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INTRACELLULAR TRAFFICKING AND BIOLOGICAL IMPACT OF *LEISHMANIA* VIRULENCE FACTORS ON MACROPHAGES

By

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

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To my mother Leyda Libia, for whom I hold utmost admiration and gratitude

To my family

“Béchamp avait raison, le microbe n'est rien, le terrain est tout”

Louis Pasteur – 1822-1895

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If I were asked whether I would do it all over again, I would say **yes!** And I do not mean that lightly, because this great journey for the pursuit of knowledge and self-discovery has been facilitated by very remarkable people.

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ABSTRACT

Leishmania parasites cause a spectrum of debilitating diseases found worldwide. The *Leishmania* life cycle is digenetic, and starts with sandflies that inoculate metacyclic promastigotes into a vertebrate host. Promastigotes are internalized by tissue phagocytes where they transform into amastigotes. Phagosomes mature into highly microbicidal phagolysosomes via membrane exchanges with lysosomes, and with organelles in the secretory pathway. Remarkably, *Leishmania* remodels phagolysosomes into parasitophorous vacuoles that promote parasite growth. To achieve this feat, the parasite employs an armament of abundant surface-bound glycoconjugates that include the GP63 metalloprotease and lipophosphoglycan. GP63 cleaves multiple host substrates, thereby enabling *Leishmania* to subvert phagocyte functions such as transcription, translation, lipid metabolism, cytokine secretion, LC3-associated phagocytosis and antigen cross-presentation. Lipophosphoglycan, a complex glycophospholipid, promotes parasite survival by inhibiting phagolysosomal maturation, hence quenching the microbicidal power of the phagosome.

The effector functions of a macrophage rely on a very active endomembrane trafficking system that regulates how the cell responds to environmental stimuli. Vesicle fusion is regulated by members of the soluble *N*-ethylmaleimide-sensitive factor activating protein receptor, and Synaptotagmin families. In this work, we characterized Syt XI, an inhibitory member of the Synaptotagmin family whose function had not been reported. We discovered that Syt XI is a recycling endosome and lysosome-associated protein that controls phagocytosis, as well as the phagosome's killing capacity **[primary article no. 1]**. We found that Syt XI dampens TNF and IL-6 secretion. Importantly, Syt XI is degraded by GP63 and excluded from parasitophorous vacuoles via lipophosphoglycan. Additionally, *Leishmania*-infected macrophages were found to secrete TNF and IL-6 in a GP63-dependent fashion. To demonstrate that this release depends on Syt XI degradation, siRNA knockdown of Syt XI before infection revealed that the effects of siRNA knockdown and GP63 degradation are not cumulative. To bring these findings into the *in vivo* context, we showed that injection of GP63-containing parasites into mice

also leads to increased TNF and IL-6 secretion and to an augmented influx of neutrophils and inflammatory monocytes to the infection site **[primary article no. 2]**.

Unlike bacteria, *Leishmania* is not known to inject its virulence factors across the phagosome membrane. This raises the question of how the parasite's virulence effectors reach their targets. Due to the reported importance of the host's secretory pathway on parasite survival, we hypothesized that *Leishmania*'s virulence factors co-opt this pathway in order to egress from the phagosome into the host cell cytoplasm. Using biochemistry and microscopy-based assays, we demonstrated that GP63 and the phosphoglycans are rapidly redistributed throughout the cytoplasm in vesicles containing markers of the endoplasmic reticulum, and of the endoplasmic reticulum-Golgi intermediate compartment. Importantly, chemical inhibition of the secretory pathway hinders the redistribution of GP63 and the phosphoglycans, thereby impeding the cleavage of GP63 targets. This prompted us to study the role of Sec22b, which regulates endoplasmic reticulum-Golgi trafficking, on the intracellular trafficking of GP63 and phosphoglycans. We found that Sec22b promotes the redistribution of these virulence factors and enables GP63 to reach host proteins **[primary article no. 3]**.

In sum, the work presented in this dissertation sheds light into how *Leishmania*'s virulence molecules enter the host cell in order to exert their functions. We also revealed that GP63 induces TNF and IL-6 release *in vitro* and *in vivo*, thereby contributing to the accrual of inflammatory phagocytes to the infection site. Remarkably, GP63 and the phosphoglycans access their targets by hijacking cellular organelles and their resident vesicle fusion molecules. These findings provide important insight into how *Leishmania* sabotages macrophage biology.

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LIST OF ABBREVIATIONS

ADRP: Adipose differentiation related protein

AP-1: Activator protein-1

Arf6: ADP-ribosylation factor 6

BFA: Brefeldin A

BMM: Bone marrow-derived macrophages

CNS: Central nervous system

CNX: Calnexin

CRT: Calreticulin

CTD: C-terminal domain

DAPI: 4',6-diamidino-2-phenylindole

DC: Dendritic cells

DIC: Differential intensity contrast

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

Dvl: Dishevelled

EEA1: Early endosome antigen 1

EM: Electron microscopy

EMT: Epithelial-mesenchymal transition

EPO: Erythropoietin

ER: Endoplasmic reticulum

ERGIC: Endoplasmic reticulum-Golgi intermediate compartment

ETEC: Enterotoxigenic *Escherichia coli* K88

FAM129B: Family with sequence similarity 129 member B

FBS: Fetal bovine serum

GABARAPL2: GABA(A) receptor-associated protein like 2

GIPL: Glysylinositolphospholipid

GM-CSF: Granulocyte-macrophage colony-stimulating factor

GP63: Major surface glycoprotein 63; leishmanolysin; major surface peptidase

GPI: Glycosylphosphatidylinositol

GRP: Glucose-regulated protein

GTPase: Guanosine triphosphatase

HCS: High-content screening

HDM: House dust mite

HMGCoA: Hydroxymethylglutaryl-coenzyme A

HOPS: Homotypic fusion and protein sorting complex

HSPG2: Heparan sulfate proteoglycan 2

IFN: Interferon

IGF: Insulin-like growth factor

IL: Interleukin

IP: Immunoprecipitation

IPEC-J2: Intestinal columnar epithelial cells

IRE1α: Inositol requiring enzyme 1α

iTRAQ: Isobaric tags for relative and absolute quantification

LAMC2: Laminin gamma 2

LAMP: Lysosomal-associated membrane protein

LC3B: Microtubule-associated protein1 light chain 3

***LC-MS/MS*:** Liquid chromatography coupled to tandem mass spectrometry

LD: Lipid droplet

LDL: Low-density lipoprotein

LPG: Lipophosphoglycan

LPS: Lipopolysaccharide

MCS: Membrane contact site

M-CSF: Macrophage colony-stimulating factor

MHC: Major histocompatibility complex

MIG: Monokine induced by gamma interferon, or CXCL9

MIP-2α: Macrophage inflammatory protein 2-α, or CXCL1/2

miRNA: Micro RNA

mROS: Mitochondrial reactive oxygen species

MV: Microvesicle

NADPH: Nicotinamide adenine dinucleotide phosphate

NET: Neutrophil extracellular trap

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NO: Nitric oxide

NOX2: NADH oxidase 2

NTD: N-terminal domain

PAMP: Pathogen-associated molecular pattern

PDI: Protein disulfide isomerase

PG: Phosphoglycan(s)

$PGF_{2\alpha}$: Prostaglandin F2α

***Phox*:** Phagocytic oxidase

$PI(3,4,5)P_3$: Phosphatidylinositol-3,4,5-*tris*phosphate

$PI(4,5)P_2$: Phosphatidylinositol-4,5-*bis*phosphate

PI3K: Phosphoinositide 3-kinase(s)

PKC: Protein kinase C

PPG: Proteophosphoglycan

PRR: Pathogen recognition receptor

PV: Parasitophorous vacuole

RANTES: Regulated upon activation normal T cell expressed and secreted, or CCL5

RE: recycling endosome

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

SEM: Scanning electron microscopy

shRNA: Small hairpin RNA

siRNA: Small interfering RNA

SNARE: Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors

Stx: Syntaxin

Syt: Synaptotagmin

TACE: TNF-α-converting enzyme

TAM: Tumour-associated macrophage

TCIRG1: T-cell immune regulator 1

TCL: Total cell lysate

TEM: Transmissible electron microscopy

Tfr1: Transferrin receptor 1

TGEV: Transmissible gastroenteritis virus

TGF: Transforming growth factor

TGN: *Trans*-Golgi network

TNF: Tumour necrosis factor

VAMP: Vesicle-associated membrane protein

VL: Visceral leishmaniasis

WT: Wild type

CHAPTER 1: LITERATURE REVIEW

1 THE ROLE OF PHAGOCYTES IN IMMUNITY

1.1 Introduction and discovery

Phagocytes are cells of myeloid origin that circulate in the blood and reside in all animal tissues. They are endowed with the capacity to perform phagocytosis, the process by which phagocytes engulf and destroy microorganisms, cellular debris and particulate foreign bodies (Desjardins *et al.*, 2005, Gordon, 2016). The term phagocyte comes from the term *phagein* (to eat) and *–cito* (cell). Many cell types are capable of performing phagocytosis, with immune system phagocytes being crucial in organismal development and antimicrobial defense (Banoub *et al.*, 2017). In mammals, phagocytes have evolved specialized functions that depend on the microenvironment in which they are found. Indeed, one litre of human blood is estimated to contain $6x10^9$ of these cells, and many more reside in all organs (Banoub *et al.*, 2017). Phagocytes are classified into professional (Figure 1) and non-professional (Figure 2) depending on the specificity and rapidity with which they engulf particles (Banoub *et al.*, 2017, Rabinovitch, 1995, Tauber, 2003).

Phagocytes were originally a theory of Russian zoologist Ilya Ilich Metchnikoff (*Илья Ильич Мечников*, 1845-1916) who suspected the existence of cells that defend against microbial infections (Silverstein, 2011, Tauber, 2003). In 1882, Metchnikoff observed that when he pricked starfish larvae, a series of amoebal cells were recruited to and attached to the pricking agent at the wounding site. Upon presentation of his observations in Vienna, his colleague Carl Friedrich Claus suggested the term 'phagocyte' to denote those cells (Chernyak *et al.*, 1988, Silverstein, 2011, Tauber, 2003). Metchnikoff then extended his studies onto other systems. He observed that fungal spores were attacked and destroyed by specialized cells that are present in the model organism *Daphnia*, and that *Bacillus anthracis* spores were engulfed by phagocytic cells in mammals. These data confirmed Metchnikoff's initial observation that phagocytes served as defenders of the organisms that harbour them.

At the time, Metchnikoff's work was met with skepticism and strife, which was in part due to the work of Paul Ehrlich in the 1890s (Kaufmann, 2008). The latter postulated that immunity was dependent on the binding of specialized host cell-surface molecules to specific chemical groups on toxins. Ehrlich's theory was that if host cells survived an initial encounter with a toxin, the cells would start liberating some the molecule that initially bound and neutralized the toxin, thence allowing the organism to survive future encounters with the same toxin. Such molecules are nowadays known as antibodies (Kaufmann, 2008). In 1904, Almroth Wright helped to bridge Metchnikoff's and Ehrlich's work by suggesting that the phagocytosis of bacteria was increased in the presence of opsonins and antibodies coating the microorganisms (Gordon, 2016, Kaufmann, 2008, Silverstein, 2011). Although Metchnikoff's work was accepted at the beginning of the 20th century, the roles of phagocytes within the immune system started – and still continue – to be understood in the 1980s. Along with Paul Ehrlich, Metchnikoff's seminal discoveries were celebrated with the 1908 Nobel Prize in Physiology or Medicine (Kaufmann, 2008).

1.2 Professional phagocytes

Professional phagocytes are characterized for their enhanced phagocytic efficiency and for their central role in antimicrobial defence and organismal development. This family is constituted by monocytes, macrophages, dendritic cells, neutrophils and mast cells (Gordon, 2016, Rabinovitch, 1995). Most of these phagocytes descend from a myeloid precursor in the bone marrow (Figure 1). Additionally, embryonic stem cells from the yolk sac have been shown to give rise to fetal monocytes that differentiate into early tissue macrophages and dendritic cells (van de Laar *et al.*, 2016) (Cybulsky *et al.*, 2016) (Figure 1).

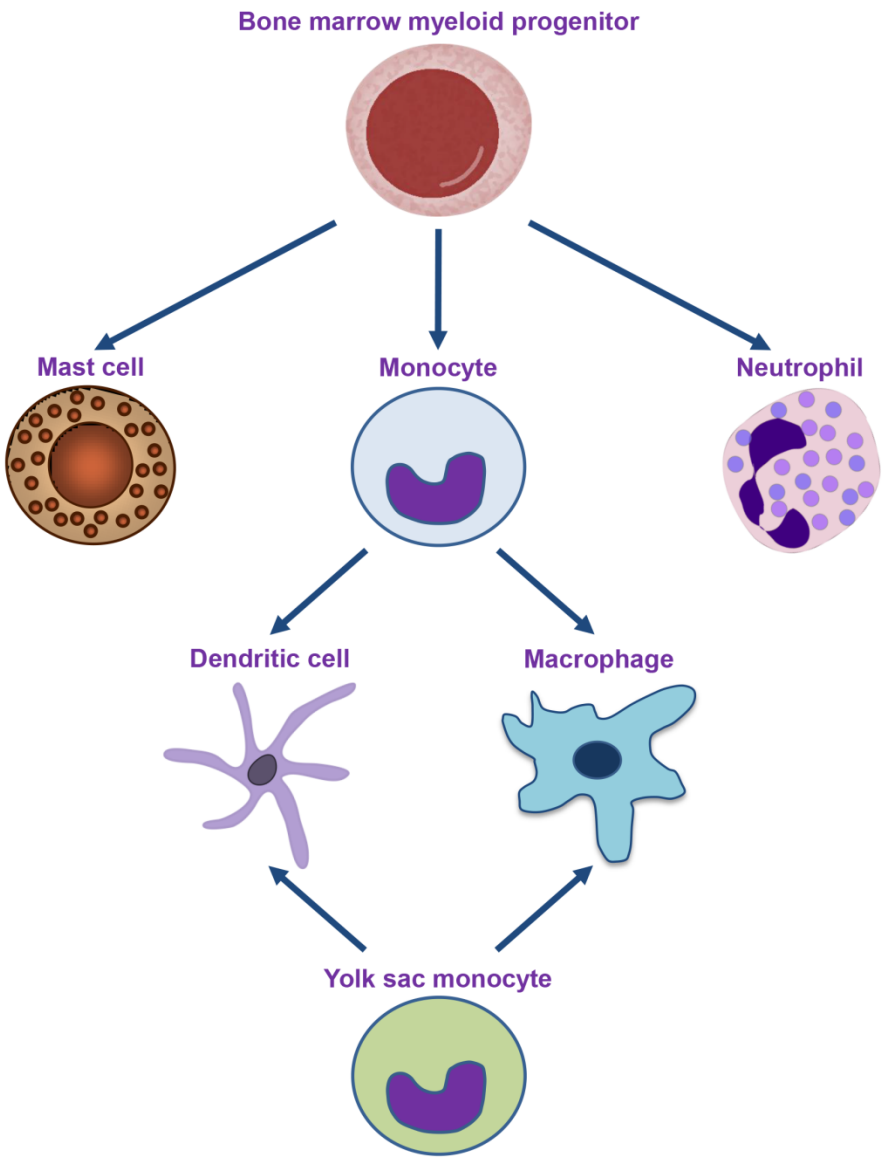


Figure 1 of literature review. The ontogeny of professional phagocytes. Professional phagocytes descend from a myeloid precursor in the bone marrow or from stem cells in the developing embryo's yolk sac. Monocytes are usually found in the blood stream. Upon encounter with antigens or inflammatory stimuli, they can develop into macrophages or dendritic cells, depending on the context. The phagocytic efficiency of professional phagocytes is enhanced by the presence of surface receptors that recognize a wide variety of endogenous and exogenous particles.

This section introduces professional phagocytes in the context of their particular functions in the host. Although these cells differ widely in the context-specific functions that they perform, they are attracted to and migrate to tissues when they come in contact with endogenous chemoattractants such as chemokines and pathogen molecules. They then mediate the development of inflammation, the elimination of the

inflammatory insult and the resolution of the response (Banoub *et al.*, 2017, Cybulsky *et al.*, 2016, Faurschou *et al.*, 2003, Hellebrekers *et al.*, 2018).

1.2.1 Neutrophils

Neutrophils (9-12 µm) are highly abundant (60-70% of circulating phagocytes) and short-lived granulocytic cells whose primary function is the active ingestion of invading bacteria and fungi (Hellebrekers *et al.*, 2018). They are very motile and possess a characteristic multi-lobed nucleus of highly compacted chromatin (Figure 1). The abundant granules in their cytoplasm contain lytic enzymes and antimicrobial molecules that help degrade phagocytosed microbes (Faurschou *et al.*, 2003, Hellebrekers *et al.*, 2018). Their abundance and small size allow them to rapidly infiltrate – when needed – tissues via diapedesis. Indeed, neutrophils are the first responders during an infection, where they are attracted by the cytokines, chemokines and histamine liberated by tissue macrophages and mast cells (Hellebrekers *et al.*, 2018). Those molecules include TNF, IL-6 and IL-8, which in turn trigger chemokine secretion and the expression of selectins on endothelial cells (Starckx *et al.*, 2002). Neutrophils bind to selectins, which permits the extravasation of neutrophils from the blood to the site of infection. Once there, neutrophils recognize their target via toll-like receptors (TLRs), which bind to microbial molecules such as lipopolysaccharide (LPS) and peptidoglycan (Gouwy *et al.*, 2004). Neutrophils can also externalize their chromatin in order to form neutrophil extracellular traps (NETs) that tangle and kill microbes, or hold them in place so that macrophages can come and ingest them (Sollberger *et al.*, 2018).

1.2.2 Mast cells

Mast cells are myeloid-derived cells that harbour numerous histamine- and heparin-containing metachromatic granules (Figure 1). They are mostly found in the skin and in the mucosa of respiratory and digestive organs (Arthur *et al.*, 2016). The heparin and histamine in their granules act as vasodilators that augment vascular permeability and

tissue remodelling. This allows for cells such as neutrophils to emigrate into inflammatory sites (Sollberger *et al.*, 2018). In allergy and anaphylaxis, excess histamine causes persistent inflammation and tissue damage. Mast cells display TLRs and other recognition receptors at their plasmalemma, which in turn facilitate the clearance of damaged erythrocytes (Sharma *et al.*, 2018) and the killing of pathogens (Lin *et al.*, 1999). They can also present antigens to lymphocytes, and improve the antigen-presenting capacity of dendritic cells via small extracellular vesicles known as exosomes (Skokos *et al.*, 2003).

1.2.3 Monocytes

Monocytes are mononuclear phagocytes characterized by a kidney-shaped nucleus (Figure 1) and measuring up to 18 µm (Ginhoux *et al.*, 2014). They originate in the bone marrow and the yolk sac, and enter the circulation to infiltrate organs such as the liver, spleen and lungs (Ginhoux *et al.*, 2014, van de Laar *et al.*, 2016, Yang *et al.*, 2014). There, they differentiate into tissue macrophages or dendritic cells. Monocytes can be inflammatory ($Ly6C^{hi}$) and anti-inflammatory ($Ly6C^{lo}$), a dichotomy that allows them to originate very distinct types of macrophages. Under physiological conditions, inflammatory monocytes monitor extravascular tissues and present antigens to lymphocytes. Stress by sterile or non-sterile inflammatory stimuli triggers the release of the CCR2 chemokine, which triggers migration of inflammatory monocytes to the injury site (Ginhoux *et al.*, 2014). There, these cells develop into highly inflammatory and microbicidal ‘M1’ macrophages (Yang *et al.*, 2014). On the other hand, CCR7/8-dependent migration to the lymph nodes can trigger differentiation into dendritic cells (Qu *et al.*, 2004). Non-inflammatory monocytes patrol the luminal site of the endothelium and eliminate dying cells and debris (Thomas *et al.*, 2015). Although they express low levels of CCR2, they can travel to inflamed tissues to become anti-inflammatory and reparative ‘M2’ macrophages. Inflammatory monocytes can also become non-inflammatory macrophages, illustrating the fact that monocyte development is highly plastic and context-dependent (Ginhoux *et al.*, 2014, Yang *et al.*, 2014).

1.2.4 Dendritic cells

Dendritic cells are characterized by their neuronal-like protrusions that can measure up to 10 μm (Figure 1) (Banoub *et al.*, 2017, Collin *et al.*, 2018, Cybulsky *et al.*, 2016, Steinman *et al.*, 1973). They are found in great quantities in the skin, intestines, and other mucosal sites (Figure 2). Although dendritic cells are specialized in phagocytosis, their main function is to use phagocytosis as a way of processing antigens for subsequent presentation at the cell surface. Antigens are loaded onto class II major histocompatibility complexes (MHC) and presented to specialized lymphocytes, B and T cells, in order to mount an adaptive immune response to the antigen (Mellman, 2013). In this manner, dendritic cells link innate and adaptive immunity in eukaryotes. They mediate strong adaptive responses to foreign agents while maintaining a tolerogenic response to self (Mellman, 2013). These cells exist in various states of maturity, with immature dendritic cells patrolling peripheral sites for antigen. Once an antigen is phagocytosed, dendritic cells are activated and migrate towards lymph nodes. There, they mature and augment their expression of MHC molecules and other costimulatory ligands that are required for antigen presentation. They can also secrete various cytokines such as TNF, IL-1 and IL-12 (Collin *et al.*, 2018, Mellman, 2013, Steinman *et al.*, 1973).

Dendritic cells were originally observed by Paul Langerhans in 1868 when he was studying human cutaneous epithelium (Steinman *et al.*, 1994). Langerhans mistook these cells for neurons, and it was not until the 1970s that Ralph M. Steinman and Zanvil A. Cohn correctly characterized them via phase-contrast microscopy and functional assays. In 1973, Steinman and Cohn coined the term ‘dendritic cell’; they observed that these cells were phagocytic, motile, and able to induce the cytotoxic functions of T cells (Steinman *et al.*, 1973, Steinman *et al.*, 1994).

1.2.5 Macrophages

Macrophages, a term derived from the Greek ‘large eaters’, are monocyte-derived cells that exist in most tissues (Figures 1 and 2) (Cybulsky *et al.*, 2016, Sieweke *et al.*, 2013). They are the primary destroyers of particulate matter, as well as bacteria, fungi, protozoans, senescent cells and apoptotic bodies (Arandjelovic *et al.*, 2015, Gordon, 2016). As mentioned, when monocytes infiltrate tissues, they can transform into macrophages via the action of the granulocyte macrophage colony-stimulating factor (GM-CSF) in conjunction with other cytokines such as IL-10, IL-12 and other factors such as apoptotic cells (Ginhoux *et al.*, 2014, Sieweke *et al.*, 2013). When monocytes become macrophages, cell size, phagocytic capacity, and intraphagosomal antimicrobial properties increase dramatically (Ginhoux *et al.*, 2014). Although tissue macrophages are usually found in a quiescent state, they can be activated by a variety of stimuli during the immune response. Contact with and phagocytosis of antigens serves as the initial stimulus. This can include microbial molecules such as LPS, which binds to macrophages via the LPS-binding protein to TLR4 on the macrophage surface (Cybulsky *et al.*, 2016). Activation and microbicidal capacity are further amplified by cytokines such as interferon gamma (IFN-γ), which are produced by T cells (Biswas *et al.*, 2010, Nathan *et al.*, 1983, Sieweke *et al.*, 2013, Tam *et al.*, 2014).

Macrophages were originally observed by Metchnikoff during his experiments with starfish. However, the term ‘macrophage’ was coined by Aschoff in 1924 when he was studying these cells as part of the reticulo-endothelial system (Silverstein, 2011, Tauber, 2003). In the 1960s, the role of macrophages was defined more precisely by the work of Zanvil A. Cohn and colleagues (Steinman *et al.*, 1994). They characterized the macrophage as a secretory cell able to kill inside (through phagocytosis) and outside of the cell, and capable of releasing over 50 products. The latter include numerous molecules that participate in inflammation, such as cytokines, prostaglandins and leukotrienes (Cybulsky *et al.*, 2016, Steinman *et al.*, 1994).

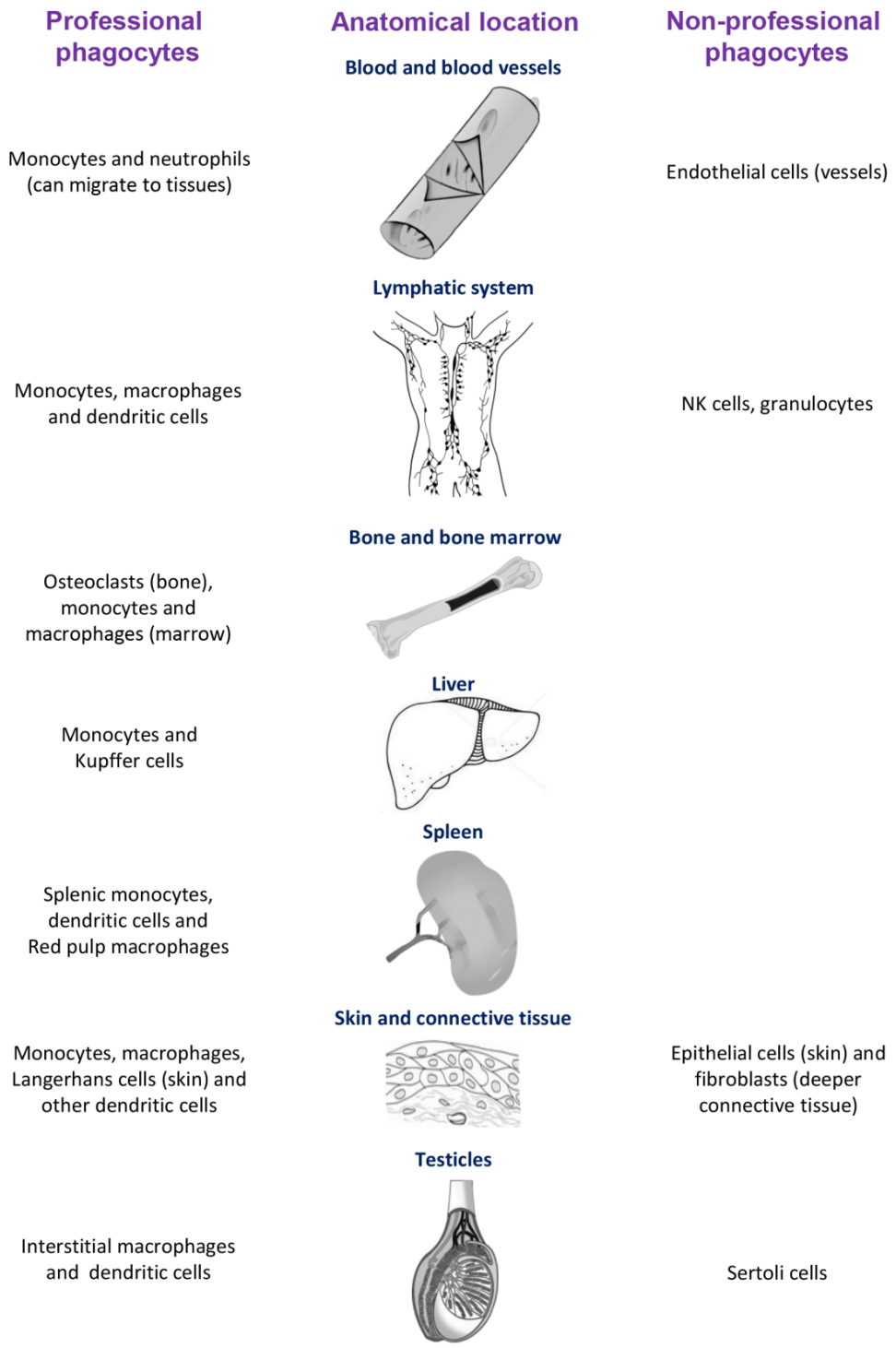


Figure 2 of literature review. Diversity of professional and non-professional at selected anatomical sites. Professional and non-professional phagocytes are present in a variety of anatomical locations with vastly different microenvironments that influence phagocyte function. For instance, osteoclasts are a specialized type of macrophage that absorbs bone. In the skin and mucosa, professional phagocytes internalize a variety of environmental antigens and microbes. In the case of immunoprivileged sites such as the testes, macrophages and dendritic cells are only present in the interstitium, while sertoli cells, which are non-professional phagocytes, ingest senescent germ cells. The images used in this figure were labeled for reuse.

1.3 Non-professional phagocytes

Non-professional phagocytes are non-immune cells whose main function is not phagocytosis. They have evolved very specific functions that are crucial in homeostatic regulation and tissue remodelling at sites such as the eye and the testis, where it is detrimental to have inflammatory professional phagocytes (Figure 2) (Penberthy *et al.*, Rabinovitch, 1995). Non-professional phagocytes are comprised of endothelial, epithelial cells and fibroblasts, which clear dead cells and debris in the tissues where they reside (Figure 2). For instance, testicular sertoli cells phagocytose senescent germ cells; in the eye, retina pigment epithelial cells clear used photoreceptor outer segments (Penberthy *et al.*). Non-professional phagocytes cells do not possess the recognition receptors or the intracellular machinery necessary for efficient phagocytosis. They produce low levels of reactive oxygen species (ROS) or antimicrobial peptides compared to professional phagocytes (Penberthy *et al.*, 2018, Rabinovitch, 1995).

Recently, it was discovered that professional and non-professional phagocytes communicate via the insulin growth factor 1 (IGF-1), a pleiotropic hormone involved in muscle building (Han *et al.*, 2016). Lung epithelial cells phagocytose apoptotic bodies and are in constant contact with allergens such as house dust mite (HDM). HDM is internalized and induces inflammation in lung epithelial cells, which then triggers the release of IL-4 and IL-13 by immune cells. Those cytokines then elicit the release of anti-inflammatory extracellular vesicles and IGF-1 by lung macrophages (Han *et al.*, 2016). IGF-1 then signals lung epithelial cells to stop ingesting apoptotic cells and to start the uptake of anti-inflammatory macrophage-derived extracellular vesicles (Bourdonnay *et al.*, 2015, Han *et al.*, 2016), thus ensuing in the control of airway inflammation. Much remains to be discovered about how professional and non-professional phagocytes collaborate to promote organismal homeostasis (Penberthy *et al.*, 2018).

2 THE MOLECULAR MACHINERY THAT REGULATES VESICULAR TRAFFIC IN EUKARYOTIC CELLS

2.1 Introduction and historical context

Eukaryotic cells are factories that produce and secrete a multitude of molecules of essential function, from hormones and neurotransmitters to cytokines (Blank *et al.*, 2014, Südhof, 2012). In the year 2013, Drs. James E. Rothman, Randy W. Schekman and Thomas C. Südhof were awarded the Nobel Prize in Physiology or Medicine in commemoration for their discoveries on the molecular machinery that regulates vesicular traffic, an intracellular transport system of pivotal importance in health and disease. Starting in the 1970s and 1980s, the three laureates worked on separate aspects of the mechanisms by which molecules such as enzymes and neurotransmitters are transported within, and out of cells. In the 1970s, Dr. Schekman worked on budding yeast and identified the genes that produce the proteins necessary for the transport of these vesicles. Using classical genetic analysis, he characterized 23 genes involved in the vesicular transport that takes place from the perinuclear region of the endoplasmic reticulum (ER) to the Golgi apparatus and the cell membrane (Novick *et al.*, 1980, Novick *et al.*, 1979). On the other hand, Dr. Rothman worked with mammalian cells. His group characterized the *N*-ethylmaleimide-sensitive fusion (NSF) protein, which allows the transport of proteins to the Golgi apparatus, the synaptosomal-associated protein (SNAP), and the NSF attachment protein receptor (SNARE) (Wilson *et al.*, 1989). He discovered how proteins make it into vesicles that reach and attach onto a target membrane, ensuring that they are delivered to the right compartment (Rothman, 1994). The last mechanism of this cellular transport pathway was to identify how a vesicle, now in juxtaposition with its target membrane, fuses to allow its contents to be delivered. This mechanism was characterized by Dr. Südhof and colleagues, who used neurons as a model system. Using biochemical and functional assays, Dr. Südhof and collaborators described the calcium influx-dependent mechanism that allows vesicle fusion to occur (Pang *et al.*, 2010, Südhof, 2012, Südhof *et al.*, 2011). Of notable

importance is their discovery of Synaptotagmin (Syt), which catalyzes this fusion step (Perin *et al.*, 1990, Südhof, 2012).

Cellular products must be transported, with great precision, from their site of synthesis to the extracellular milieu. For these reasons, the organization of cellular trafficking is fundamental, and is a process that is carried out by vesicles and their molecular adaptors. Indeed, eukaryotic cells are characterized by the orderly and precise distribution of molecules in different cellular compartments (Pang *et al.*, 2010, Südhof, 2012). If the vesicular transport system essential for its functioning and survival does not work, the cell ceases to be a complex and precise biological machine and collapses into chaos. Vesicles act as vehicles that transport molecules among cellular organelles, and from organelles to the plasma membrane. Vesicles execute inter-organelle communication by carrying molecules as part of their lipid bilayer or lumen. Vesicular transport is spatiotemporally regulated by a wide array of membrane proteins that reside in the various cellular organelles. In turn, such molecules define the identity and content of those cellular organelles (Jahn *et al.*, 2006, Malsam *et al.*, 2011). For example, acidic pH and hydrolytic enzymes should be present only in lysosomes. However, when a cell ingests a particle via phagocytosis, the vacuole is acidified via a vesicle fusion process that leads to the accrual and fusion of lysosomes with phagosomes (Levin *et al.*, 2016). Vesicles are formed in a source compartment and loaded with cargo molecules that must be transported. Once released into the cytosol, the vesicles are specifically directed towards a target compartment to which they eventually finally fuse with. Intracellular vesicles can originate at virtually all cellular organelles (Jahn *et al.*, 2006, Malsam *et al.*, 2011). Oftentimes, these vesicles originate at the Golgi and the ER or at the plasma membrane (Figure 3) (Bonifacino *et al.*, 2004, Jahn *et al.*, 2006). Indeed, crossing the plasma membrane is one of the main ways in which compounds enter or leave the cell.

2.2 The import route: endocytosis

Endocytosis is the process by which large macromolecules and small particles enter the cell in an ATP-dependent manner (Figure 3) (Bonifacino *et al.*, 2004). In this process, material that enters the cell induces an invagination in the plasma membrane that leads to the formation of a vesicle encasing the material. Endocytosis is subdivided according to the nature of the internalized material. In receptor-mediated endocytosis, the internalization process is initiated by recognition of extracellular ligands by receptors on the cell surface. Recognition of the ligand starts a signalling program that induces the internalization of the ligand-receptor complex in a vesicle. This signalling leads to the active recruitment of vesicle coat proteins known as clathrin and caveolae, which facilitate membrane curvature and budding (Nichols *et al.*, 2001, Traub, 2011). Caveolae are lipid microdomains that consist of small cholesterol-rich invaginations at the plasma membrane. These structures are delineated by caveolin-1, possess the ganglioside GM-1, and contain receptors such as CR3 (Harris *et al.*, 2002, Kenworthy, 2002). Endocytosis contributes to the recycling of those cell membrane receptors, which can be triggered by the attachment of a single ubiquitin molecule to the receptor (Bonifacino *et al.*, 2004, Ghaddar *et al.*, 2014). On the other hand, pinocytosis refers to the endocytosis of liquid and any solutes found therein. It is a dominant feature of cells in the intestinal mucosa, which specialize in nutrient acquisition (Doherty *et al.*, 2009). Pinocytosis can occur independently of clathrin coats through the constitutive uptake of micropinosomes (Nichols *et al.*, 2001). Internalization of large particles is termed phagocytosis (Desjardins *et al.*, 2005, Gordon, 2016) and will be discussed in section ‘3’ of this Chapter. Once endocytosed vesicles are formed in the cytoplasm, they may fuse with lysosomes, which induce digestion of intravesicular contents. The products of digestion are then recycled in the cell (Bonifacino *et al.*, 2004, Levin *et al.*, 2016).

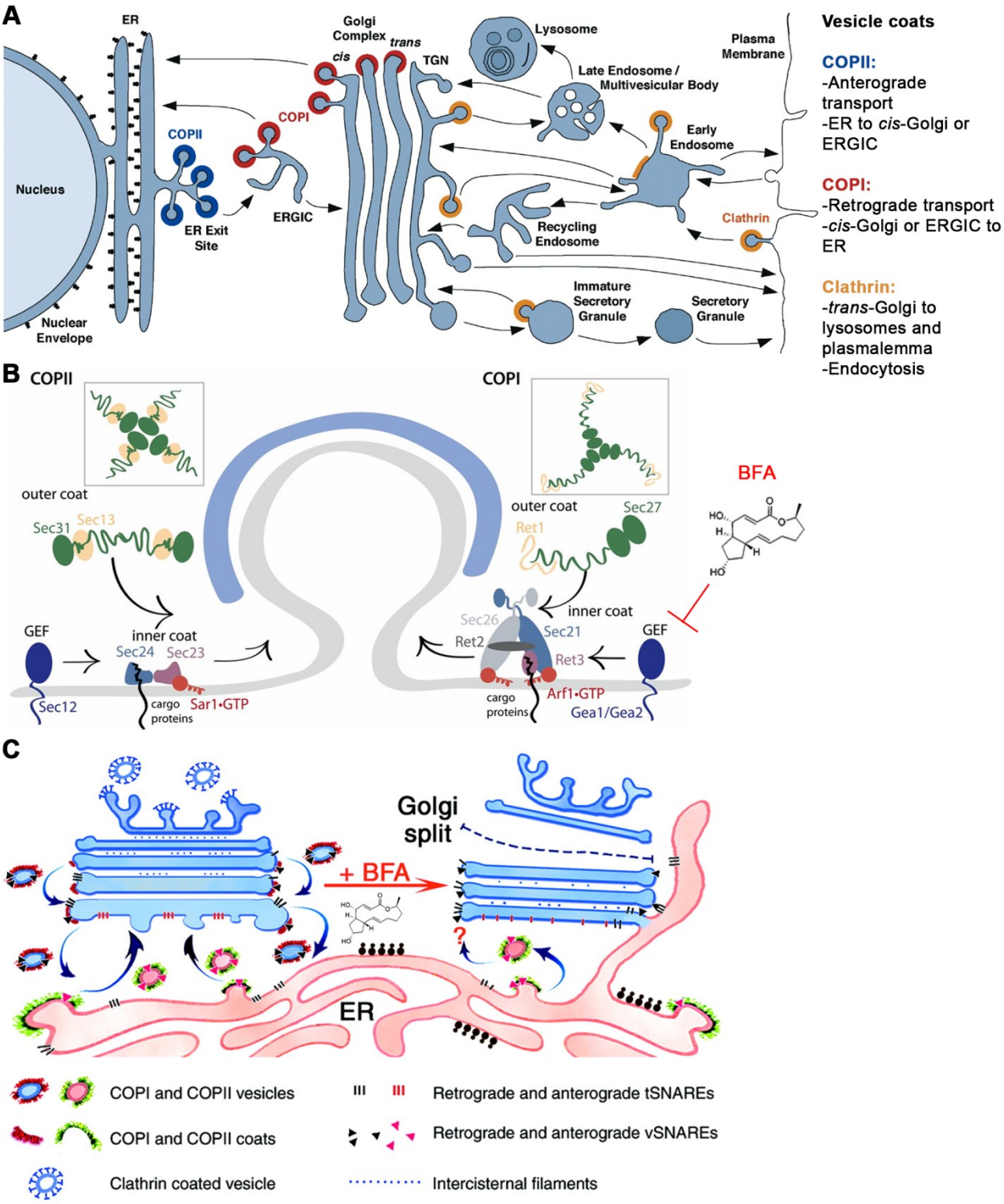


Figure 3 of literature review. Vesicle trafficking in the secretory pathway. (A) The secretory pathway is constituted by a group of organelles through which synthesized proteins and lipids are transported in or on vesicles. This transport process is spatiotemporally regulated by coat proteins (COPI, COPII and clathrin) and vesicle fusion proteins that regulate cargo destination. **(B)** In retrograde and anterograde transport, vesicle buddying from the ER is initiated by GTPase-mediated recruitment and assembly of COPI and II complexes, respectively. This leads to cargo selection, membrane curvature and vesicle pinching. **(B, C)** Brefeldin A is a reversible inhibitor that blocks the GTPase activity required for COPI and COPII coat assembly. BFA stops retrograde transport by inhibiting the GEF

activity of Gea1/Gea2, and may also block anterograde transport. **(C)** This blockage halts ER-Golgi traffic, eventually causing the Golgi to collapse onto the ER. Adapted with permission from (Barlowe *et al.*, 2013, Bonifacino *et al.*, 2004, Nebenführ *et al.*, 2002).

2.3 The secretory pathway: exocytosis

Exocytosis is the process by which cytoplasmic molecules are secreted outside the cell (Figure 3), and is a key feature of secretory cells such as neurons, pancreatic islets and cells of the immune system (Bonifacino *et al.*, 2004, Huynh *et al.*, 2007b, Stow *et al.*, 2013). In this highly regulated process, vesicles encasing cellular products usually originate from the Golgi network, travel to the plasma membrane, and fuse to unload their contents. After proteins are processed in the ER, they are transported to the Golgi network via COPII-coated vesicles in a process known as anterograde transport (Bonifacino *et al.*, 2004, Stow *et al.*, 2013). There, proteins mature as they become post-translationally modified in the various cisternae of this organelle. Eventually, proteins reach the *trans*-Golgi network (TGN), where they are packaged and sorted into clathrin-coated vesicles (Traub, 2011), and sent to different cellular compartments or the outside of the cell (Bonifacino *et al.*, 2004, Huynh *et al.*, 2007b, Stow *et al.*, 2013). There are two types of exocytosis – constitutive and regulated. Constitutive exocytosis is feature of all eukaryotic cells. In this process, vesicles are secreted in a continuous fashion, making it essential for the renewal of the plasmalemmal membrane and its proteins (Bonifacino *et al.*, 2004, Huynh *et al.*, 2007b). In macrophages, the secretion of cytokines such as TNF is a constitutive process that is triggered by cellular activation (Beutler, 1999, Stow *et al.*, 2013). In regulated exocytosis, proteins are synthesized and stored in a vesicle until the cell receives a regulatory signal to initiate secretion; this is a feature of cells that secrete hormones such insulin (Bonifacino *et al.*, 2004, Huynh *et al.*, 2007b, Stow *et al.*, 2013). In the immune system, mast cells and neutrophils also secrete their cytoplasmic granules via regulated exocytosis (Arthur *et al.*, 2016, Hellebrekers *et al.*, 2018, Stow *et al.*, 2013).

2.3.1 The machinery involved in anterograde and retrograde transport

When vesicles bud from a donor compartment, they are coated with proteins that are necessary for inducing the membrane deformity required for vesicle formation, for selecting the cargo of the vesicle, and for determining the delivery site. Assembly of these proteins induces membrane buckling and budding. Eventually, the protein coat comes off when the vesicle fuses with its target compartment (Bonifacino *et al.*, 2004, Nichols *et al.*, 2001, Traub, 2011). Many of these proteins were initially found in the yeast-based genetic screens performed in Dr. Schekman's laboratory. Mutations in the components of this machinery gave rise to swollen intracellular compartments and hindered secretion (Novick *et al.*, 1980, Novick *et al.*, 1979). The blockages occurred in cellular locations that were later traced to where these proteins exert their functions (Bonifacino *et al.*, 2004).

Anterograde transport, or forward pathway, describes the passage that takes place from the ER to the ER-intermediate compartment (ERGIC) and onto the *cis*-Golgi (Barlowe *et al.*, 2013, Bonifacino *et al.*, 2004). Vesicle formation at the ER starts with recruitment of the Sar1/2 GTPase, whose function is to recruit adaptors that are involved in cargo selection and coat formation (Figure 3). Sar1/2 is initially recruited in its inactive GDP-bound form, and through interaction with the guanine exchange factor (GEF) Sec12, Sar is activated into its GTP-bound form. This activation triggers a conformational change in Sar1/2 that unmasks a myristylated anchor, which permits Sar1/2-GTP to anchor itself into the vesicle's membrane. Sar1/2 initiates membrane deformation and mediates the recruitment of the adaptor complex Sec23-Sec24. Those proteins possess binding sites for vesicular cargo, which can be membrane-bound or luminal. The assembling Sec23-Sec24 complexes recruit Sec13-Sec31 dimers, which form the outer part of the vesicle's coat and induce further membrane curvature. Eventually, loaded vesicles bud off. Coat proteins are shed when Sar1/2-GTD is hydrolyzed into Sar1/2-GDP by GTPase-activating proteins (GAPs) (Barlowe *et al.*, 2013, Bonifacino *et al.*, 2004).

In contrast, retrograde transport describes the traffic that originates in the Golgi or ERGIC and moves to the ER (Figure 3). It is essential for recycling vesicle trafficking-associated proteins, such as SNAREs and coat proteins, back to the ER. It also returns misfolded proteins for chaperone-assisted quality control in the ER lumen. Similar to retrograde transport, cargo selection and coat assembly is initiated by the GEF-mediated switch of Arf1-GDP into its GTP-bound form. This mediates the recruitment of inner coat complex Sec21-Sec26-Ret1-Ret3, which leads to accrual of outer coat proteins Ret1 and Sec27. This assembly forms a triskelion-like complex that mediates vesicle pinching (Barlowe *et al.*, 2013, Bonifacino *et al.*, 2004). The importance of vesicle trafficking in the secretory pathway is exemplified by the fact that it can be targeted by several toxins of fungal and synthetic origin. Brefeldin A (BFA) is a toxin from the *Eupenicillium brefeldianum* fungus that blocks the secretory pathway and is used to study endomembrane trafficking and protein transport (Nebenführ *et al.*, 2002, Sciaky *et al.*, 1997) (Figure 3). BFA is a reversible and non-competitive inhibitor of the GEF activity of GBF1 (Gea1/Gea2), thereby blocking the activation of Arf1 into its GTP-bound form. The ensuing blockage at the ERGIC/*cis*-Golgi leads to an accumulation of SNAREs and other vesicle fusion proteins. This leads to abnormal fusion of the ERGIC and Golgi with the ER, thereby triggering the unfolded protein response (Nebenführ *et al.*, 2002). In immune cells, BFA effectively inhibits cytokine secretion and autophagosome biogenesis, which are actively dependent on the secretory pathway (Ge *et al.*, 2013, Stow *et al.*, 2013). It can also inhibit the transit of several viruses whose proteins undergo maturation in the host cell's ER and Golgi (Tamura *et al.*, 1968).

Vesicle movement is dependent on Rab GTPases, which are part of the Ras family of monomeric G proteins (Bonifacino *et al.*, 2004, Chavrier *et al.*, 1990, Murray *et al.*, 2014). Rabs are found as soluble inactive proteins in the cytosol that become membrane-bound when their activation induces the unmasking of a myristyl moiety (Murray *et al.*, 2014). Rabs are involved in vesicle transport and recognition of target membranes, the specificity of which is attested by the presence of over 60 Rabs that are expressed in the various cellular compartments (Chavrier *et al.*, 1990, Murray *et al.*,

2014). Indeed, it has been found that vesicular transport by motor proteins requires Rab activity. The next step in vesicle trafficking is the tethering and fusion that must occur when vesicles arrive at their destination (Bonifacino *et al.*, 2004, Murray *et al.*, 2014).

2.4 The role of SNAREs in vesicle targeting and docking

The destination to which a vesicle travels is determined by components of the vesicle such as coat proteins, Rab effectors and importantly, by proteins that catalyze membrane fusion (Bonifacino *et al.*, 2004, Stow *et al.*, 2006). Indeed, vesicle docking and fusion rely on members of the SNARE family. In mammalian cells, over 60 SNAREs make up this group of conserved membrane proteins that mediate membrane traffic in most cellular organelles (Figure 4) (Bonifacino *et al.*, 2004, Hong *et al.*, 2014, Rothman, 1994, Stow *et al.*, 2006). The SNARE family can be subdivided in two subfamilies, vesicular (v-) SNAREs and target (t-) SNAREs, the latter being present in acceptor or target membranes (Rothman, 1994, Wilson *et al.*, 1989). Data suggest that t-SNAREs form stable subcomplexes that pair with incoming v-SNAREs in order to form a SNARE complex prior to fusion. SNAREs possess a characteristic SNARE motif that consists of at least 72 amino acids arranged in repetitive heptads, giving rise to a helical domain. T- and v-SNAREs interact through their SNARE domains to form a right-handed four-helix bundle known as a *trans*-SNARE complex. The quaternary structure of the bundle is highly conserved among species (Hong *et al.*, 2014). Since multiple SNAREs are found on both vesicles and target membranes, SNAREs were classified according to their structural characteristics. In that nomenclature, R-SNAREs contribute an arginine (R) to the core of an assembled *trans*-SNARE helix bundle; R-SNAREs are often v-SNAREs such as the vesicle-associated membrane protein 8 (VAMP8) and Sec22b (Fasshauer *et al.*, 1998, Hong *et al.*, 2014). Q-SNAREs are often t-SNAREs and contribute a glutamine (Q) to the core of a complex. Q-SNAREs can be further classified into Qa, Qb and Qc depending on the location of the Q in a SNARE complex. Examples include the syntaxins (Stx) and SNAP-25 (Fasshauer *et al.*, 1998, Hong *et al.*, 2014). Interacting SNAREs form a Qa-Qb-Qc-R four-helix bundle whose core position is known as the 0

layer, which is the site where the Q and R residues interact (Golebiewska *et al.*, 2014, Hong *et al.*, 2014). The function of many SNAREs was initially characterized in neuronal cells where their absence translated into crippling neurotransmission defects. Indeed, the importance of SNAREs in vesicle fusion is exemplified by the disease phenotypes observed when the expression or integrity of these proteins is compromised (Fasshauer *et al.*, 1998). For example, synaptic vesicle transmission, which is mediated by SNAP-25 and VAMP1/2, can be inhibited by the botulinum toxin-mediated cleavage of those SNAREs (Blasi *et al.*, 1993). Moreover, the tetanus toxin is a zinc metalloprotease that cleaves VAMP2 and blocks exocytosis of vesicles that cluster at the synapse (Verderio *et al.*, 1999). These examples also allude to the fact that several microbes have evolved the capacity to inhibit SNARE function to promote their survival. In the case of dendritic cells, cleavage of the SNARE VAMP8 by the *Leishmania* GP63 metalloprotease inhibits antigen cross-presentation (Matheoud *et al.*, 2013).

2.4.1 SNARE complex assembly and dissociation

Vesicle fusion is a process that requires energy in order to overcome the electrostatic forces of repulsion between fusing membranes, and to form a fusion pore adjoining the two membranes (Hong *et al.*, 2014, Südhof, 2012). SNARE proteins generate energy through their protein-protein and protein-lipid actions that synergize to promote membrane fusion. In the assembly stage of a SNARE complex, the adoption of a *trans* complex among Q- and R-SNAREs is necessary in order to bring two fusing membranes in close physical proximity to one another. As previously mentioned, SNARE complex assembly requires the intertwining of SNARE domains into a helical bundle (Golebiewska *et al.*, 2014, Hong *et al.*, 2014, Südhof, 2012) (Figure 4). The assembly process is usually inhibited by the Munc18 protein, which binds to the SNARE domain of Stx proteins and hinders interactions with other SNAREs. This inhibitory complex is opened when the Munc13-RIM complex mediates the GTP-dependent dissociation of Munc18. It has been observed that Munc18 can also promote fusion by staying associated with the N-terminal of Stx, thereby stabilizing SNARE complexes.

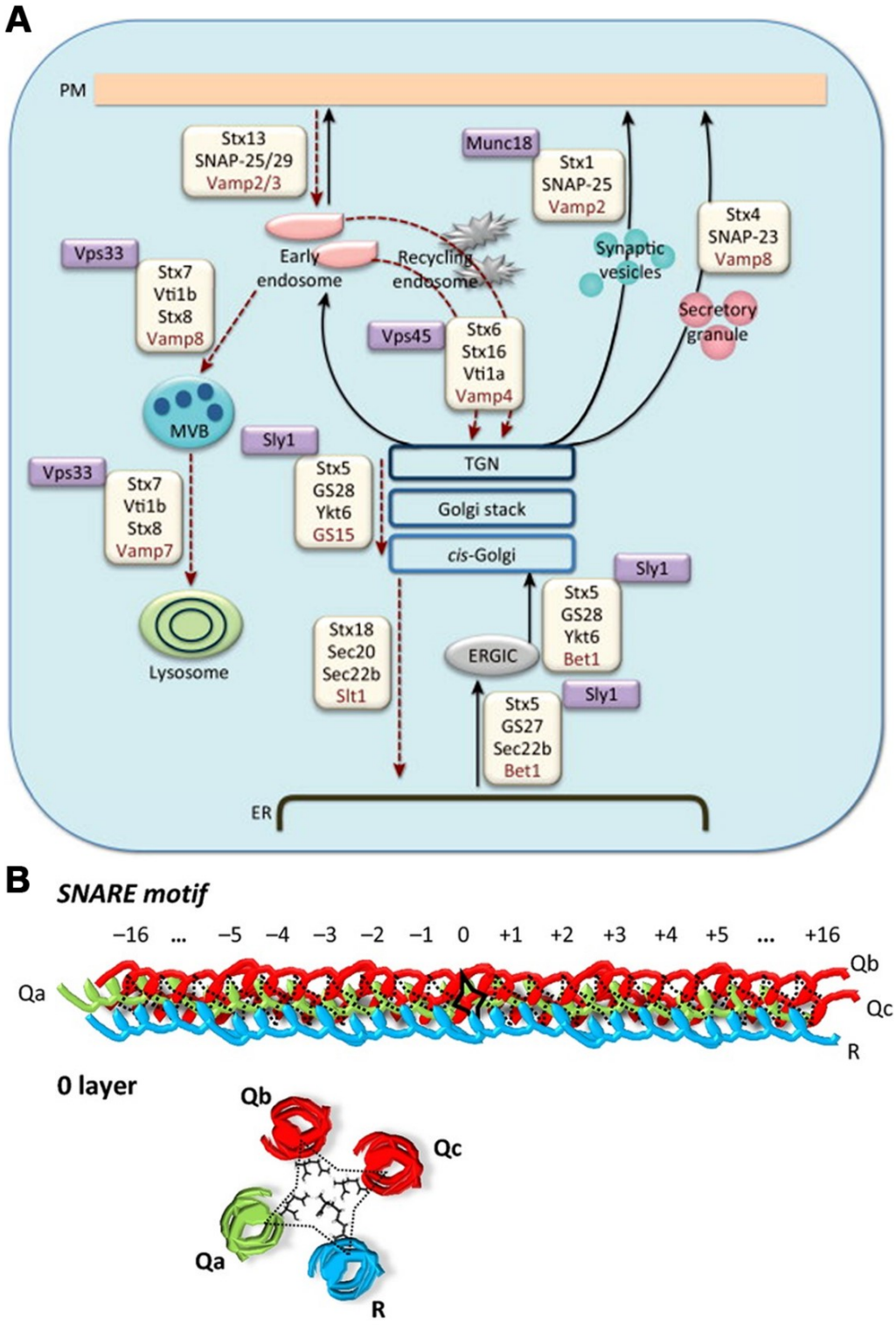


Figure 4 of literature review. SNARE proteins promote vesicle fusion through quaternary structure formation. **(A)** SNARE proteins are diverse and reside in cellular organelles to mediate specific vesicle fusion events. V-SNAREs are in red and t-SNAREs in black; Munc18 and proteins stabilizing SNARE complex formation are noted in purple boxes. **(B)** Vesicle docking is mediated by the interaction of multiple SNAREs on donor and receptor membranes. Interacting cognate SNAREs form a four-helix coiled coil structure whose core contains interacting Q and R residues in a 0-ionic layer. Adapted with permission from (Golebiewska *et al.*, 2014, Hong *et al.*, 2014).

