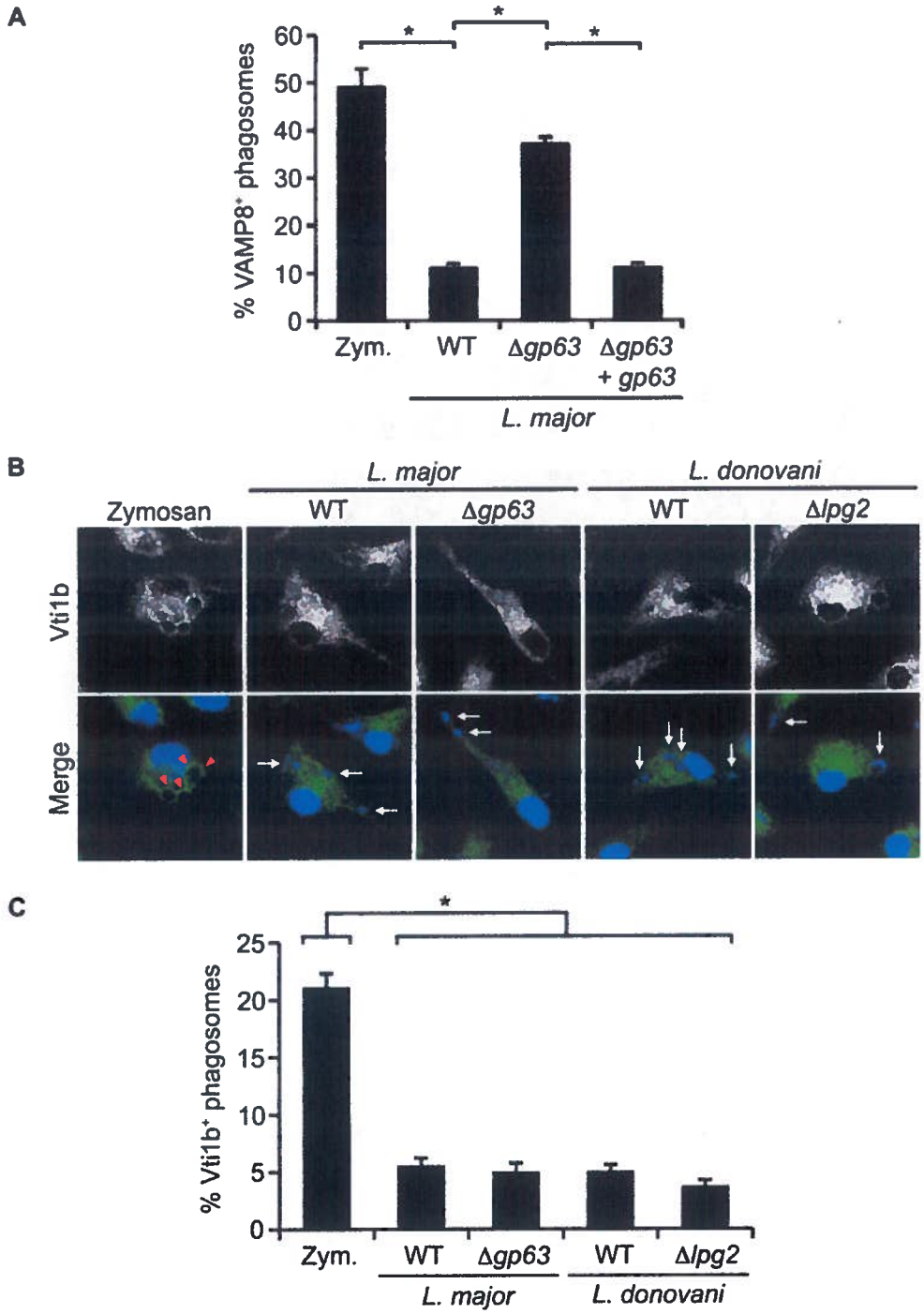
# Figure 2


A
L. major
Ctl
WT
Δgp63
Δgp63+gp63
-LC3-I
-LC3-II
β-actin
B
L. major WT
L. major Δgp63
Ctl
1
2
6
1
2
6
(h)
p62
β-actin
C
L. major
WT
Δgp63
Δgp63+gp63
LC3
Merge
D
% LC3+ phagosomes
20
15
10
5
0
*
*
WT
Δgp63
Δgp63 + gp63