In order to generate the force necessary for fusion, Q- and R-SNAREs must form a *trans* complex (Golebiewska *et al.*, 2014). Indeed, part of the energy liberated during the formation of a *trans*-SNARE complex is stored as mechanical energy in the semi-rigid zones that connect the SNAREs' transmembrane domains with the helical bundle in a SNARE complex (Golebiewska *et al.*, 2014, Hong *et al.*, 2014, Südhof, 2012). That energetically unfavourable conformation is relieved when the vesicle and target membranes align with the SNARE complex (Figure 5). In this manner, this decrease in tension helps overcome the energy barrier for fusion, and the two membranes come together. At this point, *trans*-SNARE complexes are further stabilized in order for fusion to occur. This is mediated by complexin, which binds to the groove between the helices formed by Q- and R-SNAREs and stabilize the C-terminal region of the complex (Südhof, 2012, Südhof *et al.*, 2011). In turn, this allows Syts (Syt) to trigger membrane fusion, an event that depends on Ca^{2+} levels. Syts bind Ca^{2+}, which induces a conformational change that displaces complexin from the SNARE complex (Figure 5). Calcium binding also induces Syt to interact with the target membrane in order to form a fusion pore. After fusion occurs, SNARE complexes must be dissociated. This event is mediated by NSF, a member of the AAA+ family of ATPases. In conjunction with α-SNAP, NSF mediates the disassembly of *trans*-SNARE complexes in an ATP-dependent manner, a process that allows R-SNAREs to be recycled for future fusion events (Südhof, 2012, Südhof *et al.*, 2011).

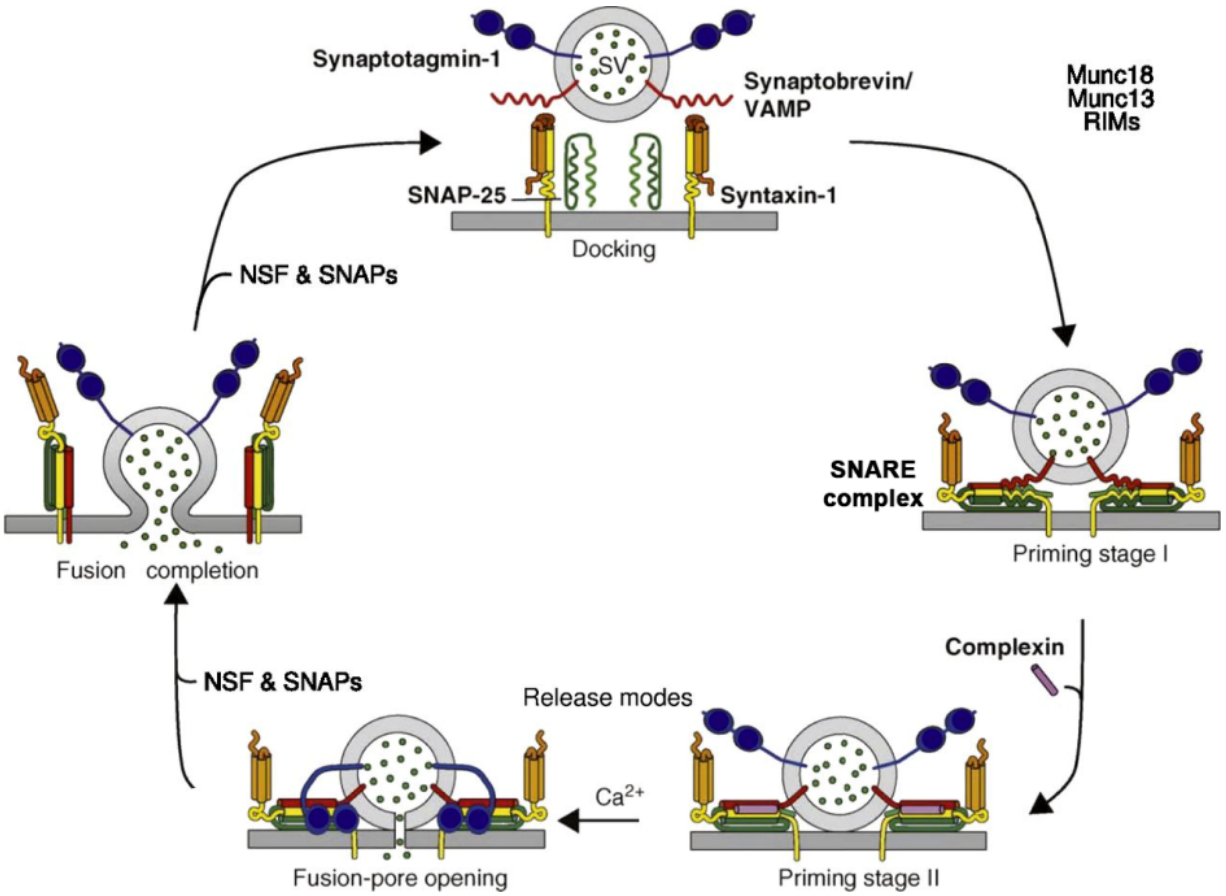


Figure 5 of literature review. The role of SNAREs in the vesicle fusion cycle. The natural repulsion that occurs when two membranes are in close proximity is overcome by SNARE interactions. Munc18 and complexin proteins mediate and stabilize *trans*-SNARE complex formation. This interaction brings the apposed membranes into very close proximity. Subsequently, a calcium influx activates Syt, which mediates fusion pore formation and content mixing. Following fusion, SNARE complexes are untangled by NSF and SNAP proteins, thereby permitting those SNAREs to participate in future fusion cycles. Adapted with permission from (Rodrigues *et al.*, 2016, Südhof, 2012).

2.4.2 SNARE complexes that regulate ER ↔ Golgi transport

Trafficking among the ER, ERGIC and the Golgi apparatus is finely attuned by protein complexes that dictate the specificity and directionality of vesicle fusion events (Figures 4 and 6) (Appenzeller-Herzog *et al.*, 2006, Malsam *et al.*, 2011). Vesicle reconstitution assays have revealed the possibility of at least 147 Q/R-SNARE complexes that could regulate fusion among these organelles, of which five have been experimentally validated (Malsam *et al.*, 2011, Parlati *et al.*, 2002). In anterograde transport from the ER, COPII vesicle components have been found to interact with and select the SNAREs that are recruited to those vesicles (Mossessova *et al.*, 2003). Indeed, the Sec23-Sec24

components have been found to bind to peptide signals in the v-SNARE GOS28. When GOS28 is complexed with Stx5, the peptide signal is no longer accessible and cannot be recruited by COPII (Mossessova *et al.*, 2003). Passage from the ER to the ERGIC and Golgi is governed by two complexes (Malsam *et al.*, 2011, Volchuk *et al.*, 2004). The first one is the Stx5-Membrin-Sec22b t-SNARE subcomplex, which pairs with v-SNARE rBet1. The second one regulates retrograde transport within the Golgi cisternae, and is constituted by Stx5-GOS28-Ykt6 (t-SNARE subcomplex) and Gs15 (v-SNARE).

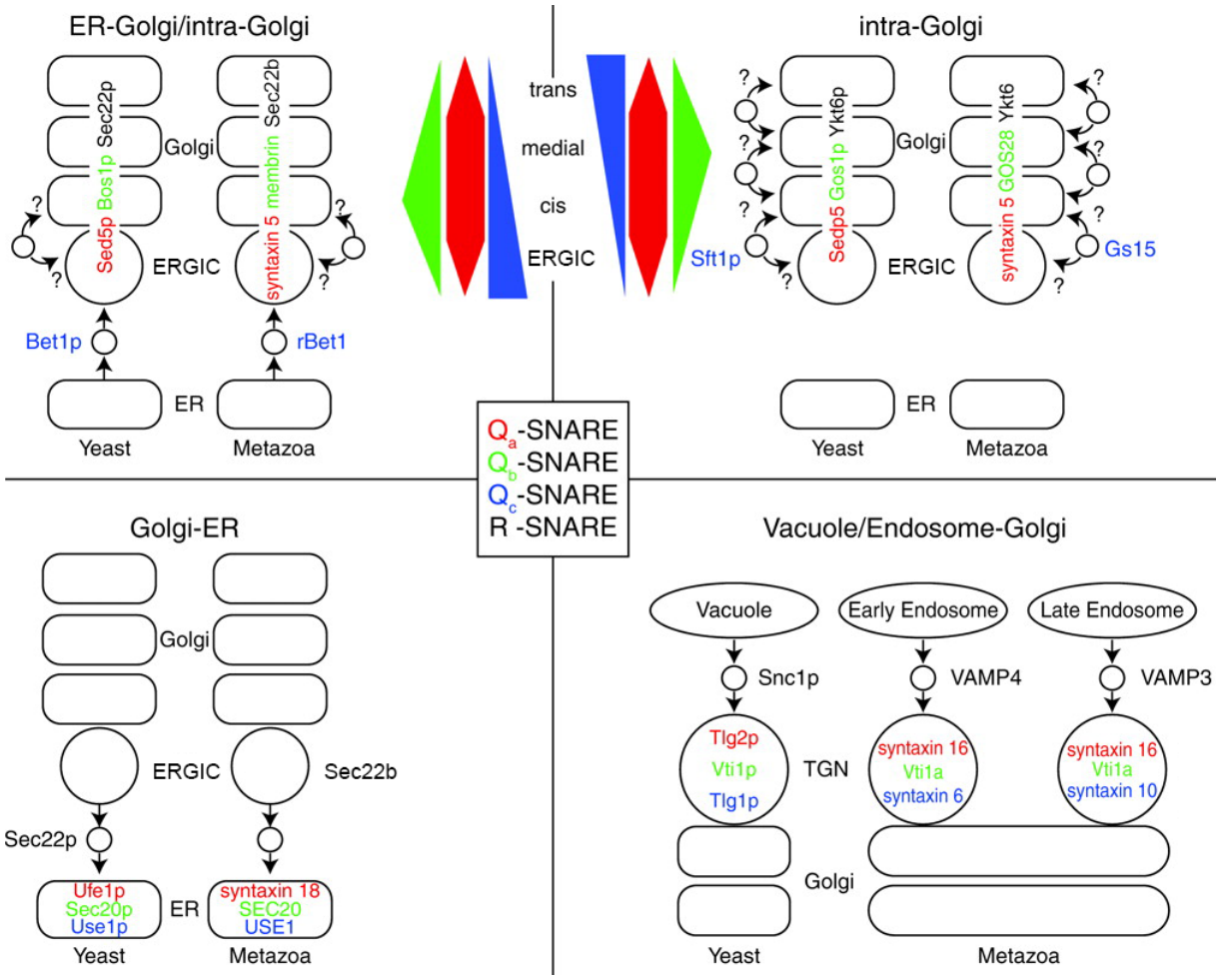


Figure 6 of literature review. SNARE-mediated regulation of ER-Golgi membrane trafficking. The importance of the ER, ERGIC and Golgi organelles is highlighted by the multiple SNARE complexes that regulate vesicle trafficking among these membranous compartments. Many of these complexes are conserved from yeast to metazoans. Of notable importance is the SNARE Sec22b, which can act as a t-SNARE in ER → Golgi traffic and as t-SNARE in the Golgi → ER route. The Golgi contains polarized concentrations of various SNAREs, as colour-coded by the shapes in the upper panels. Adapted with permission from (Malsam *et al.*, 2011), copyright to Cold Spring Harbor Laboratory Press.

In anterograde transport, COPI vesicle components have also been found to mediate the accrual of SNAREs. In particular, Rein and colleagues showed that the Arf1 GTPase recruits GOS28, and Sec22b; the latter acts as a v-SNARE in anterograde transport (Rein *et al.*, 2002, Spang *et al.*, 1998). In this regard, Sec22b pairs with Stx5/Stx18/Sec20/USE1 as possible cognate t-SNAREs (Dilcher *et al.*, 2003, Spang *et al.*, 1998). Finally, arrival of material from early and late endosomes is packaged in VAMP3 and VAMP4-positive vesicles, respectively. Those v-SNAREs pair with t-SNARE complex at the TGN and consist of Stx16-Vti1a-Stx10 (VAMP3) and Stx16-Vti1a-Stx6 (VAMP4) (Figure 6) (Malsam *et al.*, 2011).

2.1 The role of Syts in vesicle fusion

As mentioned previously, the SNARE-mediated targeting and docking of two membranes is not enough for vesicle fusion to occur (Fukuda, 2007, Pang *et al.*, 2010, Südhof, 2012). Data from studies on neurotransmission has revealed that vesicles fuse with the synaptic cleft when an action potential occurs. This voltage stimulus triggers the opening of Ca^{2+} channels, which in turn increases intracellular [Ca^{2+}] up to 100 nM, or ~15000X the concentration of extracellular calcium. This [Ca^{2+}] is sensed by the Syt protein, which in turn drives vesicle fusion at the synaptic cleft (Perin *et al.*, 1990, Südhof, 2012). Syts regulate vesicle fusion in processes ranging from the exocytosis of synaptic vesicles and cytokines to phagocytosis and autophagy (Bento *et al.*, 2016, Pang *et al.*, 2010, Vinet *et al.*, 2008). In neurons, Syt I and II are the most abundant. The importance of Syt is highlighted by knockout experiments where absence of Syt I/II severely decreased neurotransmission in fruit flies, worms and mice (Fukuda, 2007, Südhof, 2012). Indeed, Syts compose a family of more than 20 proteins that exist in eukaryotic organisms including plants and animals (Figure 7).

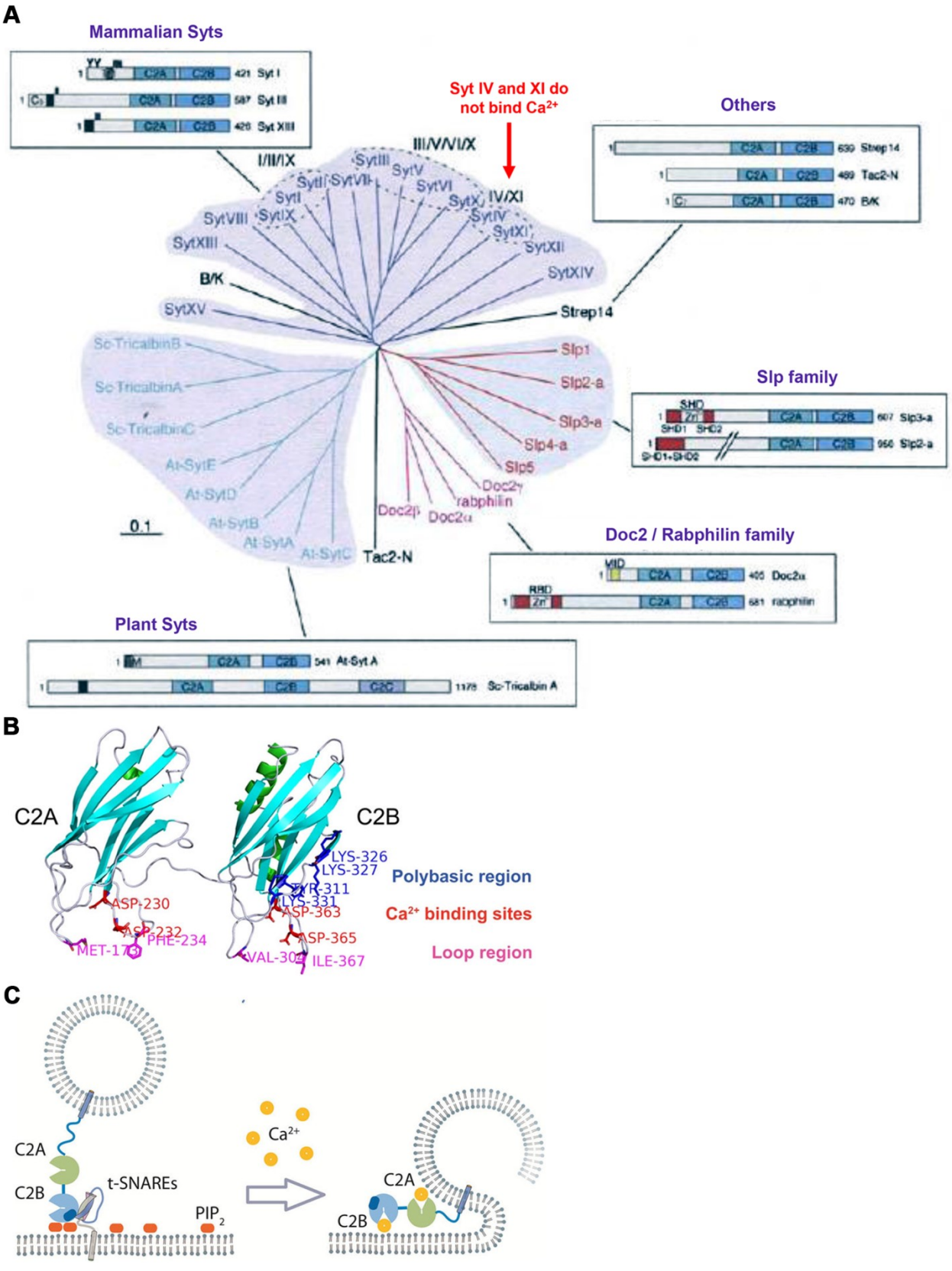

A
Mammalian Syts
Syt IV and XI do not bind Ca2+
Others
I/II/IX
III/V/VI/X
IV/XI
SytIII
SytV
SytVII
SytVI
SytI
SytX
SytIV
SytVIII
SytIX
SytXI
SytXIII
SytXII
SytXIV
B/K
SytXV
Strep14
Sc-TricalbinB
Sc-TricalbinA
Sc-TricalbinC
At-SytE
At-SytD
At-SytB
At-SytA
At-SytC
Tac2-N
Slp1
Slp2-a
Slp3-a
Slp4-a
Slp5
Doc2γ
rabphilin
Doc2β
Doc2α
0.1
Slp family
Doc2 / Rabphilin family
Plant Syts
421 Syt I
587 Syt III
426 Syt XIII
639 Strep14
489 Tac2-N
470 B/K
607 Slp3-a
960 Slp2-a
405 Doc2α
681 rabphilin
541 At-Syt A
1178 Sc-Tricalbin A
B
C2A
C2B
LYS-326
LYS-327
TYR-311
LYS-331
ASP-363
ASP-365
ASP-230
ASP-232
PHE-234
MET-17
VAL-30
ILE-367
Polybasic region
Ca2+ binding sites
Loop region
C
C2A
C2B
t-SNAREs
PIP2
Ca2+
C2A
C2B

Figure 7 of literature review. Syts are conserved type I membrane proteins that drive membrane fusion. (A) Evolutionary tree of Syts and Syt-like proteins (Slp) showing the presence of calcium-binding C2A and C2B domains. Exceptionally, the C2 domains of Syt IV and XI do not bind calcium. **(B)** Structure of the C2A and C2B domains showing the calcium-binding regions, and the lipid-binding polybasic region. **(C)** Simplified mechanism of action by which Syt mediates calcium-mediated membrane fusion. Adapted with permission from (Fukuda, 2007, Lai *et al.*, 2015).

All Syts are type I membrane proteins with their N-terminal domain (NTD) in the vesicle's lumen (Fukuda, 2007, Pang *et al.*, 2010, Perin *et al.*, 1990, Südhof, 2012). The NTD is followed by a membrane-spanning α-helix that connects to its C-terminal domains (CTD) in the cytoplasmic side. The CTD in Syts is composed of two β-sandwich C2 domains, C2A and C2B (Figure 7) (Lai *et al.*, 2015). These domains were originally identified in protein kinase C (PKC) and are connected by a nine amino acid linker (Perin *et al.*, 1990). C2A binds to three Ca^{2+} cooperatively, whereas C2B domains bind to two Ca^{2+} ions (Lai *et al.*, 2015, Südhof, 2012). Calcium binding to Syts induces an increase in the electrical potential of the C2B domain, thereby allowing it to bind to SNAREs and to the negatively-charged lipid phosphatidylinositol-4,5-*bis*phosphate ($PI(4,5)P_2$) at the target membrane (Bai *et al.*, 2003). Lipid interactions are mediated by a polybasic region on the C2B domain (Lai *et al.*, 2015, Perin *et al.*, 1990). Moreover, calcium binding also allows the C2A domain to bind to phosphatidylserine (PS) on the target membrane. These CTD-lipid interactions allow Syt to bind to the target membrane, which causes curvature stress and membrane buckling. This membrane deformation is thought to induce contact and fusion of the two interacting membranes (Figure 7) (Perin *et al.*, 1990, Südhof, 2012, Südhof *et al.*, 2011).

2.1.1 Syt XI: an inhibitory Syt that does not bind calcium

Most members of the Syt family function as Ca^{2+} sensors of vesicle fusion by virtue of the C2 domains present in these Syts. However, Syts IV and XI possess a conserved serine in their C2A domain that precludes it from binding to Ca^{2+} and phospholipids (Pang *et al.*, 2010, von Poser *et al.*, 1997, Wang *et al.*, 2010) (Figures 7 and 8). A Kyte-Doolittle plot revealed that murine SYTXI had a hydrophobicity profile similar to that of

other previously characterized Syts; of note is the highly hydrophobic N-terminal TM domain (Figure 8).

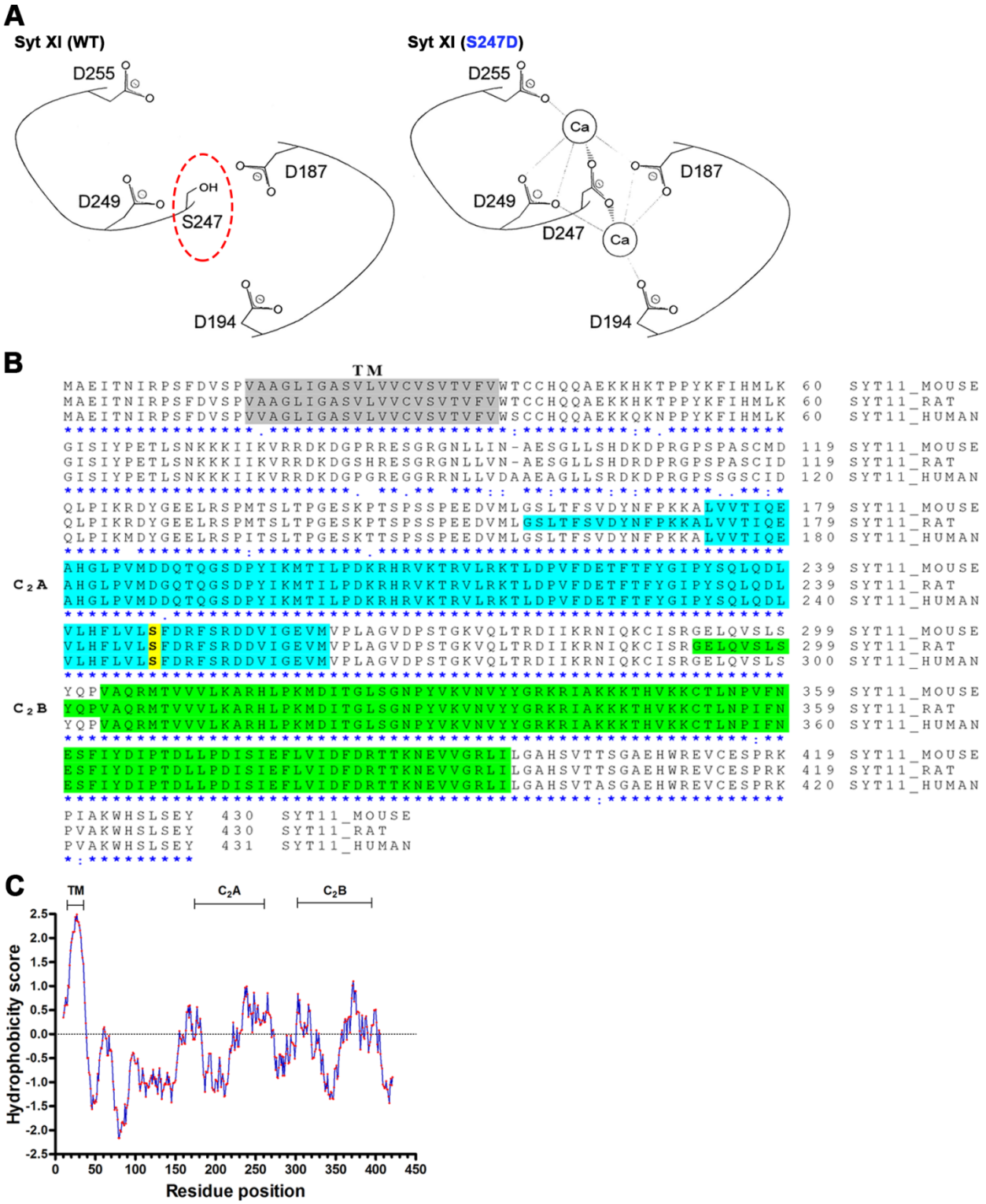


Figure 8 of literature review. Syt XI does not bind Ca^{2+} and is conserved across species. (A) Structure of the WT and S247D-mutated C2A domain of Syt XI. S247 inhibits calcium coordination and binding by Syts IV and XI. When this residue is mutated to an aspartic acid, C2A acquires the capacity to bind calcium. The displayed conformation consisting of five aspartic acids (lower structure) is the WT state of calcium-binding Syts. **(B)** ClustalW sequence alignment (http://services.uniprot.org/clustalw) showing similarities among murine, rat and human SYTXI. Identical and similar amino acids are depicted with an asterisk and a colon, respectively. The predicted TM domain is highlighted in grey, and the C2A and C2B domains in turquoise and green, respectively. Serine 247, which prevents Ca^{2+} binding to the C2A domain of Syt XI, is highlighted in yellow. **(C)** Kyte-Doolittle hydrophobicity plot for murine SYTXI along with the predicted location of its TM, C2A and C2B domains. Positive hydrophobicity scores indicate membrane-interacting regions. Sequences and domain predictions were obtained from the ExPASy server (http://www.expasy.ch/cgi-bin/protscale.pl). Panel (A) was adapted with permission from (von Poser *et al.*, 1997).

A ClustalW sequence alignment shows that the S247 mutation in the C2A domain of SYTXI is conserved in mice, rats and humans, much like the rest of the protein (Figure 8). Following the discovery of Syts IV and XI, it was thought that these proteins had evolved the capacity to inhibit vesicle fusion or to mediate Ca^{2+}-independent vesicle trafficking processes (von Poser *et al.*, 1997). Indeed, Wang and Chapman showed that even in the presence of calcium and appropriate phospholipids, Syt XI inhibited the fusion of liposomes containing cognate SNAREs (Wang *et al.*, 2010).

After the discovery of Syt XI, Huynh and colleagues used the yeast two-hybrid system to find substrates for the Parkin ubiquitin ligase, thereafter discovering that Syt XI was a substrate (Huynh *et al.*, 2003). Parkin facilitates the turnover of proteins involved in the functioning of the central nervous system (CNS). Parkin mutations result in the pathological accumulation of proteins, which eventually ensues in the onset of Parkinson's disease. Interestingly, mutated versions of Parkin lead to the accumulation of Syt XI in Lewy bodies in the brain (Huynh *et al.*, 2003). Additionally, recent epidemiological studies have shown that particular single nucleotide polymorphisms in Parkin and Syt XI are associated with the development of Parkinsonism (Sesar *et al.*, 2016). On the other hand, the promoter of Syt XI is composed of 33 bp repeats that act as binding sites for the Sp1 transcription factor (Inoue *et al.*, 2007). Inoue and colleagues demonstrated that a high number of those repeats induces excessive Syt XI expression, and is linked to the development of Schizophrenia (Inoue *et al.*, 2007).

Considering the implication of Syt XI in CNS pathologies, one can hypothesize that it has a role in regulating synaptic transmission in the brain. This argument is supported by the observation that Syt IV, which is very homologous to Syt XI, was found to negatively regulate the secretion of brain-derived neurotrophic factor (Dean *et al.*, 2009). Syt XI was also found to interact with KIF1A, a brain-associated molecular motor, and with the dishevelled (Dvl) signalling protein (Park *et al.*, 2005). Looking at the subcellular localization of Syt XI, Fukuda and colleagues found that it was located in the Golgi and neurite buttons of the rat pheochromocytoma cell line PC12 (Fukuda *et al.*, 2001). In macrophages, colocalization studies revealed that Syt XI resides in recycling endosomes, lysosomes and even autophagosomes (Bento *et al.*, 2016, Wang *et al.*, 2018). Since those compartments are important for the secretion of immunomodulatory molecules (Murray *et al.*, 2014), Syt XI may be involved in the regulation of that processes.

3 PHAGOCYTOSIS – AT THE CORE OF MACROPHAGE FUNCTION

In higher metazoans, phagocytosis partakes in functions ranging from body plan development and antimicrobial defence, to the clearance of apoptotic cells and tissue repair (Figure 9) (Arandjelovic *et al.*, 2015, Desjardins *et al.*, 2005, Flannagan *et al.*, 2012, Gordon, 2016). Phagocytosis is a complex and highly regulated process that starts when a particle of >0.5 μm comes in contact with the plasma membrane of a phagocytic cell. The particle is first recognized by specific receptors that dictate the mode of internalization and the associated signalling program (Coppolino *et al.*, 2001, Flannagan *et al.*, 2012). Then, a phagocytic cup is formed around the particle, a process that depends on membrane contributions by multiple organelles within the macrophage (Gagnon *et al.*, 2002, Huynh *et al.*, 2007b). The internalized particle, now inside a phagosome, goes through a series of vesicular exchanges that promote the maturation of the phagosome into a phagolysosome (Desjardins, 1995). The maturing phagosome is initially a non-lytic compartment that turns into an acidified and highly degradative organelle through membrane contributions from lysosomes and organelles in the secretory pathway (Figure 9) (Flannagan *et al.*, 2012, Gagnon *et al.*, 2002, Garin *et al.*, 2001, Gordon, 2016). When intraphagosomal cargo is degraded, the resulting antigens may be presented at the cell's surface for subsequent presentation to lymphocytes (Gordon, 2016, Houde *et al.*, 2003). Contingent on the nature of the particle and its interactions with phagocytic receptors, phagocytosis may initiate an inflammatory or anti-inflammatory response (Arandjelovic *et al.*, 2015, Gordon, 2016, Poon *et al.*, 2014).

Since the work presented in this thesis is primarily concerned with macrophages, the information herewith presented in this section will focus on macrophages, unless specified otherwise.

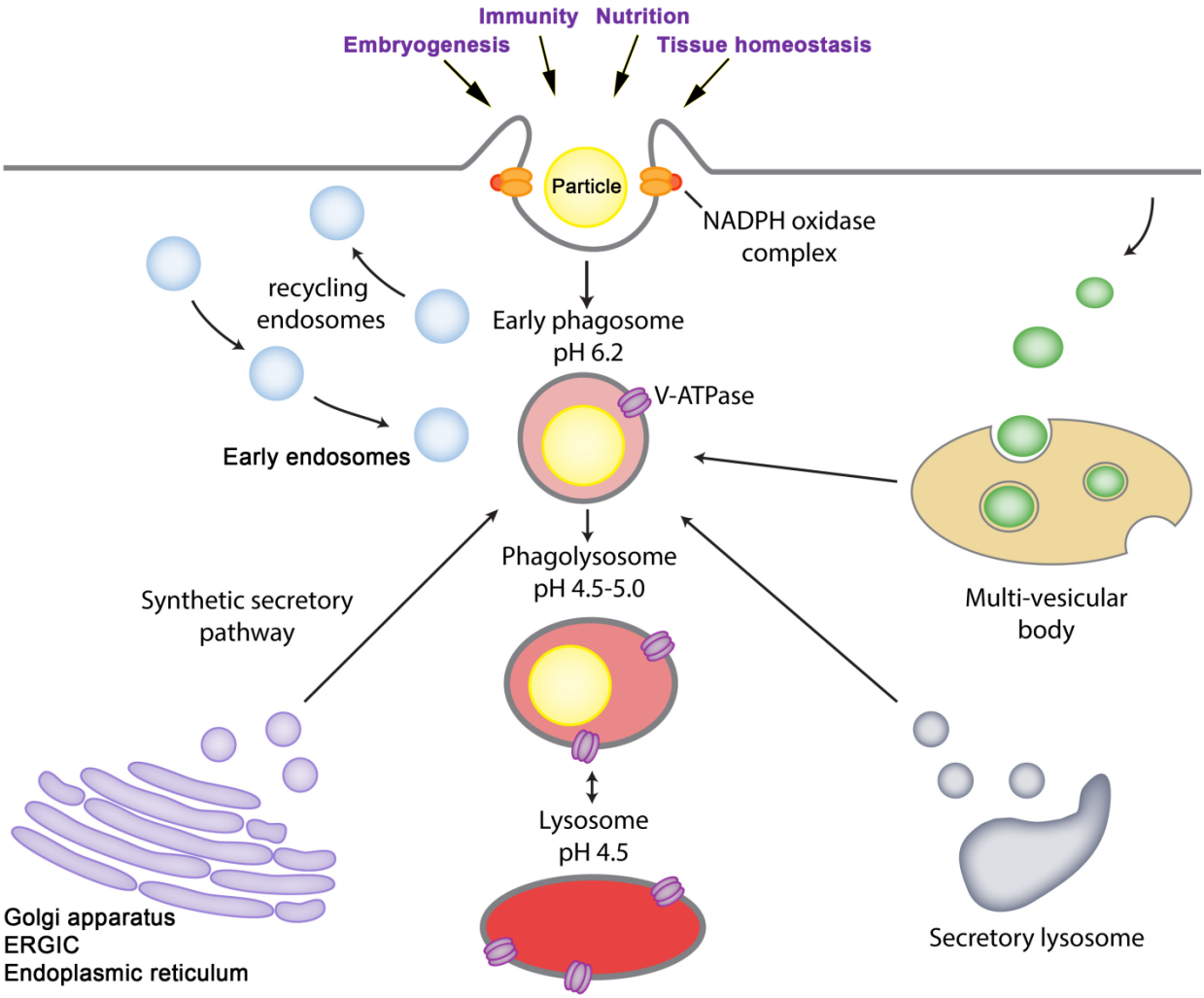


Figure 9 of literature review. Phagocytosis is a multifunctional and highly dynamic process. Metazoans have evolved the capacity to use phagocytosis in embryogenesis, immunity, and in tissue homeostasis through ingestion of apoptotic and senescent cells. Particles are internalized in a vacuolar organelle known as the phagosome. This organelle undergoes sequential and transient interactions with early endosomes, lysosomes and organelles in the secretory pathway. These interactions give rise to the phagolysosome, which has the capacity to digest its internalized cargo. Adapted with permission from (Desjardins *et al.*, 2005, Gordon, 2016).

3.1 Particle adherence and recognition

Macrophages have developed diverse receptors to recognize and internalize the large array of particles that they find in their environment (Figure 10) (Barth *et al.*, 2017, Flannagan *et al.*, 2012, Gordon, 2016). These receptors recognize molecules present exclusively on the particle, which can include pathogen molecules or epitopes that are displayed on apoptotic cells. Recognition receptors can also detect and bind to particles

that have been coated by other immune system molecules. Once these receptors are engaged, they cluster and synergize to commence a signalling program that leads to actin polymerization and phagosome formation (Flannagan *et al.*, 2012, Gordon, 2016, Levin *et al.*, 2016).

Particle identification and recognition triggers the local aggregation of internalization receptors into a phagocytic synapse (Barth *et al.*, 2017, Coppolino *et al.*, 2001, Gordon, 2016). Although receptors move laterally prior to aggregation, their aggregation is held by membrane pickets such as CD44, which constrains the synapse via formin-induced actin filaments (Freeman *et al.*, 2018). The phagocytic synapse promotes the relay of potent phagocytic signalling into the cell (Dushek *et al.*, 2012). Those signals are initiated at the receptors themselves. In the case of Fcγ receptors (FcγR), the first major step is activation of Src-family kinases such as Syk (Crowley *et al.*, 1997). These proteins then recruit adaptor proteins that activate the phosphatidylinositiol 3-kinase (PI3K), which generates phosphatidylinositol-3,4,5-*tris*phosphate ($PI(3,4,5)P_3$) from $PI(4,5)P_2$ (Flannagan *et al.*, 2012, Marshall *et al.*, 2001) (Figure 10). This leads to the recruitment and activation of GTPases such as Rac, which enable the activation of actin nucleation protein Arp2/3. The latter promotes actin polymerization and formation of the phagocytic cup (May *et al.*, 2000)

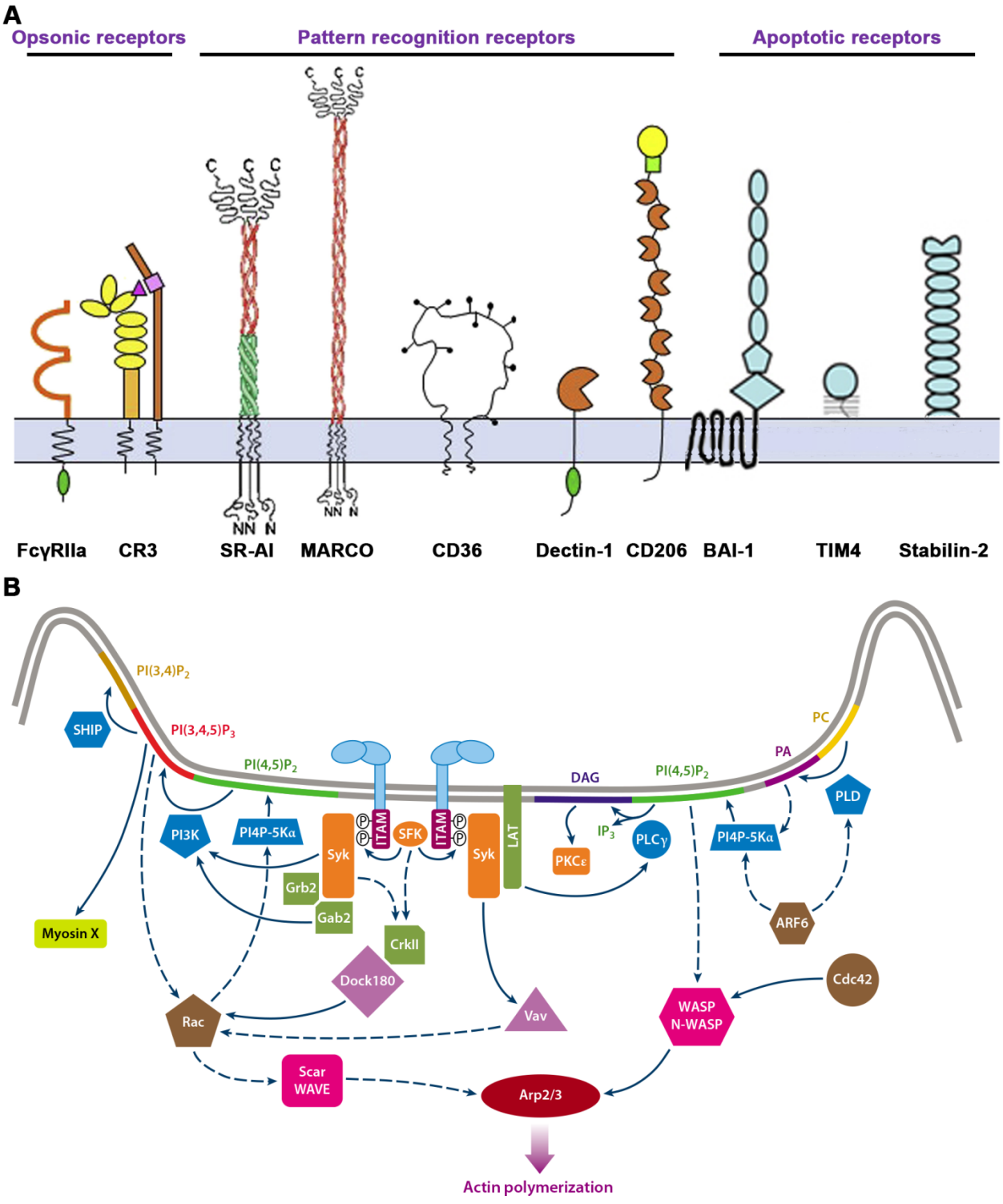


Figure 10 of literature review. Receptor-mediated recognition of phagocytic particles induces actin polymerization and phagosome formation. (A) Phagocytes decode particle diversity by using receptors that recognize PAMPs, apoptotic molecules and immune system opsonins. **(B)** Receptor engagement activates signalling pathways that induce phospholipid modifications. In the case of FcγR-mediated phagocytosis, $PI(3,4,5)P_3$ helps recruit Rac via its phospholipid-binding domain. Rac recruits and activates Arp2/3, which promotes actin nucleation and phagocytic cup formation. Adapted with permission from (Barth *et al.*, 2017, Flannagan *et al.*, 2012, Gordon, 2016).

3.1.1 Pattern recognition receptors (PRRs)

PRRs (Figure 10) recognize pathogen-associated molecular patterns (PAMPs), which are small molecules that are exclusively present on the surface of bacteria, fungi and protozoans (Gordon, 2016, Takeuchi *et al.*, 2010). PAMPs include polysaccharides present on LPS and lipoteichoic acid, and β1,3-glucan, which is present on yeast cell walls. The latter are recognized by the scavenger receptor A1 (SR-A1) and by dectin-1, respectively. Other receptors such as MARCO can bind whole bacteria and LPS, aside from other endogenous ligands (Gordon, 2016, Takeuchi *et al.*, 2010). On the other hand, mannose receptors such as CD206 can recognize glycans on the surface of microorganisms such as *Pneumocystis carinii* (Fraser *et al.*, 2000) and *Leishmania* (Wilson *et al.*, 1988), and CD36 can recognize *Plasmodium falciparum*-infected erythrocytes and oxidized lipids (Cojean *et al.*, 2008). Interestingly, expression of dectin-1, or other phagocytic receptors, on non-phagocytic cells allows them to internalize particles bearing the target PAMP (Flannagan *et al.*, 2012, Herre *et al.*, 2004). Furthermore, the ample diversity in PRR-pathogen interplay is still being discovered, and is illustrative of a continuing evolutionary battle.

3.1.2 Opsonic receptors

Opsonins are soluble molecules of the immune system that act as phagocytosis coadjuvants and that circulate in the blood, lymph and mucosa (Hiemstra *et al.*, 1998). These molecules include immunoglobulin G (IgG), which are produced by B cells and recognize foreign antigens. Components of the complement system such as C3b, iC3b and C4b are synthesized by the liver and also act as opsonins (Hiemstra *et al.*, 1998, Ross *et al.*, 1992). These molecules recognize and enrobe particles in order to flag them for internalization. Opsonins are then recognized by diverse opsonic receptors (Figure 10) found on macrophages and other phagocytes, thereby enabling indirect binding to the target particle. For example, IgGs are recognized by Fc receptors such as

FcγRIIa and FcγRIIb, and complement product iC3b is bound by complement receptors (CR) 3 and 4 (Anderson *et al.*, 1990, Ross *et al.*, 1992). As with PRRs, transfecting opsonic receptors into non-phagocytic cells endows them with the capacity to phagocytose (Anderson *et al.*, 1990, Ross *et al.*, 1992).

3.1.3 Apoptotic cell receptors

Apoptosis is a process that generates corpses that must be cleared by phagocytes. The phagocytosis of apoptotic cells leads to a non-inflammatory response that promotes immunological tolerance (Arandjelovic *et al.*, 2015, Barth *et al.*, 2017). Apoptotic cells display molecules such as oxidized lipids and high amounts of phosphatidylserine (PS), a lipid usually found on the inner leaflet of the plasmalemma. Apoptotic cell recognition is then mediated by specialized receptors (Figure 10) such as BAI-1, TIM-4, and stabilin-2 have evolved to recognize PS; moreover, CD36 has been found to recognize oxidized PS (Barth *et al.*, 2017). Absence of apoptotic receptors such as BAI-1 results in increased number of apoptotic cells, which leads to inflammation in the colon and defective muscle repair (Poon *et al.*, 2014).

3.2 Phagosome maturation

3.2.1 The early phagosome

Following particle internalization, the formed phagosome immediately goes through a series of biochemical changes where the phagosome exchanges membrane and intraluminal contents with other organelles within the cell (Desjardins, 1995, Gordon, 2016) (Figure 11). This trafficking is spatiotemporally regulated by Rab GTPases (which alter between an active GTP-bound state and an inactive GDP-bound state), and vesicle fusion molecules. Early phagosomes acquire Rab5, which is accrued from the plasma membrane and early endosomes (Chavrier *et al.*, 1990, Duclos *et al.*, 2000,

Vieira *et al.*, 2003). Rab5 is critical to phagosomal development, since its absence hinders phagosome maturation into phagolysosomes.

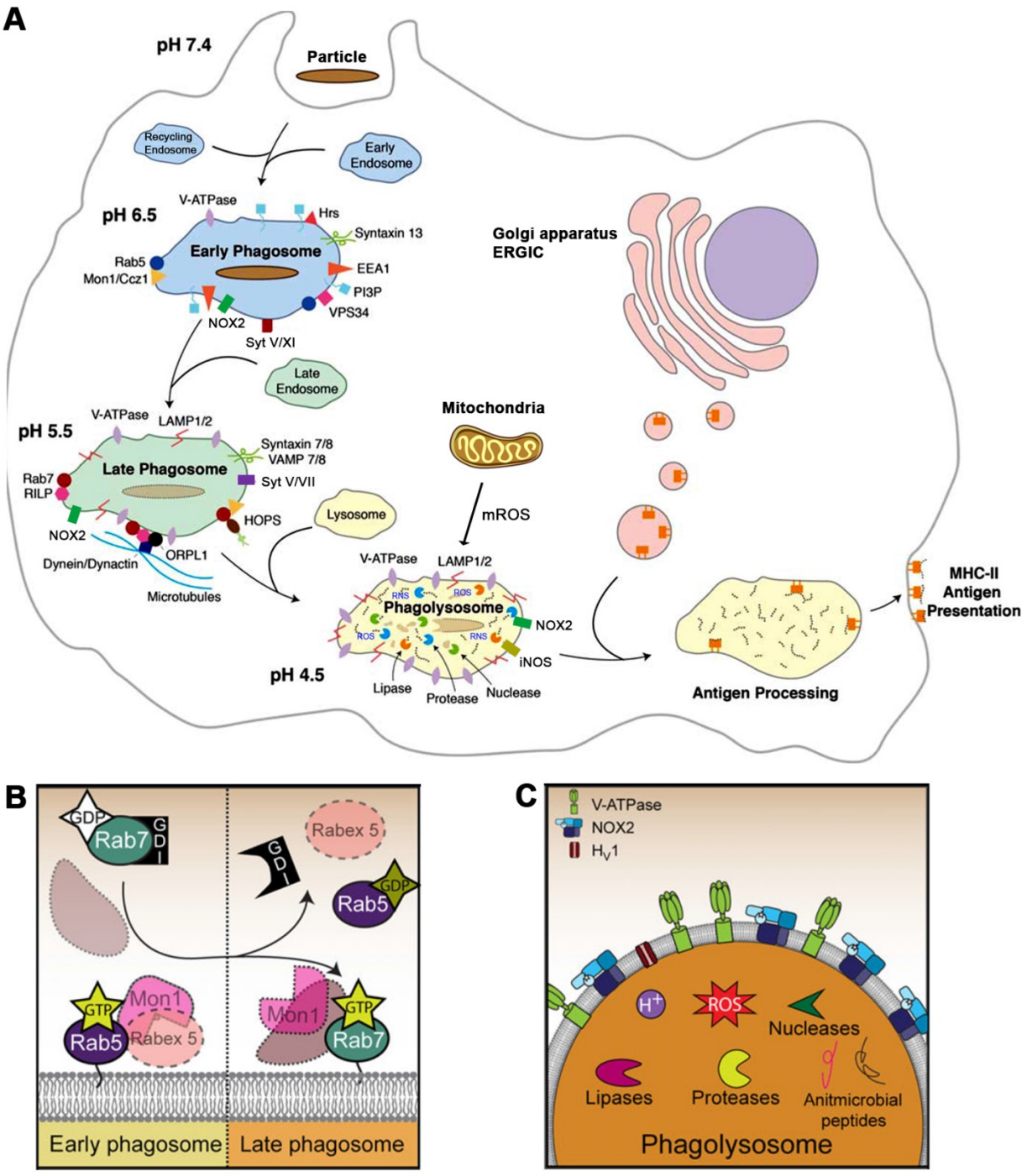


Figure 11 of literature review. The molecular machinery regulating the transition from early phagosome to phagolysosome in macrophages. (A) Internalized phagosomes accrue several effectors from early phagosomes including Rab5, Mon5 and EEA1. They also recruit proteins from recycling endosomes such as Syt V and XI, which

promote the acquisition of the V-ATPase and NOX2, respectively. The switch from early to late phagosome **(A, B)** happens when the Rab5 GTPase is exchanged to Rab7, a process that depends on Mon1 and Ccz-1. Late phagosomes are increasingly acidified by fusing with lysosomes. These organelles also endow the resulting phagolysosome **(A, C)** with the capacity to degrade lipids, proteins and nucleic acids. Of particular importance is the role of ROS (produced by NOX2 and by mitochondrial recruitment) and RNS (produced by iNOS) in killing internalized pathogens. Adapted with permission from (Levin *et al.*, 2016, Poirier *et al.*, 2015).

Conversely, expression of a GTP-locked mutant leads to enlarged phagosomes that are less microbicidal (Duclos *et al.*, 2000). Rab5 promotes the transient recruitment of the p150-Vps34 complex, which promotes formation of phosphatidylinositol 3-phosphate (PI(3)P) (Vieira *et al.*, 2001). This phospholipid helps recruit the NOX2 NADPH oxidase, the early endosomal antigen 1 (EEA1) effector, Mon1 and other proteins (Flannagan *et al.*, 2012, Levin *et al.*, 2016). EEA1 interacts with the SNARE Stx13 and promotes docking and fusion of early endosomes with phagosomes (Christoforidis *et al.*, 1999).

3.2.2 The late phagosome and phagolysosome

Early phagosomes are transient organelles that evolve into more acidic organelles (pH 5.5-6.0) that are termed late phagosomes. Acidification is mediated by increased recruitment of the vacuolar ATPase (V-ATPase), which transports H^+ into the phagosomal lumen in an ATP-dependent fashion (Cotter *et al.*, 2015, Flannagan *et al.*, 2012). In fact, recruitment of the V-ATPase starts in early phagosomes and is modulated by Syt V, a membrane fusion regulator present in recycling endosomes and lysosomes (Vinet *et al.*, 2008). Knockdown of Syt V by RNA interference (RNAi) decreases the macrophage's phagocytic capacity, especially under conditions of high membrane demand (Vinet *et al.*, 2008, Vinet *et al.*, 2011). Importantly, decreased Syt V expression inhibits phagosomal accrual of the V-ATPase and of the cathepsin D hydrolase. Syt V-deficient phagosomes fail to acidify, implying that their microbicidal activity is also reduced (Vinet *et al.*, 2009).

The crucial element in the early-to-late phagosome transition is the switch in GTPase effectors that takes place. Namely, Rab7 is recruited to late phagosomes with a concomitant loss of Rab5 (Levin *et al.*, 2016, Poteryaev *et al.*, 2010, Vieira *et al.*, 2001)

(Figure 11). This exchange is mediated by the early phagosome-associated protein Mon1, which recruits Ccz-1 to the phagosome membrane. Ccz-1 acts by displacing Rab5 from the early phagosome and by acting as a tether for GDP-bound Rab7 (Poteryaev *et al.*, 2010). The homotypic fusion and protein sorting complex (HOPS) mediates the switch from a GDP- to a GTP-bound state, allowing Rab7 to exert its effector functions. Rab7 activates the oxysterol-binding protein-related protein 1 (ORPL1), which mediates microtubule-dependent interactions between the late phagosome and the dynein/dynactin molecular motors (Johansson *et al.*, 2005). This allows fusion with lysosomes, hence giving origin to the phagolysosome. Fusion with lysosomes is a Ca^{2+}-dependent event mediated by the Stx7–VAMP7–Syt VII complex (Czibener *et al.*, 2006, Ward *et al.*, 2000). Interaction with lysosomes promotes further acidification (pH 4.5-5.0) of the phagosome and promotes the accrual of the lysosome-associated membrane proteins (LAMP-) 1 and 2, which are not only important for lysosomal integrity, but also for the acquisition of microbicidal properties (Huynh *et al.*, 2007a, Huynh *et al.*, 2007b).

3.2.3 The killing machinery of the phagolysosome

The phagolysosome is a highly degradative organelle that is particularly adept at killing microbes (Figure 11). The lysosome contributes hydrolytic enzymes that degrade lipids, carbohydrates and proteins (Flannagan *et al.*, 2009, Flannagan *et al.*, 2012). Indeed, the acidic environment inside the phagolysosome has important consequences for the activity of these enzymes, with cathepsin peptidases being particularly more active in low pH (Flannagan *et al.*, 2012, Turk *et al.*, 2001). The phagolysosome also contains microbicidal cationic lipids and channels that purge the phagolysosomal lumen of metal ions (Flannagan *et al.*, 2012). Importantly, the increased $[H^+]$ in the phagolysosome stimulates the activity of the natural resistance-associated macrophage protein 1 (NRAMP1), which extrudes Zn^{2+} and Mn^{2+} from the phagolysosome (Jabado *et al.*, 2000, Searle *et al.*, 1998). This ouster of vital mineral nutrients was found to fetter the growth of bacteria and parasites such as *Leishmania* (Gruenheid *et al.*, 1997, Searle *et al.*, 1998, Vidal *et al.*, 1995).

The phagolysosomal lumen also contains a high concentration of ROS and reactive nitrogen species (RNS) that synergize to damage the nucleic acids and proteins of ingested microorganisms (Bogdan, Flannagan *et al.*, 2012). The phagolysosomal membrane is an assembly site for the NOX2 NADPH oxidase complex, which is crucial for the killing of intracellular pathogens. The NADPH oxidase complex is normally inactive in the cell, and assembles on the cell membrane and on the phagosome membrane (Shatwell *et al.*, 1996). NOX2 assembly and activity starts in early phagosomes and continues throughout phagocytosis. The importance of this complex is illustrated by the fact that humans with a defective oxidase suffer from chronic granulomatous disease, a disease characterized by recurrent life-threatening infections (Dinauer *et al.*, 1992, Shatwell *et al.*, 1996). The complex is constituted by six subunits. These subunits are a Rho guanosine triphosphate (GTPase), usually Rac1 or Rac 2 and five phagocytic oxidase (*phox*) subunits. These *phox* subunits are the membrane-bound flavocytochrome b_{558}, which is made up of $gp91^{phox}$ and $p22^{phox}$, and the cytoplasmic proteins $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$. Phosphorylation of $p47^{phox}$ by PKC is required for interaction with $p67^{phox}$/$p40^{phox}$ heterodimers (Brown *et al.*, 2003, Perisic *et al.*, 2004); the oxidase is activated when a heterotrimer of $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ is recruited to the membrane-associated flavocytochrome b_{558} (Nauseef, 2004). NOX2 assembly starts at the nascent phagosome at the plasma membrane (Dingjan *et al.*, 2017), with Rab11-associated recycling endosomes mediating the trafficking of the b_{558} subunit from the site of synthesis in the ER to the cell surface (Casbon *et al.*, 2009) where phagosomes are born and acquire b_{558} (Dingjan *et al.*, 2017). Subunit $gp91^{phox}$ is also delivered through to phagosomes in a fusion process that depends on the concerted action of SNAREs SNAP23 (Sakurai *et al.*, 2012), VAMP8 (Matheoud *et al.*, 2013) and Stx7 (Dingjan *et al.*, 2017). If the delivery of $gp91^{phox}$-positive recycling endosomes to phagosomes is regulated by Syt XI, then it could contribute to the antibacterial capacity of the phagosome. The NOX2 complex transports electrons across the phagosome membrane to form O_2^-, which can be transformed into H_2O_2. Reaction of that molecule with Cl^- gives rise to the highly cytotoxic HClO. In addition to NOX2 at the phagolysosome, mitochondria also contribute ROS (mROS) to the

phagolysosome (West *et al.*, 2011). Engagement of TLR1, 2 and 4 mediates mitochondrial recruitment to the periphery of the phagosome through translocation of the tumour necrosis factor receptor-associated factor 6 (TRAF6) from the ER to the mitochondria (West *et al.*, 2011).

The inducible nitric oxide synthase 2 (iNOS) is responsible for the formation of RNS in the phagosome (Bogdan, 2015). Indeed, iNOS and RNS participate in the killing of bacteria and *Leishmania* parasites (Bogdan, Qadoumi *et al.*, 2002). Generation of ROS precedes that of RNS, since induction of iNOS requires engagement of TLRs followed by the proinflammatory program that ensues thereafter. Although iNOS assembles as a homodimer that transforms L-arginine to NO• radicals (Tzeng *et al.*, 1995), not much is known about its trafficking to the phagolysosome. Other NOSs are active in the Golgi (Fulton *et al.*, 2002), which in theory could deliver NOS-positive vesicles to the phagosome.

3.3 Roles of the ER/ERGIC and the SNARE Sec22b on phagosome biology

The membrane transactions that occur in the secretory pathway are critical for the post-translational processing and delivery of proteins in the cell. Immune cells are particularly dependent on this pathway for the processing and release of inflammatory mediators (Murray *et al.*, 2014, Stow *et al.*, 2006). Phagosome biogenesis requires an ample supply of membrane that originates from multiple organelles in the cell (Braun *et al.*, 2006, Dingjan *et al.*, 2018, Huynh *et al.*, 2007b). The dynamic nature of ER-Golgi membrane trafficking raised the interesting possibility that these organelles might be involved in the biogenesis and maturation of phagosomes (Gagnon *et al.*, 2002). Indeed, Gagnon and colleagues found that the ER has a direct interaction of membrane and ER proteins to the phagosome. The phenomenon was observed on phagosomes containing inert beads and intracellular microbes *Salmonella* and *Leishmania* (Gagnon *et al.*, 2002). Using immunogold labelling and lysates from isolated phagosomes, they

found that phagosomes displayed the ER marker calnexin as well as the typical lysosomal marker LAMP-1 (Huynh *et al.*, 2007a, Huynh *et al.*, 2007b). Recruitment of the ER to the phagosome was found to be dependent on PI3K and on the V-ATPase (Gagnon *et al.*, 2002). These findings were vociferously contested by other laboratories claiming that the plasma membrane was the main membrane contributor to nascent phagosomes (Gagnon *et al.*, 2005, Touret *et al.*, 2005). However, knockdown of the v-SNARE VAMP3, which regulates recycling endosome fusion to the phagosome, showed that phagocytosis still occurred (Allen *et al.*, 2002). This supported the possibility that the ER, being one of the largest organelles within cell, exported membrane for phagosome formation (Becker *et al.*, 2005). Accumulating evidence points to a model where many cell organelles, including the ER and the ERGIC, contribute membrane and effector proteins during phagosome biogenesis and maturation (Figures 9 and 11). To gain a global overview of all phagosomal proteins, Garin and colleagues used proteomics to find that the phagosome was enriched in ER chaperones and other proteins that could confer an antigen-processing capacity to the phagosome (Brunet *et al.*, 2003, Garin *et al.*, 2001). It was found that the phagosome used many of those proteins to process exogenous antigens for subsequent cross-presentation on MHC class I molecules (Desjardins *et al.*, 2005, Houde *et al.*, 2003).

These findings had raised the question of what membrane fusion proteins regulate ER-phagosome traffic. Sec22b acts as a v- and t-SNARE that resides and traffics in the ER-ERGIC-Golgi circuit (Figures 4, 6 and 12) (Cebrian *et al.*, 2011, Dingjan *et al.*, 2018, Malsam *et al.*, 2011). Becker and colleagues found that Sec22b regulated the delivery of ER membrane to large phagosomes, raising the possibility that it might act as a v-SNARE during phagocytosis. They also found that the Golgi was not recruited to the phagosome (Becker *et al.*, 2005). From the involvement of Sec22b in phagocytosis (Becker *et al.*, 2005, Hatsuzawa *et al.*, 2009), it was inferred that this SNARE also played a role in phagosome maturation and function. Using immunofluorescence and immunoprecipitation, Cebrian and colleagues expanded upon these findings by discovering that Sec22b, a resident of the ER and the ERGIC, was recruited to

phagosomes. There, Sec22b was found to interact with the t-SNARE Stx4 (Cebrian *et al.*, 2011).

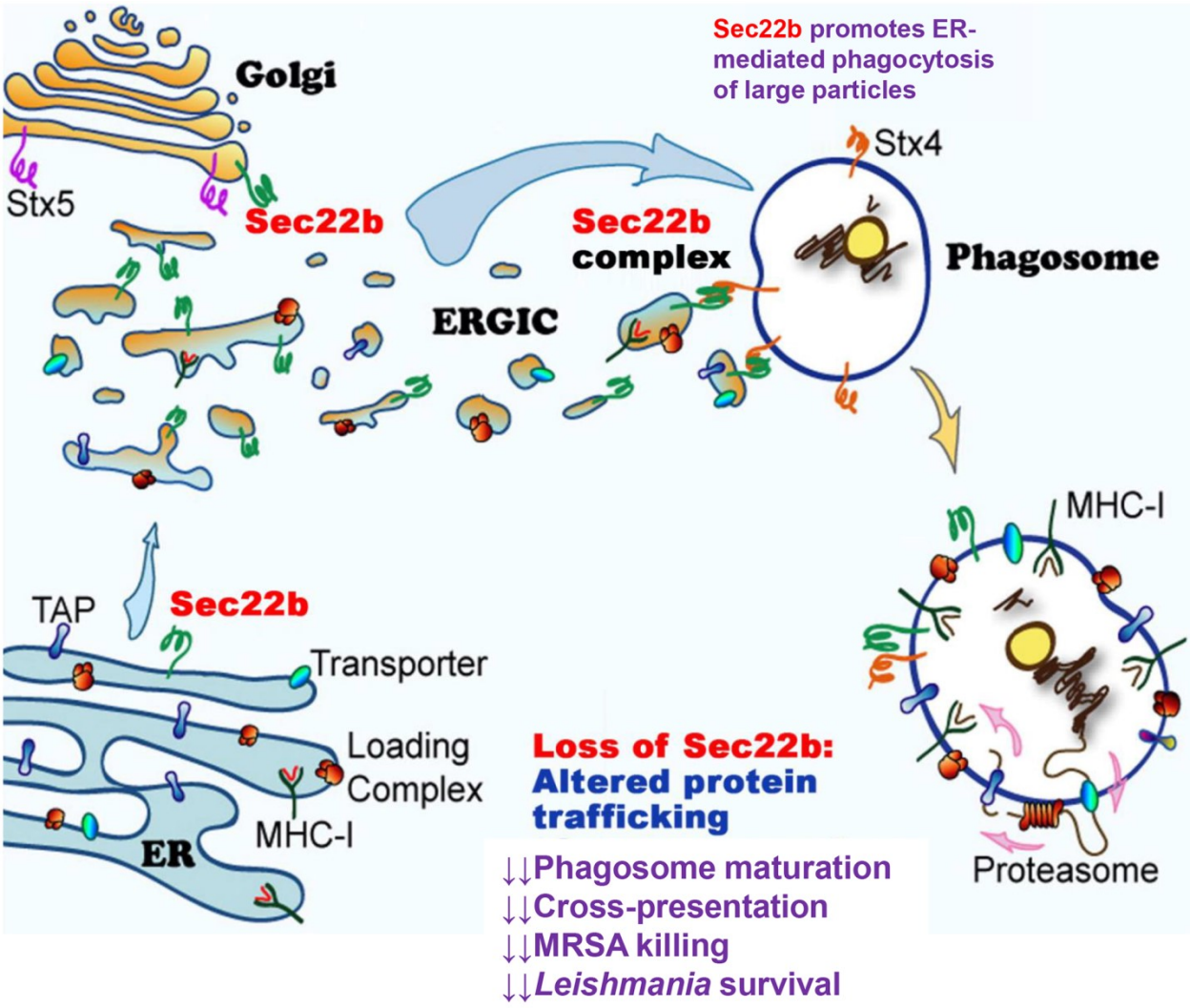


Figure 12 of literature review. The ER/ERGIC-resident SNARE Sec22b modulates phagosomal biogenesis and function. Phagosome biogenesis is a process that requires the accrual of endosomal and ER/ERGIC membranes. The SNARE Sec22b, which resides in these organelles, control the recruitment of membranes and effector proteins to the phagosome. Absence of Sec22b from phagosomes results in immature phagosomes with decreased antimicrobial capacity. Adapted with permission from (Cebrian *et al.*, 2011).

Phagosome maturation in dendritic cells and macrophages differs in that dendritic cell phagosomes acidify to a higher pH compared to that of macrophage and neutrophil phagosomes (Blander, 2018). A higher pH is required for antigen cross-presentation, since a lower pH would lead to augmented antigen degradation by intraphagosomal

cathepsins (Blander, 2018). Importantly, the SNARE VAMP8 was found to regulate the recruitment of NOX2 to the phagosome (Matheoud *et al.*, 2013). NOX2 acidifies the phagosome by importing protons into the phagosomal lumen when it generates ROS. Hence, decreased levels of VAMP8 ensue in a lower pH and in decreased antigen cross-presentation (Matheoud *et al.*, 2013). Cebrian *et al.* and others used Sec22b knockdown (KD) dendritic cells to show that absence of Sec22b also drastically inhibited antigen cross-presentation (Cebrian *et al.*, 2011, Nair-Gupta *et al.*, 2014). They found that Sec22b modulates the recruitment of lysosomes, and lysosomal cathepsins to the phagosome. In dendritic cells, this is particularly important since accelerated lysosomal recruitment would lead to decreased antigen presentation. Sec22b KD indeed inhibited the accrual of ER/ERGIC proteins that is required for the cross-presentation of antigens coming from pathogens such as *Toxoplasma* (Cebrian *et al.*, 2011). The *in vivo* importance of Sec22b on this pivotal phagosome function was brought to the fore by Alloati and colleagues (Alloatti *et al.*, 2017). Using mouse DC-specific knockouts of Sec22b, they discovered that this SNARE was important for antigen cross-presentation and immunity against melanoma antigens. Mice lacking Sec22b in their DCs also died faster when afflicted by melanoma (Alloatti *et al.*, 2017).

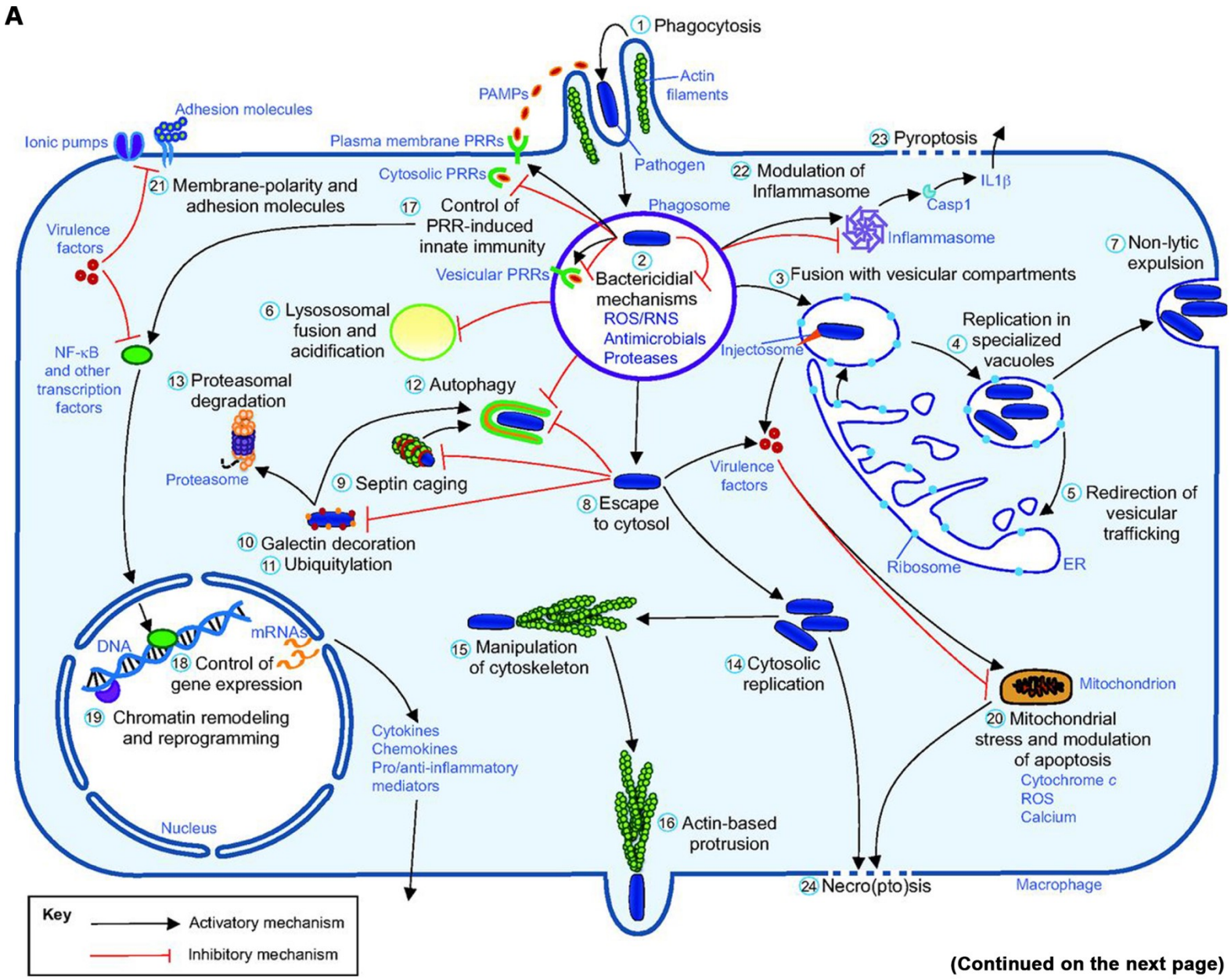
The involvement of Sec22b in phagosome biology (Figure 12) also highlights the possibility that it may regulate the microbicidal activity of the phagosome. To demonstrate this, Abuaita and colleagues knocked down Sec22b and its cognate t-SNARES Stx4 and 5 (Abuaita *et al.*, 2015). Using bacterial killing assays, they observed that a KD of those SNAREs led to decreased killing of methicillin-resistant *Staphylococcus aureus* (MRSA). In addition, they found that Sec22b was needed for the sustained generation of ROS in phagosomes harbouring MRSA (Abuaita *et al.*, 2015). In stark contrast to MRSA, Sec22b and its cognate SNAREs promote the survival of the *Leishmania* parasite in infected macrophages (Canton *et al.*, 2012a) [see section ‘5’ of this Chapter]. This finding points to the prospect that ER/ERGIC-derived vesicles may control the intracellular survival of certain pathogens.

3.4 Phagocytosis by neutrophils

Neutrophils possess powerful microbicidal mechanisms for the elimination of microorganisms, which makes them essential to the early immune response against pathogens. Neutrophils are recruited to the site of infection via chemical effectors released by resident macrophages and mast cells (Nathan, 2002, Nordenfelt *et al.*, 2011). Once at the site of infection, neutrophils unleash their arsenal of microbicidal mechanisms by phagocytosing and killing invading microorganisms. In contrast to the endosomal pathway-dependent phagocytic process of macrophages, phagocytosis in neutrophils proceeds by the fusion of distinct sets of cytoplasmic granules with the maturing phagosome (Faurschou *et al.*, 2003, Nordenfelt *et al.*, 2011). Azurophilic granules contain myeloperoxidase, elastase and other proteases; these granules are recruited to nascent and already-formed phagosomes. Specific granules, which contain NADPH oxidase components and lactoferrin, are also recruited to neutrophil phagosomes and are crucial in the killing of pathogens (Faurschou *et al.*, 2003, Nordenfelt *et al.*, 2011). Furthermore, neutrophils secrete cytokines that mediate the development of adaptive immunity. Once neutrophils are no longer needed, they senesce, undergo apoptosis and are rapidly phagocytosed by macrophages (Savill, 1994, Savill *et al.*, 2002).

3.5 How pathogens evade killing by the phagolysosome

Although phagocytosis evolved as a defense mechanism against infectious diseases, it is also the process by which many facultative intracellular pathogens evade the immune response to ensure their survival (Alix *et al.*, 2011, Poirier *et al.*, 2015, Weber *et al.*, 2018). The phagosome offers membrane-bound seclusion from immune system effectors such as antibodies and complement molecules. In order to avoid the lethal maelstrom that is the phagosomal lumen, a plethora of microbes have devised numerous cunning stratagems to thrive therein (Figure 13) (Alix *et al.*, 2011, Poirier *et al.*, 2015, Weber *et al.*, 2018).



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B

Biological event targeted	Pathogen	Bacterial effector	Host target
Endosomal trafficking	*Mycobacterium tuberculosis*	ManLAM	Reduces intracellular Ca^{2+} concentration
		SapM	Hydrolyzes PI3P into PI
	Legionella pneumophila	VipD	Interacts with GTP-Rab5 and GTP-Rab22a
		VipA	Interacts with EEA1 and SNAREs
		SopB	
	Salmonella enterica	SopE	Recruits Rab5
			Recruits and activates Rab5
Phagosome and lysosome fusion		SifA	
		SipC	
	S. enterica	SopB	Uncouples Rab7 from RILP
			Inactivates Hook3
		PtpA	Hydrolyzes $PI(4,5)P_2$ into PI5P, reducing the recruitment of Rab8, Rab13, Rab23, and Rab35
		EsxG/H	
	M. tuberculosis		
		EspB	Rab13, Rab23, and Rab35
			Dephosphorylates VPS33B
		Cord factor	Form a complex that targets Hrs, a component of the ESCRT machinery.
		Unknown	Inhibits phagolysosome fusion when cosecreted with ESAT-6 and CFP-10
Phagosome acidification	*Yersinia pestis*	PtpA	Creates a steric block to fusion and/or increases the hydration force between two phospholipid bilayers
	M. tuberculosis	SidK	
	L. pneumophila	Unknown	Resides and replicates in a phagolysosome-like vacuole
	Yersinia pseudotuberculosis	SseB/C/D	Binds subunit H of V-ATPase and prevents assembly of the proton pump
	S. enterica		
Cytoskeleton Remodeling		SipA	Binds subunit A of V-ATPaseand inhibits ATP hydrolysis and proton translocation
	S. enterica	SipC	
		SseI	Decreases the activity of the V-ATPase pump
		SspH2	Forms a complex that helps in the translocation of T3SS effectors across the bacterial membrane
		SpvB	
		SopB	Catalyzes actin polymerization and bundling of actin filaments
		PipB2	Stabilizes SifA via its actin modification effects
		SifA	Bundles and nucleates actin filaments
		SseJ	Interacts with filamin A and promotes cross-linking of F-actin by filamin A
		SseF/G	Interacts with filamin A and promotes cross-linking of F-actin by filamin A
		VipA	Interacts with profilin-1 and prevents the interaction of profilin-1 with G-actin
		Unknown	
	L. pneumophila	ActA	Depolymerizes and disrupts the actin cytoskeleton by modifying G-actin
	Mycobacterium marinum	IcsA	
	Listeria monocytogenes	Unknown	Indirectly recruits SNX3, which forms tubules for the movement of the phagosome to the perinuclear region
	Shigella flexneri	IpgD	
		IpaB	Interacts with kinesin-1 and forms Sifs
Vacuolar Membrane Lysis	*M. marinum*	IpaC	Interacts with SKIP, forms Sifs, promotes phagosomal tubulation, and uncouples Rab7 from RILP
	S. flexneri		
		IpaH7.8	
		LLO	Rab7 from RILP
		PI/PC-PLC	Interacts with SKIP and GTPase RhoA and promotes phagosomal tubulation

Figure 13 of literature review. Intracellular bacteria have evolved diverse strategies to attenuate the phagosome's microbicidal power. (A) Vacuolar pathogens can circumvent every intricacy of the phagocytic process. From preventing fusion with the host cell's endomembrane system to detoxifying the phagosomal lumen **(B)**, every step can be targeted by a pathogen-derived effector molecule. Adapted with permission from (Poirier *et al.*, 2015, Torraca *et al.*, 2014).

These diverse strategies reflect the intense evolutionary pressure on pathogens to adapt and conquer their host. To avoid intraphagosomal killing, the most obvious strategy is to avoid phagocytosis. Bacteria that produce polysaccharide capsules, such as *Streptococcus pneumoniae* and *Haemophilus influenza,* can resist phagocytosis, thus making them more virulent than strains that do not encapsulate (Cress *et al.*, 2014). Fortunately, the host organism can secrete specific antibodies against these capsules; this targets the bacterium for phagocytosis.

Internalized pathogens have evolved many protein effectors to target every step of the phagosome maturation pathway (Figure 13). For instance, *Mycobacterium tuberculosis* expresses proteins that inhibit phagosome maturation and detoxify intraphagosomal ROS and RNS (Poirier *et al.*, 2015, Torraca *et al.*, 2014). The mycobacterial molecule ManLAM is released into the cytoplasm of the infected macrophage; this inhibits the recruitment of Rab5 onto the phagosome, hence arresting its development at the early stage (Chatterjee *et al.*, 1998). Preventing intraphagosomal acidification is also key in avoiding the phagosome's hydrolytic enzymes. To this end, bacteria such as *M. tuberculosis* and *Legionella pneumophila* employ effectors PtpA and SidK, respectively, to inhibit the assembly of the V-ATPase complex (Xu *et al.*, 2010, Zhou *et al.*, 2015). Other pathogens produce effectors to directly increase intraphagosomal pH. In the case of *H. pylori*, ureases and VacA quench acidification. This bacterium also induces homotypic phagosome fusion, which induces the formation of megasomes that harbor many bacteria (Allen, 2007). Other pathogens use their effector molecules to break away from the phagosome into the host cell cytoplasm, thereby preventing any interaction with the lysosome (Alix *et al.*, 2011, Poirier *et al.*, 2015). For instance, *Listeria* produces listeriolysin O and various phospholipases that allow it to destabilize and break down the phagosomal membrane (Portnoy *et al.*, 1988).

4 CYTOKINE SECRETION BY MACROPHAGES

4.1 Introduction to cytokine biology

The development of an effective immune response depends on the highly regulated crosstalk that occurs among immune and non-immune cells. The complex interactions among these cells are mediated by a series of low molecular weight (~5-20 kDa) secreted proteins that are collectively known as cytokines (Dinarello, 2007, Nathan, 2002). These small proteins function as chemical messengers that regulate the intensity and duration of the immune response. Cytokines regulate the growth, development, metabolism, maturation, activation and lifespan of several cells (Dinarello, 2007, Nathan, 2002). Cytokines are secreted in response to an exogenous or endogenous stimulus, and act on target cells that express the plasmalemmal receptor for that cytokine (Dinarello, 2007). Multiple cytokines can signal through different receptors, a phenomenon that conveys and evokes distinct responses from their target cells (Duitman *et al.*, 2011, Owen *et al.*, 2013). In fact, there are over 50 cytokines and a similar number of cytokine receptors. Cytokines are subdivided into structural subfamilies that encompass interferons, tumour necrosis factors, interleukins and hematopoietins (Figure 14) (Duitman *et al.*, 2011, Owen *et al.*, 2013).

The binding of a cytokine to its membrane receptor relays a signal to the cell that leads to changes in gene expression (Dinarello, 2007, Owen *et al.*, 2013). In addition, soluble cytokine receptors have been detected in the serum and their function is to sequester their target cytokine to inhibit its function (Duitman *et al.*, 2011). Cytokines can act on many different cellular targets and their action can be autocrine, meaning that the cytokine acts on the same cell that secreted it. Cytokines can also act in a paracrine fashion to modulate the biology of nearby cells. As with hormones, cytokines can function in an endocrine fashion to influence the function of distant cells. In addition, the biological impact of cytokines can be pleiotropic, meaning that a single cytokine may have varied effects that depend on the target cell and signalling receptor. The action of these proteins may also be redundant, as groups of cytokines can exert identical

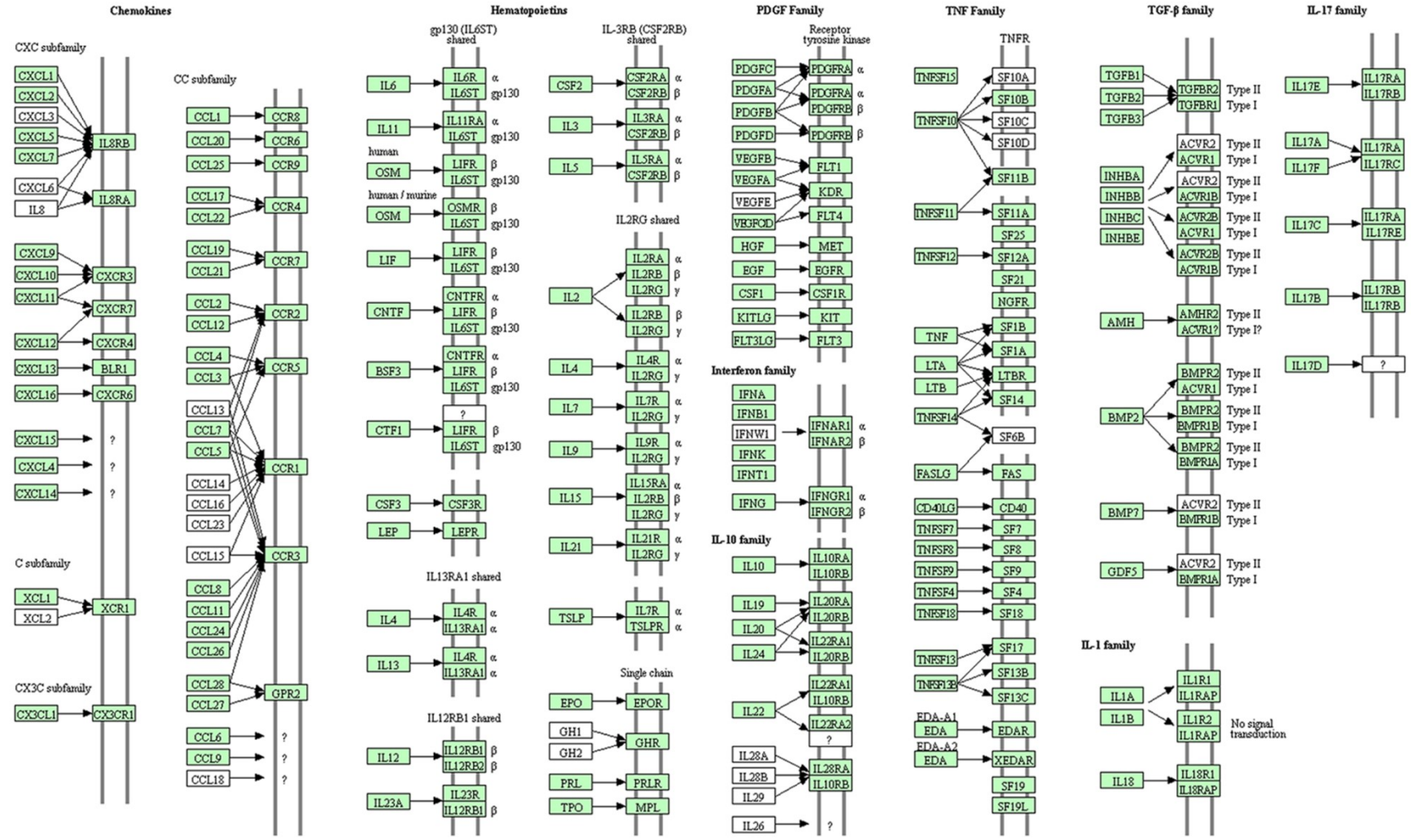
biological outcomes and even signal through the same receptors (Dinarello, 2007, Owen *et al.*, 2013). As an example, IL-4 and IL-13 are both strong inducers of TH2 immunity. Cytokine action can be synergistic, since many cytokines act in conjunction to augment their intended response. Cytokines can also antagonize each other. For instance, IL-1 and IL-10 are proinflammatory and anti-inflammatory, respectively (Stenvinkel *et al.*, 2005). From a functional point of view, cytokines can be grouped as mediators of innate and adaptive immunity, chemotactic agents and as modulators of hematopoiesis (Figure 14) (Schmitz *et al.*, 2011).

4.1.1 Cytokines involved in innate and adaptive immunity

The main cytokines that intervene in the innate response are IL-1, IL-6, IL-12, IL-16, TNF and type I (-α, -β) and II (-γ) IFNs. These cytokines are mainly produced by macrophages, NK cells, and by other non-immune cells such as fibroblasts and endothelial cells (Greenberg *et al.*, 2002, Owen *et al.*, 2013). They play a fundamental role in immunity by inducing the killing of intracellular parasites and viruses. This is achieved by phagocytes such as macrophages, whose killing capacities are increased by T cell-derived cytokines such as IFN-γ (Cybulsky *et al.*, 2016, Goldsby *et al.*, 2002, Huber *et al.*, 1981). These cytokines can also induce the release of proinflammatory cytokines TNF, IL-6 and IL-12, which contribute to the development of fever and to the activation of T cells into TH1 cells (Dorman *et al.*, 2000, Hurst *et al.*, 2001, Schmitz *et al.*, 2011).

The transition from innate to adaptive immunity is mediated by a series of cytokines that mediate the differential activation of resting T and B cells. Lymphocytes are differentiated into two TH1 and TH2 subpopulations depending on which cytokines they receive from neighbouring phagocytes and lymphocytes (Duitman *et al.*, 2011, Nathan, 2002, Stow *et al.*, 2009). Receipt of IL-4 and IL-5 induces differentiation into TH2 cells that in turn secrete immunoregulatory cytokines TGF-β and IL-12 (Croxford *et al.*, 2014, Travis *et al.*, 2014).

A



(Continued on the next page)

B

functional class	major physiological or pathophysiological effects	examples
cytokines regulating adaptive immune functions		
lymphocyte growth and differentiation factors	clonal expansion of T-cells, ↑ Th1/TH2/TH17 responses, Th1/Th2/Th17 polarization, B-cell activation, 8-cell growth, auto-immune responses	IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, IL-15, IL-17, IL-18, IL-23, IL-25, IL-33, IL-35, IFNγ
cytokines positively regulating innate immune functions		
pro-inflammatory cytokines	↑ inflammatory mediators,↑ innate immune responses, ↑ activation of most cell types, pro metastatic,↑ bone-resorption, ↑ acute phase proteins	IL-1 α, IL-1 β, TNFα, IL-6, IL-12, IL-18, IL-23, IL-32, IL-33, IL-34, IL-36, MIF, CD40L, RANKL
chemokines	↑ cellular emigration, ↑ leucoyte infiltration, ↑ neovascularization, ↑ activation of many cell types, pro-metastatic	IL-8 (CXCL8), MCP1 (CCL2), MIP1α (CCL3), Rantes (CCL5), Groα/β/γ (CXCL1,2,3), MIF, others
interferons		
type II IFN	macrophage activation, ↑ MHC class II	IFNγ
type I IFN	anti-viral, ↑ MHC class I, anti-inflammatory, anti-angiogenic	IFNα, IFNβ
cytokines downregulating innate immune functions		
anti-inflammtory cytokines	↓ inflammatory mediators, ↓ cytokine-mediated lethality, ↓ autoimmune and autoinflammatory disease, ↑ fibrosis, anti-tumor effects	IL-10, IL-13, TGFβ, IL-22, IL-37 IL-1 Ra, IFNa/β

Figure 14 of literature review. Cytokines are pivotal regulators of intercellular communication and function. Cytokine function is dictated by the receptors to which they bind. The map in **(A)** groups cytokines by structural subfamily, and was generated with the KEGG website (http://www.genome.jp/kegg/kegg2.html, pathway mmu04060). **(B)** The immune response is attuned by a portfolio of cytokines that regulate processes ranging from macrophage activation to cell migration. Panel (B) was adapted with permission from (Schmitz *et al.*, 2011).

Moreover, cytokines such as IFN-γ inhibit the conversion to and proliferation of TH2 cells, while IL-4 and IL-10 inhibit the proliferation of TH1 cells (Oswald *et al.*, 1992). Activation of a B lymphocyte at rest requires antigen binding to the B cell receptor (Shukla *et al.*, 2015). This recognition amplifies the expression of cytokine receptors for IL-1, which is produced by macrophages, and for IL-4 and IL-5, which are mainly produced by TH2 cells (Owen *et al.*, 2013, Schmitz *et al.*, 2011). Cytokines also dictate the type of immunoglobulin secreted by B cells. For instance, activated B cells release IgE in response to IL-4 (Shukla *et al.*, 2015).

4.1.2 Chemokines: cytokines that induce cell migration

Chemokines are a family of structurally related and very low molecular weight cytokines. They are produced by different immune and non-immune cells that have a strong migratory capacity (Figure 14) (Dinarello, 2007, Owen *et al.*, 2013). These proteins are subdivided into the C, CC, CXC and CX3C by virtue of disulfide bridges that link the β-sheets within their structure (Addison *et al.*, 2000, Gijsbers *et al.*, 2004, Maghazachi *et*

al., 1996). These subfamilies also have specific target cells, with CC chemokines such as RANTES acting on monocytes and CXC chemokines such as IL-8 acting on neutrophils. Chemokines control the migration of cells in the development of organs and lymphoid tissues. Additionally, chemokines promote or hinder the development of blood vessels. During an immune response, chemokines that are secreted in response to an inflammatory stimulus direct the migration of myeloid and lymphoid cells to the site of injury. (Keeley *et al.*, 2008). Importantly, chemokine production is usually induced by cytokines that are produced at earlier stages of the immune response. TNF, which is promptly released upon encounter with an inflammatory stimulus, induces the production of neutrophil-recruiting chemokines CXCL1 and CXCL5 (Griffin *et al.*, 2012, Vieira *et al.*, 2009).

4.1.3 Hematopoietins: cytokines that modulate immune cell development

Hematopoietins play a very important role in the stimulation of bone marrow development into functional immune cells (Owen *et al.*, 2013, Varzaneh *et al.*, 2014). These molecules act on the immature populations, promoting their maturation and proliferation. They include molecules such as the stem cell factor, IL-3 and IL-7. A medically important hematopoietin is the granulocyte colony stimulator factor (G-CSF), which is given to immunosuppressed patients in order to stimulate the production of neutrophils (Mehta *et al.*, 2015).

4.2 Macrophage cytokines

In addition to their phagocytic ability, macrophages direct a large portion of their membrane traffic towards processing and secreting cytokines (Huynh *et al.*, 2007b, Murray *et al.*, 2014, Stow *et al.*, 2009). When a proinflammatory ligand binds to a macrophage receptor, the macrophage secretes a wave of proinflammatory cytokines in response to the stimulus. These cytokines are produced in a rapid fashion, implying that a large portion of intracellular trafficking in macrophages is directed towards the release

of these molecules. Following a proinflammatory stimulus, the first batch of cytokines to be released includes TNF, IL-1β and IL-6; they exert a synergistic role in starting an effective immune response (Carmi *et al.*, 2009, Lee *et al.*, 2004, Stenvinkel *et al.*, 2005). They increase vascular permeability, which facilitates the recruitment and entry of lymphoid and myeloid cells to the inflammation site (Keeley *et al.*, 2008). These cytokines also modulate the function of their neighboring cells. For instance, the action of TNF and IL-6 on endothelial cells makes them release the chemokines CXCL2 and CCL2, which mediate the recruitment of neutrophils and monocytes, respectively (Griffin *et al.*, 2012, Vieira *et al.*, 2009). Albeit the fact that proinflammatory cytokines promote the effective clearance of an offending endogenous or exogenous stimulus, the response is self-contained and highly controlled. Sixteen to 24h after the start of the stimulus, these cytokines also stimulate the production and release of anti-inflammatory cytokines such as IL-10 and TGF-β (Cunha *et al.*, 1992, Defrance *et al.*, 1992, Fiorentino *et al.*, 1991). In macrophages, those cytokines act in an autocrine and paracrine fashion to promote their own production, and to reduce the secretion of proinflammatory effectors. They also act in an endocrine fashion to alleviate inflammation. Macrophages are exposed to a wide variety of stimuli that range from bacterial compounds to apoptotic cells and extracellular parasites (Ginhoux *et al.*, 2014, Hamrick *et al.*, 2000, Huber *et al.*, 1981, Levin *et al.*, 2016, Owen *et al.*, 2013). The nature of the compound or particle deeply influences the development of the macrophage. Inflammatory stimuli bias macrophage development towards the M1 subtype (Figure 15) (Biswas *et al.*, 2010, Lu *et al.*, 2013).

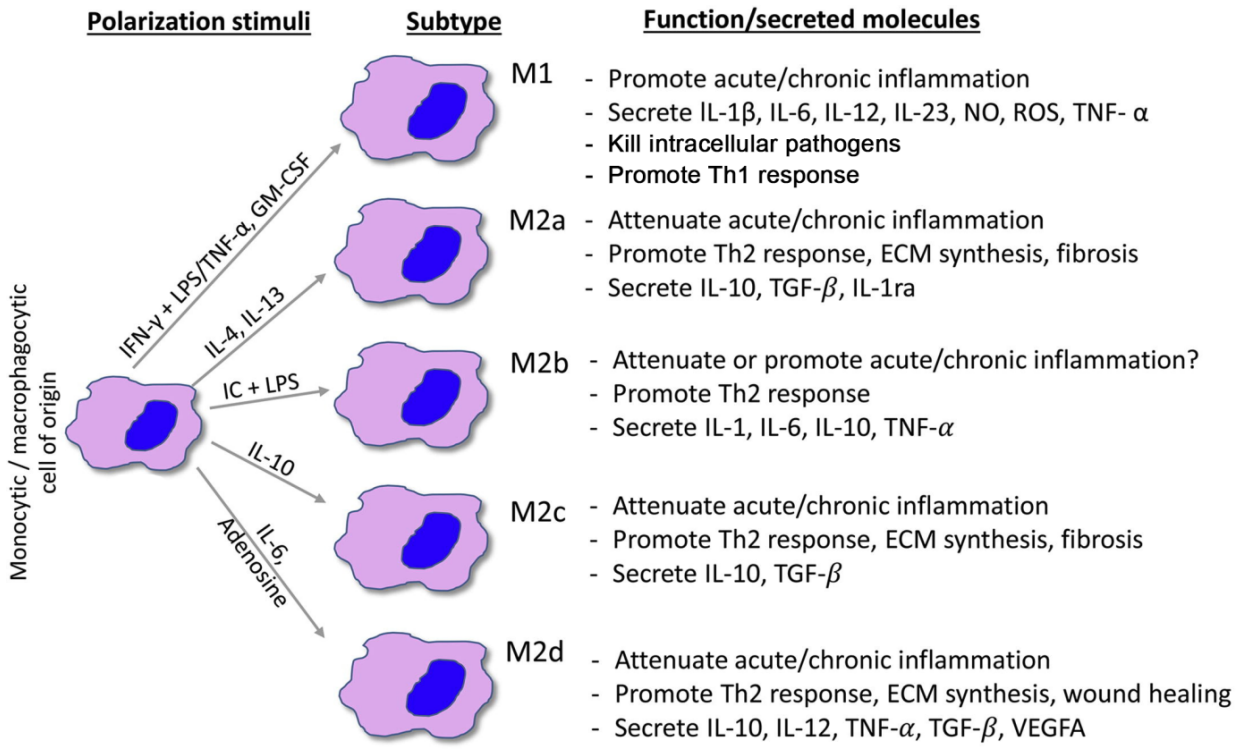


Figure 15 of literature review. The phenotypic plasticity of macrophages dictates the type of cytokines that they secrete. Macrophages are under constant exposure to environmental signals such as lymphocyte-derived cytokines and dead cells. These stimuli induce monocyte development into M1 or M2 macrophages. These macrophages undergo a metabolic rewiring that allows them to secrete cytokines that specialize in promoting (M1) or mitigating (M2) inflammation. Adapted with permission from (Klopfleisch, 2016).

M1 macrophages kill intracellular pathogens and secrete cytokines that favour a proinflammatory phenotype, such as the induction of TH1 T cells. In contrast, apoptotic cells and anti-inflammatory cytokines induce the development of M2 macrophages, which promote wound healing and tissue repair and the induction of TH2 T cells (Biswas *et al.*, 2010, Lu *et al.*, 2013). These phenotypes are reversible; for instance, exposure of M2 macrophages to IFN-γ and TNF can induce them to switch to the M1 phenotype (Biswas *et al.*, 2010, Klopfleisch, 2016). Diversity in the microenvironment has led scientists to discover additional M2 macrophage subtypes that specialize in mediating processes such as extracellular matrix formation and wound healing (M2a,c,d). The M2b and M2d subtypes secrete proinflammatory and anti-inflammatory cytokines, and their role is to control acute and chronic inflammation (Biswas *et al.*, 2010, Klopfleisch, 2016).

4.3 Vesicle fusion proteins that regulate cytokine secretion in macrophages

Macrophages respond to stimulatory signals with a cytokine response that is coordinated at the transcriptional, translational and post-translational levels. Failure in this regulation contributes to pathologies involving excessive inflammation, or results in a response that fails to clear the offending agent (Beutler, 1999, Duitman *et al.*, 2011, Nathan, 2002). In contrast to granulocytic cells of the immune system such as mast cells and neutrophils, cytokine secretion by macrophages occurs mostly via the constitutive pathway (Duitman *et al.*, 2011, Stow *et al.*, 2013). Following activation by a microbial molecule such as LPS, proinflammatory cytokine synthesis and secretion occurs rapidly and in a continuous manner. For this to occur, several proteins of the membrane trafficking machinery must also be upregulated. As a matter of fact, LPS stimulation leads to the upregulation of SNAREs Stx6, Stx7 and Vti1b at the Golgi; VAMP3 at recycling endosomes; and Stx4 and SNAP23 at the plasmalemma (Murray *et al.*, 2005a, Murray *et al.*, 2005b, Stanley *et al.*, 2012). In addition to SNAREs, several Rabs and PI3Ks are involved in regulating the passage of cytokines through the secretory pathway (Murray *et al.*, 2014). The development of fluorescence tags and imaging techniques has spurred research into how macrophage cytokines transit from their port of synthesis to the extracellular milieu.

4.3.1 The trafficking and release of model cytokines TNF, IL-6 and IL-10

The importance of the Golgi in membrane trafficking is exemplified by studies where chemical or genetic perturbation of Golgi function blocks protein secretion to the outside of the cell (Misumi *et al.*, 1986, Novick *et al.*, 1980, Novick *et al.*, 1979). This blockage is often accompanied by an accumulation of proteins in the Golgi. In LPS-stimulated macrophages, the cytokines TNF, IL-6 and IL-10 have been observed to colocalize with Golgi components such as p230, which associates with these cytokines in vesicles that bud from the TGN (Murray *et al.*, 2014). In order to unravel the molecular machinery involved in this process, Murray and colleagues employed a microarray approach to find

which genes were upregulated upon LPS stimulation of macrophages (Murray *et al.*, 2005b). Examination of the data oriented them to focus on the SNAREs Stx6 and Vti1b, which were found to colocalize and cofractionate with Golgi markers and TNF. Immunoprecipitation (IP) experiments revealed that the SNARE Vti1b interacted with Q-SNAREs Stx6 and Stx7, which raised the importance of this complex in cytokine release (Murray *et al.*, 2005b). Interestingly, overexpression of these TGN SNAREs potentiates TNF release.

REs are important membrane compartments that recycle proteins between plasma membrane and the TGN (Hsu *et al.*, 2010, Murray *et al.*, 2005a). In many secretory cells, they also act as sorting stations where molecules are redistributed to cellular compartments. REs have been found to colocalize with diverse vesicle coat proteins and Rabs, which speaks for the multifunctional nature of these sorting compartments in the cell (Hsu *et al.*, 2010, Manderson *et al.*, 2007, Murray *et al.*, 2005a). If recycling endosomes are chemically inactivated, the release of TNF, IL-6 and IL-10 is hampered (Murray *et al.*, 2005a). Rab11 serves as an LPS-inducible marker of REs, and its knockdown also lowers cytokine secretion (Murray *et al.*, 2005a). Interestingly, Murray and colleagues found that the LPS-inducible SNARE VAMP3 colocalized with REs and formed a complex with the Golgi Q-SNAREs Stx6, and Vti1b (Murray *et al.*, 2005a). Whereas overexpression of VAMP3 ensues in augmented cytokine secretion, its knockdown leads to decreased transport to the plasmalemma. In conjunction with these data, live cell imaging was used to track cytokine release. It was found that cytokine-containing vesicles migrated from the perinuclear compartment to plasma membrane microdomains that bear the SNAREs Stx4 and SNAP23 (Pagan *et al.*, 2003). Inactivation of Stx4 function also disrupts cytokine secretion, hence demonstrating the importance of that SNARE in RE-mediated cytokine delivery (Manderson *et al.*, 2007, Murray *et al.*, 2005a, Pagan *et al.*, 2003).

A key feature of membrane trafficking in macrophages is its efficiency and promptness. Aside from delivering cytokines to the plasma membrane, REs were shown to fuse with nascent phagosomes (Murray *et al.*, 2005a). Since phagosomes require large amounts

of membrane to engulf particles, REs supply part of that demand while concomitantly delivering cytokines (Huynh *et al.*, 2007b, Murray *et al.*, 2005a, Murray *et al.*, 2014). Murray and colleagues showed that TNF is, to this date, the only cytokine delivered at the phagocytic cup (Manderson *et al.*, 2007, Murray *et al.*, 2005a, Stanley *et al.*, 2012). Since Stx4 is involved in phagosome formation (Dingjan *et al.*, 2018), it may serve as a t-SNARE that docks TNF-containing vesicles to the nascent phagosome. In contrast, IL-6 and IL-10 are carried by REs to the plasma membrane (Manderson *et al.*, 2007) and it was later demonstrated that IL-10 can traffic directly from the TGN to the plasmalemma (Murray *et al.*, 2014, Stanley *et al.*, 2012). The passage of TNF from REs to the plasma membrane is also regulated by SNARE-interacting proteins such as SCAMP5 (Han *et al.*, 2009). Han and colleagues found that silencing of SCAMP5 resulted in decreased secretion of TNF and the chemokine CCL5. SCAMP5 also interacts with Syt I and II (Han *et al.*, 2009), and albeit the fact that the roles of those Syts in macrophages are not known, SCAMP5 may be involved in regulating Syt-mediated vesicle fusion at the plasma membrane.

The roles of Syts in cytokine secretion have only started to be elucidated. Since Syt XI is associated with REs and other organelles, it was necessary to address whether this Syt regulated cytokine secretion and phagocytosis. Prior to its functional characterization, Syt XI was hypothesized to inhibit exocytic processes in macrophages and other cells.

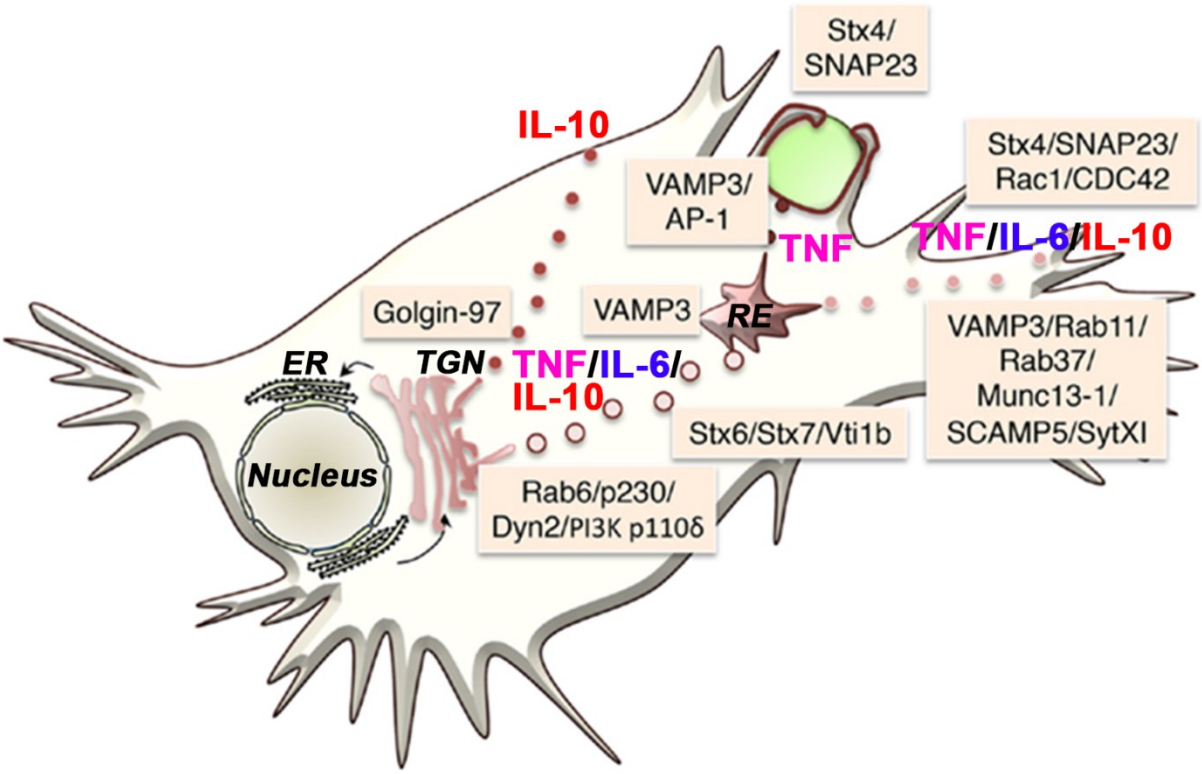


Figure 16 of literature review. Membrane trafficking pathways and molecules involved in the release of TNF, IL-6 and IL-10 in macrophages. Macrophage cytokine secretion is largely dependent on trafficking from the TGN to REs that ferry these cytokines to the phagocytic cup or the plasma membrane (TNF and IL-6). Direct delivery from the TGN to the plasma membrane also occurs (IL-10). Adapted with permission from (Murray *et al.*, 2014).

5 THE *LEISHMANIA* PARASITE AND ITS INTERACTION WITH THE HOST MACROPHAGE

5.1 Introduction and epidemiology

Protozoans of the *Leishmania* genus cause the leishamaniases, a group of anthropozoonoses of great medical and veterinary importance that are transmitted by hematophagous sandflies (Hartley *et al.*, 2014, Killick-Kendrick *et al.*, 1981, WHO, 2018). Depending on the species and geographical context, the leishmaniases manifest themselves as cutaneous lesions, or as debilitating mucocutaneous lesions that leave life-long scars. Leishmaniasis may also affect the viscera and is fatal if untreated (Figure 17) (Alvar *et al.*, 2012, Hartley *et al.*, 2014, WHO, 2018). *Leishmania* parasites belong to the Kinetoplastida order and Trypanosomatidae family, which can be subdivided into two subgenres, *Viannia* and *Leishmania* (Figure 17). This subgenre division is related to parasite development in the insect vector. In the *Leishmania* subgenus, the promastigote form of the parasite colonizes the anterior, middle and posterior intestine, while in the subgenus *Viannia*, only the anterior and middle intestine are colonized (Sacks *et al.*, 2001b).

The leishmaniases have a wide geographic distribution, being endemic in five continents and 98 countries (Alvar *et al.*, 2012, WHO, 2018). There are ~20 reported *Leishmania* species in the world. Nonetheless, only 12 species, including those of the *Viannia* subgenre, exist in the Americas (Figure 17). Furthermore, the leishmaniases are neglected diseases that primarily affect the poorest regions of developing countries. According to the World Health Organization (WHO), it is estimated that >1 billion people are at risk of infection in the world, with 650000-2 million new cases occurring per annum (WHO, 2018). Recent epidemiological studies estimate that 50000-90000 new cases of visceral leishmaniasis occur annually, whereas 600000-1 million new cutaneous leishmaniasis cases occur. Only three countries account for over 90% of mucocutaneous leishmaniasis cases, while 10 countries account for 75% of cutaneous

leishmaniasis. On the other hand, 90% of cases of visceral leishmaniasis are concentrated mainly in India, Bangladesh, Ethiopia, Kenya, Sudan and Brazil (Alvar *et al.*, 2012, WHO, 2018). Although cutaneous and mucocutaneous leishmaniasis make up most cases of leishmaniasis in the Americas, visceral leishmaniasis affects humans and animals, and has a very high fatality rate (Alvar *et al.*, 2012, WHO, 2018).

Treatment of the leishmaniases is dependent on the clinical manifestation, and on local expertise regarding treatment availability, administration, local guidelines and drug resistance (Alvar *et al.*, 2012, WHO, 2018, Zulfiqar *et al.*, 2017). Modes of treatment include chemotherapy, surgery, thermotherapy, cryotherapy and electrotherapy. Unfortunately, no vaccine is available. Some of the most widely used pharmaceuticals include pentavalent antimonials such as sodium stabigluconate and meglumine antimoniate. These drugs are highly toxic and difficult to administer; this has led to a surge in drug resistance (WHO, 2018). Repurposing of the antifungal drug amphotericin B has been used with success for the treatment of antimony-resistant visceral leishmaniasis. Other drugs such as miltefosine, paramomycin, and fluconazole have been used with mixed success. The worldwide incidence of drug resistance has spurred many international initiatives to develop new medicines that are effective, accessible and easy to administer (WHO, 2018, Zulfiqar *et al.*, 2017). Detailed knowledge of the host-parasite interphase combined with molecular modeling and bioinformatics is necessary to guide current and future efforts in the fight against this disease. Since the leishmaniases primarily occur in highly impoverished areas of the world, they are not of great interest to the pharmaceutical industry due to the low income of the customer base (WHO, 2018). In addition, the study of these diseases is negatively affected by the insufficient funding received from research and development agencies. To end this paragraph on a brighter note, someone who studies the *Leishmania* parasite is affectively called a ‘leishmaniac’.