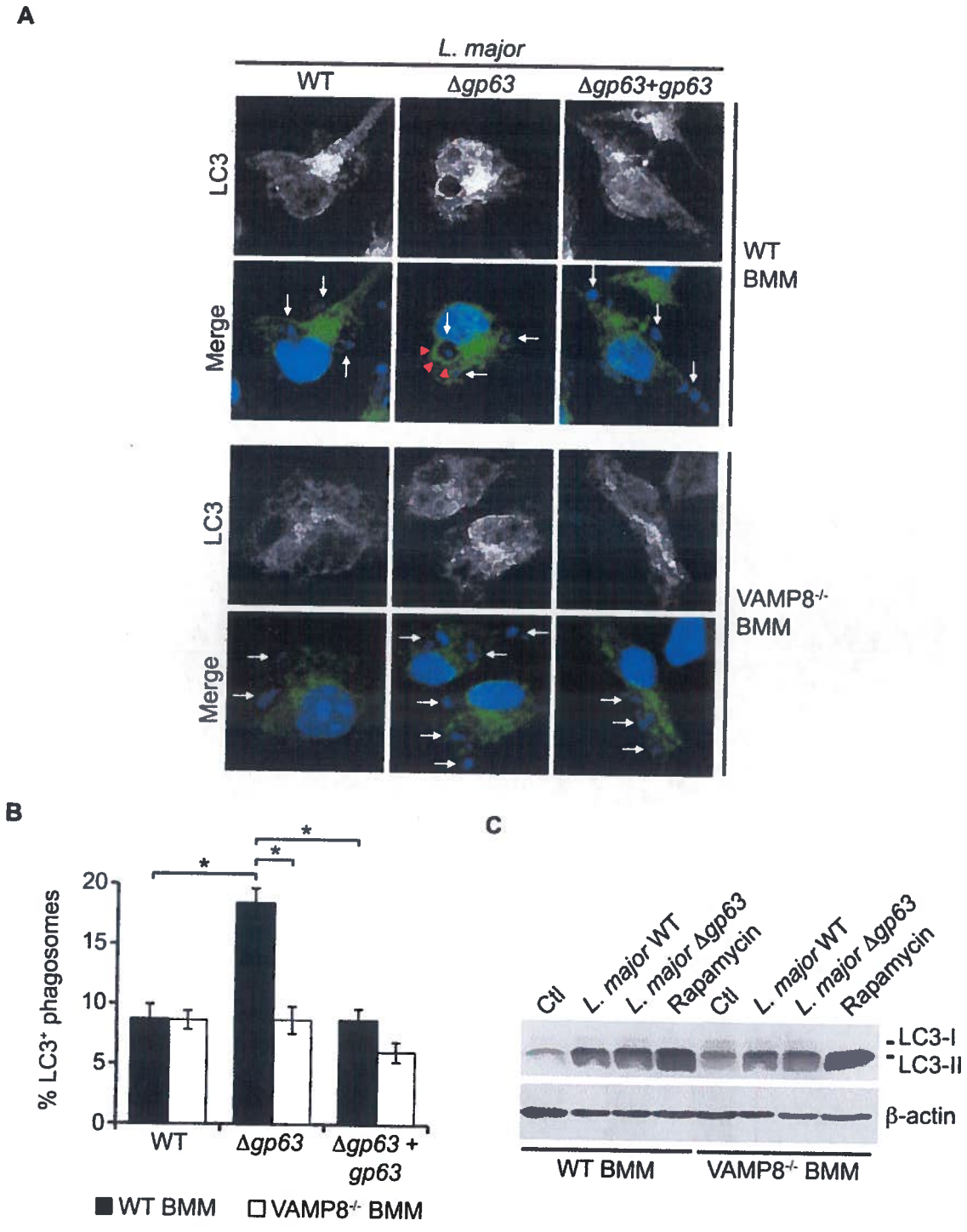
# Figure 3


L. major
Ctl
WT
Δgp63
Δgp63+gp63
VAMP8
Vti1b
(short)
Vti1b
(long)
VAMP7
β-actin
#
#

# Figure 4


A
% VAMP8+ phagosomes
60
50
40
30
20
10
0
*
*
*
Zym.
WT
Δgp63
Δgp63 + gp63
L. major
B
L. major
L. donovani
Zymosan
WT
Δgp63
WT
Δlpg2
Vti1b
Merge
C
% Vti1b+ phagosomes
25
20
15
10
5
0
*
Zym.
WT
Δgp63
WT
Δlpg2
L. major
L. donovani

# Figure 5


A
L. major
WT
Δgp63
Δgp63+gp63
LC3
Merge
WT BMM
VAMP8-/- BMM
B
% LC3+ phagosomes
20
15
10
5
0
WT
Δgp63
Δgp63 + gp63
WT BMM
VAMP8-/- BMM
C
Ctl
L. major WT
L. major Δgp63
Rapamycin
LC3-I
LC3-II
β-actin
WT BMM
VAMP8-/- BMM