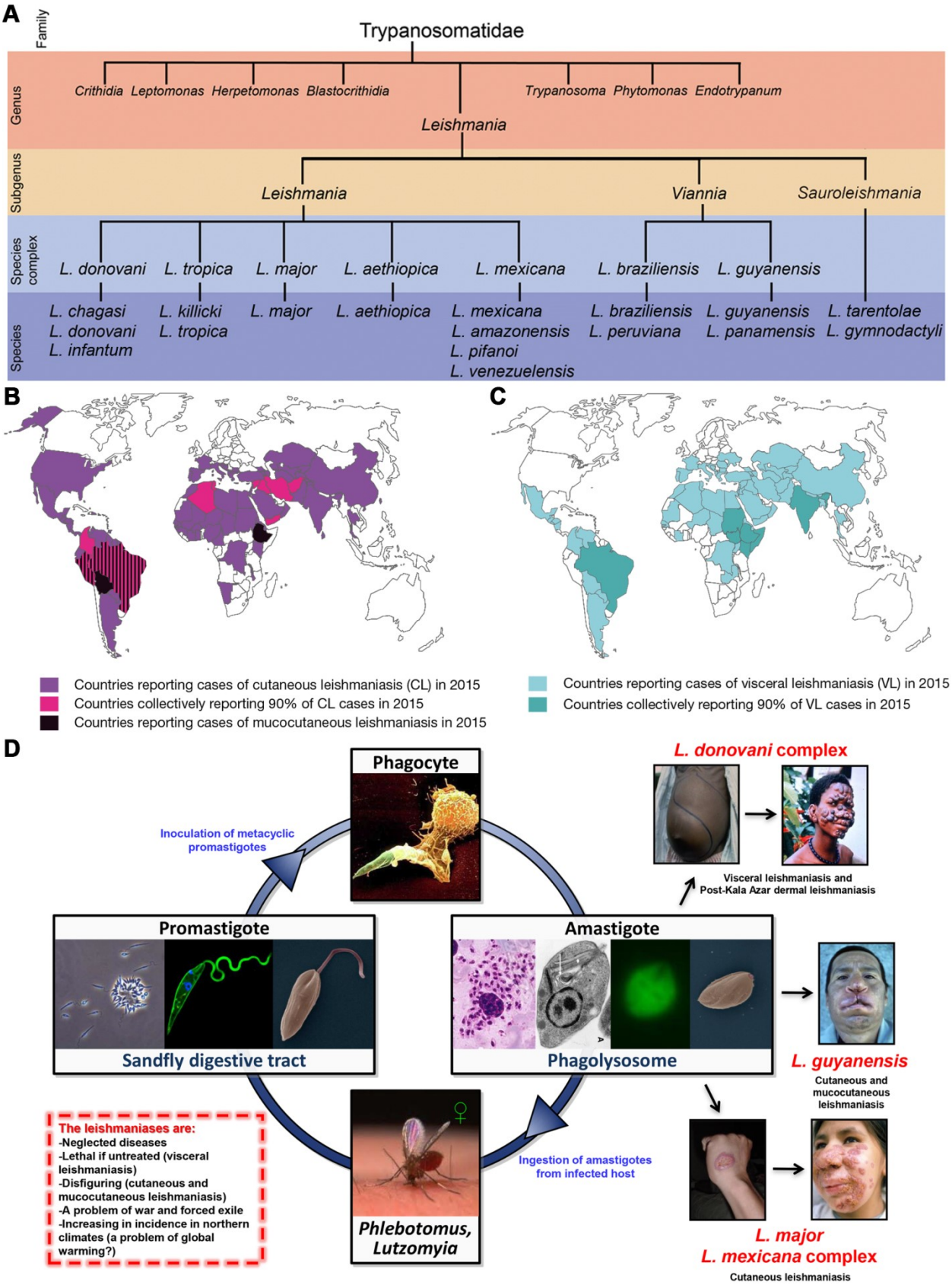

A
Family
Trypanosomatidae
Genus
Crithidia Leptomonas Herpetomonas Blastocrithidia
Trypanosoma Phytomonas Endotrypanum
Leishmania
Subgenus
Leishmania
Viannia
Sauroleishmania
Species complex
L. donovani
L. tropica
L. major
L. aethiopica
L. mexicana
L. braziliensis
L. guyanensis
Species
L. chagasi
L. donovani
L. infantum
L. killicki
L. tropica
L. major
L. aethiopica
L. mexicana
L. amazonensis
L. pifanoi
L. venezuelensis
L. braziliensis
L. peruviana
L. guyanensis
L. panamensis
L. tarentolae
L. gymnodactyli
B
C
Countries reporting cases of cutaneous leishmaniasis (CL) in 2015
Countries collectively reporting 90% of CL cases in 2015
Countries reporting cases of mucocutaneous leishmaniasis in 2015
Countries reporting cases of visceral leishmaniasis (VL) in 2015
Countries collectively reporting 90% of VL cases in 2015
D
Phagocyte
Inoculation of metacyclic promastigotes
Promastigote
Sandfly digestive tract
Amastigote
Phagolysosome
Ingestion of amastigotes from infected host
Phlebotomus, Lutzomyia
L. donovani complex
Visceral leishmaniasis and Post-Kala Azar dermal leishmaniasis
L. guyanensis
Cutaneous and mucocutaneous leishmaniasis
L. major
L. mexicana complex
Cutaneous leishmaniasis
The leishmaniases are:
-Neglected diseases
-Lethal if untreated (visceral leishmaniasis)
-Disfiguring (cutaneous and mucocutaneous leishmaniasis)
-A problem of war and forced exile
-Increasing in incidence in northern climates (a problem of global warming?)

Figure 17 of literature review. Overview of the *Leishmania* parasite and the leishmaniases. (A) Taxonomical classification of *Leishmania* protozoa. This family tree shows fifteen medically important species that cause disease in humans and animals. The latest WHO maps that illustrate the distribution of cutaneous & mucocutaneous **(B)**, and visceral leishmaniasis **(C)** in the world. Note that these diseases affect most countries in the tropical and neotropical belts, placing >1 billion people at risk of contracting the parasite. **(D)** Life cycle of the *Leishmania* parasite. Infected female sandflies transmit metacyclic promastigotes to humans and animals. In the host, promastigotes transform into the non-flagellated amastigotes within PVs. These amastigotes propagate and disseminate, which eventually leads to the diverse species-dependent pathologies that comprise the leishmaniases. When an uninfected sandfly ingests blood containing amastigotes, the life cycle is renewed. Panel (A) was adapted with permission from (Real *et al.*, 2013, WHO, 2018). Photo credits in panels (B-D): H. I. Al-Mohammed *et al.* (Al-Mohammed *et al.*, 2005), J. Berger, P&R Fotos, E. Myburgh, R. Wheeler, WHO/TDR/El-Hassan.

5.2 The *Leishmania* life cycle

Leishmania parasites are transmitted to vertebrate hosts via the bite of female phlebotomine sandflies (Figure 17) (Killick-Kendrick *et al.*, 1981). These insects inoculate virulent metacyclic promastigotes into the vertebrate host. Promastigotes are phagocytosed into parasitophorous vacuoles (PV) by the host's phagocytes, primarily by tissue macrophages. Within the host, temperature and intraphagosomal acidification are thought to promote the differentiation of promastigotes into the ovoid and non-flagellated amastigote forms (Sacks *et al.*, 2001b, Zilberstein *et al.*, 1994). Amastigotes reproduce via binary fission until the host cell bursts. With the death of the cell, numerous amastigotes are released and phagocytosed by other macrophages, thence allowing the parasite to disseminate. More recently, Real and colleagues found that intercellular amastigote transmission occurs when uninfected macrophages internalize infected apoptotic macrophages (Real *et al.*, 2014). This mode of infection also induces the release of anti-inflammatory cytokines TGF-β and IL-10, which promote intracellular survival (Belkaid *et al.*, 2001).

The *Leishmania* life cycle is perpetuated when an uninfected phlebotome takes a blood meal from an infected vertebrate host. This blood may contain either infected macrophages or free amastigotes. During the passage through the digestive tract of the sandfly host, macrophages rupture and release amastigotes. The phlebotome's intestine, which is a fibrous network composed of chitin and several proteins, provides a lower temperature and neutral pH. Those signals induce the differentiation of

amastigotes into procyclic promastigotes, which multiply intensely and develop into metacyclic promastigotes (McConville *et al.*, 1992, Sacks, 1989). The process of transformation into metacyclic promastigotes is known as metacyclogenesis. Importantly, metacyclic promastigotes are the infectious form of the *Leishmania* parasite that initiates infection in vertebrate hosts. Morphologically, they are smaller, more slender and have a longer flagellum when compared to procyclic promastigotes. Metacyclics are also non-dividing and are highly mobile. During a blood meal, metacyclic promastigotes migrate into the sandfly's esophagus and pharynx, thence lodging in the proboscis where they block further blood intake. A contraction of the proboscis muscle causes sandflies to regurgitate newly ingested blood containing metacyclic promastigotes (Kamhawi, 2006, Sacks *et al.*, 2001b).

5.3 How *Leishmania* settles in the host macrophage

5.3.1 Life in the vertebrate host after inoculation

Metacyclic promastigotes are accompanied by vector- and parasite-derived effectors that are co-inoculated into the host (Atayde *et al.*, 2016, Rogers *et al.*, 2003). Those molecules play a pivotal role in the establishment of infection. Sandfly saliva contains molecules that exert an immunomodulatory role on the host. For example, the Maxadilan peptide from *Lutzomia* sandflies promotes vasodilation and the establishment of infection (Rogers *et al.*, 2003). *Leishmania* also secretes microvesicles (MV), which are small extracellular vesicles that contain parasite molecules ranging from RNA to proteins (Silverman *et al.*, 2011). The sandfly's inoculate, which contains *Leishmania*-derived MVs, induces IL-17-mediated inflammation at the bite site (Atayde *et al.*, 2015). The immunomodulatory properties of sandfly saliva provoke local inflammation and the recruitment of immune cells to the infection site (Atayde *et al.*, 2016). Paradoxically, it is those very same cells that are supposed to eliminate the parasite. Similar to other intracellular parasites, *Leishmania* has evolved a complex strategy to hijack phagocytes in order to avoid the immune response to ensure reproductive success (Sibley, 2011).

5.3.2 *Leishmania* entry into host macrophages

As discussed, phagocytosis employs a panoply of receptors that mediate particle uptake (Flannagan *et al.*, 2012, Levin *et al.*, 2016). Promastigote internalization involves the ligation of receptors on the phagocyte's surface with intrinsic ligands, opsonins and immunoglobulins on the parasite's surface. *Leishmania* employs the highly abundant glycoconjugates lipophosphoglycan (LPG) and the GP63 metalloprotease to bind to complement proteins C3b and iC3b, as well as galectins and the mannose binding protein (Blackwell, 1985, Puentes *et al.*, 1988). On the macrophage surface, complement receptors (CR) 1 and 3 mediate binding to complement units (Elhay *et al.*, 1990, Mosser *et al.*, 1993). The mannose-fucose receptor and p150,95 also mediate binding with promastigotes, though the function of those receptors has not been elucidated (Blackwell, 1985). Albeit required for parasite survival, LPG is dispensable for binding and internalization by macrophages. Importantly, binding of parasites to the aforementioned receptors avoids macrophage activation (Kane *et al.*, 2000).

The opsonization status of the promastigote dictates how it is internalized into the macrophage. The Rho family of guanosine triphosphatases controls the process of internalization by regulating actin cytoskeleton dynamics (Chimini *et al.*, 2000). Non-opsonized *L. donovani* promastigotes, which bind to FcγR and CR3 (Mac-1), recruit Rac1 and elicit formation of Cdc42-containig pseudopods that help to internalize the promastigote (Coppolino *et al.*, 2001, Lodge *et al.*, 2005). Recruitment of the Wiscott-Aldrich syndrome protein (WASP) to the forming PV depends on the interaction of WASP with GTP-bound Cdc42, which activates the Arp2/3 complex and promotes polymerization of periphagosomal F-actin (Coppolino *et al.*, 2001). In contrast, opsonized *L. donovani* promastigotes bind to CR3 and entry is mediated by RhoA, Rho kinase and myosin II, leading to Arp2/3 polymerization of F-actin. Opsonized promastigotes do not induce the formation of the big pseudopods that form around non-opsonized promastigotes. Since circulating promastigotes are likely to be opsonized,

the dominant mode of internalization is biased towards being RhoA-dependent (Lodge *et al.*, 2005). Promastigotes also use caveolae to gain entry into macrophages (Rodriguez *et al.*, 2006). Experiments with virulent opsonized and non-opsonized *L. infantum chagasi* promastigotes demonstrated that parasites localize to caveolae and follow an internalization pathway that delays phagolysosome formation and promotes parasite survival (Rodriguez *et al.*, 2006). Caveolin-1 is recruited persistently to PVs even 24h post-infection, something that is not observed with phagosomes containing latex beads or non-virulent promastigotes. Parasitophorous vacuoles containing virulent promastigotes also recruit Rab5 and EEA1 transiently, as expected for caveolae-mediated entry (Rodriguez *et al.*, 2006). Intact lipid microdomains are required for internalization and survival of virulent *L. infantum chagasi* promastigotes. Interestingly, disruption of caveolae with the methyl-*β*-cyclodextrin (M*β*CD) detergent inhibits phagocytosis of opsonized and non-opsonized promastigotes. Moreover, caveolae disruption avoids recruitment of caveolin-1 and results in augmented fusion of lysosomal compartments with PVs. Hence, entry into caveolae may be essential for routing the PV to a pathway that promotes parasite viability (Rodriguez *et al.*, 2011, Rodriguez *et al.*, 2006). Promastigote phagocytosis is also dependent on the growth phase of the parasite (Ueno *et al.*, 2009). Promastigotes in logarithmic (log) and metacyclic phase bind to CR3 on the macrophage's surface. However, promastigotes in log phase also bind to mannose receptors whereas metacyclic promastigotes do not. Entry of metacyclic promastigotes is marked by persistent association of caveolin-1, which also colocalizes with CR3. While metacyclic promastigotes delay the recruitment of LAMP-1 and retard phagolysosome biogenesis, PVs containing log promastigotes recruit LAMP-1 rapidly. Metacyclic promastigotes eventually survive and replicate efficiently in macrophages. Hence, the entry of virulent metacyclic promastigotes is mediated by CR3 in caveolae, which ensues in a PV that upholds parasite proliferation.

In contrast to promastigotes, amastigotes enter macrophages in a Rac1/Arf6-dependent manner. Expression of inactive versions of Rac1 and Arf6 leads to a severe decrease in the internalization of splenic amastigotes. Blocking of FcγR-II/III and expression of inactive Cdc42 do not affect the rate of amastigote internalization, meaning that

ingestion depends on Rac1 and Arf6 (Lodge *et al.*, 2006a). Moreover, amastigote ligation with phosphatidylserine receptors results in secretion of anti-inflammatory cytokines TGF-β and IL-10, which promote parasite survival (Kane *et al.*, 2000). Furthermore, phagocytosis of *L. infantum chagasi* amastigotes does not depend on caveolae. PVs containing amastigotes do not colocalize with caveolin-1 and disruption of caveolae with M*β*CD does not lower internalization rates. Independent of lipid microdomain integrity, amastigote-containing PVs accrue LAMP-1 shortly after phagocytosis. Nonetheless, caveolae disruption negatively affects parasite survival ~72h post-infection, meaning that lipid microdomains somehow influence the capacity of the phagolysosome to sustain parasite proliferation. Hence, amastigotes enter macrophages through a caveolae-independent pathway that results in a PV that fuses rapidly with lysosomes. This modified phagolysosome allows amastigotes to replicate and propagate infection (Rodriguez *et al.*, 2011).

5.3.3 Contribution of the ER/ERGIC to the biogenesis of *Leishmania* PVs

Accumulating evidence indicates that the secretory pathway organelles and their molecules participate in PV formation. Various reports have shown that *Leishmania* phagosomes in macrophages and neutrophils contain ER/ERGIC markers (Garin *et al.*, 2001, Gueirard *et al.*, 2008, Houde *et al.*, 2003, Kima *et al.*, 2005, Ndjamen *et al.*, 2010). By employing confocal immunofluorescence and electron microscopy, Ndjamen and co-workers demonstrated persistent recruitment of ER proteins calnexin and Sec22b to *L. donovani* and *L. pifanoi* PVs containing promastigotes or amastigotes. In addition to Sec22b, Canton and colleagues found that the ER/ERGIC SNAREs Stx5, Stx18 and D12 were also recruited to PVs (Canton *et al.*, 2012a, Canton *et al.*, 2012b). This recruitment was nearly absent in zymosan phagosomes or in PVs containing dead parasites (Canton *et al.*, 2012a, Canton *et al.*, 2012b, Ndjamen *et al.*, 2010). Persistent recruitment of ER/ERGIC SNAREs (Aoki *et al.*, 2008) indicates that the *L. donovani* PV has an intimate relationship with the secretory pathway. This claim was substantiated by employing ricin, a toxin that traffics through the TGN and the ER lumen before unloading in the cytoplasm (Audi *et al.*, 2005, Slominska-Wojewodzka *et al.*, 2006). The

authors observed that over time, ricin accumulates in the lumen of PVs, which implies that the ER lumen exchanges contents with *Leishmania*-harbouring phagosomes (Ndjamen *et al.*, 2010). Interestingly, it was found that Retro-2-mediated inhibition of the retrograde pathway hinders PV expansion parasite survival (Canton *et al.*, 2012a). The functional importance of secretory pathway fusion molecules on PV biogenesis was exemplified when Canton and colleagues used RNAi to silence ER/ERGIC SNAREs (Canton *et al.*, 2012a, Canton *et al.*, 2012b). The KD of Sec22b, Stx5 and Stx18 and D12 gave rise to smaller PVs that contained a reduced number of amastigotes in comparison to controls. A similar effect was observed upon transfection of inactive SNAREs. The emerging role of the ER/ERGIC in PV biogenesis and parasite survival raises the question of why *Leishmania* needs those organelles for intracellular survival.

5.4 LPG and its inhibitory roles on phagosome maturation

5.4.1 The structure of LPG

A protozoan's cell surface is covered by glycoconjugates that have a direct interaction with their vertebrate and invertebrate hosts. The most studied glycoconjugate in *Leishmania* is LPG, which plays a crucial role in parasite survival both intracellularly and in the sandfly's digestive tract (Descoteaux *et al.*, 1999, Descoteaux *et al.*, 1991, Moradin *et al.*, 2012, Sacks *et al.*, 2001b, Sacks *et al.*, 2000, Turco *et al.*, 1992). Indeed, LPG is the most abundant molecule at the surface of promastigotes. Conversely, *Leishmania* amastigotes express very low amounts of LPG (McConville *et al.*, 1991). The structure of LPG (Figure 18) consists of a heteropolymer of repeating Gal(*β*1,4)Man(*α*1)-PO_4 subunits attached to a glycan core that is composed of the heptasaccharide Gal(*α*1,6)Gal(*α*1,3)Gal_f(*β*1,3)[Glc(*α*1)-PO_4]Man(*α*1,3)GlcN(*α*1,3). Linkage to the plasma membrane is mediated by the unusual 1-*O*-alkyl-2-*lyso*-phosphatidyl(*myo*)inositol GPI-type anchor (Descoteaux *et al.*, 1999, Turco *et al.*, 1992). The structure is capped by an oligosaccharide structure that varies among species. Nonetheless, the lipid anchor and glycan core are conserved (Descoteaux *et al.*, 1999, Turco *et al.*, 1992). Structural analysis of LPG has revealed that the lipid anchor is

completely conserved across *Leishmania* species, while the number, as well as the sugar composition of the repeating subunits varies (Orlandi *et al.*, 1987). This inherent variability is important in regards to promastigote attachment to the digestive tracts of different sandfly species (Sacks *et al.*, 2001b, Sacks *et al.*, 2000). The diversity in LPG structure also dictates its immunogenicity, which consequently influences the inflammatory response of the vertebrate host (Nogueira *et al.*, 2016, Passero *et al.*, 2015). Aside from its variability among different *Leishmania* species, the structure of LPG varies according to the life stage of the parasite. In the highly infectious metacyclic promastigotes, LPG is longer and has different sugar ramifications with a greater number of galactose and mannose residues when compared to procyclic promastigotes (Coelho-Finamore *et al.*, 2011, McConville *et al.*, 1992). There are other parasite molecules bearing domains that are similar to those found in LPG such as the secreted proteophosphoglycan (PPG), which are made up of proteins that are linked to a repeating Gal-Man disaccharide subunit. The secreted phosphoglycan (PG) and the glysylinositolphospholipids (GIPL) are also examples (Descoteaux *et al.*, 1999, Sacks *et al.*, 2000). In fact, some of the enzymes that participate in the biosynthesis of LPG also partake in the synthesis of other PG-containing molecules. While the *LPG1* gene participates in the synthesis of LPG's glycan core, the *LPG2* gene is involved in the synthesis of the Gal-Man-PO_4 heteropolymers in LPG, PG, and in proteins that contain these moieties such as PPG (Descoteaux *et al.*, 1995, Descoteaux *et al.*, 1994, Descoteaux *et al.*, 1999). Hence, deletion of *LPG2* leads to absence of PG-containing molecules including LPG; in contrast, deletion of *LPG1* leads to the absence of LPG only (Figure 18) (Lázaro-Souza *et al.*, 2018, Privé *et al.*, 2000, Sacks *et al.*, 2000, Zhang *et al.*, 2004).

5.4.2 The importance of LPG in intracellular survival

One of the first demonstrations that LPG was essential for parasite survival comes from the observation that LPG-deficient *L. major* and *L. donovani* promastigotes are killed by phagolysosomes. This defect was rescued by genetic complementation of LPG (McNeely *et al.*, 1990, Späth *et al.*, 2000). A step in early phagocytosis is the

accumulation of periphagosomal F-actin around the site the phagocytic cup. Such accumulation is also accompanied by the activation of kinases such as the type I PI3K, which is involved in PI(3,4,5)P_3 generation. As phagosome maturation progresses, the F-actin cup disappears (Greenberg, 1999, May *et al.*, 2001). However, in the case of *L. donovani,* F-actin accumulates progressively in an LPG-dependent manner. Such F-actin accumulation is accompanied by retention of proteins such as Cdc42, Arp2/3, WASP, α-actin, Nck and Myosin II (Holm *et al.*, 2001, Lodge *et al.*, 2005). Accumulation of these proteins promotes polymerization of F-actin and may sterically hinder recruitment of signal transducers that mediate phagosome maturation. Phagosomes containing amastigotes or LPG-deficient parasites do not show retention of F-actin and mature rapidly into phagolysosomes (Holm *et al.*, 2001, Lodge *et al.*, 2008). In addition, LPG prevents recruitment of PKC-α, which also results in F-actin accumulation and delayed recruitment of LAMP-1. In phagosomes containing inert particles or LPG-deficient promastigotes, PKC-α is recruited to nascent phagosomes and promotes F-actin depolymerisation. Hence, accumulation of F-actin and associated proteins around *L. donovani* promastigote phagosomes is dependent on LPG-mediated inhibition of PKC-α (Medina-Acosta *et al.*, 1989).

In both *L. donovani* and *L. major*, LPG also hampers recruitment of Rab7, which promotes fusion of phagosomes with late endosomes (Desjardins *et al.*, 1997, Scianimanico *et al.*, 1999). For LPG to stall phagosome maturation, intact lipid microdomains are required (Dermine *et al.*, 2005, Winberg *et al.*, 2009a). Disruption of these M*β*CD followed by infection with WT *L. donovani* promastigotes reveals a significant decrease in F-actin retention (Winberg *et al.*, 2009b). Phagosomal recruitment of LAMP-1 was even higher in M*β*CD-treated cells, raising the possibility that disruption of lipid microdomains may promote acidification of phagosomes and destruction of the parasite (Winberg *et al.*, 2009b). In the case of *L. mexicana* and *L. amazonensis*, LPG plays no role in phagosome maturation and intracellular proliferation. Phagosomes of both WT and LPG-deficient promastigotes progress to phagolysosomes, resist killing and allow differentiation into amastigotes (Courret *et al.*, 2002, Ilg, 2001). *In vivo* studies in mice show that *L. mexicana* promastigotes deficient

in LPG are no less virulent than their WT counterparts (Ilg *et al.*, 2001). It is hypothesized that the large communal vacuoles in which *L. mexicana* / *L. amazonensis* parasites dwell (Antoine *et al.*, 1998) might protect the parasites against phagolysosomal degradation (Ilg *et al.*, 2001, Wilson *et al.*, 2008). These data show that the *L. mexicana* / *L. amazonensis* LPG is required neither for virulence nor for survival, and that the functions of LPG may be species-dependent. Overall, LPG-dependent retardation of PV maturation is a process that requires intact lipid microdomains. Lipophosphoglycan insertion into these lipid microdomains leads to their disruption, which may alter the fusogenic properties of the phagosomal membrane (Dermine *et al.*, 2005, Miao *et al.*, 1995, Tolson *et al.*, 1990). This in turn leads to a stalled phagosome that provides promastigotes with an appropriate environment to transform into amastigotes.

5.4.3 LPG inhibits intraphagosomal oxidation and acidification

Leishmania promastigotes and amastigotes have evolved the ability to inhibit NOX2 assembly on their PVs, albeit through different mechanisms (Lodge *et al.*, 2006a, Lodge *et al.*, 2006b). Promastigotes use an LPG-dependent mechanism to block the recruitment of $p47^{phox}$, and $p67^{phox}$ to the phagosomal membrane. However, LPG does not block phosphorylation of $p47^{phox}$, or the association of $p47^{phox}$ and $p67^{phox}$. As a consequence to lack of NADPH oxidase assembly at the PV, wild type (WT) *L. donovani* promastigotes do not induce ROS production within their PVs (Lodge *et al.*, 2006a). On the contrary, promastigotes lacking LPG recruit $p47^{phox}$ and $p67^{phox}$ and induce ROS production within the parasite's phagosome. Although LPG does not affect the macrophage's capacity to produce ROS, *Leishmania* promastigotes inhibit intraphagosomal ROS production (Lodge *et al.*, 2006a).

Intraphagosomal acidification is important for activating its degradative hydrolases (Levin *et al.*, 2016), and Syt V regulates this process by mediating the recruitment of the V-ATPase to the phagosome (Vinet *et al.*, 2009). Upon their internalization by

macrophages, *Leishmania* promastigotes exclude Syt V from the phagosome membrane in an LPG-dependent manner; insertion of LPG into the phagosomal membrane leads to disorganization of lipid rafts (Dermine *et al.*, 2005, Vinet *et al.*, 2009, Winberg *et al.*, 2009b), and to the exclusion of Syt V from the phagosome. This in turn abrogates recruitment of V-ATPase and cathepsin D and thereby impedes phagosome acidification (Vinet *et al.*, 2010, Vinet *et al.*, 2009). Amastigotes and LPG-deficient *L. donovani* promastigotes do not preclude Syt V recruitment to phagosomes or inhibit their acidification, consistent with other reports in the literature (Desjardins *et al.*, 1997, Rasmusson *et al.*, 2004, Scianimanico *et al.*, 1999, Vinet *et al.*, 2009). Exclusion of Syt V from phagocytic cups induced by WT *L. donovani* promastigotes, also results in a decreased phagocytic capacity that is more pronounced under high membrane demand. A similar reduction in phagocytic capacity was observed in cells treated with siRNA to Syt V. On the other hand, a more severe inhibition of phagocytosis is not observed when macrophages are treated with siRNA to Syt V and fed with LPG-coated zymosan, meaning that the effects of both siRNA to Syt V and LPG are not cumulative. Altogether, these data support a Syt V-dependent mechanism where LPG abrogates the phagocytic capacity of the macrophage, while delaying acidification of existing PVs (Vinet *et al.*, 2009, Vinet *et al.*, 2011). The induction of decreased phagocytic capacity by LPG may also explain the observation that virulent *L. infantum chagasi* metacyclic promastigotes are phagocytosed less efficiently than log promastigotes (Ueno *et al.*, 2009); LPG may be responsible for the eventual reproductive success of metacyclic promastigotes. Similar to Syt V, the phagosomal recruitment of other membrane fusion regulators may also be blocked by LPG. In sum, LPG plays an essential role in parasite survival by inhibiting the recruitment of the molecular machinery that endows the phagosome with antimicrobial properties (Figure 18).

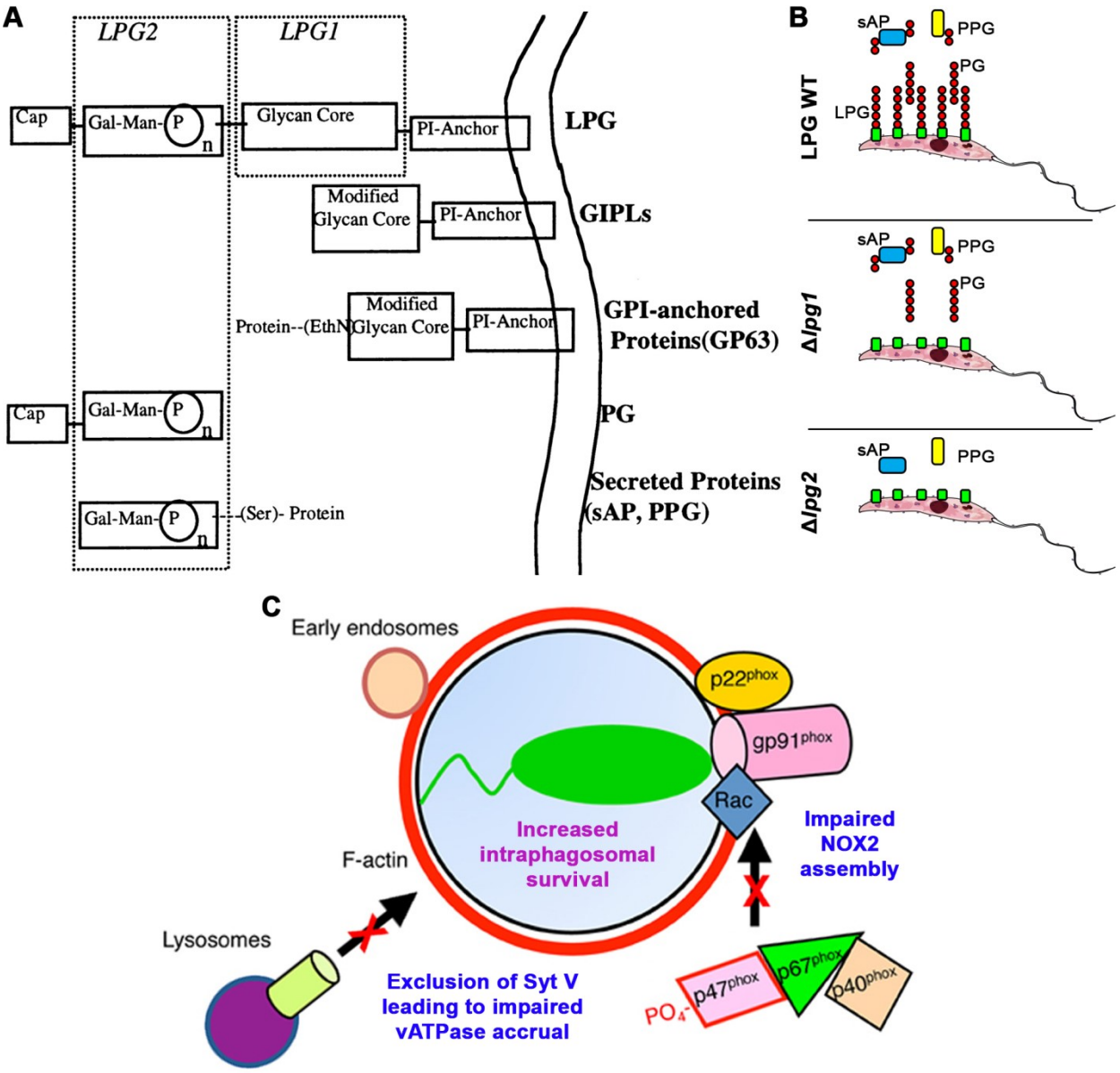


Figure 18 of literature review. The structure and function of LPG and related glycoconjugates. (A) Schematic diagram of the most abundant glycoconjugates on the surface of *Leishmania* promastigotes. The function of the *LPG1* and *LPG2* genes on the biosynthesis of these molecules is shown. Copyright (2000) National Academy of Sciences and panel. **(B)** The functions of the *LPG1* and *LPG2* genes have been discovered via the use of knockout mutants. The impact of these deletions on glycoconjugate production is shown schematically. **(C)** Diagram depicting the roles of LPG on the detoxification of the PV. The inhibition of NOX2 and V-ATPase assembly convenes in enhanced intravacuolar survival. Panels (A) and (C) were adapted with permission (Moradin *et al.*, 2012, Sacks *et al.*, 2000).

5.5 The GP63 metalloprotease

5.5.1 Discovery and biochemical properties

In the 1980s, a protein that exhibited cross-reactivity with anti-*Leishmania* serum was identified in promastigotes of several species (Bouvier *et al.*, 1985, Etges *et al.*, 1986, Fong *et al.*, 1982, Lepay *et al.*, 1983). The protein was found to be a ~63 kDa Zn^{2+}-dependent metalloprotease that comprised nearly 1% of all proteins in promastigotes (Bouvier *et al.*, 1985). This protein is known as leishmanolysin, surface acid peptidase, promastigote surface peptidase, major surface peptidase or major surface glycoprotein 63 (GP63). This protease belongs to the EC 3.4.24.36 enzymatic class (M8 endopeptidase family), and shares several common features with mammalian matrix metallopeptidases (Yao, 2010, Yao *et al.*, 2003). When the *GP63* gene was cloned, a study of its DNA and translated amino acid sequences revealed that the gene is translated as a precursor peptide (Button *et al.*, 1988). Indeed, GP63 is synthesized in the parasite's ER, where its signal sequence is cleaved after translation. An N-linked carbohydrate is added and the C-terminal peptide (about 25 amino acids) is a glycosylphosphatidylinositol (GPI) membrane anchor. During its biogenesis in the parasite, subsequent proteolytic processing involves cleavage of a propeptide to produce the mature GP63 polypeptide chain (McGwire *et al.*, 1996, Yao, 2010). The predicted propeptide contains a cysteine residue that is conserved in all GP63 molecules across species. In *L. major*, this residue appears to contribute to the modulation of Zn^{2+} ion binding at the active site and thus regulates enzymatic activity. This mechanism may be used by the parasite to prevent self-damage by active GP63 (Macdonald *et al.*, 1995a). The crystal structure of the GPI-anchored form of this protease revealed that it has a relatively compact secondary structure consisting of three domains with a high content of *β* sheets (Figure 19) (Schlagenhauf *et al.*, 1998). The NTD forms a surface indentation that harbours the active-site cleft. There, a HEXXH motif in an *α* helix contributes to Zn^{2+} ion coordination through the motif's His residues (Schlagenhauf *et al.*, 1998). In terms of post-translational modifications, the *L. major* GP63 was predicted to be glycosylated at three sites; however, biochemical analyses showed that the protein harbours two aminoacids with an *N*-linked

$GlcMan_6GlcNAc_2$ moiety that is terminally glucosylated (Figure 19) (Funk *et al.*, 1997). Although GP63 is mainly found in its GPI-anchored form (Etges *et al.*, 1986), techniques such as flow cytometry and electron microscopy have helped identify secreted forms of this protease (Yao, 2010). In addition, membrane-associated or free GP63 are released by promastigotes into the extracellular milieu. This release was demonstrated to be decreased in the presence of the divalent metal chelator 1,10-phenanthroline or when there is a mutation at the Zn^{2+} binding site (McGwire *et al.*, 2002), suggesting that the release of GP63 is dependent on autoproteolysis.

Several *Leishmania* species contain distinct classes of *GP63* genes that are differentially regulated during all stages of the life cycle, including amastigotes, and procyclic and metacyclic promastigotes (Hsiao *et al.*, 2008, Medina-Acosta *et al.*, 1989, Olivier *et al.*, 2012, Yao, 2010). For example, the expression of membrane bound GP63 in the amastigote form is greatly reduced, although a soluble form of GP63 is localized in the parasite lysosome. Hence, GP63 may play a vital role in the different stages of the *Leishmania* life cycle (Frommel *et al.*, 1990, Medina-Acosta *et al.*, 1989, Olivier *et al.*, 2012, Yao, 2010). GP63 homologues have also been identified in *T. cruzi*, *T. brucei* and *T. rangeli* (Jackson, 2016). In the *T. cruzi* genome, genes encoding 33 GP63-lik eproteins are extensively amplified (>420 genes and pseudogenes) when compared to the genome of *L. major* and *T. brucei*. Nonetheless, there is an indirect correlation between gene expansion and proteolytic activity. For example, *T. cruzi* is by far the protozoan with the highest number of *GP63* genes, while its metallopeptidase activity is difficult to detect by zymography. In contrast, *Leishmania* parasites express a lower number of *GP63* genes with high metalloprotease activity. Nonetheless, in cell extracts of the non-virulent *L. tarentolae*, which has been classified in the *Sauroleishmania* subgenus, the *GP63* gene is highly expanded with 49 possible copies compared to seven in *L. major*, seven in *L. infantum* and 29 in *L. braziliensis* (Button *et al.*, 1988, Raymond *et al.*, 2012, Yao, 2010). Since the GP63s in *L. tarentolae* have no metalloprotease activity, it is hypothesized that the high nucleotide variability in its *GP63* genes may lower their peptidase activity (Raymond *et al.*, 2012).

5.5.2 The impact of GP63 on host cell biology

The impact of GP63 on macrophage function has been extensively investigated and a large number of functions have been described (Figure 19) (Olivier *et al.*, 2012, Podinovskaia *et al.*, 2015, Yao, 2010). One of the first studies on GP63 function by the Chang laboratory found that the protease was active over a wide range of temperatures and pH, indicating that it may be active in a variety of intracellular compartments (Chaudhuri *et al.*, 1988). It was found that GP63 inhibits parasite lysis by the complement system through the cleavage of the C3 component protein, as well as the conversion of C3b to its inactive iC3b form, which also promotes parasite internalization into PVs (Brittingham *et al.*, 1996, Chaudhuri *et al.*, 1988). Another study found that GP63 cleaves the substrate-related cytosolic proteins for PKC rich in myristoylated alanine residues (MARCKS), which supports the paradigm that GP63 cleaves signaling molecules that affect parasite survival (Corradin *et al.*, 1999).

The abundance and properties of this enzyme inspired scientists to delve deeply into its roles in manipulating host cell biology. Nonetheless, it was not until 2002 that Joshi and colleagues created the first complete *GP63* knockout in *L. major* (Joshi *et al.*, 2002). After deletion of all seven *GP63* genes, the authors found that promastigotes were sensitive to complement-mediated lysis and exhibited severely diminished virulence *in vivo* (Joshi *et al.*, 2002). GP63 also contains a SRYD sequence that is antigenically related to the fibronectin RGDS sequence, suggesting the potential interaction of GP63 with fibronectin receptors on macrophages (Soteriadou *et al.*, 1992). This study led to the finding that GP63 facilitates tissue invasion since it degrades components of the extracellular matrix such as type IV collagen and fibronectin (McGwire *et al.*, 2003). This promotes connective tissue degradation, thereby helping the parasite to migrate and disseminate. Remarkably, GP63 mediates the shutdown of signalling pathways that control microbial elimination (Gómez *et al.*, 2009, Hallé *et al.*, 2009), and the deregulation of transcription (Contreras *et al.*, 2010) and translation (Jaramillo *et al.*,

2011). Aside from macrophages, GP63 also cleaves plasmalemmal CD4 on T cells, which may decrease their response to infection (Garcia *et al.*, 1997). Moreover, GP63 enters hepatocytes via parasite-derived MVs, where it inhibits lipid biosynthesis via DICER1 cleavage (Ghosh *et al.*, 2013). The ensuing cholesterol deficiency favours parasite growth and dissemination (Descoteaux *et al.*, 2013, Ghosh *et al.*, 2013).

The critical role of membrane trafficking proteins in macrophage biology makes these proteins great targets for proteolytic attack by GP63 [Figure 19] (Matte *et al.*, 2016b). Up to date, GP63 has been found to target SNAREs and Syts that control antigen cross-presentation (Matheoud *et al.*, 2013) and LC3-associated phagocytosis via cleavage of VAMP8 (Matte *et al.*, 2016a). How GP63 and LPG access their substrates has not been elucidated and multiple hypotheses have been proposed (Figure 19). Both GP63 and LPG have been shown to traffic from PV to the cytoplasm of infected macrophages. Immunofluorescence data show that GP63 is widely distributed in the cytoplasm of infected macrophages (Matheoud *et al.*, 2013) and localizes to lipid rafts (Gómez *et al.*, 2009, Gómez *et al.*, 2010). Moreover, GP63 has been observed at the periphery of cell nuclei of B10R macrophages (Contreras *et al.*, 2010). There, GP63 is believed to interact and degrade nuclear pore-associated proteins, henceforth gaining accesses to the nucleus and cleaving a myriad of nuclear proteins (Isnard *et al.*, 2015) including the activator protein 1 (AP-1) (Contreras *et al.*, 2010). It has been hypothesized that GP63 could transfer, via its GPI anchor, from the phagosomal membrane to endosomes and thence to other secretory pathway organelles, or leave the phagosome via protein transporters (Figure 19) (Gómez *et al.*, 2010, Matte *et al.*, 2016b). In the case of LPG, trafficking from the PV to the cytoplasm has been observed in macrophages infected with *L. major* or *L. mexicana*, or fed with LPG-coated zymosan (Ilg, 2000, Späth *et al.*, 2003, Vinet *et al.*, 2009). Hence, research on this issue is needed to understand how *Leishmania*'s virulence molecules access their host cell targets. This important issue is addressed in Chapter 3 of this dissertation.

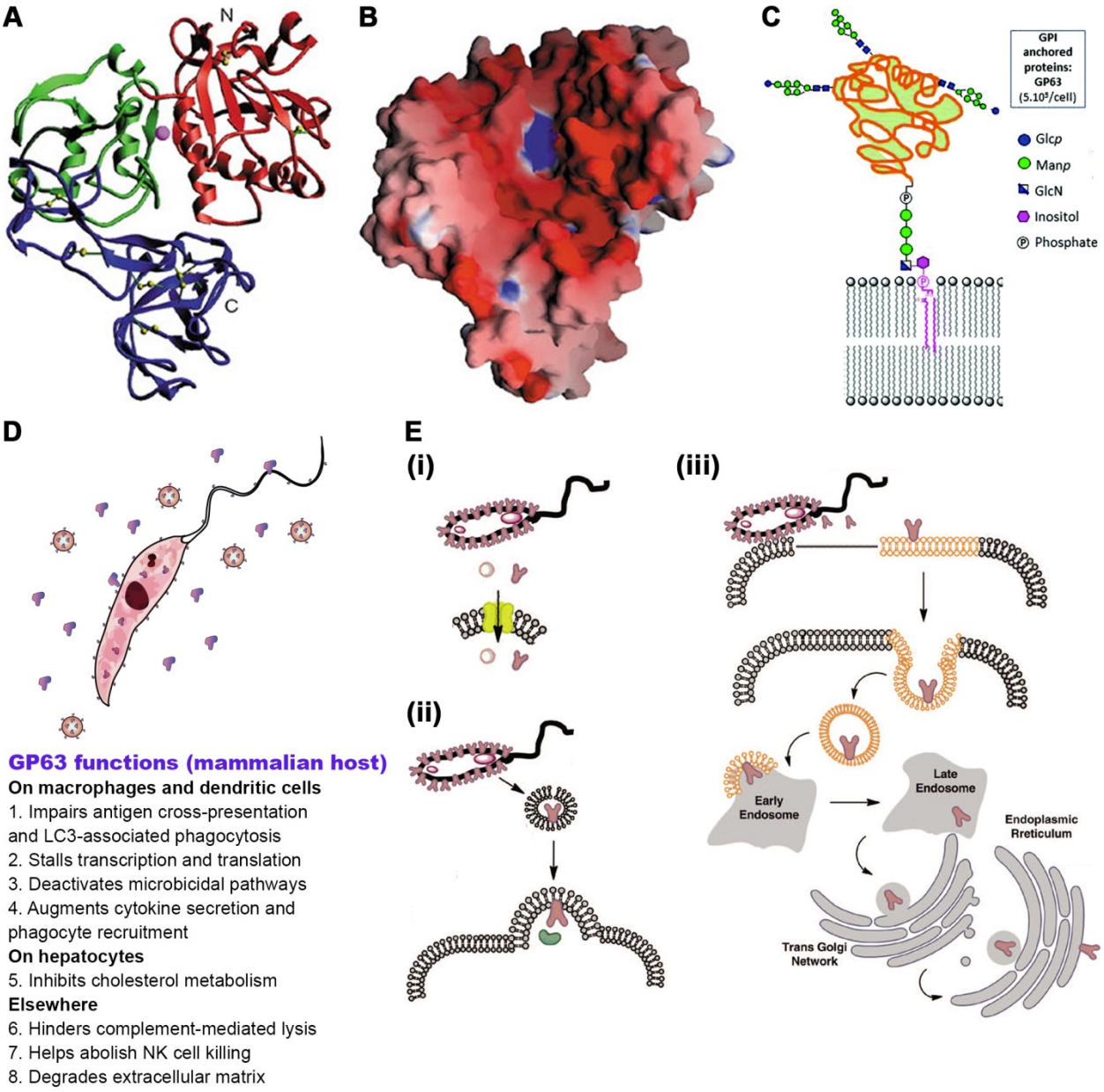


Figure 19 of literature review. The structure and function of the GP63 metalloprotease. Ribbon **(A)** and surface charge **(B)** diagrams depicting the surface-anchored *L. major* GP63. Note the three lobes and the coordination of the Zn^{2+} ion by the helix in the NTD. The surface charge diagram **(B)** shows a compact and negatively charged structure (red) with a positively charged pocket (blue) where the active site lies. **(C)** Schematic diagram depicting the GPI-mediated membrane anchoring of GP63 and its glycosylated moieties. **(D)** Impact of GP63 on mammalian host cell function. **(E)** Hypotheses regarding the possible mechanisms by which GP63 spreads in the host cell cytoplasm. GP63 has been thought to enter the host cell via specialized channels **(i)**, in parasite-derived MVs **(ii)**, or through lipid raft-mediated entry into the host cell secretory pathway **(iii)**. Adapted with permission from (Cabezas *et al.*, 2015, Gómez *et al.*, 2010, Schlagenhauf *et al.*, 1998).

CHAPTER 2: HYPOTHESES AND OBJECTIVES

Intracellular parasites and their mammalian hosts have waged a molecular war that has lasted several millennia and continues to this day. Through convergent evolution, these parasites have evolved strategies whose overarching goal is to hijack host cell processes in order to ensure parasite persistence and dissemination. The strategy employed by *Leishmania* involves entering the host cell in a phagosome, which the parasite remodels into a PV of its own. In this scenario, the PV may serve as an entry point for the parasite's molecules, which may in turn co-opt the host cell's trafficking machinery for their dissemination and function. The work that has been carried out in this dissertation addresses two interconnected research questions in the field.

First, it is known that *Leishmania* parasites induce the secretion of inflammatory cytokines and attract phagocytic cells to the infection site. However, the connection between these two phenomena and the host and parasite molecules therein involved had not been previously characterized. In this regard, I **hypothesize** that:

> ***Leishmania* targets a previously uncharacterized negative regulator of cytokine secretion in order to bolster TNF and IL-6 release post-infection. Augmented levels of these proinflammatory cytokines would then lead to increased recruitment of inflammatory phagocytes to the infection site.**

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

(a) To elucidate the role of Syt XI, a possible negative regulator of exocytosis, on cytokine secretion and phagocytosis by macrophages.

(b) To investigate whether the *Leishmania* GP63 metalloprotease is responsible for the release of TNF and IL-6 that occurs post-infection, and whether this phenomenon is linked to Syt XI degradation.

(c) To investigate whether in the *in vivo* context, the presence of GP63 is linked to the early accrual of inflammatory phagocytes to the inoculation site.

These objectives will be addressed in primary articles no. 1 and 2 (Chapter 3).

Second, *Leishmania* promastigotes have been known to use their virulence-associated glycoconjugates GP63 and LPG to hijack a multitude of processes in the mammalian host. Nonetheless, the mechanisms by which those parasite molecules egress from the phagosome into the host cell cytoplasm had not been discovered. Since *Leishmania* has no known secretion system, the question emerges as to whether the parasitized host cell aids the intracellular dissemination of *Leishmania* effectors. On this matter, I **hypothesize** that:

> **GP63 and LPG are redistributed throughout the cytoplasm of infected cells in vesicles whose trafficking is mediated by host organelles. Indeed, the host cell secretory pathway and its membrane fusion molecules may mediate the egress of these molecules out of the phagosome.**

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

(a) To elucidate whether the entry of GP63 and LPG into infected cells occurs via phagocytosis.

(b) To investigate whether the host cell secretory pathway is implicated in the intracellular trafficking of GP63 and LPG.

(c) To elucidate whether the SNARE Sec22b, a vesicle fusion regulator mediating ER-ERGIC-Golgi traffic, mediates the redistribution of GP63 and LPG in infected cells.

These objectives will be addressed in primary article no. 3 (Chapter 3).

CHAPTER 3: PRIMARY ARTICLES

ARTICLE NO. 1: SYNAPTOTAGMIN XI REGULATES PHAGOCYTOSIS AND CYTOKINE SECRETION IN MACROPHAGES

Synaptotagmin XI regulates phagocytosis and cytokine secretion in macrophages

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1 ABSTRACT

Synaptotagmins (Syts) are a group of type-I membrane proteins that regulate vesicle docking and fusion in processes such as exocytosis and phagocytosis. All Syts possess a single transmembrane domain, and two conserved tandem Ca^{2+}-binding C2 domains. However, Syts IV and XI possess a conserved serine in their C2A domain that precludes these Syts from binding Ca^{2+} and phospholipids, and from mediating vesicle fusion. Given the importance of vesicular trafficking in macrophages, we investigated the role of Syt XI in cytokine secretion and phagocytosis. We demonstrated that Syt XI is expressed in murine macrophages, localized in recycling endosomes, lysosomes, and recruited to phagosomes. Syt XI had a direct effect on phagocytosis and on the secretion of TNF and IL-6. Whereas siRNA-mediated knockdown of Syt XI potentiated secretion of these cytokines and particle uptake, overexpression of a Syt XI construct suppressed these processes. Additionally, Syt XI knockdown led to decreased recruitment of $gp91^{phox}$ and LAMP-1 to phagosomes, suggesting attenuated microbicidal activity. Remarkably, knockdown of Syt XI ensued in enhanced bacterial survival. Altogether, our data reveal a novel role for Syt XI as a regulator of cytokine secretion, particle uptake, and macrophage microbicidal activity.

2 INTRODUCTION

Macrophages are multifunctional cells specialized in the phagocytosis and destruction of pathogens, apoptotic cells, and foreign particles (Huber *et al.*, 1981, Huynh *et al.*, 2007b). When macrophages are activated by host cytokines such as IFN-γ, or by microbial factors such as LPS, they generate a highly microbicidal environment (Haas, 2007). Macrophages also secrete a panoply of pleiotropic cytokines – such as TNF and IL-6 – that are responsible for unleashing an effective immune response, and for mediating the transition between innate and adaptive immunity (Huynh *et al.*, 2007b, Stow *et al.*, 2009, Unanue *et al.*, 1976). In macrophages, the formation and maturation of phagosomes are sequential processes that necessitate extensive remodelling of the cytoskeleton and exchanges with multiple organelles in a process coined as 'kiss and run' (Desjardins, 1995). The maturation of phagosomes is controlled by Rab proteins, which are a subtype of GTPases, with Rab5 and Rab7 governing fusion events of phagosomes with early and late endosomes, respectively (Scott *et al.*, 2003). As phagosomes mature, they become very acidic and acquire a panoply of highly microbicidal molecules such as cathepsins and other acid hydrolases (Botelho *et al.*, 2011). Late phagosomes acquire markers such as the lysosomal-associated membrane proteins ($LAMP^3$)-1 and -2, which are required for acquisition of Rab7 (Huynh *et al.*, 2007a) and of microbicidal properties (Binker *et al.*, 2007). Crucial to the killing of many intracellular pathogens is the generation of reactive oxygen species (ROS) in the phagosome, which is produced mostly by the NADPH oxidase 2 complex (NOX2) (Lam *et al.*, 2010, Shatwell *et al.*, 1996) and mitochondria (West *et al.*, 2011). The complex, which is normally inactive in the cell, is constituted by six subunits. These subunits are the Rho guanosine triphosphate (GTPase), usually Rac1 or Rac2, and five phagocytic oxidase (*phox*) subunits. *Phox* subunits are the membrane-anchored $gp91^{phox}$ and $p22^{phox}$, which together form the vesicular flavocytochrome b_{558}; and the cytoplasmic components $p40^{phox}$, which interacts with heterodimers of $p47^{phox}$ and $p67^{phox}$ (Perisic *et al.*, 2004). The oxidase becomes functional when an activated $p40^{phox}$-$p47^{phox}$-$p67^{phox}$ heterotrimer is recruited to a membrane-bound flavocytochrome b_{558}, which then produces ROS by oxidizing NADPH to $NADP^+$ in the cytoplasmic region of $gp91^{phox}$. This reaction liberates one H^+ ion and 2 electrons that travel through the

flavocytochrome b_{558} into the phagosomal lumen to react with oxygen molecules and produce superoxide ions. The preponderance of ROS, acid hydrolases and other microbicidal molecules create a hostile intraphagosomal milieu that fetters the survival of most pathogens.

A highly organized orchestration of vesicular trafficking is essential for the proper spatiotemporal execution of cytokine secretion and phagocytosis. This trafficking is regulated by members of the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) such as the vesicle-associated membrane protein 3 (VAMP3), which promotes trafficking of TNF-containing vesicles to the cell membrane and to nascent phagosomes (Murray *et al.*, 2005a, Stow *et al.*, 2009). Constitutive exocytosis is responsible for the secretion of TNF and IL-6. SNARE proteins Syntaxin-6, -7, and Vti1b control trafficking from the Golgi complex to recycling endosomes. From recycling endosomes, VAMP3-bearing vesicles are delivered to the plasma or phagosome membrane where Syntaxin-4 – a cognate SNARE of VAMP3 – is present. Though TNF is delivered to forming phagosomes, IL-6 is not (Manderson *et al.*, 2007). Failure to regulate cytokine secretion results in pathologies where excess TNF and IL-6 leads to chronic inflammation (Beutler, 1999, Nishimoto *et al.*, 2004).

The action of SNAREs is regulated by Synaptotagmins (Syts), which are a large group of membrane proteins that regulate vesicle docking and fusion in processes such as the exocytosis of synaptic vesicles and hormones (Pang *et al.*, 2010), phagocytosis (Beutler, 1999, Vinet *et al.*, 2008), mast cell degranulation, and acrosome exocytosis in sperm cells (Südhof, 2012). All Syts possess a single transmembrane domain, and two conserved tandem C2 domains, which were originally identified in protein kinase C (Perin *et al.*, 1990). Eight members of the Syt family function as Ca^{2+} sensors of vesicle fusion by virtue of the C2 domains present in these Syts. Syts IV and XI, however, belong to a different Syt family where a conserved serine in the C2A domain precludes these Syts from binding Ca^{2+} and phospholipids (Pang *et al.*, 2010, von Poser *et al.*, 1997). In fact, murine Syts IV and XI inhibit vesicle fusion (Wang *et al.*, 2010), and may

mediate vesicle trafficking processes that do not depend on Ca^{2+} (von Poser *et al.*, 1997, Wang *et al.*, 2010). Indeed, Syt IV has been found to regulate synaptic function by negatively regulating brain-derived neurotrophic factor release (Dean *et al.*, 2009), among others. As of today, a function had not been assigned to Syt XI. Nonetheless, Syt XI exists in different vesicular compartments of the rat pheochromocytoma cell line PC12 (Fukuda *et al.*, 2001, Huynh *et al.*, 2003). It was also discovered that Syt XI binds to KIF1A, a kinesin-related molecular motor in brain (Park *et al.*, 2005), and with the dishevelled (Dvl) protein, which plays a major role in the Wnt signalling axis (Nusse, 2012). Interestingly, Syt XI is degraded by Parkin, a protein that is often mutated in Parkinson's disease. Defective Parkin leads to accumulation of Syt XI at the core of Lewy bodies (Huynh *et al.*, 2003). Also, excess of Syt XI in the human brain has been linked to Schizophrenia (Inoue *et al.*, 2007). All of these results have led authors to postulate that Syt XI may have a role in modulating synaptic transmission in brain. In macrophages, only Syt V and Syt VII have been found to be expressed. Syt V is a positive regulator of phagocytosis that regulates acquisition of the V-ATPase to maturing phagosomes (Vinet *et al.*, 2008, Vinet *et al.*, 2009), and Syt VII mediates lysosomal membrane delivery to nascent phagosomes (Czibener *et al.*, 2006). We hypothesized that Syt IV and Syt XI may be expressed in macrophages and have an inhibiting function in cytokine secretion and phagocytosis. Our initial experiments revealed that only Syt XI was expressed in macrophages.

In this work, we examined the role of Syt XI in macrophages. We showed that this phagosome-associated Syt controls negatively particle ingestion and the release of TNF and IL-6. Although lack of Syt XI led to augmented particle intake, phagosomes appeared weakened by their impaired recruitment of $gp91^{phox}$ and LAMP-1. We demonstrated that Syt XI knockdown lead to diminished bactericidal activity, likely as a consequence of less microbicidal phagosomes.

3 MATERIALS AND METHODS

3.1 Ethics statement

Mice were manipulated in strict accordance to protocol 0811-09, approved by the *Comité Institutional de Protection des Animaux* of the INRS-Institut Armand-Frappier. This protocol respects guidelines on good animal practice provided by the Canadian Council on animal care.

3.2 Antibodies and plasmids

The rabbit polyclonal antibody targeting the C2A domain of Syt XI was produced and purified by affinity chromatography (Fukuda *et al.*, 1999). The rat monoclonal anti-transferrin receptor 1 (Tfr1) was obtained from Cedarlane Laboratories. The rat monoclonal anti-early endosomal antigen 1 (EEA1) antibody and the mouse monoclonal anti-gp91^{phox} antibody were from BD transduction laboratories. The rat monoclonal anti-lysosome-associated membrane protein 1 (LAMP-1) antibody was developed by J. T. August (1D4B) and purchased through the Developmental Studies Hybridoma Bank at the University of Iowa, and the National Institute of Child Health and Human Development. The rabbit polyclonal antibody against p38 was obtained from Cell Signalling. The rabbit anti-GFP antibody was from Santa Cruz. The FLAG-Syt XI-GFP construct (Saegusa *et al.*, 2002) was inserted into the *Not*I site of the pCIN4 expression vector (Rees *et al.*, 1996) using conventional cloning techniques.

3.3 Cell culture

The mouse macrophage cell line RAW 264.7 and the PC12 neuroendocrine cell line were cultured in complete DMEM (Life Technologies) with L-glutamine, supplemented with 10% heat-inactivated FBS (PAA Laboratories), 10 mM HEPES at pH 7.4, and

antibiotics (Life Technologies) in a 37ºC incubator with 5% CO_2. Bone marrow-derived macrophages (BMM) were extracted from the bone marrow of 6- to 8-week old female BALB/c mice (Charles River), and differentiated with complete DMEM with L929 cell-conditioned medium (15% v/v) as a source of colony-stimulating factor 1 (Descoteaux *et al.*, 1989). Prior to experiments, BMM were transferred to tissue-culture treated petris containing complete DMEM with no L929-conditioned medium for 16 h.

3.4 Transfections

For small interfering RNA (siRNA) transfections, RAW 264.7 macrophages in the second passage were reverse-transfected in 24-well plates with the Lipofectamine® RNAiMAX Reagent (Life Technologies) as per the manufacturer's protocol. The final concentration of siRNA was 25 nM in a final volume of 600 µl; incubation in transfection medium lasted 60 h. Prior to experiments, macrophages were cultured for an extra 6 h in DMEM containing 10% FBS. Macrophages were mock-transfected, transfected with siRNA to GFP (Flandin *et al.*, 2006), or with the ON-TARGETplus SMARTpool siRNA to Syt XI (Thermo Scientific), which contains four siRNAs with the following target sequences: Sequence 1: CGAUCGACUACUAAGAAU. Sequence 2: GAGAGAGGUCUGCGAGAGU. Sequence 3: AUGUCAAGGUGAACGUCUA. Sequence 4: GCACAGUCUGAGCGAGUAC. BLAST searches were conducted to ensure that these sequences targeted only the Syt XI mRNA.

The pCIN4 plasmid containing the FLAG-Syt XI-GFP construct, or empty pCIN4, was electroporated into RAW 264.7 macrophages (Stacey *et al.*, 1993). Stably transfected macrophages were selected in complete DMEM containing 500 µg/ml G418, and individual clones were expanded and assayed for FLAG-Syt XI-GFP expression by Western blot and confocal immunofluorescence microscopy.

3.5 RT-PCR

TRIzol (Life Technologies) was used to extract RNA from adherent BMM, RAW 264.7 macrophages, or PC12 cells, as per the manufacturer's instructions. RNA was reverse-transcribed (Matte *et al.*, 2010), and PCR was performed with a DNA thermal cycler (Perkin-Elmer Corporation, version 2.3) with the following primer pairs, and an annealing temperature of 55ºC. Hypoxanthine phosphoribosyltransferase (HPRT): F-AD55: 5'-GTTGGATACAGGCCAGACTTTGTTG-3', R-AD56: 5'-GATTCAACTTGCGCTCATCTTAGGC-3'; Syt IV: F-AD476: 5'-CACCTACCGAAATCTGATGTGTC-3', R-AD477: 5'-GACCAACCGTCCAATCACCTC-3'; Syt XI: F-AD445: 5'-CAATGCGTTTTCTGCCGTAGTAGA-3', R-AD446: 5'-CTGACCAGGGACATCATCAAGAG-3'. Samples were run in 1.5% (w/v) agarose gels and the Alpha Imager 3400 (Alpha Innotech Corporation) machine was used to photograph gels.

3.6 Cytokine secretion measurements

RAW 264.7 macrophages were stimulated with LPS from *Escherichia coli* Serotype O127:B8 (Sigma) at a final concentration of 100 ng/ml. After stimulation, cell culture supernatants were collected and centrifuged to remove debris. ELISA kits were used as per the manufacturers' protocols to quantify murine TNF (Ready-SET-Go! Mouse TNFα Kit, eBiosciences) and IL-6 (BD OptiEIA, BD Biosciences) secretion.

3.7 Phagosome isolation and phagocytosis assays

For the isolation of purified phagosomes, 40 x 10^7 RAW 264.7 macrophages were seeded overnight in 150 x 20 mm tissue culture dishes. Cells were then incubated at 4ºC for 10 min in the presence of 10 ml of complete DMEM containing 200 µl of magnetic beads (Stapor) of a 3 µm diameter. Cells were then transferred to 37ºC to

trigger internalization for the required phagocytosis time. Cells were then washed three times with cold PBS prior to scraping and collection in cold PBS. Cells were then centrifuged at 2000 rpm for 5 min at 4ºC, and the pellet was resuspended in purification buffer (50 ml PBS 10X, 2 ml EDTA at 0.5 M and pH 8, and 445 ml H_2O. Solution was adjusted to pH 7.2). Cells were centrifuged again at 2000 rpm for 5 min at 4 ºC. Macrophages were then homogenized in 1 ml of purification buffer with protease inhibitors: cells were lysed with a 1 ml syringe using a 22G needle until 90% were lysed without major nuclei breakage (as monitored by light microscopy). Homogenates were centrifuged at 2000 rmp for 5 min at 4ºC, and the pellet resuspended in 1 ml of purification buffer with protease inhibitors. Homogenates were then placed on a magnet for 10 min to isolate phagosomes. Thereafter, supernatants were aspirated, and isolated phagosomes were washed with purification buffer containing protease inhibitors. Subsequently, isolated phagosomes were lysed with ice-cold lysis buffer containing 1% NP-40, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and protease and phosphatase inhibitors (Roche). Protein concentrations were measured using the Pierce BCA protein assay (Fisher Scientific).

Zymosan was opsonised with mouse serum (Lodge *et al.*, 2006a). For synchronized phagocytosis using opsonised zymosan, macrophages were incubated, at 4ºC for 15 min, using the zymosan-to-cell ratio required by the experiment. Macrophages were washed with cold complete DMEM to remove excess particles, and internalization was triggered by transferring cells to 37ºC for the required times (Vinet *et al.*, 2008). Cells were then washed with PBS and stained using the HEMA 3 stain set (Fisher), or prepared for confocal immunofluorescence microscopy.

3.8 Bacteria killing assays

RAW 264.7 macrophages transfected with siRNA were infected with non-opsonised *Escherichia coli* DH1 (OD_{600} = 0.6) at a ratio of 20:1. Infections were carried out

according to the protocol outlined by Hamrick *et al.* (Hamrick *et al.*, 2000). Briefly, bacteria were added in 20 µl of PBS, and plates were centrifuged at 1000g for 1 min. Then, plates were incubated at 37ºC for 20 min prior to four washes with 1 ml PBS. Complete DMEM with 5 µg/ml of gentamicin (Life Technologies) was then added for 20 min (zero time point) or for an additional 4 h. After these time points, macrophages were washed once with 0.5 ml of PBS, and lysed with a solution of 1% Triton X-100 (v/v) in PBS. Lysates were diluted, plated in agar plates, and incubated for 18 h at 37°C. Bactericidal activity was assessed by counting colonies in agar plates, and results were expressed as the $\log_{10}$ of CFU per ml (CFU/ml). Infections were done in 24-well plates in triplicate.

3.9 Confocal immunofluorescence microscopy

Macrophages in coverslips were fixed with 2% paraformaldehyde (Canemco & Miravac) for 10 min. Thereafter, cells were permeabilized and blocked for 15 min with a solution comprised of 0.1% Triton X-100, 1% BSA, 2% goat serum, 6% non-fat milk, and 50% FBS. This was followed by a two-hour incubation with primary antibodies diluted in PBS. Then, cells were incubated with the appropriate combination of secondary antibodies (anti-rabbit AlexaFluor 488, anti-rat 568, and anti-mouse 568; Molecular Probes), cholera toxin subunit B linked to AlexaFluor 594 (Ctx) to stain lipid rafts (Molecular Probes), and DRAQ5 to stain DNA (Biostatus) for 30 min. Coverslips were washed three times with PBS after every step. After staining, coverslips were mounted on glass slides with Fluoromount-G (Southern Biotechnology Associates), and sealed with nail polish. Macrophages were imaged with the oil immersion 63X objective of an LSM 780 microscope (Carl Zeiss Microimaging GmbH). Images were taken in simultaneous scanning mode and processed via the ZEN 2011 software (Carl Zeiss Microimaging GmbH).

SDS-PAGE and Western blotting

Prior to lysis, adherent macrophages were washed with PBS containing 1 mM sodium orthovanadate (Sigma). Cells were scraped in the presence of lysis buffer containing protease and phosphatase inhibitors. After a 10 min incubation on ice, lysates were sonicated and centrifuged for 10 min to remove insoluble matter. After protein quantification, 30 µg of sample was boiled in SDS sample buffer, migrated in 10% SDS-PAGE gels, and analyzed by Western blotting (Vinet *et al.*, 2008).

3.10 Data analysis

Statistical significance was assessed using an unpaired two-tailed Student's *t*-test. '*' denotes $p \leq 0.05$, '**' denotes $p \leq 0.01$, and '***' $p \leq 0.001$. Error bars represent the standard error of the mean. Graphs were plotted with Microsoft Excel or SigmaPlot.

4 RESULTS

4.1 Macrophages express Syt XI

Up to date, Syt IV and Syt XI have only been found to be expressed in neuronal tissues. The capacity of these Syts to inhibit vesicle fusion (Tucker *et al.*, 2003, Wang *et al.*, 2010) prompted us to assay the expression of these proteins in macrophages, since vesicle fusion is essential for processes such as cytokine secretion and phagocytosis (Huynh *et al.*, 2007b). By employing RT-PCR, we assayed the expression of Syt IV and Syt XI in BMM, RAW 264.7 macrophages, and in the neuroendocrine cell line PC12. We found that both types of macrophages expressed Syt XI, but not Syt IV (Fig. 1A). Next, we immunostained macrophages with an antibody specific for the C2A domain of Syt XI. Immunofluorescence revealed that Syt XI was expressed in vesicles distributed in the cytoplasm of BMM (Fig. 1B); interestingly, Syt XI accumulated at the extremities of long protrusions that BMM sometimes extended (see inset). Expression of a FLAG-Syt XI-GFP construct in RAW 264.7 macrophages displayed a vesicular distribution pattern similar to that of endogenous Syt XI in BMM (Fig. 1C). Based on these data, we pursued further the characterization of Syt XI.

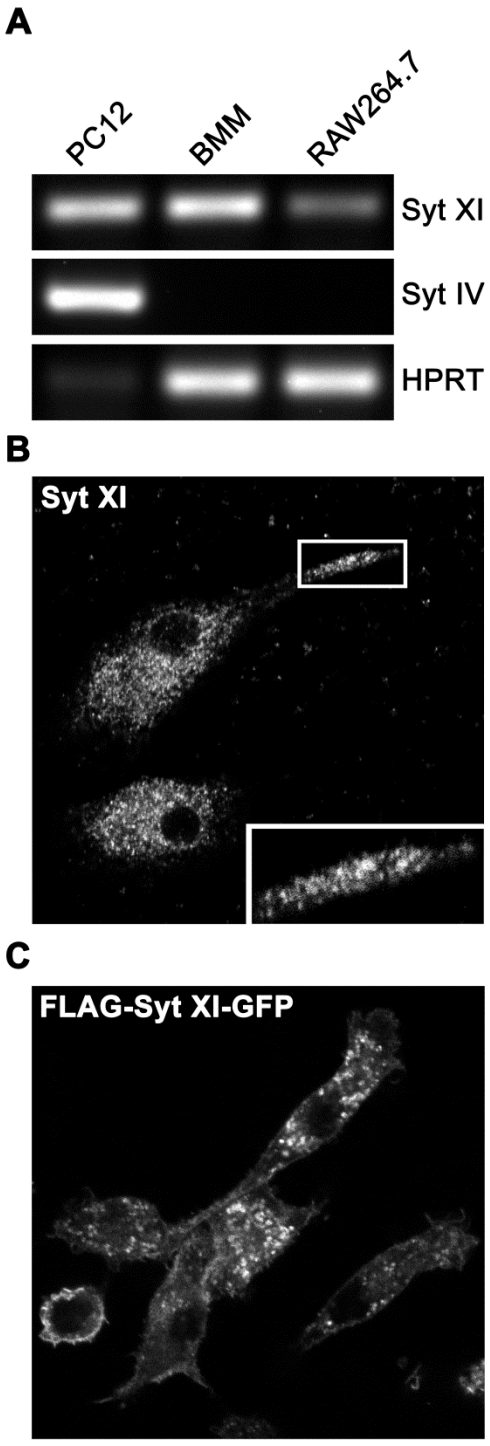

Figure 1 of article 1. Syt XI is expressed in macrophages. (A) Syt XI, but not Syt IV, is expressed in macrophages as demonstrated by RT-PCR. **(B)** As observed via immunofluorescence microscopy, BMM express Syt XI in vesicles distributed throughout the cell, and accumulates at the extremities of long protrusions emanating from the cytoplasm (inset). **(C)** RAW 264.7 macrophages expressing a FLAG-Syt XI-GFP construct. Results are representative of at least three independent experiments.

4.2 Syt XI localizes to recycling endosomes and lysosomes

Syt XI associates with the Golgi apparatus of non-stimulated PC12 cells (Fukuda *et al.*, 2001), and with the perinuclear region and neurite tips of nerve grown factor (NGF)-treated PC12 cells (Huynh *et al.*, 2003). We hypothesized that Syt XI could have a similar vesicular distribution in macrophages. We employed confocal immunofluorescence microscopy to image resting macrophages co-stained for Syt XI, DNA, and for established markers of lipid rafts (Ctx) (Chazotte, 2011), early/recycling endosomes (Tfr1) (Chavrier *et al.*, 1990), early endosomes (EEA1) (Mu *et al.*, 1995), and lysosomes (LAMP-1) (Chavrier *et al.*, 1990). In BMM, we observed that Syt XI colocalized mostly with Tfr1 and LAMP-1 (Fig. 2A), indicating that Syt XI was present in recycling endosomes and lysosomes. A very similar colocalization pattern was observed for FLAG-Syt XI-GFP in RAW 264.7 macrophages (Fig. 2B). These findings suggested a possible role for Syt XI in the processes of phagocytosis and cytokine secretion, since recycling endosomes traffic cytokines to the cell surface (Manderson *et al.*, 2007, Stow *et al.*, 2009), while providing membrane for phagosome formation (Bajno *et al.*, 2000, Manderson *et al.*, 2007, Stow *et al.*, 2009); on the other hand, lysosomes also provide membrane and antimicrobial effectors to the maturing phagosome (Binker *et al.*, 2007, Czibener *et al.*, 2006, Huber *et al.*, 1981).

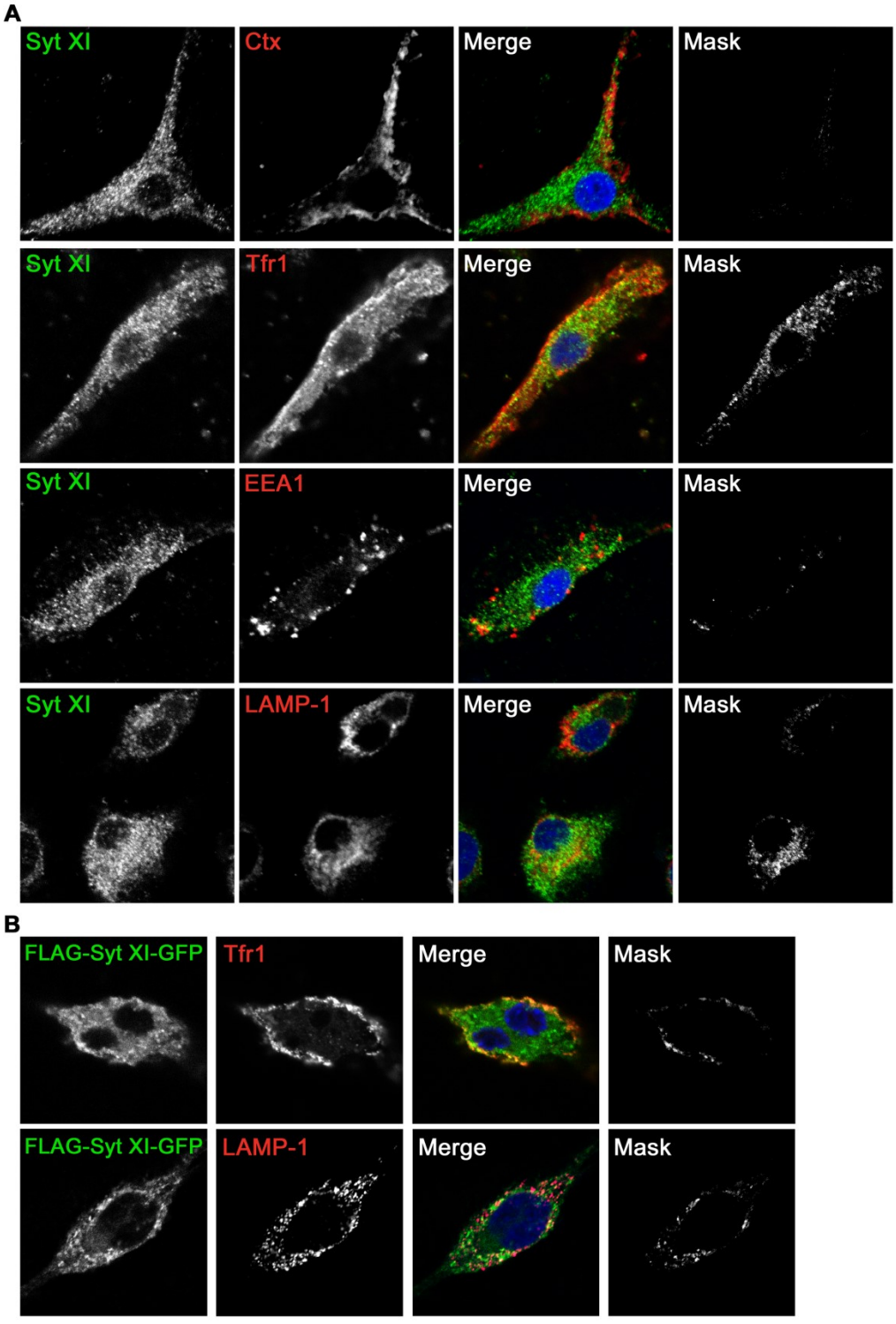

Figure 2 of article 1. Syt XI associates with recycling endosomes and lysosomes. In resting BMM, Syt XI (green) colocalized extensively with Tfr1 and LAMP1 (red), which are markers of early/recycling endosomes and lysosomes, respectively. **(A)** Resting BMM were fixed, immunostained with the antibody combinations shown, and stained with Ctx and DRAQ5 to demarcate lipid rafts (red) and nuclei (blue), respectively. **(B)** RAW 264.7 macrophages stably expressing the FLAG-Syt XI-GFP construct were fixed and stained with the indicated antibodies. Cells were visualized by confocal immunofluorescence microscopy. Panels showing colocalized pixels (mask) are shown. Results are representative of at least three independent experiments.

4.3 Phagosomes recruit Syt XI

The involvement of Syts V and VII in phagocytosis (Czibener *et al.*, 2006, Vinet *et al.*, 2008), and the finding that Syt XI is found in recycling endosomes and lysosomes, prompted us to investigate the association of Syt XI to phagosomes. To assess phagosomal recruitment of Syt XI, we isolated magnetic bead-phagosomes from RAW 264.7 macrophages (Lutz *et al.*, 1993). Purified phagosomes were lysed, and protein extracts were analysed by Western blot (Fig. 3A). We observed that Syt XI was enriched in phagosomes 1 h post-internalization. However, Syt XI levels decreased after 4 h of bead internalization. To further elucidate the phagosomal recruitment of Syt XI, we incubated BMM with opsonised zymosan at a ratio of 10 particles to 1 macrophage. Macrophages were incubated at 4ºC to synchronize phagocytosis prior to particle internalization at 37ºC for the indicated time points. Cells were then fixed, stained for Syt XI and DNA, and imaged by confocal microscopy. We observed that 15 min post-internalization, 55.7% (±1) of phagosomes were positive for Syt XI, whereas 1 h post-internalization, 27.6% (±2.4) of phagosomes were positive (Fig. 3B). In addition, we observed that Syt XI colocalized with recycling endosomes at the phagocytic cup (Fig. 3C). Given the crucial roles of recycling endosomes in phagocytosis and cytokine secretion (Murray *et al.*, 2005a, Stow *et al.*, 2009), this observation strengthened our hypothesis about a role for Syt XI in these processes. Together, these data showed that Syt XI is recruited to early phagosomes, and that such recruitment diminishes with time.

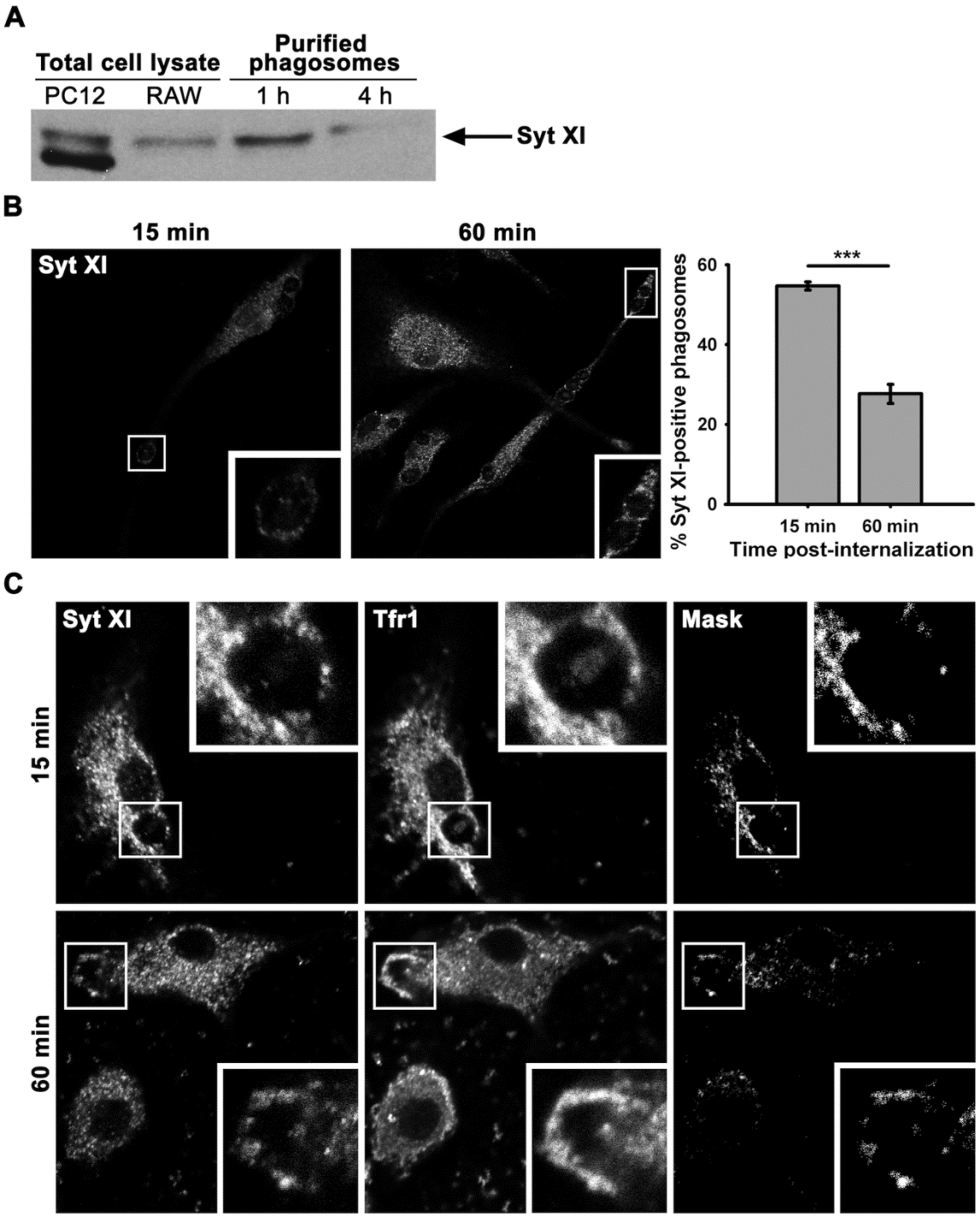

Figure 3 of article 1. Syt XI is recruited to early phagosomes. (A) RAW 264.7 macrophages are **temporarily** enriched in Syt XI, 1 h after phagocytosis. We performed synchronized phagocytosis on RAW 264.7 macrophages with magnetic beads, and isolated phagosomes by magnetic force. PC12 lysate was used as positive control; the lower band in the PC12 lane is non-specific. Phagosomal fractions were analyzed for Syt XI content via Western blot. **(B)** In BMM, recruitment of Syt XI to phagosomes containing opsonised zymosan (10:1 ratio) occurred early and decreased significantly 1 h post-internalization, as evidenced by confocal immunofluorescence microscopy. Recruitment was quantified on the rightmost panel of **(B)**. **(C)** Syt XI colocalized with Tfr1-positive recycling endosomes at the phagosome 15 min and 1 h post-internalization. This experiment was repeated twice in triplicate, and quantifications were performed for 100 phagosomes per triplicate. ***, $p \leq 0.001$.

4.4 Knockdown of Syt XI leads to an increase in cytokine secretion and phagocytosis

Given that Syt XI is found in recycling endosomes and that it is recruited to the phagosome, we set out to assay the role of Syt XI in the secretion of TNF and IL-6, and in phagocytosis. To this end, we knocked down Syt XI by transfecting RAW 264.7 macrophages with a pool of three siRNAs. This resulted in a ~70% decrease in Syt XI levels in total cell lysates, and in decreased immunostaining (Fig. 4A and B). After siRNA transfection, we stimulated macrophages with LPS for various time points, and quantified the secretion of TNF and IL-6 via ELISA analysis of culture supernatants. SiRNA-mediated knockdown of Syt XI resulted in a notable increase in TNF and IL-6 secretion, especially after 12 h of stimulation (Fig. 4C and D). To assay phagocytosis, siRNA-treated macrophages were synchronously fed with opsonised zymosan particles in various proportions. Syt XI knockdown led to an increased percentage of macrophages ingesting at least one particle, regardless of the cell/particle ratio used (Fig. 4E). These data demonstrate that decreased Syt XI levels lead to an increase in cytokine secretion from LPS-stimulated macrophages, and to augmented phagocytosis.

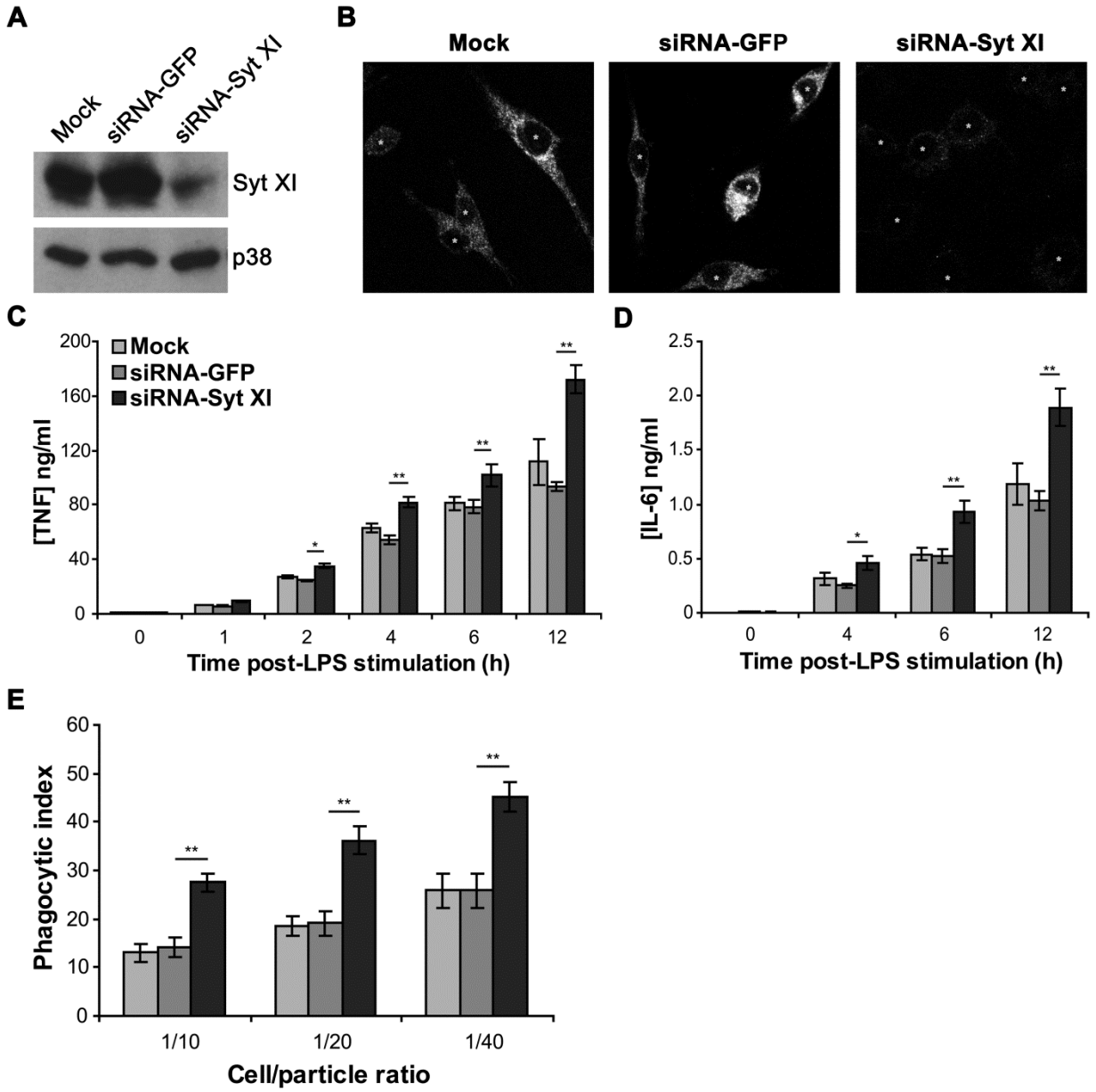

Figure 4 of article 1. Knockdown of Syt XI leads to increased cytokine secretion and phagocytosis. Knockdown of Syt XI in RAW 264.7 macrophages was evaluated by Western blot, and by confocal immunofluorescence. RAW 264.7 macrophages were subjected to reverse transfection with Lipofectamine RNAiMAX, lysed, and Syt XI levels were analyzed by Western blot **(A)**. Also, macrophages were fixed, immunostained for Syt XI, and visualized by confocal immunofluorescence microscopy **(B)**. White asterisks denote the location of cells in the panels. **(C, D)** Transfected macrophages were stimulated with LPS for the indicated time points, and cytokine secretion in culture supernatants was assessed by ELISA. Results represent the mean of two independent experiments done in triplicate. **(D)** Synchronized phagocytosis with different ratios of opsonised zymosan was performed after transfection. After 10 min of particle internalization, macrophages were washed and stained via the HEMA-3 kit. The percentage of macrophages with at least one ingested particle was quantified for at least 100 macrophages via light microscopy. Results represent the mean of three independent experiments done in triplicate. *, $p \leq 0.05$; **, $p \leq 0.01$.

4.5 Overexpression of Syt XI leads to a decrease in cytokine secretion and phagocytosis

To confirm the results that we obtained by employing siRNA, we overexpressed Syt XI in RAW 264.7 macrophages. We constructed stable cell lines expressing FLAG-Syt XI-GFP in the pCIN4 vector, and expression was assessed by analysis of total cell lysates by Western blot, and by immunofluorescence (Fig. 5A and 1C). For subsequent experiments, we used two independent clones (G and H) to eliminate possible clonal effects. LPS stimulation of macrophages overexpressing FLAG-Syt XI-GFP led to a marked decrease in TNF and IL-6 secretion (Fig. 5B and C). Moreover, when these macrophages were synchronously fed with opsonised zymosan particles, a decreased phagocytic index was observed (Fig. 5D). Together with the siRNA data, our findings indicate that Syt XI negatively regulates the processes of TNF and IL-6 secretion, and phagocytosis.

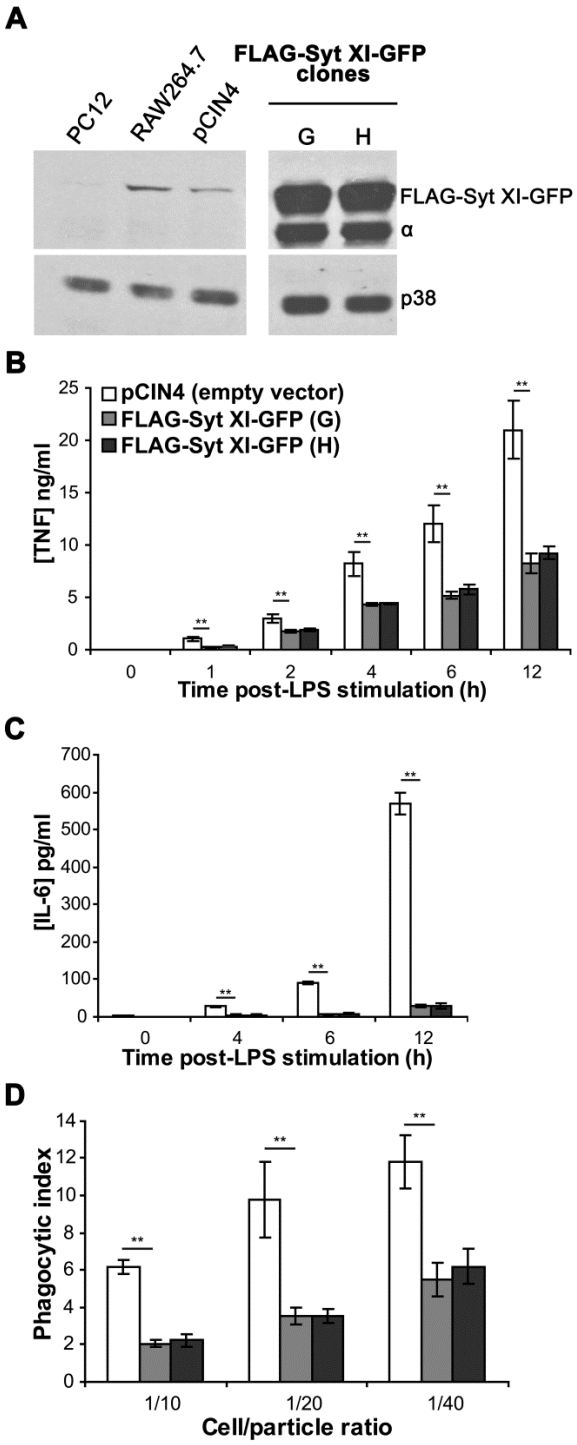

Figure 5 of article 1. Overexpression of Syt XI leads to decreased cytokine secretion and phagocytosis. (A) RAW 264.7 macrophages were stably transfected with the FLAG-Syt XI-GFP construct in the pCIN4 vector. Expression of this construct was assessed via Western blot, using an antibody recognizing GFP. The bands under the RAW 264.7 and pCIN4 (empty) lanes are non-specific, and 'α' denotes degradation products. In both FLAG-Syt XI-GFP clones, the expression pattern is similar as visualized by confocal microscopy (see Fig. 1C). **(B, C)** Stably transfected macrophages were stimulated with LPS for the indicated durations. ELISA was used to quantify cytokine secretion in culture supernatants. **(D)** Opsonised zymosan, in different proportions, was fed to stably transfected

macrophages. Ten minutes post-internalization, particle internalization was quantified for at least 100 macrophages. All results shown are the mean of three independent experiments done in triplicate.

4.6 Syt XI regulates the recruitment of gp91^{phox} and LAMP-1 to the phagosome

After having established that Syt XI was a negative regulator of particle uptake in macrophages, we hypothesized that the absence of Syt XI could also affect the recruitment of proteins that affect phagosomal maturation. Two of these proteins, namely gp91^{phox} and LAMP-1, affect the ability of the phagosome to produce ROS and acquire lysosomal characteristics, respectively. Interestingly, Syt XI colocalized with both of the aforementioned proteins in the cytoplasm, as well as in contouring phagosomes of BMM (Fig. 6A and B). These observations prompted us to assay the effect of Syt XI knockdown on the recruitment of gp91^{phox} and LAMP-1 to the phagosome. After siRNA treatment of RAW 264.7 macrophages, synchronized phagocytosis with opsonised zymosan was performed and cells were fixed, stained, and visualized by confocal microscopy. After quantification, we found that cells treated with Syt XI siRNA recruited significantly less gp91^{phox} and LAMP-1 to the phagosome (Fig. 6C and D). Given that gp91^{phox} and LAMP-1 have profound effects on the microbicidal capacity of the phagosome (Binker *et al.*, 2007, Lam *et al.*, 2010), our findings indicated that Syt XI could have a direct impact on the ability of macrophages to control intracellular pathogens.

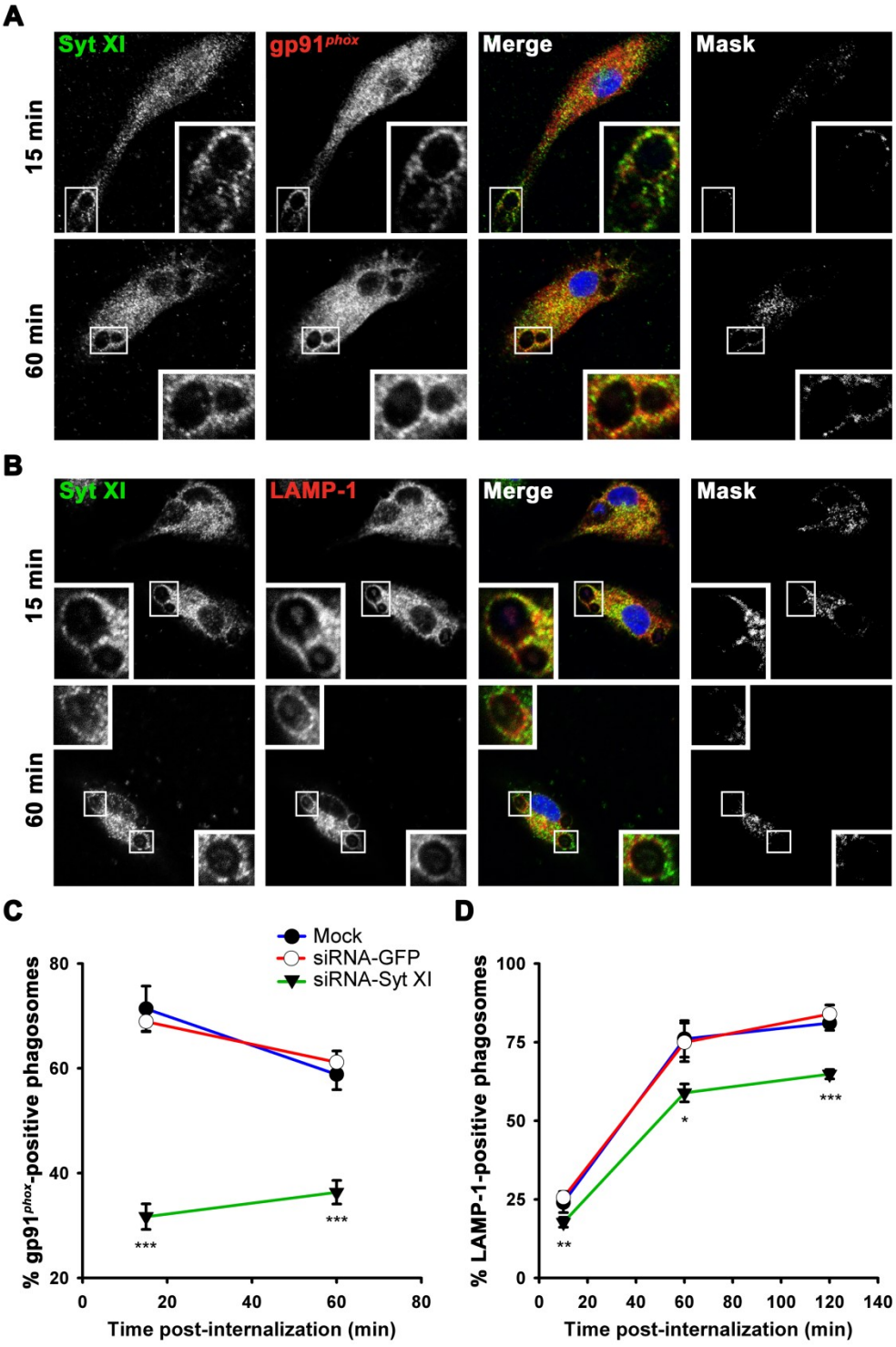

Figure 6 of article 1. Syt XI regulates the recruitment of gp91^{phox} and LAMP-1. (A, B) Syt XI colocalizes with gp91^{phox} and LAMP-1 in phagosomes. By confocal immunofluorescence microscopy, we found that Syt XI (green) colocalized with gp91^{phox} and LAMP-1 (red) in resting BMM and in BMM with zymosan phagosomes. Colocalization was observed in the cytoplasm and on phagosomes (see insets and colocalization masks). RNAi-mediated knockdown of Syt XI leads to decreased recruitment of gp91^{phox} and LAMP-1 to phagosomes. After siRNA treatment, RAW 264.7 macrophages were fed with zymosan, fixed, stained with a gp91^{phox} or LAMP-1 antibody and the number

of gp91^{phox}- **(B)**, or LAMP-1-positive **(C)** phagosomes was quantified. Experiments were done twice in triplicate and quantifications were performed for 100 phagosomes per triplicate. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

4.7 Knockdown of Syt XI leads to increased intracellular survival of *E. coli*

Syt XI knockdown led to an increase in phagocytosis, and to phagosomes that showed decreased recruitment of gp91^{phox} and LAMP-1. These observations prompted us to hypothesize that macrophages with decreased Syt XI levels formed phagosomes with a weakened capacity to kill intracellular pathogens. To test this hypothesis, mock or siRNA-treated macrophages were infected with non-opsonised *E. coli* DH1. After infection, cells were lysed, and diluted lysates were plated in agar plates. After the zero time point, we found that there were significantly more ($p < 0.01$) CFU from macrophages treated with siRNA to Syt XI compared with macrophages treated with siRNA to GFP (Fig. 7). This is probably due to an initial increase in phagocytosis as observed for zymosan-fed macrophages (Fig. 4E). Remarkably, macrophages treated with siRNA to Syt XI were not able to clear *E. coli* 4 h post-infection (Fig. 7); indeed, a small increase in the number of CFU was observed from the zero to the 4 h time point. We concluded that Syt XI regulates the microbicidal activity of the phagosome.

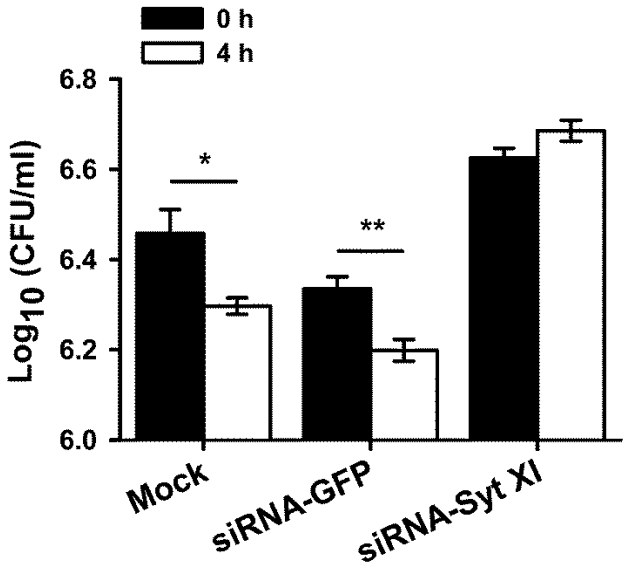

Figure 7 of article 1. Knockdown of Syt XI lowers the microbicidal activity of macrophages. Control or siRNA-treated **macrophages** were incubated with *E. coli* in a ratio of 20 bacteria to 1 macrophage. After washing, cells were

incubated with DMEM containing gentamicin for 20 min (0 h time point) or for an additional 4 h. After each time point, cells were washed and lysed; lysates were diluted and plated in agar plates. Macrophage bactericidal activity was evaluated by counting CFU. Results represent the average of two independent experiments done in triplicate. *, $p \leq 0.05$; **, $p \leq 0.01$.

5 DISCUSSION

In the present investigation, we characterized a member of the Syt family whose function had not been previously elucidated. Using macrophages, we determined that Syt XI is a vesicular protein that modulates negatively the secretion of TNF and IL-6, and phagocytosis. Though particle uptake is increased upon Syt XI knockdown, the phagosomes that are formed recruit defectively markers that are necessary for microbicidal activity in phagosomes. We showed that such phagosomes are less adept at killing bacteria. Collectively, our results identify Syt XI as a novel regulator of macrophage function.

The finding that Syt XI, and not Syt IV, is expressed in macrophages implies that only one Syt of this subfamily is required to exert a negative regulatory function in macrophage vesicular trafficking. In contrast, PC12 cells express both Syt IV and Syt XI; though Syt IV has been characterized (Dean *et al.*, 2009), the function of Syt XI in neuronal cells remains unknown. Association of Syt XI to recycling endosomes and lysosomes was an indication that Syt XI was possibly implicated in cytokine secretion and phagocytosis, since recycling endosomes are an important distributing point for the passage of cytokines to the cell surface, and both recycling endosomes and lysosomes contribute membrane to the developing phagosome (Stow *et al.*, 2009). Recruitment of Syt XI to the phagosome also prompted us to determine Syt XI's role in phagocytosis. Syt XI was present more abundantly during the early stages of phagocytosis, which implicates Syt XI in early phagocytic events. We employed two complementary approaches to determine that Syt XI was a negative regulator of both cytokine secretion and phagocytosis. Knockdown of Syt XI via siRNA showed that decreased Syt XI expression led to augmented TNF and IL-6 secretion from stimulated macrophages, especially after 12 h of stimulation; phagocytosis was increased as well. The effect of Syt XI on phagocytosis did not depend on particle load, in contrast to Syt V and Syt VII (Czibener *et al.*, 2006, Vinet *et al.*, 2008). To further substantiate these results, overexpression of a Syt XI construct diminished levels of secretion and phagocytosis. The role of Syt XI in macrophage trafficking resembles the negative role that Syt II has

on the Ca^{2+}-dependent exocytosis of lysosomes (Baram *et al.*, 1999), and on major histocompatibility complex (MHC) class II presentation in mast cells (Baram *et al.*, 2002). Though Syt XI and Syt II belong to different Syt subfamilies that have different properties, both of these Syts negatively regulate exocytic processes in cells of the innate immune system. It would be worthwhile investigating whether Syt XI modulates degranulation and cytokine secretion in other cells of the immune system. In addition, the role of Syt XI parallels the role of Syt IV as a negative regulator of brain-derived neurotrophic factor secretion (Dean *et al.*, 2009). This is expected, given the close similarity that exists between Syt XI and Syt IV (von Poser *et al.*, 1997). It has been shown that Syt XI inhibits vesicle fusion and seems to lack SNARE binding activity (Tucker *et al.*, 2003, Wang *et al.*, 2010). At the vesicle fusion step, Syt XI could act as a break that slows down the fusion of TNF- and IL-6-containing vesicles with the plasma membrane. Absence of Syt XI could lead to premature and uncontrolled release of TNF and IL-6, which could contribute, in part, to diseases where the underlying pathology includes excess release of these cytokines (Beutler, 1999, Nishimoto *et al.*, 2004).

There are a number of possible mechanisms by which Syt XI could negatively regulate vesicular trafficking. It has been shown that delivery of TNF-containing vesicles requires the fusion of VAMP3-bearing vesicles with cognate SNAREs, such as Syntaxin-4, at the plasma membrane and at the phagocytic cup (Murray *et al.*, 2005a). In light of the fact that Syt XI inhibits Ca^{2+}-triggered vesicle fusion in liposome fusion assays, Syt XI may hinder the formation of SNARE complexes by sterically hindering interactions between Q- and R-SNAREs. In addition, Syt XI could affect the recycling of vesicles once they fuse to the plasma membrane. The NSF-SNAP complex, which aids in vesicle recycling by dissociating SNARE complexes (Südhof *et al.*, 2011), could be regulated by a mechanism where Syt XI delays the liberation of SNARE complexes and thence slow down vesicle traffic. In addition, it has been shown that Syt XI acts as a receptor for the molecular motor KIF1A in neurons (Park *et al.*, 2005). If KIF1A – or a related motor – is involved in exocytosis of cytokine-containing vesicles, or in the focal delivery of membrane to phagosomes, Syt XI could act as a negative regulator of KIF1A-mediated delivery of vesicles. Additionally, it is possible that Syt XI may bind to other molecular

motors in macrophages. Since Syt XI has not yet been found to bind SNAREs, a mechanism for Syt XI function on cytokine secretion action could also depend on the regulation of signalling pathways that regulate cytokine production and release. Future research will determine how Syt XI regulates vesicular trafficking associated to phagocytosis and cytokine secretion.

Aside from augmenting phagocytosis, absence of Syt XI led to the formation of phagosomes that failed to acquire phagolysosomal features. gp91^{phox} and LAMP-1 are recruited to phagosomes from recycling endosomes and lysosomes, respectively (Casbon *et al.*, 2009, Huynh *et al.*, 2007a). We found that both gp91^{phox} and LAMP-1 colocalize with Syt XI on endosomes, lysosomes, and phagosomes. In the absence of Syt XI, we showed that recruitment of both gp91^{phox} and LAMP-1 to phagosomes was impaired, indicating that Syt XI participates in the control of phagolysosome biogenesis. Consistent with a role for Syt XI in the control of phagosome maturation, we observed that the ability of macrophages to kill *E. coli* was reduced in the absence of Syt XI. It is possible that Syt XI partners with other vesicular regulators to modulate the delivery of recycling endosomes and lysosomes containing gp91^{phox} and LAMP-1 to the phagosome. Future experiments will address the mechanisms by which Syt XI regulates the delivery of effectors to phagosomes.

Several intracellular microbes live in remodelled vacuoles that promote their survival and propagation (Alix *et al.*, 2011, Flannagan *et al.*, 2009). Many of these pathogens have evolved strategies to impede phagosome maturation by impeding the formation of ROS or by preventing acquisition of lysosomal effectors to the phagosome (Alix *et al.*, 2011, Flannagan *et al.*, 2009, Lodge *et al.*, 2008, Sibley, 2011). Our findings suggest that Syt XI could be a target for pathogens that have the capacity to exclude Syt XI from their phagosome. Parasites of phagocytes could destroy Syt XI to provoke an overproduction of cytokines that would then attract more phagocytes and facilitate parasite dissemination. Absence of Syt XI may also contribute to pathogen survival by

impeding recruitment of phagosomal effectors such as gp91^{phox} and LAMP-1 and thus prevent killing of the microorganism.

In summary, our results establish Syt XI as a novel negative regulator of cytokine secretion and phagocytosis. Moreover, Syt XI controls phagolysosome biogenesis, likely by modulating the delivery of antimicrobial effectors to the phagosome. Due to the similarity between Syt XI and Syt IV, it is reasonable to infer that Syt XI exerts analogous functions in the trafficking of other cells, including those of the immune system.

6 ACKNOWLEDGMENTS

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ARTICLE NO. 2: *LEISHMANIA* PROMASTIGOTES INDUCE CYTOKINE SECRETION IN MACROPHAGES THROUGH THE DEGRADATION OF SYNAPTOTAGMIN XI

Leishmania promastigotes induce cytokine secretion in macrophages through the degradation of Synaptotagmin XI

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Author contributions: conceived and designed the experiments: GAD and AD. Performed the experiments: GAD (90%), SS and AD. Analyzed the data: GAD, SS and AD. Contributed reagents/materials/analysis tools: MF, ST and SS. Wrote the paper: GAD and AD.

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1 ABSTRACT

Synaptotagmins (Syts) are type-I membrane proteins that regulate vesicle docking and fusion in processes such as exocytosis and phagocytosis. We recently discovered that Syt XI is a recycling endosome- and lysosome-associated protein that negatively regulates the secretion of TNF and IL-6. Here, we show that Syt XI is directly degraded by the zinc metalloprotease GP63 and excluded from *Leishmania* parasitophorous vacuoles by the promastigotes surface glycolipid lipophosphoglycan. Infected macrophages were found to release TNF and IL-6 in a GP63-dependent manner. To demonstrate that cytokine release was dependent on GP63-mediated degradation of Syt XI, siRNA-mediated knockdown of Syt XI before infection revealed that the effects of siRNA knockdown and GP63 degradation were not cumulative. In mice, intraperitoneal injection of GP63-expressing parasites led to an increase in TNF and IL-6 secretion and to an augmented influx of neutrophils and inflammatory monocytes to the inoculation site. Both of these cell types have been shown to be infection targets and aid in the establishment of infection. In sum, our data revealed that GP63 induces proinflammatory cytokine release and increases infiltration of inflammatory phagocytes. This study provides new insight on how *Leishmania* exploits the immune response to establish infection.

2 INTRODUCTION

Parasites of the *Leishmania* genus are the causative agents of the leishmaniases, a group of human neglected parasitic diseases endemic in many regions of the world (Alvar *et al.*, 2012). The life cycle of *Leishmania* is digenetic. Promastigote forms are inoculated into the mammalian host by infected sand flies and are ingested by phagocytes, where they form parasitophorous vacuoles (PV^3). Phagocytes are crucial for both immunity and development; they ingest apoptotic bodies, foreign particles, destroy microbes, and present antigens (Huber *et al.*, 1981, Huynh *et al.*, 2007b). However, many pathogens have evolved the ability of using phagocytosis to hide from the immune system and replicate intracellularly (Alix *et al.*, 2011, Flannagan *et al.*, 2009). Macrophages and other phagocytes also secrete a panoply of pleiotropic cytokines – such as TNF and IL-6 – that are responsible for establishing an effective immune response, and for linking innate and adaptive immunity (Huynh *et al.*, 2007b, Stow *et al.*, 2009, Unanue *et al.*, 1976). Within phagocytic cells, promastigotes differentiate into amastigotes that replicate in phagolysosomes. Infected phagocytes are characterized by a deactivation of their effector functions, which may contribute to the suppression of cell-mediated immunity observed during leishmaniasis. *Leishmania* parasites have the capacity of subverting phagocytosis (Turco *et al.*, 1992) and of modulating cytokine secretion (Matte *et al.*, 2002), allowing the parasite to thrive within phagocytic cells and within the organism as a whole.

A salient feature of *Leishmania* parasites is their capacity to alter phagocyte biology through pathogenicity factors such as lipophosphoglycan (LPG) and the zinc metalloprotease GP63, both of which are predominant at the promastigote stage of the parasite (Joshi *et al.*, 2002, Moradin *et al.*, 2012, Olivier *et al.*, 2012, Turco *et al.*, 1992). GP63 cleaves proteins involved in the regulation of phagocyte functions (Yao, 2010), leading to altered cell signalling, to subversion of transcription and translation (Contreras *et al.*, 2010, Gómez *et al.*, 2009, Hallé *et al.*, 2009, Jaramillo *et al.*, 2011), antigen cross-presentation (Matheoud *et al.*, 2013), lipid metabolism (Descoteaux *et al.*, 2013, Ghosh *et al.*, 2013), and likely to other unknown effects. LPG is a complex

glycophospholipid that supports parasite survival both in the sand fly gut and in the phagosome (Descoteaux *et al.*, 2002b, Turco *et al.*, 1992). In the macrophage phagosome, LPG inhibits phagolysosomal biogenesis through alteration of membrane fusogenic properties (Desjardins *et al.*, 1997). In this regard, we recently reported that *Leishmania* targets Synaptotagmin (Syt) V – a regulator of particle uptake and phagosome maturation – by impeding the recruitment of this Syt to phagocytic cups via LPG (Vinet *et al.*, 2009, Vinet *et al.*, 2011). Syts constitute a large family of membrane-associated proteins that regulate vesicle-associated processes ranging from exocytosis (Arango Duque *et al.*, 2013, Pang *et al.*) to phagocytosis (Beutler, 1999, Vinet *et al.*, 2008). Due to the roles of Syts in vesicle trafficking, they are attractive targets for pathogens. For instance, the parasite *Trypanosoma cruzi* uses Syt VII, a Ca^{2+}-dependent regulator of lysosome exocytosis, to invade target cells (Caler *et al.*, 2001). In the case of Syt V, abrogation of Syt V recruitment by LPG results in the exclusion of the V-ATPase and in reduced phagosomal acidification (Vinet *et al.*, 2009).

During phagocytosis, both IL-6 and TNF are shepherded to the macrophage surface and to the site of phagocytic cup formation via a highly orchestrated pathway that is directed by members of the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) family. From the Golgi apparatus, SNARE proteins Syntaxin-6, -7, and Vti1b control trafficking to recycling endosomes, and thence, the vesicle-associated membrane protein 3 (VAMP3) guides trafficking of TNF- and IL-6-containing vesicles from recycling endosomes to the cell surface (Murray *et al.*, 2005a, Stow *et al.*, 2009). A pivotal function of these two proinflammatory cytokines is to help recruit phagocytes to the inflammation site through modulation of chemokine release and expression of adhesion molecules (Biswas *et al.*, 1998, Griffin *et al.*, 2012, Jones *et al.*, 2006, Vieira *et al.*, 2009). Inoculation of *L. major* promastigotes has been shown to be followed by the infiltration of neutrophils and inflammatory monocytes. Both of these cell types are infection targets and important for the establishment of infection (Gonçalves *et al.*, 2011, Peters *et al.*, 2008, Ribeiro-Gomes *et al.*, 2014). Syts are regulators of SNARE complex activity and play a role in cytokine release. Indeed, we recently reported that Syt XI, which inhibits vesicle fusion (Wang *et al.*, 2010), dampens the

secretion of both TNF and IL-6 (Arango Duque *et al.*, 2013). Interestingly, it has been demonstrated that *L. major* promastigotes induce the secretion of TNF and IL-6 following their internalization by macrophages (Arena *et al.*, 1997, Karam *et al.*, 2006, Lapara *et al.*, 2010, Matte *et al.*, 2002, Wenzel *et al.*, 2012). This raises the possibility that these parasites could disrupt membrane fusion regulators to induce proinflammatory cytokine release. Given the inhibitory role of Syt XI in cytokine secretion (Arango Duque *et al.*, 2013), we hypothesized that *Leishmania* could target Syt XI to facilitate release of TNF and IL-6 post-infection. This may then regulate the infiltration of inflammatory phagocytes to the infection site.

In this research, we demonstrate that in macrophages, Syt XI was excluded from PVs via LPG and degraded by *Leishmania* promastigotes in a GP63-dependent fashion, leading to increased secretion of TNF and IL-6. Furthermore, we show that in early infection, GP63 augmented *in vivo* TNF and IL-6 release, as well as the accrual of neutrophils and inflammatory monocytes. These data indicate that GP63 enables *Leishmania* promastigotes to elicit cytokine release and inflammatory phagocyte recruitment to the site of infection, both of which may contribute to the establishment of infection.

3 MATERIALS AND METHODS

3.1 Ethics statement

Experiments involving mice and hamsters were done as prescribed by protocols 1312-03 and 1302-02, respectively, which were approved by the *Comité Institutionel de Protection des Animaux* of the INRS-Institut Armand-Frappier. These protocols respect procedures on good animal practice provided by the Canadian Council on animal care.

3.2 Antibodies and plasmids

The mouse monoclonal anti-LPG (CA7AE) antibody (Tolson *et al.*, 1989) was from Cedarlane and the mouse monoclonal antibody anti-GP63 was a gift from Dr. W. R. McMaster (University of British Columbia) (Button *et al.*, 1993, Macdonald *et al.*, 1995b). The rabbit polyclonal antibody targeting the C2A domain of Syt XI was purified by affinity chromatography (Fukuda *et al.*, 1999). Rabbit polyclonal antibodies anti-p38 and -GFP were obtained from Cell Signalling and Santa Cruz, respectively. For flow cytometrical analysis of cellular infiltrates, the following anti-mouse antibodies recognizing various antigens were used: CD11c–APC (clone HL3), CD11b-biotin (clone MI/70), MHCII-FITC (clone 2G9), and Gr1-PE (clone RB6-8C5) (all from BD Biosciences). Streptavidin V450 was from eBioscience. The anti-mouse CCR2-AlexaFluor 700 and anti-CD-23-PerCp-Cy5 (clone B3B4) antibodies were obtained from R&D Systems and BioLegend, respectively.

To express GST-Syt XI-cyto in *Escherichia coli*, the Syt XI-cyto construct was cloned into the *Bam*HI/*Not*I site of the pGEX-4T3 vector (Amersham Bioscience) as described (Itoh *et al.*, 2006).

3.3 Cell culture

The mouse macrophage cell line RAW264.7 and RAW264.7 cells expressing the FLAG-Syt XI-GFP construct (Arango Duque *et al.*, 2013) were cultured in complete DMEM with L-glutamine (Life Technologies) and complemented with 10% heat-inactivated foetal bovine serum (FBS) (PAA Laboratories), 10 mM HEPES (Bioshop) at pH 7.4, and antibiotics (Life Technologies) in a 37ºC incubator with 5% CO_2. Bone marrow-derived macrophages (BMM) were extracted from the bone marrow (Descoteaux *et al.*, 1989) of 6- to 8-week old female BALB/c mice (Charles River), and differentiated with complete DMEM supplemented with 15% v/v L929 cell-conditioned medium (LCM) as a source of colony-stimulating factor 1. To render BMM quiescent prior to experiments, cells were transferred to tissue-culture treated petris and kept for 16 h in complete DMEM without LCM.

Promastigotes were cultured in *Leishmania* medium (medium 199 (Sigma) with 10% heat-inactivated FBS, 40 mM HEPES at pH 7.4, 100 µM hypoxanthine, 5 µM hemin, 3 µM biopterin, 1 µM biotin, and antibiotics), in a 26ºC incubator with 5% CO_2. *L. major* NIH S clone A2 promastigotes (WT, Δ*gp63*, and Δ*gp63*+*gp63*) were kindly provided by Dr. W. R. McMaster (University of British Columbia), and *L. tarentolae* Parrot-TarII promastigotes were a kind gift from Dr. B. Papadopoulou (Université Laval). Amastigotes of the LV9 strain of *L. donovani* were extracted from the spleens of infected female HsdHan:AURA hamsters (Harlan Sprague Dawley Inc.) as previously described (Reiner, 1982). Incubation in *Leishmania* medium at pH 7.4, 26ºC, for 10-15 days was used to differentiate LV9 amastigotes into promastigotes. The Δ*gp63*+*gp63 L. major* and Δ*lpg2 L. donovani* LV9 promastigotes were grown in *Leishmania* medium supplemented with 50 µg/ml G418 (Life Technologies), or 100 µg/ml hygromycin (Roche), respectively. Prior to infections, promastigotes in late stationary phase were opsonised with BALB/c mouse serum (Vinet *et al.*, 2009).

3.4 Transfections, infections and cytokine quantification

For small interfering RNA (siRNA) transfections, RAW264.7 macrophages were plated in the absence of antibiotics for 16 h on glass coverslips (Fisher), or on 24-well plates. Then, macrophages were subjected to two rounds of transfection with Oligofectamine (Life Technologies), with each transfection being 24 h apart (Murray *et al.*, 2005a). Macrophages were mock-transfected, transfected with siRNA to GFP (Flandin *et al.*, 2006), or with the ON-TARGETplus SMARTpool siRNA to Syt XI (Thermo Scientific), which contains four siRNA with the following sequences: Sequence 1: CGAUCGACUACUAAGAAU. Sequence 2: GAGAGAGGUCUGCGAGAGU. Sequence 3: AUGUCAAGGUGAACGUCUA. Sequence 4: GCACAGUCUGAGCGAGUAC. BLAST searches were performed to confirm that these sequences targeted only the Syt XI mRNA. After the second transfection, macrophages were cultured for 24 h prior to stimulation or infection.

Using 24-well plates and a final volume of 300 µl, $3x10^5$ adherent BMM or RAW264.7 macrophages were stimulated with 0.2 ng/ml LPS from *E. coli* Serotype O127:B8 (Sigma), or infected with opsonised *L. major*, *L. tarentolae*, or *L. donovani* promastigotes or amastigotes. After stimulation or infection, cell culture supernatants were collected and centrifuged to remove non-internalized parasites and debris. Enzyme-linked immunosorbent assay (ELISA) kits were used as per the manufacturers' protocols to quantify murine IL-6 (BD OptiEIA, BD Biosciences) and TNF (Ready-SET-Go! Mouse TNFα Kit, eBiosciences) secretion.

3.5 Synchronized phagocytosis assays

For synchronized phagocytosis using zymosan or parasites, macrophages were incubated at 4ºC for 15 min (Arango Duque *et al.*, 2013). Macrophages were washed with cold complete DMEM to remove excess particles, and internalization was triggered by transferring cells to 37ºC for the required times (Vinet *et al.*, 2008). Cells were then

washed with PBS and prepared for confocal immunofluorescence microscopy. Zymosan was either opsonised with mouse serum (Lodge *et al.*, 2006b), or coated (Vinet *et al.*, 2009, Vinet *et al.*, 2011) with purified *L. donovani* LPG (Orlandi *et al.*, 1987, Russo *et al.*, 1992).

3.6 Confocal immunofluorescence microscopy

Macrophages on coverslips were fixed with 2% paraformaldehyde (Canemco and Mirvac) for 7 min and blocked and permeabilized for 17 min with a solution of 0.1% Triton X-100, 1% BSA, 6% non-fat milk, 2% goat serum, and 50% FBS. This was followed by a 2 h incubation with primary antibodies diluted in PBS. Then, macrophages were incubated with a suitable combination of secondary antibodies (anti-rabbit AlexaFluor 488, anti-rat 568, and anti-mouse 568; Molecular Probes) and DRAQ5 (Biostatus). Coverslips were washed three times with PBS after every step. After the final washes, Fluoromount-G (Southern Biotechnology Associates) was used to mount coverslips on glass slides (Fisher), and coverslips were sealed with nail polish (Sally Hansen). Macrophages were imaged with the 63X objective of an LSM780 microscope (Carl Zeiss Microimaging), and images were taken in sequential scanning mode. Image analysis was performed with the ZEN 2011 software. For fluorescence intensity profiles, a 1 pixel line was traced around phagocytic cups using the DIC (TPMT) channel as guide; the same microscope settings were used for all conditions.

3.7 Lyses, SDS-PAGE and Western blotting

Prior to lysis, adherent macrophages or parasites were placed on ice and washed with PBS containing 1 mM sodium orthovanadate and 2 mM 1,10-Phenanthroline. Cells were scraped in the presence of lysis buffer containing 1% NP-40, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8), 2 mM 1,10-Phenanthroline, and phosphatase and protease inhibitors (Roche). Alternatively, macrophages were lysed in 8 M Urea

supplemented with 400 U/ml Benzonase® Nuclease (Qiagen) (Chen *et al.*, 2012). After incubation at -70°C, lysates were centrifuged for 10 min to remove insoluble matter. After protein quantification, 30 µg of sample was boiled (100ºC) for 6 min in SDS sample buffer and migrated in 10% SDS-PAGE gels. Proteins were transferred onto Hybond-ECL membranes (Amersham Biosciences), blocked for 2 h in TBS1X-0.1% Tween containing 5% skim milk, incubated with primary antibodies (diluted in TBS1X-0.1% Tween containing 5% BSA) overnight at 4°C, and thence with appropriate HRP-conjugated secondary antibodies for 1 h at room temperature. Then, membranes were incubated in ECL (GE Healthcare) and immunodetection was achieved via chemiluminescence.

3.8 Protein purification and *in vitro* degradation assays

The pGEX-4T3-Syt XI-cyto plasmid was transformed into **E. coli** BL21 (λDE3), and protein production was induced in 200 ml LB (+100 µg/ml ampicillin) containing 10 mM IPTG (Roche) for 24 h. Bacteria were centrifuged and resuspended in GST binding buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA) containing 0.5% Triton X-100 and complete protease inhibitors. This mixture was frozen for 30 min at -70°C, thawed on ice, and sonicated with a Branson Sonifier 150 (three 20 sec pulses in continuous mode at intensity 5; 20 sec pause among pulses). After centrifugation, 500 µl of glutathione beads (Amersham Bioscience) was added to supernatant and incubated at 4°C with agitation for 2 h. Beads were then spun and washed thrice with 20 ml GST binding buffer. GST-Syt XI-cyto was eluted by resuspending beads – twice – in a solution containing 10 mM reduced Glutathione, 50 mM Tris-HCl pH 8.0 and 5% glycerol for 15 min at room temperature.

For *in vitro* GST-Syt XI-cyto cleavage assays, 1 µg of eluted GST-Syt XI-cyto was incubated with $30x10^6$ *L. major* promastigotes in 200 µl *Leishmania* medium, or with the equivalent amount of promastigote lysate in 200 µl binding buffer (PBS1X, 1 mM $ZnCl_2$).

Incubations were carried out in 0.6 ml tubes at 37°C with agitation. For GP63 inhibition experiments, *Leishmania* lysates were pre-incubated with 2 mM 1,10-Phenanthroline for 1 h prior to addition of GST-Syt XI-cyto. After incubations, parasites were discarded, SDS loading buffer containing 2 mM 1,10-Phenanthroline was added, and samples were boiled at 100°C for 6 min. Ten microliters of each sample was loaded on SDS-PAGE gels, and degradation was analyzed by Western blot.

3.9 Intraperitoneal infections and FACS analysis

Female BALB/c mice were inoculated intraperitoneally with $2x10^6$ *L. major* promastigotes (WT, Δ*gp63*, and Δ*gp63*+*gp63*) in late stationary phase (Gonçalves *et al.*, 2011). Promastigotes were gently resuspended in 0.5 ml of filtered HBSS (Life Technologies), and injected into the lower left abdominal quadrant using a 1 ml syringe mounted with a 26G x ½ needle. Four hours post-inoculation, mice were euthanized and intraperitoneal cavities were flushed with 5 ml of cold HBSS containing 10 mM EDTA (pH 7.4). Lavages were centrifuged, supernatants were probed for cytokine content via ELISA, and cell populations were characterized via FACS. Cells were resuspended in cold PBS containing 1% horse serum (Sigma), 0.1% NaN_3 and 5 mM EDTA (pH 8) and stained as previously described (Paun *et al.*, 2011) using the aforementioned FACS antibodies. Flow cytometric analysis was carried out using the LSRFortessa™ cytometer (Special Order Research Product, BD Biosciences), and the BD FACSDiva Software (version 6.2) was used for data acquisition and analysis.

3.10 Data analysis

Statistical significance was assessed via the two-tailed, unequal variance *t*-test, or the two-tailed Mann-Whitney U test. Error bars denote the standard error of the mean (SEM). Graphs were plotted with SigmaPlot or GraphPad Prism 5.

4 RESULTS

4.1 *Leishmania* degrades Syt XI in a GP63-dependent manner

We previously discovered that Syt XI negatively regulates cytokine secretion and modulates phagosome maturation (Arango Duque *et al.*, 2013). Since *Leishmania* affects these processes, we postulated that this parasite may alter Syt XI integrity and function. For these reasons, we sought to investigate the fate of Syt XI in macrophages infected with *Leishmania* promastigotes. To this end, we infected BMM with WT *L. major* promastigotes for 1 h (Fig. S1) or 6 h (Fig. 1A) and assessed Syt XI distribution by confocal microscopy. Since we recently discovered that *Leishmania* promastigotes cleave various SNAREs through GP63 (Matheoud *et al.*, 2013), we included the isogenic GP63 null mutant (Δ*gp63*) and its complemented counterpart (Δ*gp63*+*gp63*) in our study. As shown in Figure 1A (and Fig. S1), we observed an absence of Syt XI staining in macrophages infected with WT or Δ*gp63*+*gp63* promastigotes. This indicates that Syt XI levels were diminished in a GP63-dependent fashion. Syt XI recruitment to PVs also appeared greatly reduced in contrast to phagosomes containing zymosan. Quantification of PVs positive for Syt XI revealed that recruitment to PVs was hampered even in those containing the Δ*gp63* mutant (Fig. 1B), suggesting that other factors may also aid in excluding Syt XI from PVs. Altogether, these data suggested that Syt XI was down-modulated in response to infection with GP63-expressing parasites, and that Syt XI was excluded from PVs.

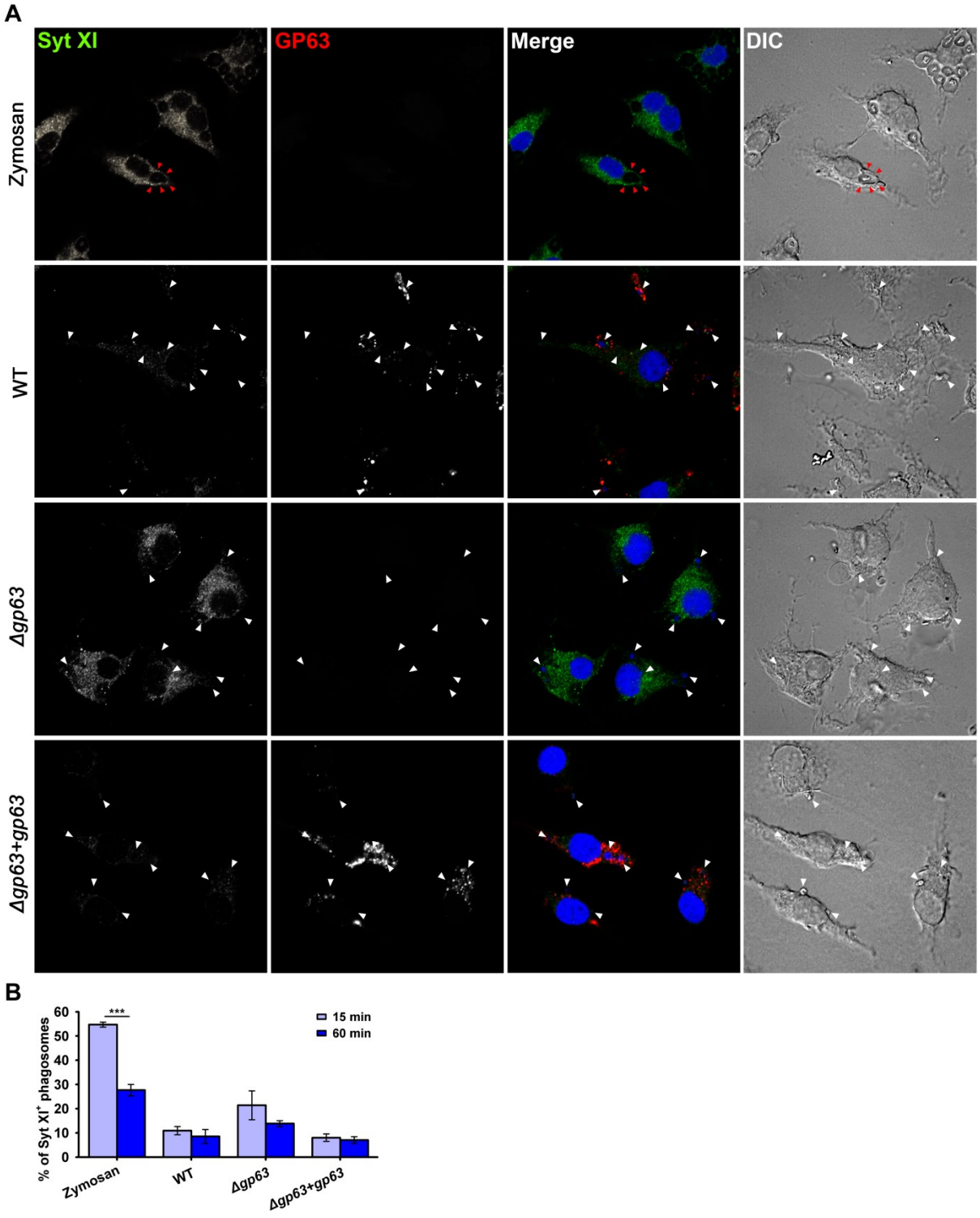
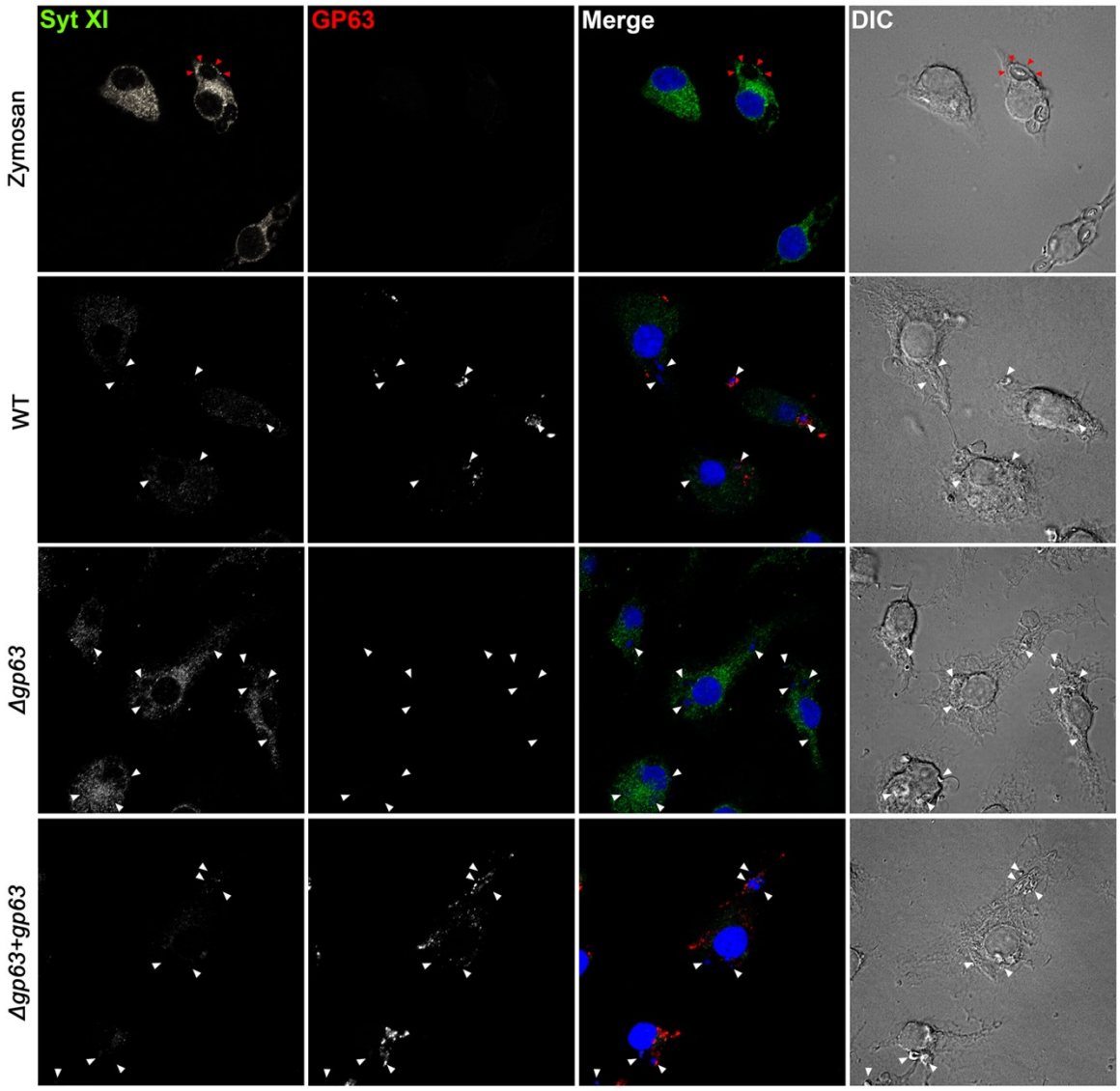


Figure 1 of article 2. GP63 lowers Syt XI levels in infected macrophages. (A) BMM were infected with opsonised *L. major* WT, Δ*gp63* or Δ*gp63+gp63* promastigotes or with opsonized zymosan for 6 h. Syt XI (green) and GP63 (red) levels were then visualized by confocal microscopy. DNA is shown in blue (DRAQ5); white and red arrowheads denote internalized parasites and sites of Syt XI recruitment around zymosan phagosomes, respectively. **(B)** PVs hindered Syt XI recruitment independently of GP63. Syt XI recruitment to PVs was quantified after 15 min or 1 h of infection for 100 phagosomes. Experiments were repeated twice in triplicate, and error bars represent the SEM. *, $p<0.05$; ** $p<0.01$. Original magnification ×63.



Supplementary Figure 1 of article 2. Syt XI levels in *Leishmania*-infected cells are modulated by GP63. BMM were infected with opsonised *L. major* WT, Δ*gp63* or Δ*gp63*+*gp63* promastigotes or with opsonized zymosan for 1 h. Syt XI (green) and GP63 (red) levels were then visualized by confocal microscopy. DNA is shown in blue (DRAQ5); white and red arrowheads denote internalized parasites and sites of Syt XI recruitment around zymosan phagosomes, respectively. Original magnification ×63.

Given that GP63 cleaves a variety of macrophage proteins (Contreras *et al.*, 2010, Ghosh *et al.*, 2013, Gómez *et al.*, 2009, Hallé *et al.*, 2009, Jaramillo *et al.*, 2011, Matheoud *et al.*, 2013), and that we observed decreased Syt XI levels upon infection by GP63-expressing parasites, we set out to determine whether GP63 was degrading Syt XI. Indeed, Western blot analyses on lysates from BMM infected with WT, Δ*gp63* and Δ*gp63*+*gp63 L. major* promastigotes revealed a degradation of Syt XI that was

dependent on the presence of GP63 (Fig. 2A). We also infected RAW264.7 macrophages expressing a FLAG-Syt XI-GFP fusion protein and 6 h post-infection, we observed that this protein was also cleaved by parasites expressing GP63 (Fig. 2B). We then assayed whether promastigotes and amastigotes from other *Leishmania* species degraded Syt XI (Fig. 2C). Infection with the viscerotropic species *L. donovani* also resulted in Syt XI degradation 6 h post-infection, albeit to a lesser degree than observed for *L. major.* Interestingly, this reduced degradation of Syt XI observed in BMM infected with *L. donovani* promastigotes correlates with lesser GP63 levels in these parasites (Fig. 2D). Consistent with the absence of detectable GP63 (Fig. 2D), we observed that *L. donovani* amastigotes did not induce Syt XI degradation (Fig. 2C) (Hsiao *et al.*, 2008, Matheoud *et al.*, 2013). Similarly, infection with promastigotes of the non-pathogenic *L. tarentolae* species did not lead to Syt XI degradation (Fig. 2C), owing to feeble levels (Fig. 2D) of inactive GP63 (Campbell *et al.*, 1992).

Having established that Syt XI was degraded by GP63, we investigated whether this degradation happened directly, or was due to an intermediate molecule. To this end, we incubated purified recombinant GST-Syt XI with either live parasites or with parasite lysates. In both cases, we observed that this recombinant Syt XI was degraded by parasites expressing GP63, and that this degradation increased with time (Fig. 3A, 3B). Moreover, we showed that the catalytic site of GP63 is important for the degradation of Syt XI since phenanthroline-mediated chelation of Zn^{2+} ions, which are critical for GP63 function, effectively inhibited cleavage by GP63-expressing promastigotes (Fig. 3B). Degradation by proteases can also be artifactual and may happen only after they are exposed to potential substrates after cell lysis (Chen *et al.*, 2012). To exclude this possibility, we lysed control and WT *L. major*-infected macrophages in 8 M urea, a chaotropic agent. After Western blot analysis, we observed that Syt XI was still degraded in infected macrophages (Fig. 3C), similar to results obtained with the NP-40-based lysis buffer containing 1,10-Phenanthroline (Fig. 2). Having established that *Leishmania* promastigotes degraded Syt XI through GP63 proteolysis, we explored the potential role of LPG as a contributor to Syt XI exclusion from PVs.

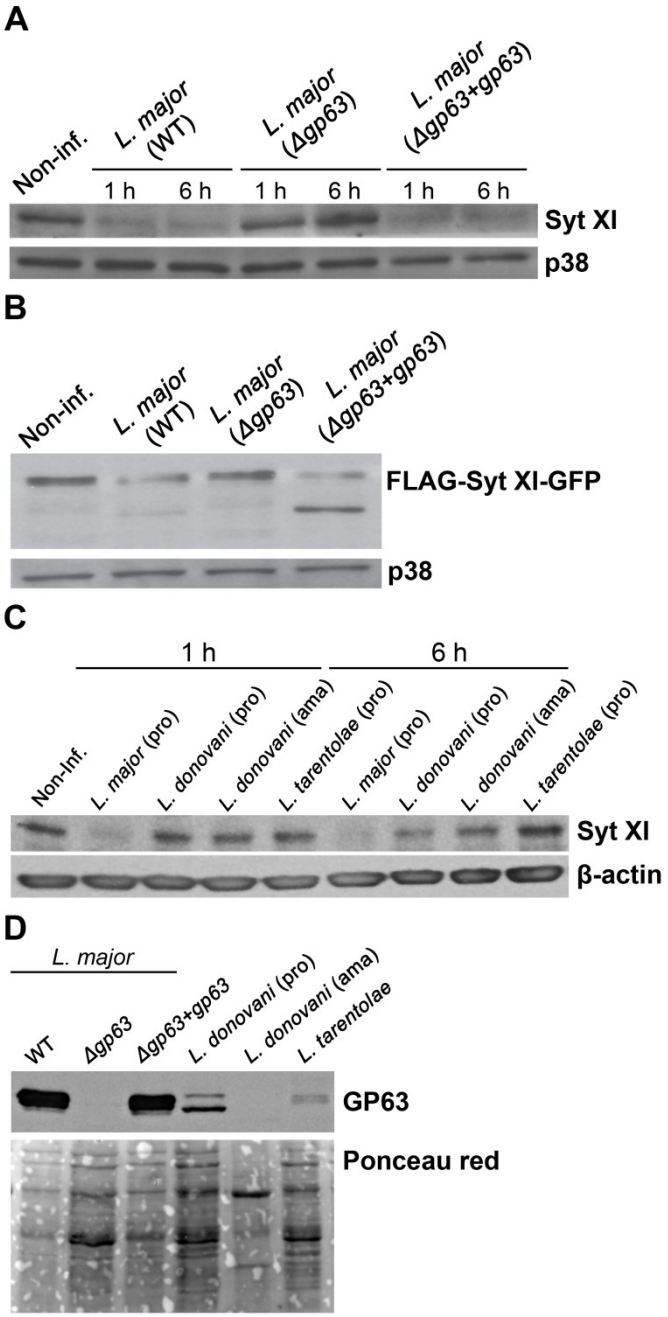


Figure 2 of article 2. *Leishmania* promastigotes use GP63 to cleave Syt XI. (A) Syt XI degradation by *Leishmania* promastigotes is dependent on the GP63 metalloprotease. BMM were infected with opsonised *L. major* WT, Δ*gp63* or Δ*gp63*+*gp63* promastigotes for 1 h or 6 h, and Syt XI levels were assessed via Western blot with an anti-Syt XI antibody. **(B)** RAW264.7 macrophages expressing FLAG-Syt XI-GFP were also infected for 6 h, and cleavage was evaluated with an anti-GFP antibody. **(C)** Syt XI degradation was also assayed in BMM infected with *L. donovani* promastigotes and amastigotes and with *L. tarentolae* promastigotes. **(D)** GP63 levels in stationary phase promastigotes and amastigotes were assessed via Western blot with an anti-GP63 antibody; a ponceau red staining of the gel was included as loading control. The lysis buffer used contained 1,10-Phenanthroline to inhibit GP63 activity during lysate processing. All experiments were repeated at least thrice. (pro), promastigotes; (ama), amastigotes.

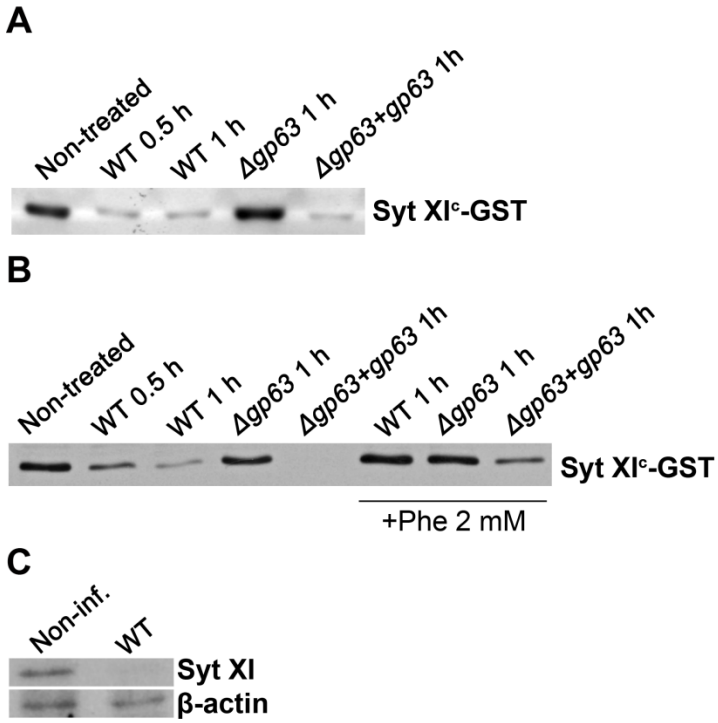


Figure 3 of article 2. Syt XI degradation is direct and does not occur during lysate processing. (A, B) GP63 degrades Syt XI directly. A GST-Syt XI^{c} fusion protein was incubated with live *L. major* WT, Δ*gp63* or Δ*gp63+gp63* promastigotes **(A)**, or with parasite lysates **(B)** and levels were assessed via Western blot with an anti-Syt XI antibody. Co-incubation of parasite lysates with 2 mM 1,10-Phenanthroline effectively blocks GP63 degradation of GST-Syt XI. **(C)** Lysates of *L. major* WT-infected BMM were prepared using 8 M urea to denature proteins during lysate processing; Syt XI degradation was assessed by Western blot. Experiments were repeated at least thrice. ^{c}, cytoplasmic domain of Syt XI.

4.2 LPG mediates exclusion of Syt XI from PVs

LPG inhibits phagolysosome maturation by impairing recruitment of antimicrobial effectors (Lodge *et al.*, 2008, Vinet *et al.*, 2009). Since cleavage by GP63 does not account for the exclusion of Syt XI from PVs, we investigated whether LPG is mediating this phenotype. To address this question, we infected BMM with either WT or phosphoglycan-defective Δ*lpg2 L. donovani* promastigotes, or with *L. tarentolae* promastigotes. Using confocal microscopy, we observed that after 1 h of phagocytosis, recruitment of Syt XI was higher in PVs containing LPG-defective Δ*lpg2 L. donovani* and the non-pathogenic *L. tarentolae* promastigotes, compared to PVs containing WT *L.*

donovani promastigotes (Fig. 4A). To directly show that LPG contributes to impaired Syt XI recruitment, we coated zymosan particles with LPG (Vinet *et al.*, 2009, Vinet *et al.*, 2011), and analysed Syt XI recruitment to phagosomes by confocal immunofluorescence microscopy. In LPG-coated zymosan phagosomes, we observed a ~2-fold decrease in Syt XI recruitment with respect to uncoated zymosan (Fig. 4B). Fluorescence intensity profile analysis of LPG-coated zymosan phagosomes revealed that LPG staining was not concomitant with Syt XI staining, implying that LPG-containing microdomains created in the membrane of phagosomes prevent Syt XI from associating to phagosomes (Fig. 4C). These data show that LPG also targeted Syt XI by excluding this protein from PVs, similar to what was found for Syt V (Vinet *et al.*, 2009, Vinet *et al.*, 2011).

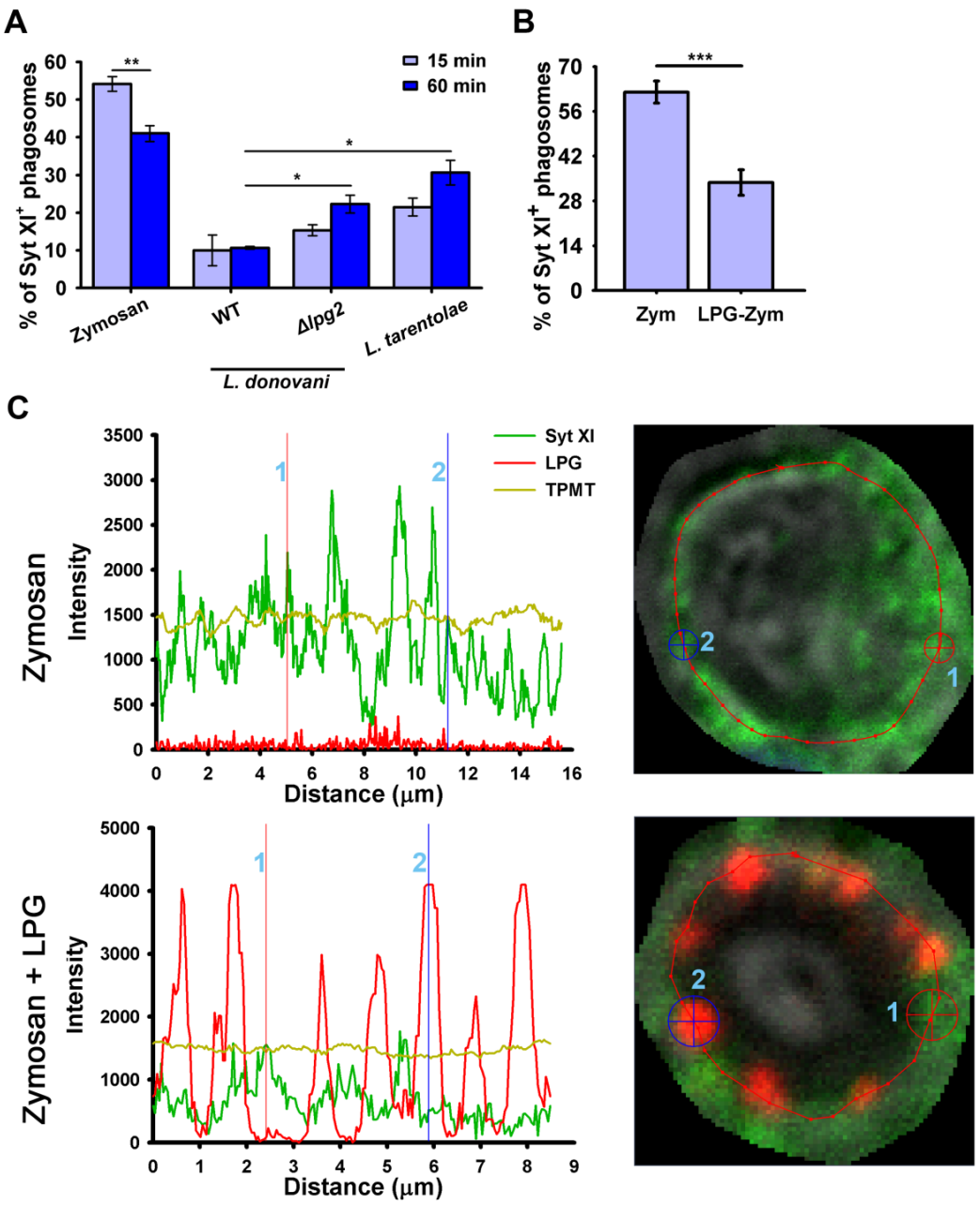


Figure 4 of article 2. LPG mediates exclusion of Syt XI from the phagosome. **(A)** LPG modulates the recruitment of Syt XI to the phagosome. BMM were given zymosan, or infected with *L. donovani* LV9 (WT or Δ*lpg2*), or *L. tarentolae* promastigotes. **(B)** To directly assess the effect of LPG on Syt XI recruitment, BMM were fed with LPG-coated zymosan for 15 min. Syt XI recruitment on LPG-coated or naked zymosan was then assessed via confocal microscopy. **(C)** LPG excludes Syt XI from the phagocytic cup. Fluorescence profiles on images of representative phagosomes revealed that LPG (red) excluded Syt XI (green) from phagocytic cups (DIC, olivaceous line). Recruitment was assessed for 100 phagosomes, and results are the average of two experiments done in triplicate. Error bars denote the SEM. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. Original magnification ×63.

4.3 *Leishmania* induces TNF and IL-6 secretion through degradation of Syt XI

Previous studies showed that *L. major* promastigotes induce TNF and IL-6 secretion in macrophages (Arena *et al.*, 1997, Lapara *et al.*, 2010, Matte *et al.*, 2002). Since GP63 degrades a negative regulator of cytokine secretion (Syt XI), we set out to investigate whether GP63 had an impact on cytokine release induced by *L. major* promastigotes. We first compared promastigotes and amastigotes for their capacity to modulate TNF and IL-6 secretion in BMM. Six hours post-infection, both TNF and IL-6 were induced by *L. major* and *L. donovani* promastigotes, though *L. major* was a stronger inducer (Fig. 5A, 5B). In contrast, *L. tarentolae* promastigotes induced neither TNF, nor IL-6, whereas *L. donovani* amastigotes failed to induce IL-6, but were able to elicit a slight increase in TNF secretion. Next, we infected BMM with *L. major* promastigotes at various doses, and secretion of TNF and IL-6 was found to be dose-dependent (Fig. 5C, 5D).

We next sought to establish whether GP63 contributes to the release of TNF and IL-6, since it had never been studied whether this protease plays a role in *Leishmania*-induced cytokine release. Wild-type and Δ*gp63*+*gp63 L. major* promastigotes induced significantly more TNF and IL-6 in BMM compared to Δ*gp63* parasites (Fig. 6A and 6B). Since decreased levels of Syt XI lead to increased TNF and IL-6 secretion (Arango Duque *et al.*, 2013), it is possible that degradation of Syt XI is responsible for the increase in cytokine release induced by GP63. To directly test this possibility, we knocked down Syt XI in RAW264.7 macrophages (blot in Fig. 6C) and measured cytokine release following infection with WT, Δ*gp63, or* Δ*gp63*+*gp63 L. major* promastigotes. Our data show that promastigotes expressing GP63 did not induce more cytokine secretion in macrophages treated with siRNA to Syt XI, with respect to macrophages transfected with siRNA to GFP, or mock-transfected (Fig. 6C and 6D). In contrast, Δ*gp63* promastigotes elicited significantly more cytokine secretion from macrophages treated with siRNA to Syt XI, than from control-transfected macrophages. In addition, the cytokine levels induced by Δ*gp63* parasites in macrophages treated with siRNA to Syt XI resembled the levels elicited by parasites expressing GP63. Hence, the

effects of siRNA and GP63 degradation did not have a cumulative impact on parasite-induced TNF and IL-6 secretion. Altogether, our data are consistent with a model where *Leishmania* promastigotes elicit TNF and IL-6 secretion through a mechanism that implicates the degradation of Syt XI by GP63 (Fig. 6E). These data also support the notion that Syt XI is a negative regulator of cytokine secretion.

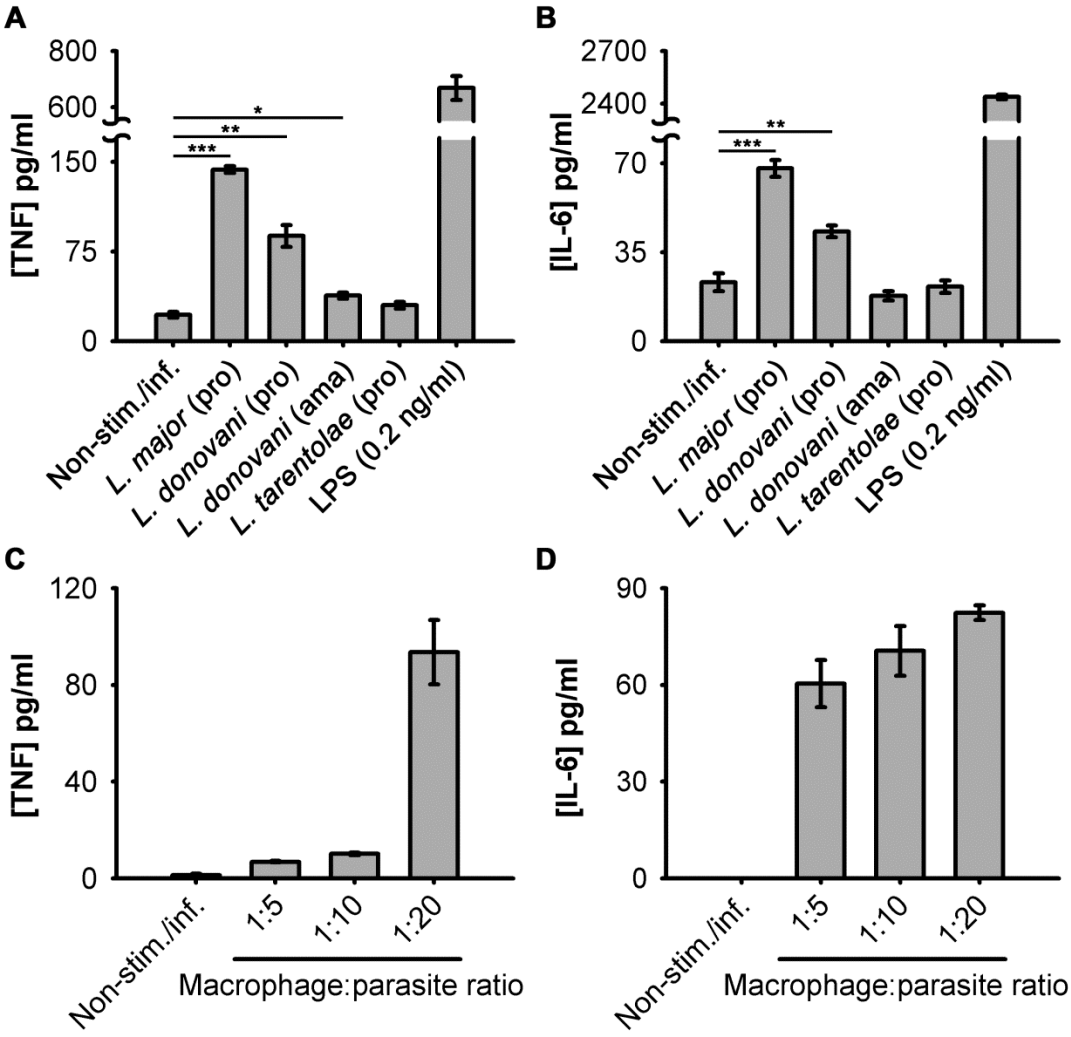


Figure 5 of article 2. *Leishmania* promastigotes induce TNF and IL-6 release. Infection with *L. major* and *L. donovani* promastigotes triggers the secretion of TNF **(A)** and IL-6 **(B)**. BMM were either stimulated with LPS (0.2 ng/ml), or infected with promastigotes or amastigotes as indicated. Cytokine release in culture supernatants was then measured by ELISA. Cytokine secretion from infected cells is dependent on parasite dose. BMM were infected with the indicated *L. major* promastigote dose for 6 h, and TNF **(C)** and IL-6 **(D)** secretion were then quantified. Results are the mean of three independent experiments done in triplicate. Error bars denote the SEM. *, $p<10^{-3}$; **, $p<10^{-5}$; ***, $p<10^{-7}$. (pro), promastigotes; (ama), amastigotes.

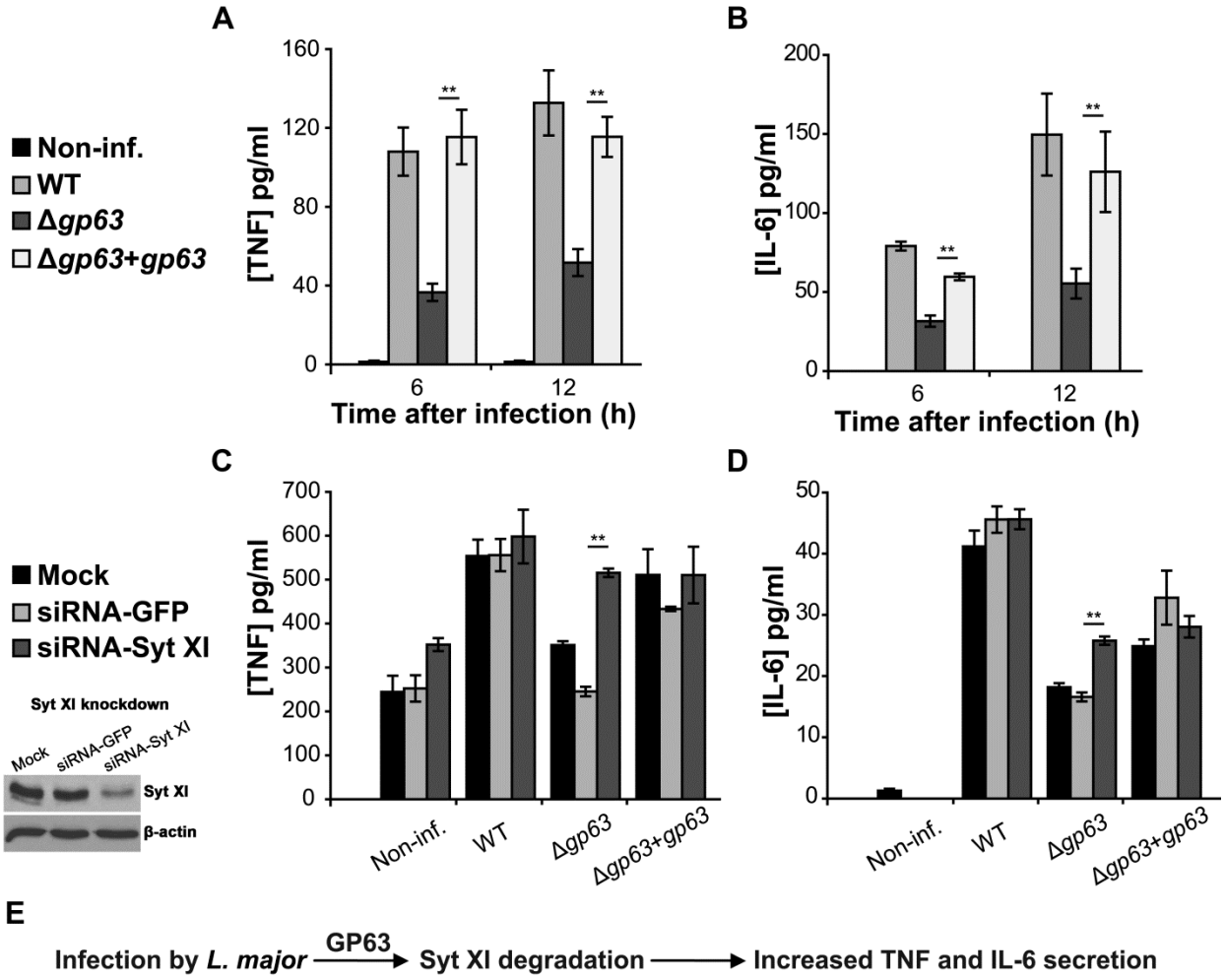


Figure 6 of article 2. ***L. major*** **induces TNF and IL-6 secretion via degradation of Syt XI**. **(A, B)** Release of TNF and IL-6 is regulated by GP63. BMM were infected with WT, Δ*gp63* or Δ*gp63+gp63 L. major* promastigotes for 6 h or 12 h. Results are the mean of three independent experiments done in triplicate. The effects of siRNA and GP63 on TNF **(C)** and IL-6 **(D)** secretion are not cumulative. After mock or siRNA transfections (Syt XI knockdown was assessed via Western blot as shown), RAW264.7 macrophages were infected with *L. major* WT, Δ*gp63* or Δ*gp63+gp63* promastigotes for 6 h. Culture supernatants were then subjected to ELISA. Results represent one representative experiment – done in triplicate – of three. Statistical significance was assessed with the Mann-Whitney U test. **(E)** Model for *L. major*-triggered secretion of TNF and IL-6 via GP63-mediated degradation of Syt XI. Error bars denote the SEM. *, $p<0.05$; **, $p<0.01$.

4.4 GP63-expressing promastigotes elicit increased TNF and IL-6 release *in vivo*, as well as increased neutrophil and inflammatory monocyte recruitment

Having shown that infection with GP63-containing promastigotes caused an increase in TNF and IL-6 secretion from cultured macrophages, we sought to determine whether this phenomenon occurred *in vivo*. To test this hypothesis, we injected $2x10^6$ stationary-phase *L. major* WT, Δ*gp63*, or Δ*gp63*+*gp63* promastigotes in the peritoneal cavities of BALB/c mice and 4 h later we assessed cytokine levels and cell content in peritoneal lavages. As demonstrated with BMM (Fig. 5 and 6), mice inoculated intraperitoneally with GP63-expressing parasites displayed augmented TNF and IL-6 (Fig. 7A and 7B) levels in comparison to mice inoculated with Δ*gp63* parasites or to control mice. TNF and IL-6 are cytokines that act on a variety cells, including those of the endothelium (Griffin *et al.*, 2012), to induce chemokine release and adhesion molecules and thence inflammatory phagocyte accrual to the inflammation site (Biswas *et al.*, 1998, Griffin *et al.*, 2012, Vieira *et al.*, 2009).Together with the fact that *L. major* promastigotes induce inflammatory phagocyte recruitment to the infection site (Gonçalves *et al.*, 2011, Ribeiro-Gomes *et al.*, 2014), we investigated whether GP63 played a role in this phenomenon. Four hours post-inoculation, we observed a GP63-dependent increase in $CD11b^+$ myeloid cell recruitment (Fig. 7C). Further characterization of this population revealed that GP63-expressing parasites induced a 2-fold increase in the percentages of neutrophils ($Gr1^{hi}CD11b^{hi}$) and inflammatory monocytes ($Gr1^+CD11b^+CCR2^+$) recruited to the infection site (Fig. 7D-G). These data support a role for GP63 in proinflammatory cytokine release and recruitment of proinflammatory phagocytes to the infection site.

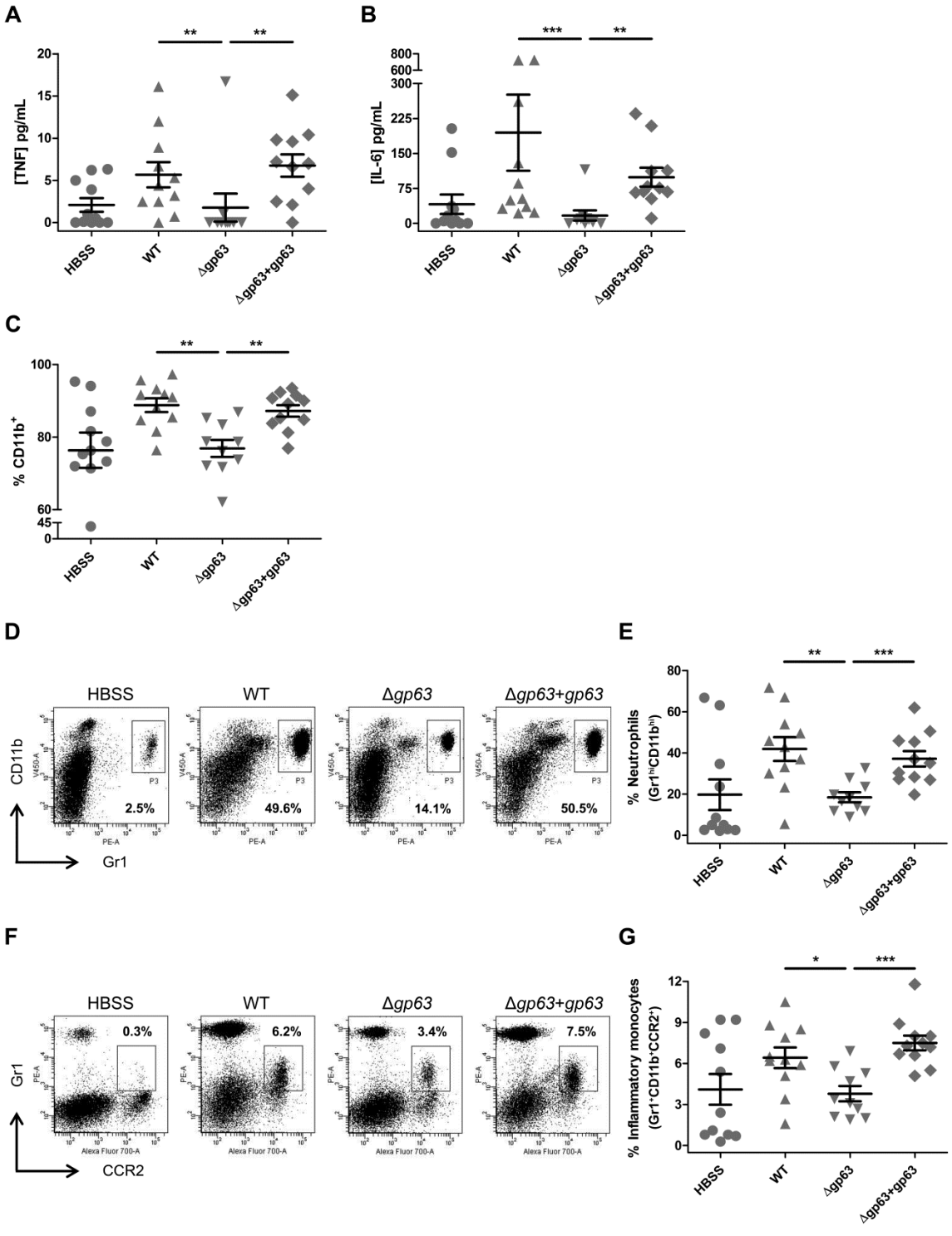


Figure 7 of article 2. ***In vivo*** **modulation of cytokine secretion and phagocyte infiltration by GP63.** Mice were inoculated intraperitoneally with WT, Δ*gp63* or Δ*gp63+gp63 L. major* promastigotes for 4 h, and TNF **(A)** and IL-6 **(B)** levels in intraperitoneal lavages were measured. The abundance of $CD11b^+$ cells was assessed by flow cytometry **(C)**. Neutrophils were analyzed by gating on live cells and then on $CD11b^{hi}$ and $Gr1^{hi}$, and inflammatory monocytes by gating on $CD11b^+$, and subsequently on $Gr1^+$ and $CCR2^+$ cells. Representative dot plots and relative percentages of neutrophils **(D, E)** and inflammatory monocytes **(F, G)** are shown. Graphs show the mean of three independent experiments – of five – performed using at least 3 mice per group; error bars denote the SEM. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

5 DISCUSSION

We previously characterized Syt XI as a negative regulator of cytokine secretion and of phagosome maturation (Arango Duque *et al.*, 2013). In this work, we demonstrated that Syt XI is both degraded by the zinc metalloprotease GP63, and excluded from the phagosome by LPG. Given that *L. major* promastigotes trigger the release of TNF and IL-6, we found that in macrophages, degradation of Syt XI by GP63 modulated this proinflammatory cytokine release. Importantly, GP63 augmented cytokine release *in vivo* and promoted infiltration of neutrophils and inflammatory monocytes to the inoculation site. Together, our results support the notion that GP63 contributes to the inflammatory response induced early during infection.

Leishmania parasites have evolved to thwart the immune response by alteration of signalling pathways that would otherwise coalesce to mount a strong microbicidal response by macrophages (Shio *et al.*, 2012). Albeit known as anti-inflammatory, some *Leishmania* species such as *L. major* can upregulate the secretion of proinflammatory cytokines (Arena *et al.*, 1997, Lapara *et al.*, 2010, Matte *et al.*, 2002). Other studies have shown that *L. major* promastigotes trigger the release of TNF and IL-6, at least during the initial stages of infection (Arena *et al.*, 1997, Karam *et al.*, 2006, Wenzel *et al.*, 2012). Furthermore, *L. major* promastigotes triggers the infiltration of inflammatory phagocytes shortly after inoculation (Gonçalves *et al.*, 2011, Peters *et al.*, 2008, Ribeiro-Gomes *et al.*, 2014). We expanded upon these findings by demonstrating that infected BMM secrete both of these cytokines and that release of TNF and IL-6 was dependent on the presence of GP63. Knowing that *L. major* promastigotes induce the secretion of proinflammatory cytokines, we hypothesized that Syt XI could also be targeted by *Leishmania*. Since Syt XI is a negative regulator of cytokine secretion (Arango Duque *et al.*, 2013), we inferred that its degradation could be a strategy used by the parasite to modulate cytokine release. Indeed, decreased levels of Syt XI in *L. major*-infected macrophages were caused by GP63-dependent cleavage. Interestingly, reduced or absence of Syt XI degradation by either *L. donovani* or *L. tarentolae* promastigotes, respectively, correlated with a smaller or no induction of TNF and IL-6 by these

parasites. When we treated macrophages with siRNA to Syt XI prior to infection with GP63-expressing promastigotes, we did not observe increased cytokine secretion in comparison with control-transfected macrophages. On the contrary, GP63-lacking promastigotes triggered significantly more cytokine release from macrophages treated with siRNA to Syt XI. These findings show that *Leishmania* uses GP63 to degrade a negative regulator of cytokine secretion in order to augment secretion of proinflammatory cytokines from macrophages. Our data confirmed that GP63 boosts TNF and IL-6 secretion *in vivo*. Inoculation of GP63-expressing parasites also resulted in augmented infiltration of inflammatory monocytes and neutrophils early in infection. *In vivo* induction of TNF and IL-6 secretion by *L. major* promastigotes may help recruit phagocytes (Matte *et al.*, 2002), since both of these inflammatory cytokines mediate monocyte and neutrophil recruitment via stimulation of chemokine production and release (Biswas *et al.*, 1998, Griffin *et al.*, 2012). TNF and IL-6 can be produced by infected resident macrophages, and these cytokines may then act on surrounding PBMCs (Biswas *et al.*, 1998) and endothelium (Griffin *et al.*, 2012) to trigger release of chemokines such as CXCL2 and CCL2. These can in turn recruit neutrophils and inflammatory monocytes to the infection site. Such an early effect of GP63 on cytokine secretion and phagocyte recruitment can have important consequences in the infection process. Infection of neutrophils can transfer infection to macrophages once infected apoptotic neutrophils are phagocytosed (Gueirard *et al.*, 2008). In addition, neutrophil depletion can severely hamper infection (Ribeiro-Gomes *et al.*, 2014). Inflammatory monocytes are also infected, and though they have been shown to kill *Leishmania in vitro* (Gonçalves *et al.*, 2011), infected cells in peritoneal infiltrates are present after 4h of infection (Ribeiro-Gomes *et al.*, 2014). Infected inflammatory monocytes and resident macrophages can also produce IL-10, which promotes the establishment of chronic infection (Belkaid *et al.*, 2001, Kane *et al.*, 2001). Hence, the finding that GP63 modulates phagocyte recruitment to the infection site may contribute to the development of pathologies associated with *L. major* infection. Whether phagocyte recruitment and release of other cytokines and chemokines are altered through GP63-mediated degradation of Syt XI *in vivo* is an issue that will deserve further investigation.

There is clear evidence that *Leishmania* promastigotes use at least two distinct mechanisms to impair phagolysosome composition and function through subversion of the membrane fusion machinery. The first mechanism consists in disrupting lipid microdomains of the phagosome membrane through the insertion of LPG (Dermine *et al.*, 2005, Winberg *et al.*, 2009b).We showed that this prevents association of Syt V with the phagosome membrane, impairing phagosomal acidification (Vinet *et al.*, 2009). The second mechanism consists in cleaving SNAREs through the action of GP63, causing a modification of phagosomal properties required for optimal antigen processing and cross-presentation (Matheoud *et al.*, 2013). The finding that Syt XI is both degraded by GP63 and excluded from the phagosome membrane by LPG indicates that these two major promastigote surface molecules can act in concert to prevent the action of this membrane fusion regulator. To our knowledge, this is the first example of a macrophage molecule targeted by those two *Leishmania* virulence factors. We recently showed that Syt XI regulates the recruitment of $gp91^{phox}$ and of the lysosomal-associated membrane protein (LAMP)-1 to phagosomes, suggesting that the LPG-mediated exclusion of Syt XI from phagosomes may contribute to the phagosome remodelling induced by *Leishmania* promastigotes (Matheoud *et al.*, 2013). Identification of Syt XI binding partners will be necessary to understand how Syt XI regulates cytokine secretion and phagolysosome biogenesis. In addition, it will be of interest to investigate whether those Syt XI interactors are also targeted by GP63 and LPG.

How GP63 accesses and cleaves Syt XI is not known. In macrophages, Syt XI is found on recycling endosomes and lysosomes (Arango Duque *et al.*, 2013) and GP63 is known to redistribute throughout the macrophage in vesicular structures throughout the cytoplasm (Figs. 1, S1 and (Matheoud *et al.*, 2013)), in lipid rafts (Contreras *et al.*, 2010, Gómez *et al.*, 2009), and in the perinuclear area (Contreras *et al.*, 2010). Because GP63 is GPI-anchored, it is likely to come into close proximity with Syt XI in membranes. Addressing this issue and characterizing the trafficking of GP63 in infected cells will result in a better understanding of how this protease accesses its multiple targets.

In summary, our data reveal that GP63 induces early TNF and IL-6 release both *in vitro* and *in vivo* and contributes to the increase in inflammatory phagocyte infiltration to the inoculation site. Improved knowledge of *Leishmania*-induced inflammation will further our understanding of how the parasite establishes infection, modulates the immune response, metastasizes, and causes pathology.

6 ACKNOWLEDGEMENTS

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This work was supported by Canadian Institutes of Health Research (CIHR) grants MOP-125990 to AD and MOP-123293 to SS. GAD was supported by a CIHR Frederick Banting and Charles Best Doctoral Award, and a *La Presse* scholarship from the *Fondation Universitaire Armand-Frappier INRS*.

ARTICLE NO. 3: THE HOST CELL SECRETORY PATHWAY MEDIATES THE EXPORT OF *LEISHMANIA* VIRULENCE FACTORS OUT OF THE PARASITOPHOROUS VACUOLE

The secretory pathway mediates the export of *Leishmania* virulence factors out of the parasitophorous vacuole

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1 ABSTRACT

To replicate within host phagocytes, the vacuolar pathogen *Leishmania* subverts microbicidal and immune processes through the action of components of its surface coat that include lipophosphoglycan and the metalloprotease GP63 (Arango Duque *et al.*, 2015, Moradin *et al.*, 2012, Olivier *et al.*, 2012). Whereas the impact of these virulence glycoconjugates on host cell function has been investigated in great detail, the mechanism by which they exit the parasitophorous vacuole and traffic within host cells remains to be elucidated (Gómez *et al.*, 2009, Tolson *et al.*, 1990). This is an intriguing issue since *Leishmania* does not possess a specialized secretion system analogous to those used by pathogenic bacteria to inject effectors into mammalian host cell cytosol (Weber *et al.*, 2018). Here, we show lipophosphoglycan and GP63 are redistributed from the parasite to the endoplasmic reticulum of infected host cells following phagocytosis. Disruption of endoplasmic reticulum-Golgi transport hindered their exit from the parasitophorous vacuole and dampened the cleavage of host proteins by GP63. Silencing of the soluble *N*-ethylmaleimide-sensitive-factor attachment protein receptor Sec22b, which regulates endoplasmic reticulum-Golgi trafficking (Cebrian *et al.*, 2011), identified this host protein as a component of the machinery mediating the spread of *Leishmania* effectors within host cells. Our findings unveil a mechanism whereby a vacuolar pathogen co-opts the host cell's secretory pathway to promote intracellular delivery of virulence factors.

2 RESULTS AND DISCUSSION

The protozoan parasite *Leishmania* causes the leishmaniases, a spectrum of human diseases ranging from a confined cutaneous lesion to a progressive visceral infection (Sacks *et al.*, 2001a). Infectious *Leishmania* metacyclic promastigotes are inoculated into mammalian hosts by phlebotomine sand flies and are internalized by phagocytes (Sacks *et al.*, 2001b). There, to establish infection, promastigotes derail phagolysosome biogenesis, shut down microbicidal pathways, and sabotage immune processes (Arango Duque *et al.*, 2014b, Contreras *et al.*, 2010, Gómez *et al.*, 2009, Jaramillo *et al.*, 2011, Matheoud *et al.*, 2013, Matte *et al.*, 2016a, Moradin *et al.*, 2012). Subversion of these host defence mechanisms is achieved in part by two major components of the *Leishmania* surface coat: the metalloprotease GP63 and lipophosphoglycan (LPG), a polymer of repeating Galβ1,4Manα1-PO_4 units attached to the promastigote surface via a glycosylphosphatidylinositol anchor (Descoteaux *et al.*, 1992, Moradin *et al.*, 2012, Olivier *et al.*, 2012). Whereas GP63 contributes to virulence by cleaving molecules that control host defence mechanisms, LPG acts by altering host cell membrane fusogenic properties and signalling pathways (Becker *et al.*, 2003, Gómez *et al.*, 2009, Moradin *et al.*, 2012). Both molecules are redistributed across infected host cells following parasite internalization, through an unknown mechanism (Arango Duque *et al.*, 2014b, Gómez *et al.*, 2009, Späth *et al.*, 2000, Tolson *et al.*, 1990). To address that issue, we infected bone marrow-derived macrophages (BMM) with *L. major* metacyclic promastigotes and assessed the fate of GP63 and of phosphoglycans (PGs), a family of surface and secreted Gal-Man-PO_4-containing glycoconjugates that comprises LPG (Turco *et al.*, 1992). Figure 1a shows that GP63 and PGs dispersed in a time-dependent fashion from the PV harboring live parasites to the cytoplasm of infected macrophages as early as 15 min of phagocytosis and remained detectable in infected cells over 72 h. Redistribution of these molecules also occurred in other phagocytes known to internalize *Leishmania*, including dendritic cells, inflammatory monocytes, and neutrophils (Fig. 1b).

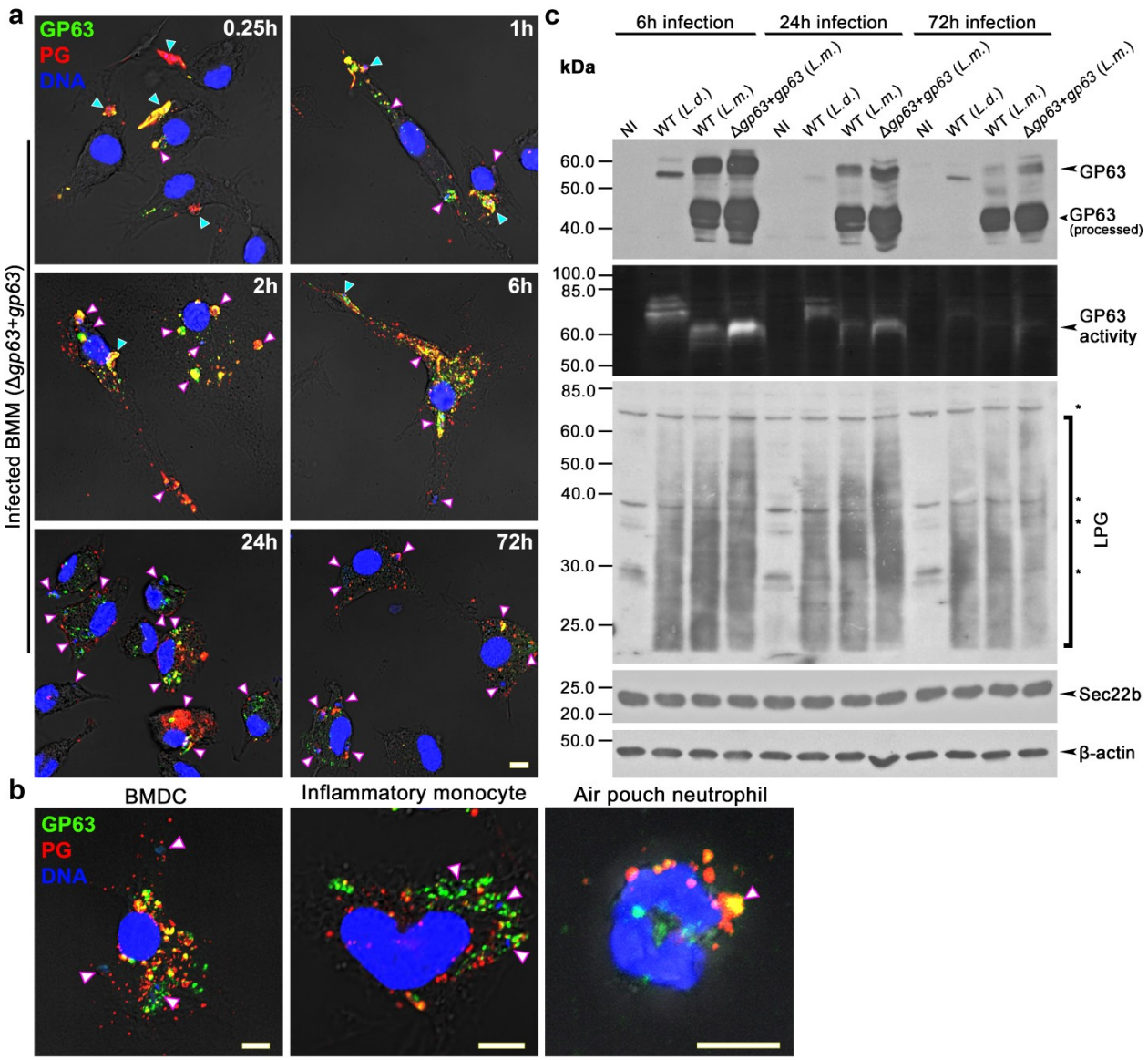


Figure 1 of article 3. GP63 and PGs are redistributed within infected cells. A, BMM were infected with opsonized *L. major (Δgp63+gp63)* metacyclic promastigotes and the distribution of GP63 (green) and PGs (red) was monitored via confocal microscopy over a period of 72 h. **B,** BMDC, inflammatory monocytes and air pouch neutrophils were infected for 6 h with *L. major (Δgp63+gp63)* metacyclic promastigotes. Redistribution of GP63 (green) and PGs (red) in the cytoplasm of infected cells was observed via immunofluorescence. DNA is shown in blue. Representative images of at least three experiments are shown. Arrowheads denote internalized parasites. Bar, 5 μm. **C,** The levels of GP63 and PGs in lysates from infected BMM were assessed by Western blot analysis. NI, non-infected. (*) indicate non-specific bands of macrophage origin (see NI lanes). The activity of GP63 was assayed via gelatin zymography. Representative images of at least two experiments are shown.

Western blot analyses showed that the 63 kDa form of GP63 was processed into a ~42 kDa catalytically inactive form whereas the levels of LPG decreased gradually over time (Fig. 1c), consistent with the down-modulation of these molecules during the

differentiation of promastigotes into the mammalian stage amastigote forms (McConville *et al.*, 1991, Medina-Acosta *et al.*, 1989). A similar redistribution pattern for PGs and GP63 was observed in BMM infected with metacyclic *L. donovani* promastigotes (Supplementary Fig. 1). Compared to *L. major*, staining for PGs was predominant in BMM infected with *L. donovani*, consistent with the significantly lower levels of GP63 in this species (Fig. 1c). Using a *L. donovani* LPG-defective mutant (∆*lpg1*) which retains the ability to express less abundant members of the PG family (such as proteophosphoglycan) we observed that these glyconconjugates also traffic out of the PV and that absence of LPG had no impact on the spreading of GP63 within infected cells (Supplementary Fig. 2). Additionally, infection of BMM with *L. major* metacyclic promastigotes expressing an inactive (Δ*gp63*+*gp63*^{E265A}) GP63 revealed that the catalytic activity of this protease is not required for the dispersal of GP63 and PGs out of the PV (Supplementary Fig. 3).

To access host cell locations, virulence factors produced by vacuolar pathogens must cross the PV membrane. To this end, bacterial pathogens have evolved complex nanomachines to inject effector proteins into the cytosol of eukaryotic cells (Weber *et al.*, 2018). In *Leishmania*, no such specialized secretion system has been described yet. Instead, an exosome-based secretion system was identified as a general mechanism for protein secretion (Silverman *et al.*, 2010).

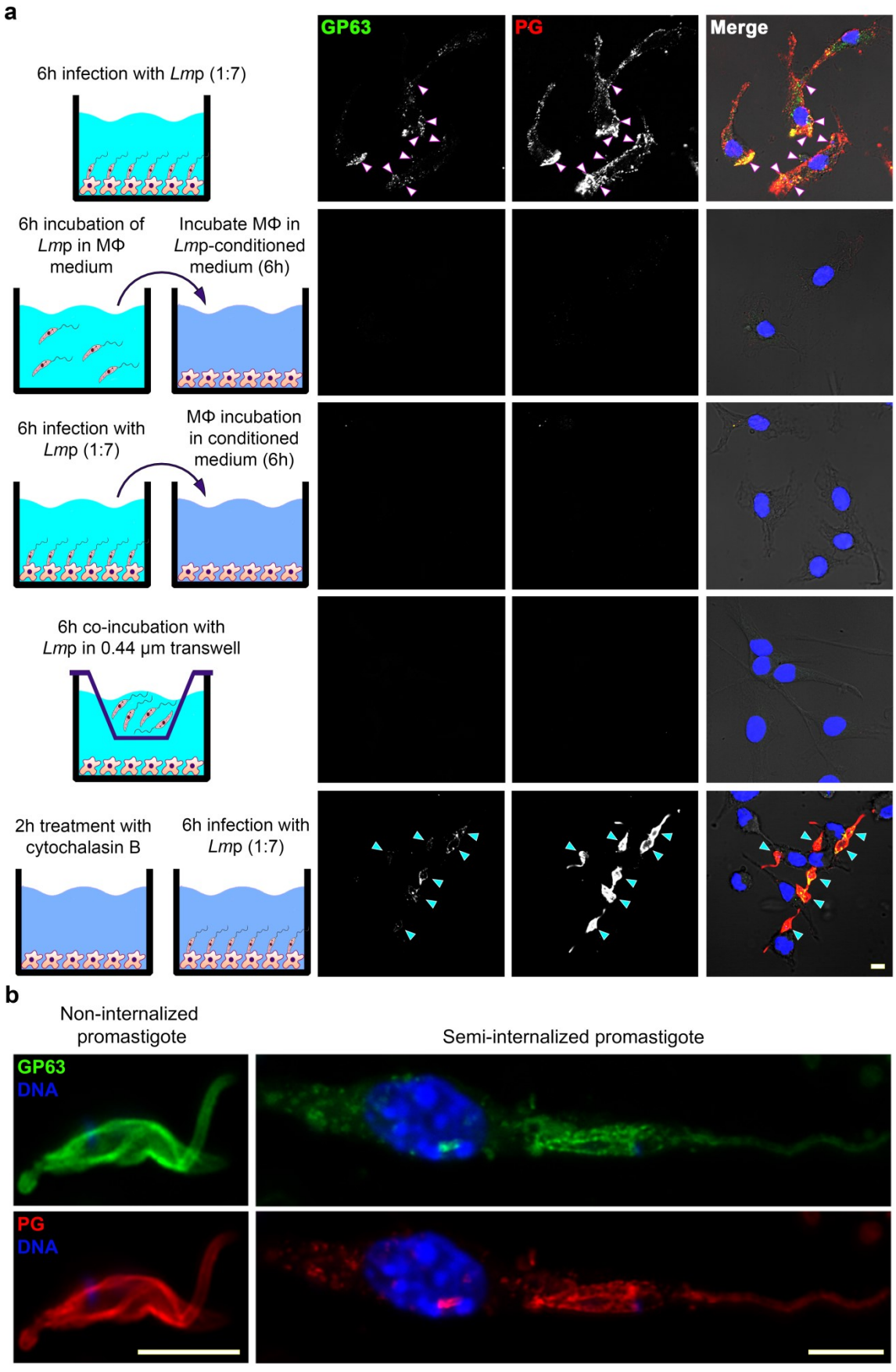

a
GP63
PG
Merge
6h infection with Lmp (1:7)
6h incubation of Lmp in MΦ medium
Incubate MΦ in Lmp-conditioned medium (6h)
6h infection with Lmp (1:7)
MΦ incubation in conditioned medium (6h)
6h co-incubation with Lmp in 0.44 µm transwell
2h treatment with cytochalasin B
6h infection with Lmp (1:7)
b
Non-internalized promastigote
Semi-internalized promastigote
GP63
DNA
PG
DNA

Figure 2 of article 3. Redistribution of PGs and GP63 requires parasite internalization. A, BMM were either infected with *L. major* metacyclic promastigotes, or incubated with conditioned medium from promastigote cultured at 37°C or from infected macrophages, for 6 h. Contact dependence was tested by incubating BMM with promastigotes separated by a 0.44 µm transwell, and requirement for entry was tested by pre-incubating BMM with cytochalasin B to inhibit phagocytosis. **B,** Enhanced resolution imaging of GP63 and PGs on non-internalized promastigotes (left panels) or semi-internalized promastigotes (right panels). These results are representative of two independent experiments. DNA is in blue; white and cyan arrowheads denote internalized and non-internalized parasites, respectively. Bar, 5 µm.

The finding that GP63 is a constituent of *Leishmania* exosomes (Silverman *et al.*, 2010) prompted us to explore the potential role of these extracellular vesicles in the spread of PGs and GP63 within host cells. As shown in Fig. 2a, we did not detect these molecules in BMM incubated with conditioned medium from promastigote cultured at 37°C or from infected macrophages, or when contact between *Leishmania* and BMM was prevented by a 0.44 µm transwell. These findings indicate that extracellular vesicles may not be the major mechanism by which *Leishmania* delivers GP63 and PGs to the cytosol of macrophages. Equally, GP63 and PGs largely remained at the parasite surface when phagocytosis was inhibited by cytochalasin B, indicating that redistribution of these molecules within host cells depends on parasite internalization. Using enhanced resolution imaging, we observed that the uniform PGs and GP63 staining on promastigotes became punctate once the parasites were phagocytosed, due to shedding from the parasite surface and transport out of the PV (Fig. 2b). These observations suggest that environmental conditions present in the PV trigger the shedding of components of the parasite surface coat through an undefined mechanism, prior to their spreading out of the PV. Clearly, future studies will be required to elucidate this process.

Previous studies revealed that both LPG and GP63 associate with lipid microdomains of host cell membranes to impair host cell processes (Contreras *et al.*, 2010, Winberg *et al.*, 2009b). Apart from the presence of GP63 in the perinuclear area of infected macrophages (Contreras *et al.*, 2010), very little is known with respect to the localization of PGs and GP63 within host cells. To determine the sub-cellular localization of these molecules in infected cells, we resolved lysates of RAW264.7 macrophages infected

with *L. major* Δ*gp63*+*gp63* or Δ*gp63* metacyclic promastigotes on discontinuous sucrose gradients. GP63 and LPG were present in fractions 2 to 8, with fractions 5 and 6 exhibiting the highest signal (Fig. 3a and Supplementary Fig. 4). This indicated that both molecules sedimented with light-density vesicles (Supplementary Fig. 4), consistent with the presence of the membrane-bound protein LC3B-II (Ge *et al.*, 2013). GP63 and LPG were also present in denser fractions that cofractionate with endoplasmic reticulum (ER) proteins calreticulin (CRT), calnexin (CNX), protein disulfide isomerase (PDI), and the ER-Golgi intermediate compartment (ERGIC) soluble *N*-ethylmaleimide-sensitive-factor attachment protein receptor (SNARE) Sec22b (Ge *et al.*, 2013). Confocal microscopy analyses revealed that GP63 and PGs colocalize with ERGIC (ERGIC53, Sec22b), and ER (CRT, PDI, Sec23) proteins in infected BMM (Fig. 3b and Supplementary Fig. 5). Colocalization of GP63 and PGs with GM1-enriched lipid rafts was also observed, as previously reported (Dermine *et al.*, 2005, Gómez *et al.*, 2009). To determine whether GP63 was oriented into the lumen of those vesicles or exposed to the cytoplasm, we incubated pelleted vesicles with proteinase K (Prot K) or phospholipase C (PI-PLC) (Fig. 3c). Although GP63 was resistant to Prot K digestion even in the presence of Triton X-100, it was released from the vesicles by PI-PLC, whereas the cytosol-facing SNARE Sec22b (Aoki *et al.*, 2008) was only sensitive to Prot K treatment. This indicates that GP63 is present on cytoplasmic face of the vesicles, consistent with the ability of this protease to cleave host cell proteins.

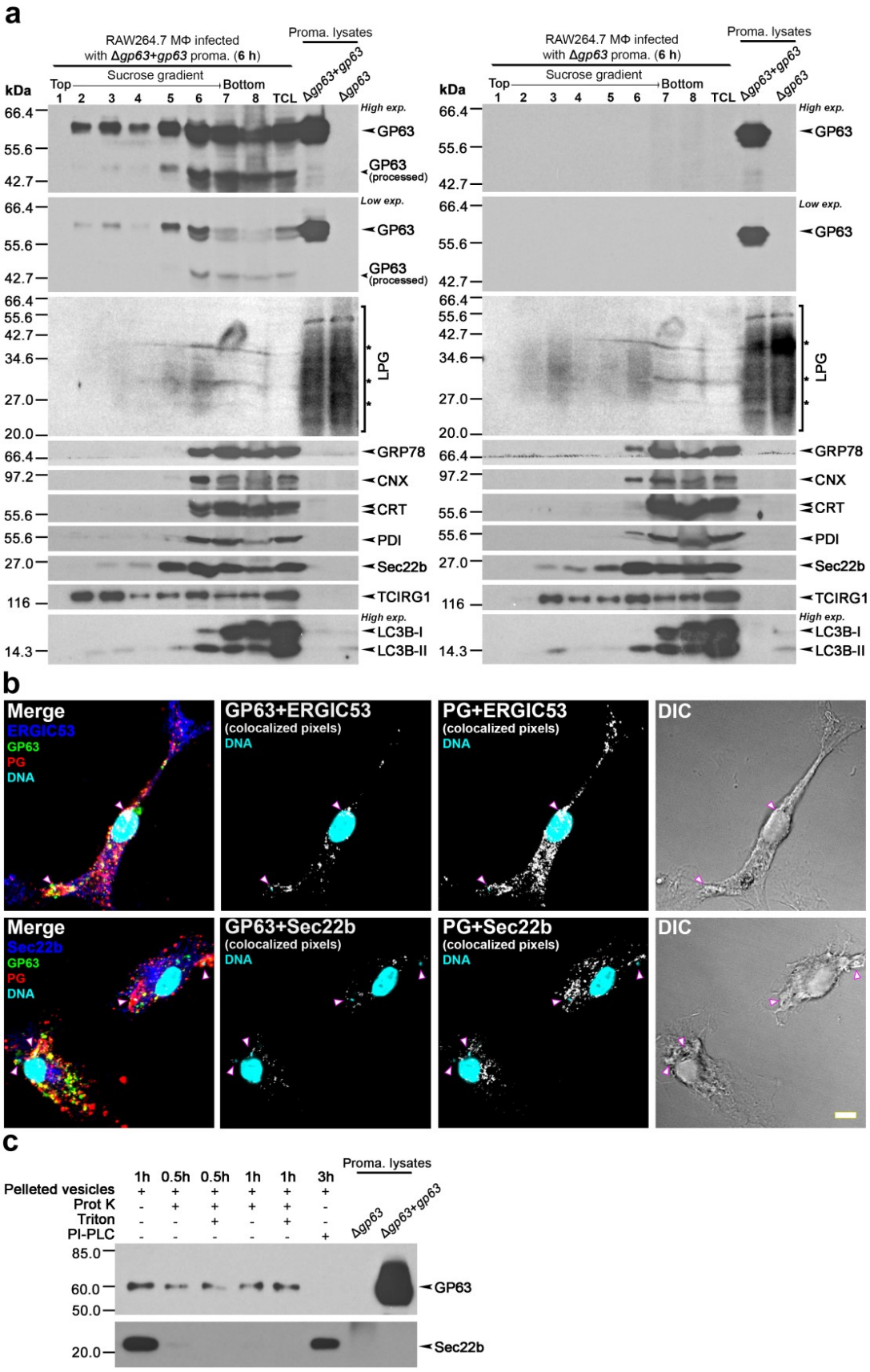

a
RAW264.7 MΦ infected with Δgp63+gp63 proma. (6 h)
Proma. lysates
Sucrose gradient
Top
Bottom
kDa
1 2 3 4 5 6 7 8 TCL Δgp63+gp63 Δgp63
High exp.
GP63
GP63 (processed)
Low exp.
LPG
GRP78
CNX
CRT
PDI
Sec22b
TCIRG1
LC3B-I
LC3B-II
b
Merge
ERGIC53
GP63
PG
DNA
GP63+ERGIC53 (colocalized pixels)
PG+ERGIC53 (colocalized pixels)
DIC
Sec22b
GP63+Sec22b (colocalized pixels)
PG+Sec22b (colocalized pixels)
c
Pelleted vesicles
Prot K
Triton
PI-PLC
1h 0.5h 0.5h 1h 1h 3h
Δgp63
Δgp63+gp63
GP63
Sec22b

Figure 3 of article 3. GP63 and PGs are present in vesicles that co-occur with ER and ERGIC markers. RAW264.7 macrophages were infected with opsonized *L. major* Δ*gp63*+*gp63* or Δ*gp63* metacyclic promastigotes for 6 h. Lysates were placed in a sucrose gradient and fractionated from the top. **A,** Western blots showing the presence of GP63 and LPG in light (2-4) or denser fractions (5-8). GRP78, CNX, CRT, and PDI were used as ER markers, Sec22b as an ERGIC marker, TCIRG1 as a maker of endosomes and lysosomes, and GRP75 as a marker of mitochondria. Light vesicle-containing fractions are delimited by the exclusive appearance of LC3B-II, which is membrane-bound. **B,** BMM were infected with *L. major* promastigotes for 6 h and the colocalization (white pixels) of GP63 (green) or PGs (red) with ERGIC markers ERGIC53 (blue) and Sec22b (blue) was assessed by confocal immunofluorescence microscopy. DNA in is cyan. Bar, 5 μm. **C,** Enzyme protection assay using vesicles that were pelleted from fraction 6 and treated with Prot K ± Triton X-100 or PI-PLC. The protection of GP63 from these enzymes was compared to that of the host's Sec22b, which faces the cytoplasmic side and is not GPI-anchored. These results are representative of at least two independent experiments.

The ER regulates the export of intraphagosomal proteins to the cytoplasm (Sacks *et al.*, 2000). To investigate the potential role of the ER and the ERGIC in the spreading of GP63 and PGs within infected cells, we employed the inhibitor of ER-Golgi trafficking Brefeldin A (Jackson *et al.*, 2000, Misumi *et al.*, 1986). Disruption of Golgi by Brefeldin A was verified by the dispersal of *cis*-Golgi marker P115 (Ge *et al.*, 2013) (Fig. 4a). In Brefeldin A-treated macrophages, trafficking of GP63 and PGs out of the PV was abrogated (Fig. 4a). Consistent with the notion that GP63 must exit the PV to cleave host cell molecules, cleavage of the macrophage membrane fusion regulator Synaptotagmin XI (Syt XI) by GP63 (Arango Duque *et al.*, 2014b) was impaired in Brefeldin A-treated macrophages (Fig. 4b,c). These results indicate that GP63 and PGs exit the PV and spread across infected cells through a mechanism involving vesicular trafficking between the PV and the ER/ERGIC.

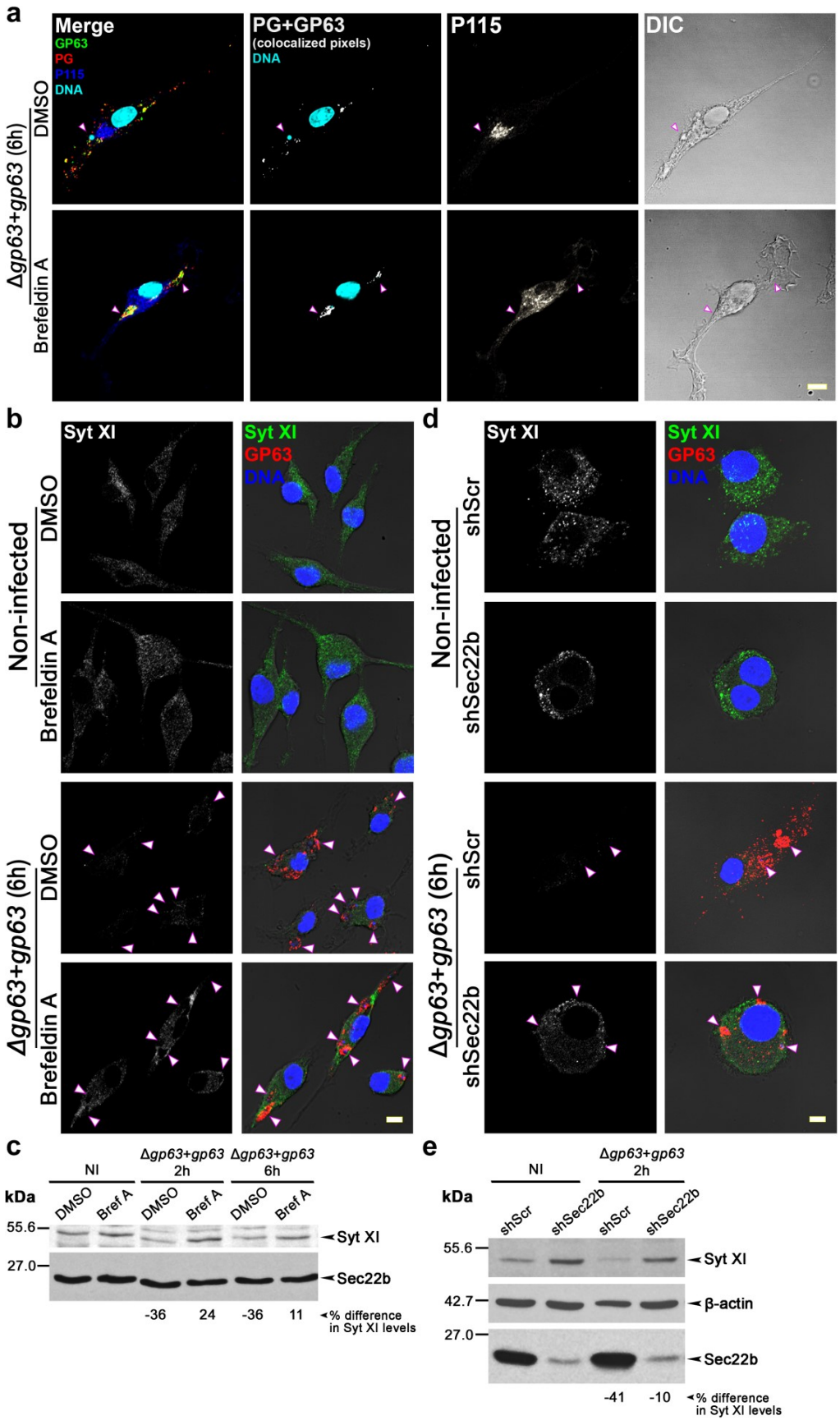

a
Merge
GP63
PG
P115
DNA
PG+GP63
(colocalized pixels)
DNA
P115
DIC
Δgp63+gp63 (6h)
DMSO
Brefeldin A
b
Syt XI
Syt XI
GP63
DNA
Non-infected
DMSO
Brefeldin A
Δgp63+gp63 (6h)
DMSO
Brefeldin A
d
Syt XI
Syt XI
GP63
DNA
Non-infected
shScr
shSec22b
Δgp63+gp63 (6h)
shScr
shSec22b
c
NI
Δgp63+gp63 2h
Δgp63+gp63 6h
kDa
DMSO
Bref A
DMSO
Bref A
DMSO
Bref A
55.6
27.0
Syt XI
Sec22b
-36
24
-36
11
% difference in Syt XI levels
e
NI
Δgp63+gp63 2h
kDa
shScr
shSec22b
shScr
shSec22b
55.6
42.7
27.0
Syt XI
β-actin
Sec22b
-41
-10
% difference in Syt XI levels

Figure 4 of article 3. Perturbation of ER-Golgi trafficking hampers the redistribution of GP63 and PGs and the cleavage of Syt XI. A, BMM were treated with Brefeldin A prior to infection with opsonized *L. major* Δ*gp63*+*gp63* metacyclic promastigotes for 6 h. GP63 is shown in green, PGs in red, P115 in blue and DNA in cyan. Colocalization between GP63 and PGs is shown in the second column (white pixels), and P115 was used as a reporter of ER-Golgi disruption. **B,** Immunofluorescence showing the impact of Brefeldin A or DMSO treatment on the degradation of GP63 (red) substrate Syt XI (green). DNA is shown in blue. **C,** Lysates from Brefeldin A-treated infected cells were examined by Western blot to evaluate the activity of GP63. The % difference in Syt XI levels represents the % difference in band intensities of infected vs. non-infected (NI) macrophages. Band intensities were normalized to Sec22b levels and a negative value is indicative of cleavage. **D,** Confocal imaging of the degradation of Syt XI by GP63 in Sec22 KD cells. **E,** Western blot showing the levels Syt XI, Sec22b, and GP63 at 2 h post-infection. The % difference in Syt XI levels represent the % difference in band intensities of 2 h-infected vs. NI cells. Band intensities were normalized to β-actin levels. A negative value is indicative of cleavage. Results are representative of two independent experiments. In **A**, **B** and **D**, white arrowheads denote internalized parasites; bar, 5 µm.

The ER/ERGIC-resident SNARE Sec22b plays a key role in phagosome maturation and function by regulating the delivery of ER and ERGIC resident proteins to phagosomes (Cebrian *et al.*, 2011, Späth *et al.*, 2003) and participates in the development of *Leishmania* PVs (Canton *et al.*, 2012b, Ndjamen *et al.*, 2010). To test whether Sec22b regulates transport of phagosome cargo to ER/ERGIC-derived vesicles, we infected the dendritic cell line JAWS-II transduced with short hairpin RNAs (shRNAs) to knock down Sec22b (Cebrian *et al.*, 2011). Cells transduced with a scrambled shRNA were used as a control. Redistribution of GP63 and PGs within infected cells as well as degradation of Syt XI were significantly impaired in Sec22b knockdown (KD) cells (Fig. 4d,e) (Supplementary Fig. 6).

The accumulation of GP63 and PGs on the PVs of Sec22b KD cells supports the notion that ERGIC provides a mobile network of vesicles that promote the export of phagosomal cargo into the cytosol (Cebrian *et al.*, 2011). The top vesicle-containing fractions of lysates from Sec22b KD cells had reduced GP63 and LPG levels compared to scrambled shRNA control cells, which is consistent with increased intraphagosomal degradation in Sec22b KD cells (Cebrian *et al.*, 2011) (Supplementary Fig. 7). Together, these data indicate that the ER/ERGIC-resident SNARE Sec22b is part of the host cell machinery that mediates the exit of *Leishmania* virulence factors from the PV.

Pathogen-containing vacuoles interact to various extents with host cell vesicles and organelles (Case *et al.*, 2016, Weber *et al.*, 2018). These selective interactions may be

finely tuned for purposes such as satisfying the metabolic needs of pathogens, supporting the membrane demand associated with pathogen replication, or modulating host cell signalling and function. Previous studies revealed that PVs harboring *Leishmania* undergo sustained interactions with the host cell's ER, allowing for the acquisition of luminal ER molecules and for the expansion of PVs (Canton *et al.*, 2012b, Ndjamen *et al.*, 2010). In the present study, we show that *Leishmania* usurps the trafficking pathway between the PV and the ER/ERGIC to deliver effectors out of the PV, where they can alter host cell process (Supplementary Fig. 8). Future studies will be required to determine whether the delivery of *Leishmania* molecules into the host cell is spatiotemporally regulated by different ER/ERGIC SNAREs, or other vesicle trafficking-associated proteins. Finally, a variety of vacuolar pathogens replicate within compartments that interact with the secretory pathway (Canton *et al.*, 2012b). It will be of interest to investigate whether the ER/ERGIC pathway is used by these pathogens to deliver effectors and modulate host cell processes. Improved understanding of this virulence strategy may help identify host factors to be potentially exploited for chemotherapy.

3 MATERIALS AND METHODS

3.1 Ethics statement

Animal work was performed as stipulated by protocols 1706-06 and 1706-07, which were approved by the *Comité Institutionel de Protection des Animaux* of the INRS-Institut Armand-Frappier. These protocols respect procedures on animal practice promulgated by the Canadian Council on animal care.

3.2 Antibodies, plasmids and inhibitors

The rabbit anti-Sec22b polyclonal antibody was obtained from Synaptic Systems; anti-ERGIC53 and -microtubule-associated protein 1 light chain 3 (LC3B) from Sigma; anti-PDI and -CNX from Enzo Life Sciences; anti-CRT from ThermoFisher; anti-glucose-regulated protein 78 (GRP78/BiP) from BD Signalling; and anti-T-cell immune regulator 1 (TCIRG1) from Abcam. The rabbit anti-Syt XI polyclonal antibody was previously described (Fukuda *et al.*, 1999). The mouse anti-phosphoglycan (Gal-Man-PO_4) CA7AE monoclonal antibody (Tolson *et al.*, 1989) was from Cedarlane. The mouse anti-GP63 monoclonal antibodies #96 (IgG2A, used in confocal microscopy) and #235 (used in Western blotting) (Button *et al.*, 1993, Macdonald *et al.*, 1995b), and the pLeishNeoGP63.1^{E265A} construct (which expresses catalytically-inactive GP63) (McGwire *et al.*, 1996) were kindly provided by W. R. McMaster (University of British Columbia). Pharmacological inhibitors Brefeldin A (35 μg/ml) (Molecular Probes), monensin (20 μM) (Sigma), and cytochalasin B (5μM) (Sigma) were reconstituted in DMSO (Bioshop).

3.3 Cell culture

BMM, bone marrow-derived dendritic cells (BMDC) and inflammatory monocytes were differentiated from the bone marrow of 6- to 8-week old female C57BL/6 mice. BMM were differentiated in complete DMEM [containing L-glutamine (Life Technologies), 10% v/v heat-inactivated foetal bovine serum (FBS) (Life Technologies), 10 mM HEPES (Bioshop) at pH 7.4, and antibiotics (Life Technologies)] supplemented with 15% v/v L929 cell-conditioned medium (LCM) as a source of macrophage colony-stimulating factor. To render BMM quiescent prior to experiments, cells were transferred to tissue culture-treated plates and kept for 16h in complete DMEM without LCM (Descoteaux *et al.*, 1989). BMDCs were differentiated in RPMI (Life technologies) containing 10% heat-inactivated FBS, 10 mM HEPES at pH 7.4, antibiotics and 10% v/v X63 cell-conditioned medium as a source of granulate-macrophage colony-stimulating factor (Ranatunga *et al.*, 2009). Inflammatory monocytes were differentiated using complete DMEM containing 15% LCM for three days. Cells were washed and incubated for 3 h with 100 U/ml IFN-γ prior to use (Hammami *et al.*, 2015). The mouse macrophage cell line RAW264.7 was cultured in complete DMEM. JAWS-II dendritic cell-like lines stably transduced with shRNA for Sec22b (Cebrian *et al.*, 2011) were a kind gift from D. S. Amigorena (Institut Curie). JAWS-II cells were cultured in RPMI containing 20% heat-inactivated FBS, 10 mM HEPES at pH 7.4, 10% X63 cell-conditioned medium and 40 µg/ml puromycin (Bioshop). All mammalian cells were kept in a humidified 37ºC incubator with 5% CO_2.

The *L. major* strains used in this study were passaged in mice to maintain their virulence. Amastigotes recovered from ear dermis lesions of infected BALB/c mice were differentiated into promastigotes (Belkaid *et al.*, 2000) in *Leishmania* medium [M199-1X (Sigma) with 10% heat-inactivated FBS, 40 mM HEPES at pH 7.4, 100 µM hypoxanthine, 5 µM hemin, 3 µM biopterin, 1 µM biotin, and antibiotics] in a 26ºC incubator. *L. major* Seidman (MHOM/SN/74) NIH clone A2 (A2WF) promastigotes (WT, Δ*gp63*, and Δ*gp63*+*gp63*) were kindly provided by W. R. McMaster (University of British Columbia). The pLeishNeoGP63.1^{E265A} construct was electroporated into Δ*gp63*

promastigotes (Veer *et al.*, 2003), selected with G418, and clones expressing similar GP63 levels to Δ*gp63*+*gp63* promastigotes were used for experiments. The Δ*gp63*+*gp63* and Δ*gp63*+*gp63*^{E265A} *L. major* promastigotes were grown in *Leishmania* medium supplemented with 100 µg/ml G418 (Life Technologies). *L. donovani* LV9 (MHOM/ET/67/Hu3:LV9) amastigotes were isolated from the spleens of infected HsdHan:AURA hamsters (Reiner, 1982) (Harlan Sprague Dawley Inc.) and were differentiated into promastigotes in *Leishmania* medium in a 26ºC incubator. WT and ∆*lpg1 L. donovani* promastigotes (Privé *et al.*, 2000) were grown in *Leishmania* medium.

3.4 Infections

Metacyclic promastigotes were enriched from cultures of freshly differentiated promastigotes in late stationary phase (Späth *et al.*, 2001). Briefly, a 2 ml cushion of 40% w/v Ficoll PM400 (GE Healthcare) was deposited on a 15 ml tube, followed by a 2 ml layer of 10% Ficoll PM400 in M199-1X. Late stationary phase promastigotes were resuspended in a 2 ml volume of DMEM with no serum, and overlaid onto the 10% Ficoll layer. After a 10 min spin, metacyclic promastigotes were collected from the DMEM-10% Ficoll interphase, which regularly contained 10% of the input population. The isolated metacyclic promastigotes express a longer form of the GPI-anchored LPG, and displayed higher levels of GP63 proteolytic activity as previously reported (Barron *et al.*, 2006, Yao, 2010) (Supplementary Fig. 9a). These promastigotes were washed and spun at 2000 *g* prior to inoculation into the ear dermis of mice (Belkaid *et al.*, 2000), or opsonised with C5-deficient serum from DBA/2 mice for *in vitro* infections (Lodge *et al.*, 2006b). Opsonised metacyclic promastigotes were resuspended in cold complete DMEM and fed to with macrophages or JAWS-II cells at 4ºC for 5 min (Arango Duque *et al.*, 2013), followed by a 2 min spin at 1200 rpm in a Sorvall RT7 centrifuge. Parasite internalization was triggered by transferring cells to 37ºC (Arango Duque *et al.*, 2014b, Vinet *et al.*, 2008). After 2 h, non-internalized parasites were washed 3X with warm medium. Cells were then washed and prepared for lysis or confocal immunofluorescence microscopy. Parasite survival was assessed by scoring

intracellular parasites in eosin-hematoxylin-stained BMM. Metacyclic promastigotes used in this study were virulent in both *in vitro* and *in vivo* infections (Supplementary Fig. 9b-d).

Air pouch exudates containing infected neutrophils were obtained from 6 to 8-week old mice. Briefly, 3 ml of sterile air was injected subcutaneously, at days 0 and 3, in the backs of anesthetized mice (Lavastre *et al.*, 2004). At day 6, 50 X 10^6 *L. major* Δ*gp63+gp63* promastigotes in 0.5 ml HBSS were injected in the air pouch. Six hours post-injection, mice were sacrificed and exudates were obtained by washing the air pouches with HBSS-EDTA. Exudate cells were washed with complete medium, spun onto poly-L-lysine coverslips (BD), and incubated at 37ºC for 20 min. Coverslips were then washed and prepared for microscopy.

3.5 Electrophoresis, Western blotting and zymography

Prior to lysis, adherent macrophages or parasites were placed on ice and washed with PBS containing 1 mM sodium orthovanadate and 5 mM 1,10-phenanthroline (Sigma). Macrophages were scraped in the presence of lysis buffer containing 1% NP-40, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8), 10 mM 1,10-Phenanthroline, and phosphatase and protease inhibitors (Roche). In the case of the semi-adherent JAWS-II cells, they were spun and pelleted 3X at 700 rpm in 5 ml of cold PBS (containing orthovanadate and phenanthroline) prior to lysis. After incubation at -70°C, lysates were centrifuged for 15 min to remove insoluble matter. After protein quantification, 30 µg of protein was boiled (100ºC) for 6 min in SDS sample buffer and migrated in SDS-PAGE gels. Proteins were transferred onto Hybond-ECL membranes (Amersham Biosciences), blocked for 2h in TBS1X-0.1% Tween containing 5% BSA, incubated with primary antibodies (diluted in TBS1X-0.1% Tween containing 5% BSA) overnight at 4°C, and thence with suitable HRP-conjugated secondary antibodies for 1h at room temperature. Membranes were incubated in ECL (GE Healthcare) and immunodetection

was achieved via chemiluminescence (Arango Duque *et al.*, 2014b). Densitometric analysis of Western blot bands was done using the AlphaEase FC software (Alpha Innotech) and heat maps were generated using R (Ge *et al.*, 2013). GP63 activity was assayed via gelatin zymography (Hassani *et al.*, 2014). Briefly, 10 µg of infected cell lysate, or 2 µg of promastigote lysate were incubated at 50°C for 5 min in sample buffer without DTT and then migrated in 10% SDS-PAGE gels containing 0.12% gelatin (Sigma). Gels were incubated for 2 h in the presence of 50 mM Tris pH 7.4, 2.5% Triton X-100, 5 mM $CaCl_2$ and 1 mM $ZnCl_2$, followed by an overnight incubation at 37°C in a buffer containing 50 mM Tris pH 7.4, 5 mM $CaCl_2$, 1 mM $ZnCl_2$ and 0.01% NaN_3. Protease activity was visualized by staining the gels with 0.5% Coomassie (Sigma).

3.6 Sucrose gradient flotation assays

Infected macrophages or JAWS-II cells ($50x10^6$) were washed and resuspended in a hypotonic solution consisting of 20 mM Tris at pH 7.33, 10 mM 1,10-Phenanthroline and 1.5X protease inhibitors and incubated on ice for 30 min. Mechanical lysis was effectuated by passing this suspension 12X through a 1 ml syringe mounted with a 27G needle (Pilar *et al.*, 2008). To this homogenate, NaCl was added to a final concentration of 0.1 M. After a 15 min centrifugation at 5000 *g* and 4°C, the postnuclear supernatant was passed through a 0.22 µm filter to eliminate unwashed parasites and phagosomes. For the flotation assay (discontinuous sucrose gradients), equal quantities (~3.4 mg) of protein were used for all conditions. The gradient was set up with 57% sucrose at the bottom (450 µl of lysate mixed with 1.1 ml of 80% sucrose), followed by 35% sucrose and then TBS at the top of the tube. The sucrose solutions contained 1.5X protease inhibitors and 1,10-Phenanthroline at 10 mM. Ultracentrifugation was carried out for 16h at 4°C (28000 rpm) in a Beckman Coulter Optima XL-I centrifuge using an SW 55 Ti rotor. After centrifugation, 650 µl fractions were carefully collected from the top. Fractions were then mixed with 0.1% sodium deoxycholate and precipitated with 17% TCA. Precipitated protein was washed with acetone, resuspended in 2X SDS sample buffer, boiled for 6 min at 100°C and loaded onto SDS-PAGE gels.

3.7 Proteinase and phospholipase protection assays

Fractions from sucrose gradients were ultracentrifuged at 49000 rpm for 1 h in order to pellet vesicles. These were then carefully resuspended in filtered TBS 1X with no additives and treated with Prot K (Bioshop) at 150 µg/ml in the presence or absence of 10% Triton-X100, for 30-60 min at 4°C. Vesicles were also treated with *B. cereus* PI-PLC (Molecular Probes) at 5 U/ml for 3 h at 37°C. Alternatively, metacyclic promastigotes were killed by incubating them for 3 min in 5% NaN_3 followed by 2 min in 0.05% formalin. After washing, promastigotes were resuspended in TBS 1X and treated with Prot K or PI-PLC. Reactions were stopped by adding protease inhibitors (1X) and PMSF (1 µM) for 5 min at 4°C, followed by sample buffer. Samples were immediately boiled at 100ºC for 6 min and loaded onto SDS-PAGE gels.

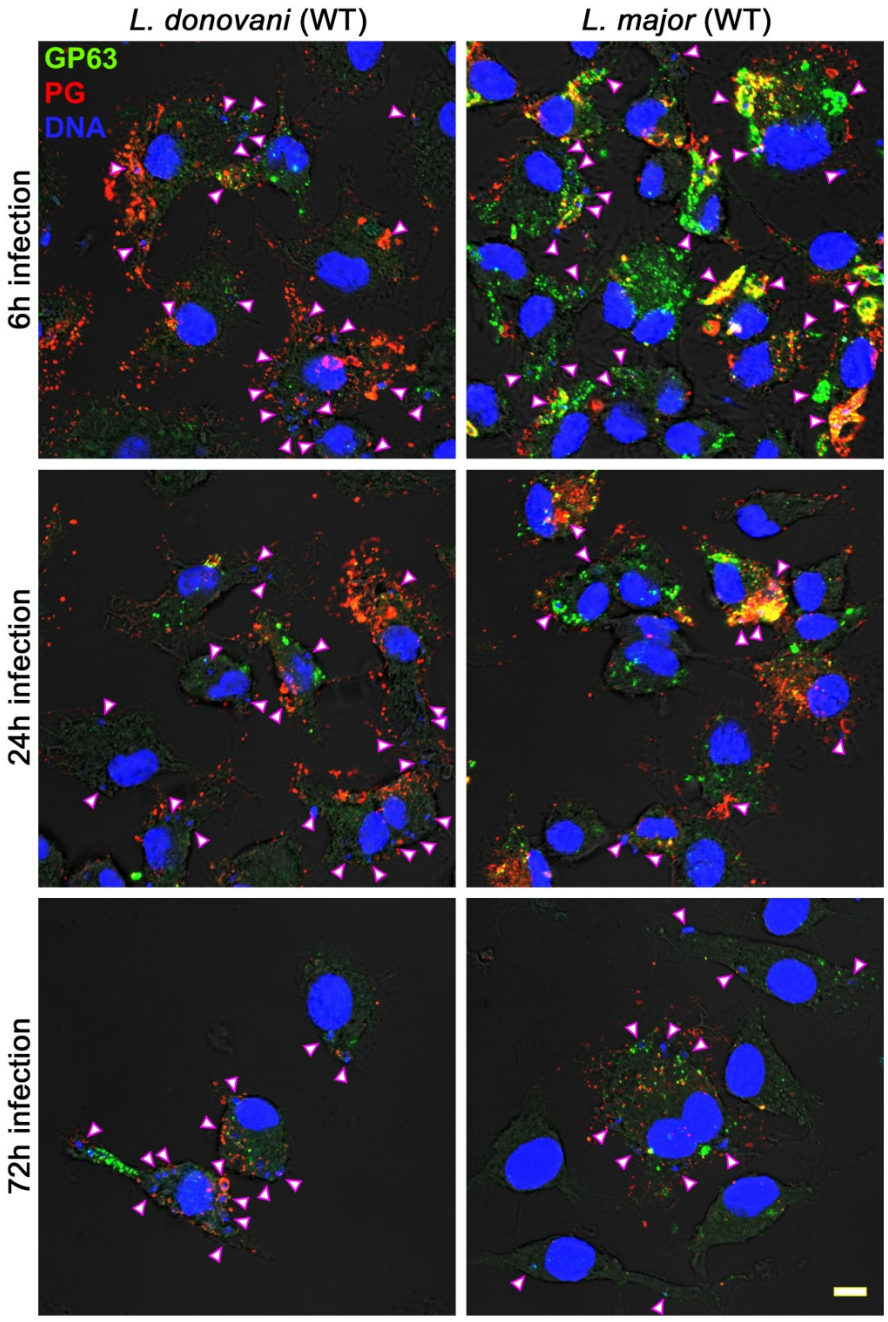
3.8 Immunofluorescence and electron microscopy

Infected cells on coverslips were fixed with 2% paraformaldehyde (Canemco and Mirvac) for 20 min and blocked and permeabilized for 17 min with a solution of 0.1% Triton X-100, 1% BSA, 6% non-fat milk, 2% goat serum, and 50% FBS. This was followed by a 2 h incubation with primary antibodies diluted in PBS. Then, macrophages were incubated with a suitable combination of secondary antibodies (anti-rabbit AlexaFluor 647, anti-rat 568, highly cross-adsorbed anti-mouse-IgG2A 488, highly cross-adsorbed anti-mouse-IgM 568; Molecular Probes) and DAPI (Molecular Probes). G_{M1}^+ lipid rafts were stained with the cholera toxin-subunit B-AlexaFluor 647 conjugate (Molecular Probes) (Vinet *et al.*, 2009). Coverslips were washed three times with PBS after every step. After the final wash, Fluoromount-G (Southern Biotechnology Associates) was used to mount coverslips on glass slides (Fisher), and coverslips were sealed with nail polish (Sally Hansen). Cells were imaged with the 63X objective of an LSM780 confocal microscope (Carl Zeiss Microimaging), and images were taken in

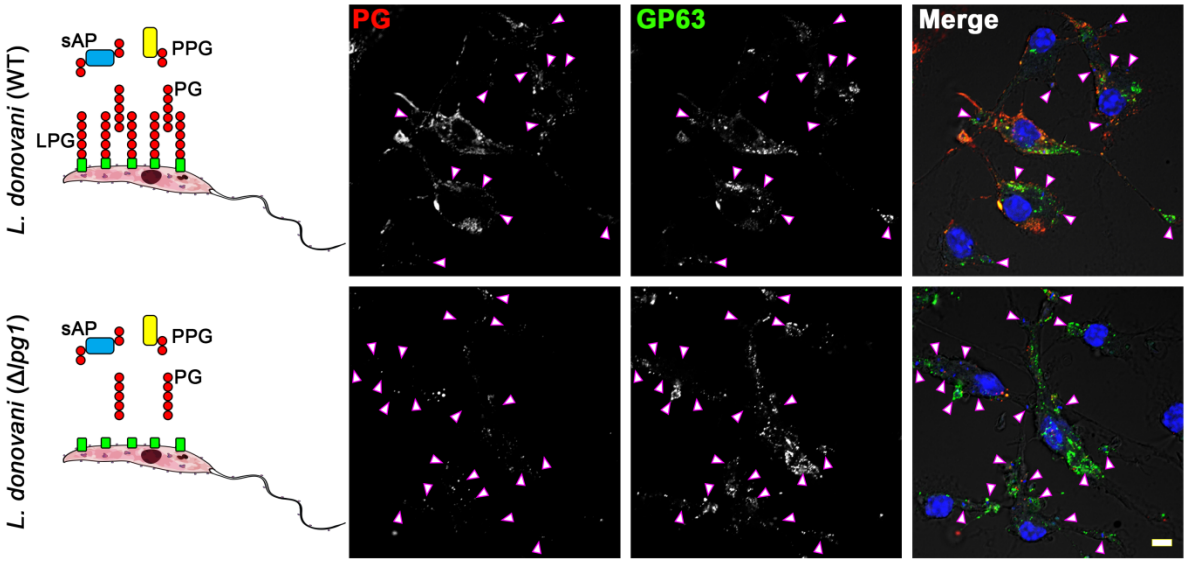
sequential scanning mode. For super resolution imaging, coverslips were imaged with an LSM880 NLO confocal microscope (Carl Zeiss Microimaging) equipped with the Airyscan module. Image processing was done with the ZEN 2012 software.

Electron microscopy (EM) was used to visualize vesicular structures in sucrose gradient-fractionated lysates. A 10 µl aliquot of each fraction was spotted onto a copper grid, spun, and negatively stained using 3% phosphotungstic acid at pH 6.0 (Berthiaume *et al.*, 1982). Structures were visualized using a Philips EM 300 electron microscope.

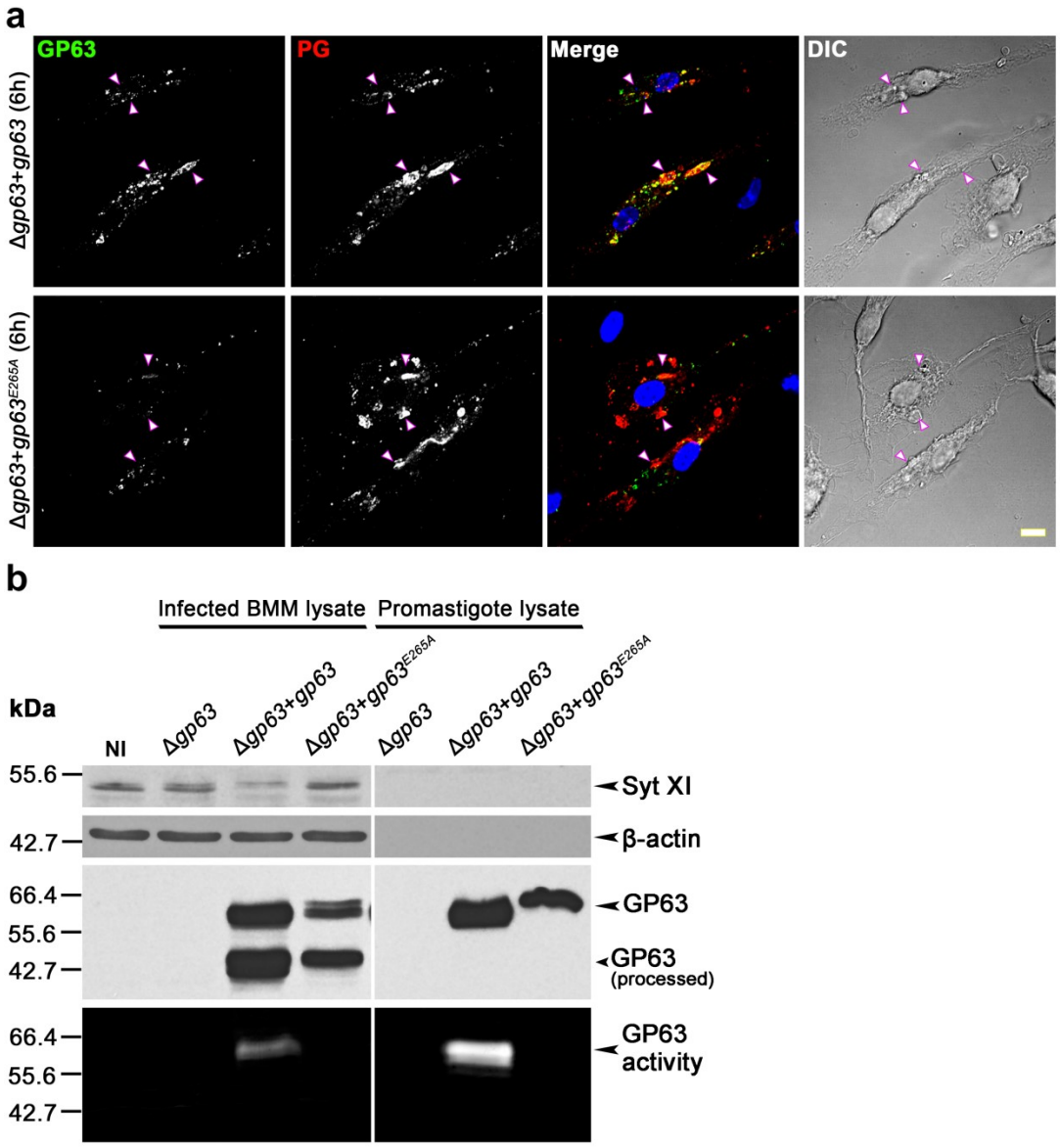
4 SUPPLEMENTARY FIGURES



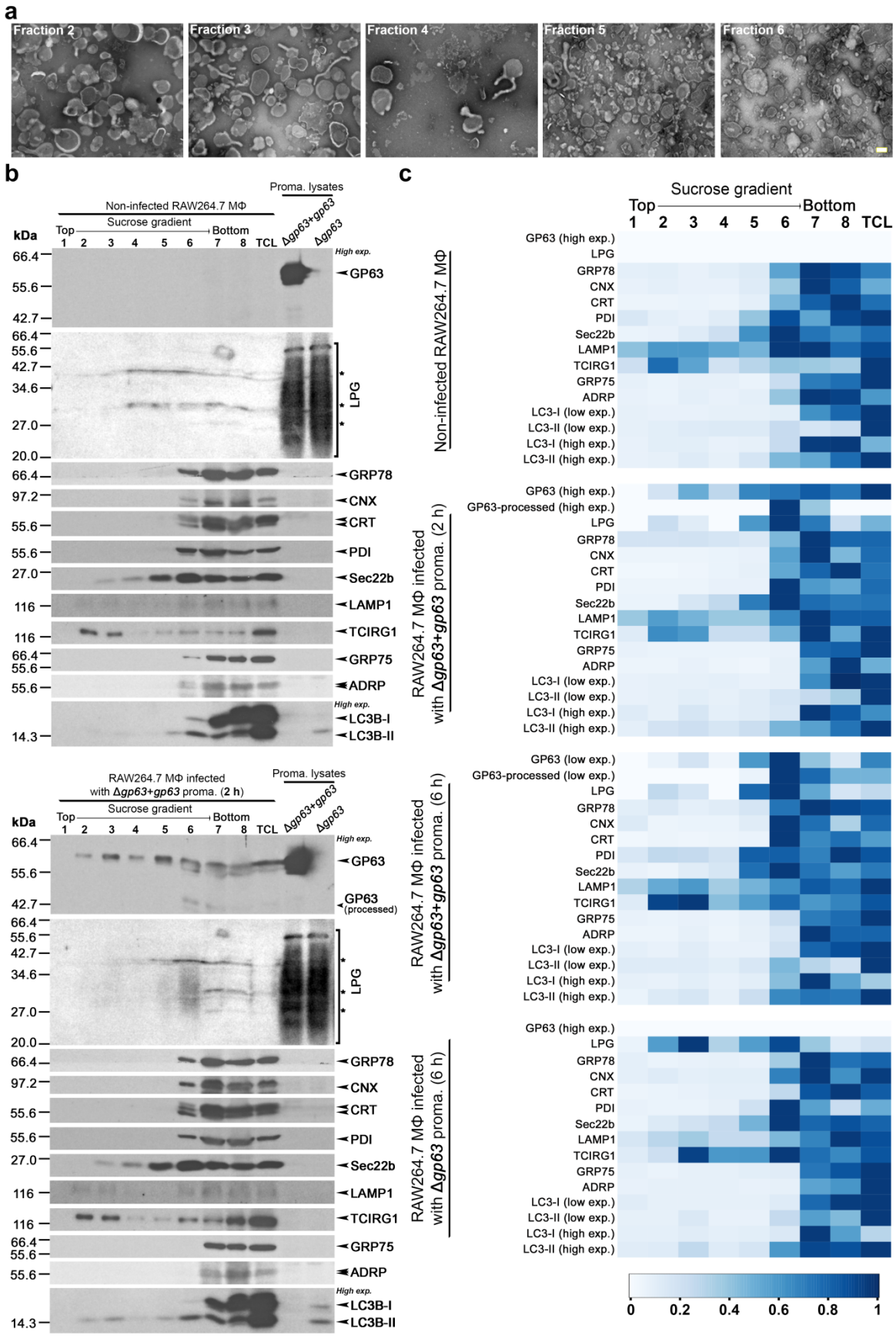
Supplementary Figure 1 of article 3. Redistribution of GP63 and PGs in macrophages infected with *L. major* and *L. donovani*. To assess the trafficking and persistence of GP63 and PGs over a period of 6 to 72h, we infected BMM with *L. major* or *L. donovani* with metacyclic promastigotes. Using immunofluorescence, the redistribution of GP63 (green) and PGs (red) was observed over the indicated time points. DNA is in blue; bar, 5 µm.



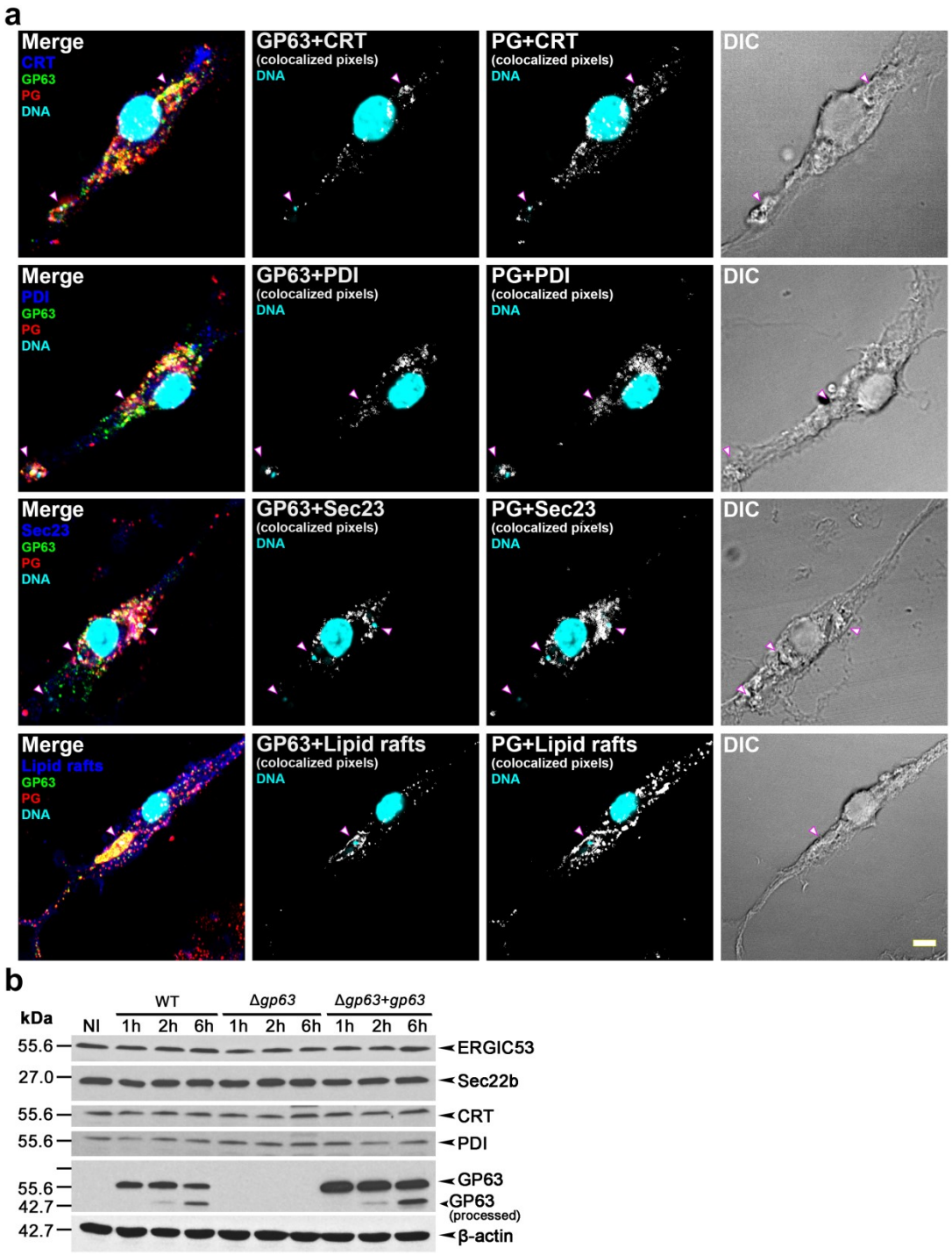
Supplementary Figure 2 of article 3. The redistribution of PGs is similar to that of LPG. We sought to elucidate whether the trafficking of PGs differs in promastigotes that predominantly express the GPI-anchored LPG versus promastigotes that only secrete the repeating disaccharide-phosphate repeats (schema on the left). We infected BMM for 6 h with WT *L. donovani* promastigotes and isogenic Δ*lpg1* mutants that do not make a functional glycan core but secrete disaccharide-phosphate repeats. PGs are shown in red, GP63 in green and DNA in blue. Images are representative of two independent experiments. White arrowheads denote internalized parasites. Bar, 5 μm.



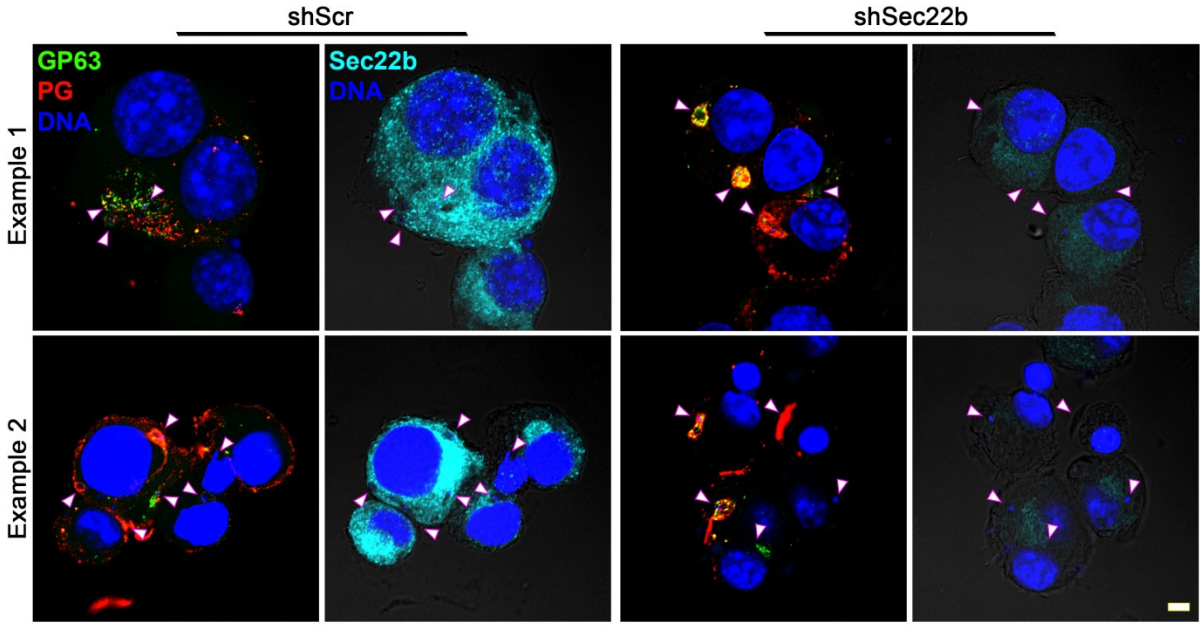
Supplementary Figure 3 of article 3. GP63 activity has no impact on the redistribution of GP63 and PGs. A, To investigate whether the catalytic activity of GP63 was required for GP63 or PGs to disperse from the PV, we infected BMM with *L. major* metacyclic promastigotes expressing catalytically active (Δ*gp63+gp63*) or inactive (Δ*gp63+gp63*^{E265A}) GP63. Six hours post-infection, cells were fixed and prepared for confocal microscopy. GP63 is shown in green, PGs in red and DNA in blue. White arrowheads denote internalized parasites. **B**, Infected cell lysates were probed by Western blot. The Syt XI blot and gelatin zymography were used to evaluate GP63 activity. These results are representative of two independent experiments. NI, non-infected; bar, 5 µm.


a
Fraction 2
Fraction 3
Fraction 4
Fraction 5
Fraction 6
b
Non-infected RAW264.7 MΦ
Proma. lysates
Sucrose gradient
Top
Bottom
kDa
1 2 3 4 5 6 7 8 TCL Δgp63+gp63 Δgp63
High exp.
GP63
LPG
GRP78
CNX
CRT
PDI
Sec22b
LAMP1
TCIRG1
GRP75
ADRP
LC3B-I
LC3B-II
RAW264.7 MΦ infected with Δgp63+gp63 proma. (2 h)
RAW264.7 MΦ infected with Δgp63+gp63 proma. (6 h)
GP63 (processed)
c
Sucrose gradient
Top
Bottom
1 2 3 4 5 6 7 8 TCL
Non-infected RAW264.7 MΦ
RAW264.7 MΦ infected with Δgp63+gp63 proma. (2 h)
RAW264.7 MΦ infected with Δgp63+gp63 proma. (6 h)
GP63 (high exp.)
GP63-processed (high exp.)
GP63 (low exp.)
GP63-processed (low exp.)
LPG
GRP78
CNX
CRT
PDI
Sec22b
LAMP1
TCIRG1
GRP75
ADRP
LC3-I (low exp.)
LC3-II (low exp.)
LC3-I (high exp.)
LC3-II (high exp.)
0 0.2 0.4 0.6 0.8 1

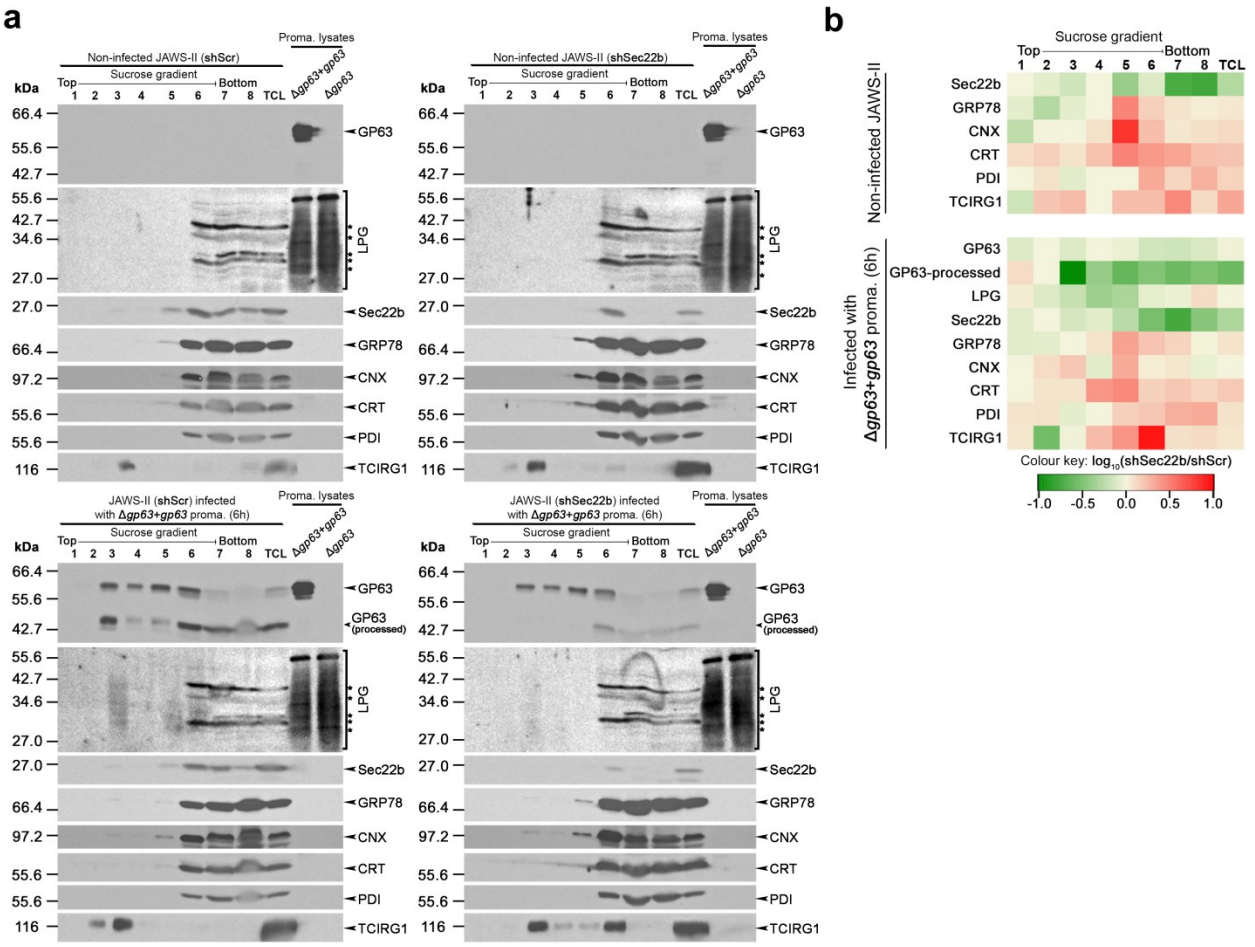
Supplementary Figure 4 of article 3. GP63 and PGs cofractionate with vesicles and ER/ERGIC markers. RAW264.7 macrophages were either non-infected or infected with opsonized *L. major* Δ*gp63*+*gp63* metacyclic promastigotes for 2-6 h. A flotation assay was performed where cells were lysed mechanically; sucrose was overlaid over lysates and samples ultracentrifuged for 18 h. Fractions were collected from the top. **A**, The presence of vesicles in the collected fractions from 6 h-infected cells (Δ*gp63*+*gp63*) was verified by electron microscopy and shown here; those from the other conditions were similar (not shown). Bar, 100 nm. **B**, Western blots show the levels of various *Leishmania* and macrophage proteins in fractionated lysates from 2 h infection; 6 h infections are show in in Fig. 2. Light vesicle-containing fractions are delimited by the exclusive appearance of LC3B-II, which is membrane-bound. The LPG band appears as a smear and asterisks (*) indicate non-specific bands of macrophage origin (see non-infected cell and promastigote lysate lanes). **C**, Densitometric analysis of flotation assay in Fig. 3a and part **B** of this Figure. To facilitate the comparison of band intensities in each condition, heat maps were produced from densitometry data. For each protein (i.e., Sec22b in non-infected cells), the band with the highest intensity was assigned a value of 1, and the other intensities in that group (fraction 1 to TCL) were normalized with respect to that band. Since there is no GP63 in non-infected cells, background from this condition was subtracted from the other conditions (infected cells). Densitometries were then normalized as above. The densitometry of the ~42 kDa fragment (GP63-processed) was also analyzed. In the case of LPG, a box encasing the smears was used to calculate the densitometries. Since there are no PGs in non-infected cells, background from this condition, including that given by the non-specific bands of macrophage origin, was subtracted from the other conditions (infected cells). TCL, total cell lysate.



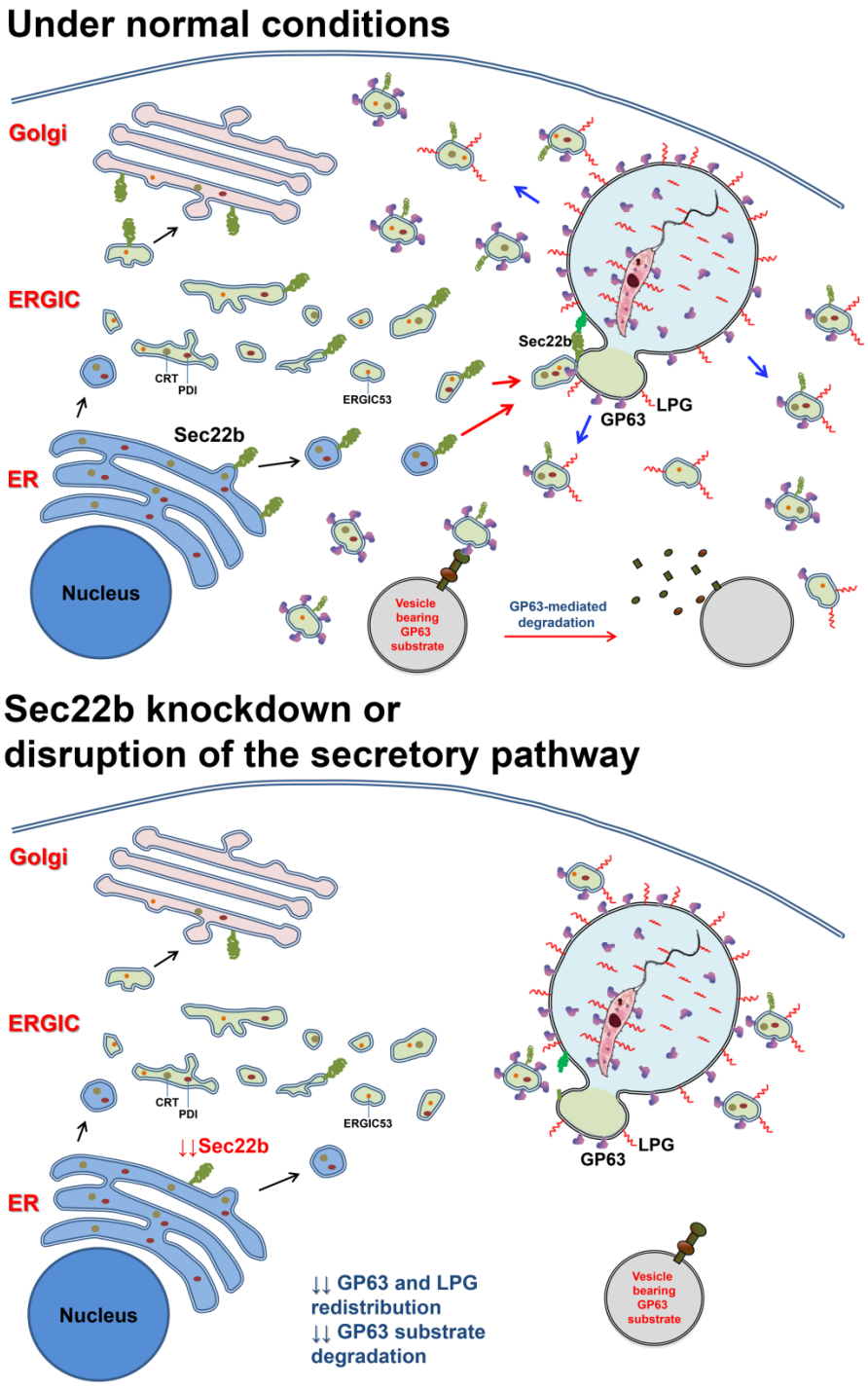
Supplementary Figure 5 of article 3. GP63 and PGs colocalize with ER markers. A. BMM were infected with opsonized *L. major* Δ*gp63+gp63* metacyclic promastigotes for 6 h, fixed, and immunostained with the antibody combinations shown and with DAPI to stain DNA (cyan). GP63 (green) and PGs (red) colocalized extensively with ER markers (blue) CRT, PDI, and Sec23. The second and third columns of panels show pairwise colocalizations (white) of GP63 or PGs with ER markers. **B**. GP63 does not cleave resident ER and ERGIC proteins. To investigate whether ER and ERGIC proteins are cleaved by GP63, BMM were infected with opsonized WT, Δ*gp63* and Δ*gp63+gp63* metacyclic promastigotes. The integrity of the various ER and ERGIC markers was assayed by Western blot. Results are representative of at least two independent experiments. NI, non-infected; bar, 5 μm.



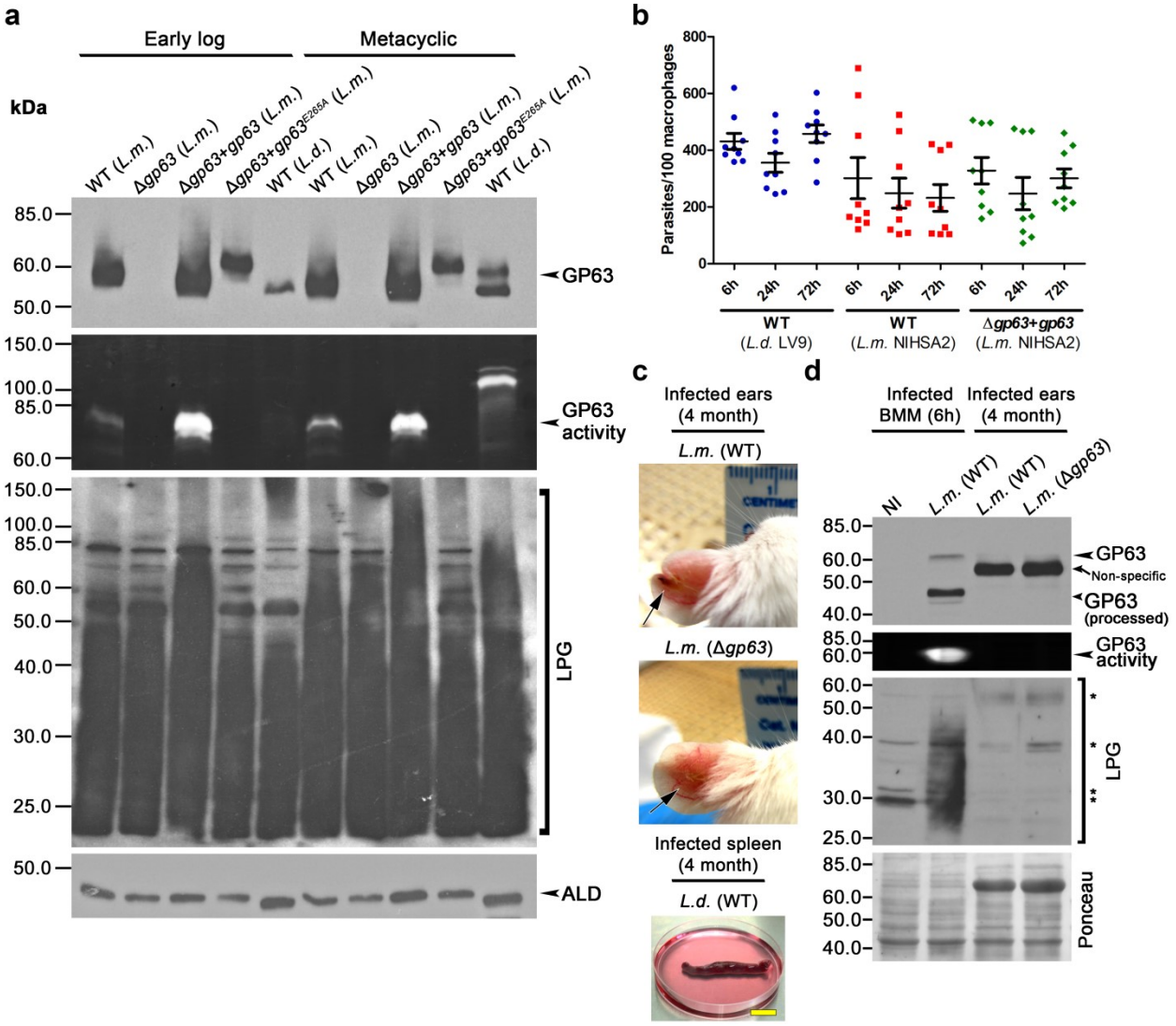
Supplementary Figure 6 of article 3. Sec22b promotes the redistribution of GP63 and PGs. Control and Sec22b KD JAWS-II cells were infected with opsonized *L. major* Δ*gp63*+*gp63* metacyclic promastigotes for 6 h. Immunofluorescence imaging shows the effect of Sec22b (cyan) knockdown on the redistribution of GP63 (green) and PGs (red). DNA is in blue; bar, 5 µm.



Supplementary Figure 7 of article 3. Flotation of GP63 and PGs in lysates of infected JAWS-II cells. JAWS-II cells were either non-infected or infected with opsonized *L. major* Δ*gp63+gp63* and Δ*gp63* metacyclic promastigotes for 6 h. A flotation assay was performed where cells were lysed mechanically; sucrose was overlaid over lysates and samples ultracentrifuged for 18 h. **A.** Western blots show the levels of various *Leishmania* and macrophage proteins in fractionated lysates. GRP78, CNX, CRT, and PDI were used as ER markers; Sec22b as an ERGIC marker; and TCIRG1 as a maker of endosomes and lysosomes. Asterisks (*) indicate non-specific bands of macrophage origin. **B**. Densitometric analysis of flotation assay shown in **A.** To facilitate the analysis of band intensities, heat maps were produced to compare densitometric data in JAWS-II cells transfected with control (shScr) or shRNA to Sec22b (shSec22b). For all studied proteins, including the processed form of GP63, densitometries in Western blots from shSec22b cells were divided by the corresponding densitometries in blots from shScr cells. The $\log_{10}$ of the ratios, varying from -1 to 1 were displayed as a colour from green to beige to red. A relative decrease is a negative value, no difference is 0, and an increase is a value greater than 0. The data are representative of two independent experiments. TCL, total cell lysate.



Supplementary Figure 8 of article 3. Host cell organelles and proteins mediate the trafficking and function of GP63 and PGs. Following promastigote internalization, GP63 and PGs traffic from the PV into the cytoplasm of the infected cell in a time-dependent manner. Redistribution is necessary for GP63 to access its substrates. This phenomenon was found to be mediated by secretory pathway, with the SNARE Sec22b playing an important role. When ER-Golgi trafficking is chemically perturbed or when Sec22b is knocked down, the redistribution of GP63 and LPG is abrogated, which leads to decreased degradation of GP63 substrates.



Supplementary Figure 9 of article 3. Metacyclic promastigotes persist in infected macrophages and cause disease in animals. A. Expression of GP63 and LPG in procyclic and metacyclic promastigotes. To compare the expression of GP63 and LPG in different developmental stages of *Leishmania* promastigotes, the lysates of procyclic (early log) and metacyclic promastigotes of all strains used in this study were analyzed. The expression of GP63, LPG and ALD were probed via Western blot and the activity of GP63 was assayed via gelatin zymography. The images shown are representative of two independent experiments. **B.** Quantification of *L. donovani* and *L. major* intracellular survival at 6, 24 and 72 h post-infection. Data are presented as mean ± s.e.m. of n = 3 experiments done in triplicate, with each point representing the number of intracellular parasites found in 100 macrophages. **C.** *L. major* (*L.m.* WT or Δ*gp63*) and *L. donovani* (*L.d.* WT) metacyclic promastigotes were used to infect mice (intradermally in ear pinna) and hamsters (intraperitoneally), respectively. The cutaneous and visceral lesions that ensued after 4 months were photographed. Black arrows point to cutaneous lesions; in lower panel, bar = 2 cm. **D.** BMM or the ear pinna of mice infected with *L. major* metacyclic promastigotes (WT or Δ*gp63*) were lyzed and probed for the presence of GP63 and LPG. NI, non-infected. Non-specific bands in the GP63 blot are noted, and asterisks (*) indicate non-specific bands of macrophage or tissue origin (see NI lane). The activity of GP63 was assayed via gelatin zymography.

5 ACKNOWLEDGEMENTS

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6 COMPETING INTERESTS

The authors declare no competing interests.

CHAPTER 4: DISCUSSION AND CONCLUSIONS

1 THE INVOLVEMENT OF SYT XI IN MACROPHAGE BIOLOGY AND BEYOND

1.1 Syts regulate the effector functions of the immune system

Syts were originally characterized as proteins that regulate neurotransmitter release in neuronal cells (Fukuda, 2007, Südhof, 2012). They achieve this function by mediating the adjoining of two membranes that have been tethered and docked by SNAREs and their regulatory proteins. Although neurotransmission is obviously very important, a great variety of homeostatic functions also depend on cellular secretion (Bonifacino *et al.*, 2004, Murray *et al.*, 2014). These functions range from the secretion of hormones and cytokines to phagocytosis and antimicrobial defense. The first studies on the involvement of Syts in the immune system started in the late 1900s and early 2000s, when it was observed that Syts I and II regulate lysosome exocytosis and antigen presentation in mast cells (Baram *et al.*, 1999, Baram *et al.*, 2002). These studies were followed by investigations that dealt with the calcium dependence of immune processes such as chemotaxis and diapedesis in neutrophils and T cells (Colvin *et al.*, 2010, Masztalerz *et al.*, 2007). By the mid-2000s, the involvement of Syts in immune defense was emerging, with studies demonstrating that Syt V and Syt VII regulated particle uptake and phagolysosome maturation (Table I) (Czibener *et al.*, 2006, Vinet *et al.*, 2008, Vinet *et al.*, 2009). Due the importance of these processes in innate immunity, we sought to characterize the roles of other Syts in macrophage function. Of particular interest was the Syt IV/XI subclade of the Syt family, since both Sys fail to bind phospholipids and calcium by virtue of their S247 residue (von Poser *et al.*, 1997, Wang *et al.*, 2010). Due to the inhibitory function of Syt IV on BDNF secretion by neurons (Dean *et al.*, 2009), we expected that these Syts functioned similarly in macrophages. To our surprise, only Syt XI was expressed in those cells (Arango Duque *et al.*, 2013).

Table I of discussion. Syts control membrane trafficking in myeloid and lymphoid cells. Multiple members of the Syt family are involved in regulating the vesicle fusion events that mediate the biological functions of cells of the immune system.

Macrophages	Dendritic cells and neutrophils	Mast cells	T cells
Syt V - Positive regulator of phagocytosis and intraphagosomal acidification	**Syt VII** - Modulator of MHCII translocation to the plasmalemma (dendritic cells)	**Syt I** - Positive regulator of lysosomal secretion (serotonin & cathepsin D)	**Syt II** - Negative regulator of chemotaxis
Syt VII - Positive regulator of phagocytosis	- Mediator of chemotaxis (neutrophils)	**Syt II** - Negative regulator of lysosomal secretion	**Syt III** - Modulator of CXCR4 recycling
- Regulator of lysosomal secretion during bone formation (osteoclasts)		- Regulator of MHC-II antigen presentation	- Positive regulator of chemotaxis
Syt XI - Negative regulator of cytokine release and phagocytosis (macrophages and microglia)		**Syt III and Syt IX** - Regulators of the intracellular trafficking of secretory granules	**Syt VII** - Positive regulator of chemotaxis
- Mediator of intraphagosomal killing			

1.2 Syt XI controls the exocytosis of proinflammatory cytokines

Inflammation is a carefully orchestrated protective response that evolved to resolve challenges such as infection and injury (Silverstein, 2011, Sozzani *et al.*, 2014, Unanue *et al.*, 1976). The process is coordinated by cytokines at the site of the immune response in order to increase vascular permeability and attract phagocytes. Additionally, those same cytokines can induce systemic effects such as the induction of fever. Deregulated inflammation often involves the excessive release of proinflammatory cytokines and leads to diseases such as fibrosis and autoimmunity (Arango Duque *et al.*, 2014a, Murray *et al.*, 2014, Scheller *et al.*, 2011). Given the detrimental effect of excessive inflammation, the immune system devised control mechanisms such as the release of anti-inflammatory cytokines. We hypothesized that the seemingly inhibitory role of Syt XI in vesicle fusion could control proinflammatory cytokine release. Indeed,

silencing this protein potentiated the release of TNF and IL-6, as well as particle ingestion (Arango Duque *et al.*, 2013). This important finding was later expanded to include IL-1β in microglia (Figure 1) (Du *et al.*, 2017). With the finding that macrophages control apoptotic cell uptake by non-professional phagocytes via IGF-1, it will be of interest to investigate whether that growth factor affects the expression of Syt XI in those cells (Arango Duque *et al.*, 2016, Han *et al.*, 2016). Since Syt XI is a negative regulator of particle ingestion (Arango Duque *et al.*, 2013, Du *et al.*, 2017), then IGF-1 may augment Syt XI levels in non-professional phagocytes.

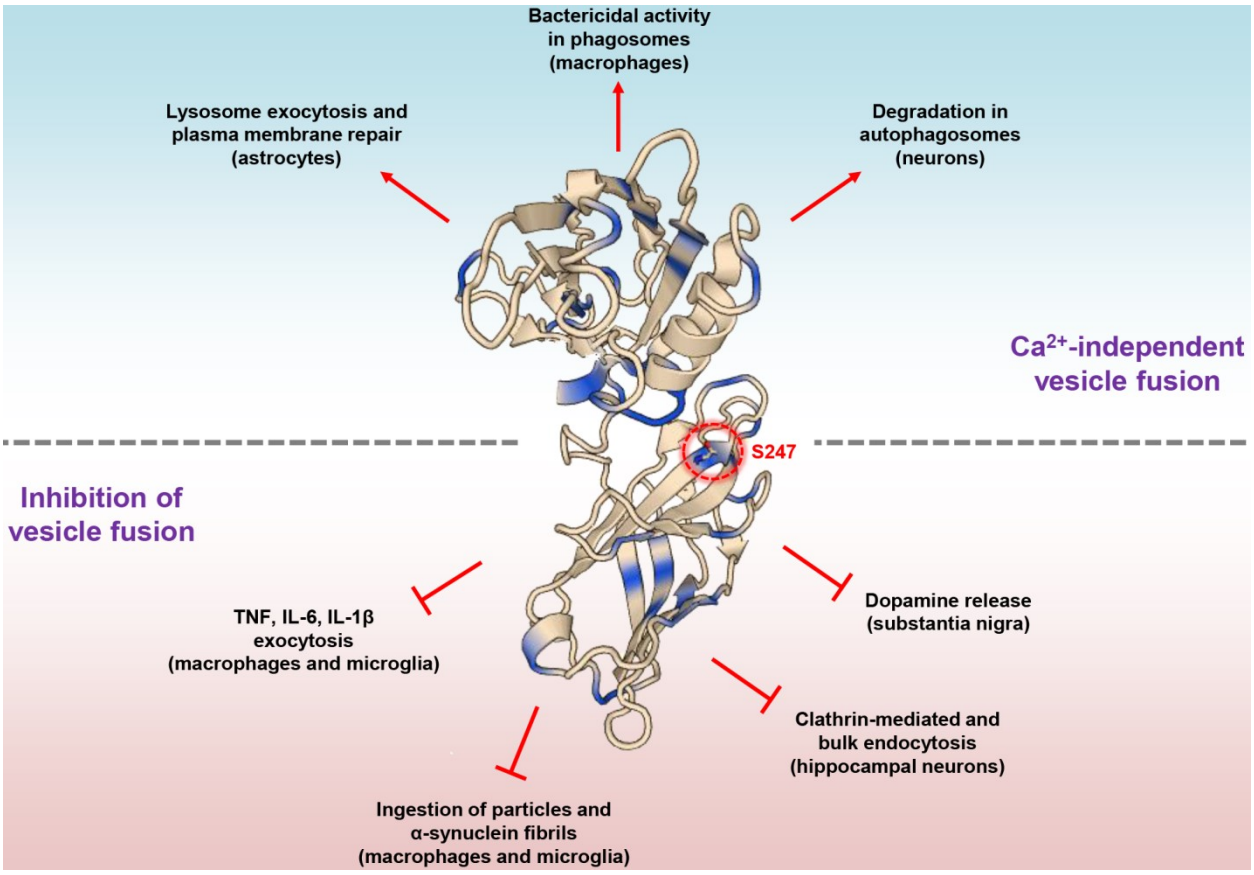

Figure 1 of discussion. Syt XI regulates processes of great importance in immunity and neurotransmission. Following the discovery of Syt XI in 1997 (von Poser *et al.*, 1997), subsequent functional studies have found that this protein controls exocytosis and endocytosis in neuronal and immune system cells. A distinguishing feature of this Syt is its S247 residue, which prevents it from binding calcium and phospholipids. Indeed, various endocytic and exocytic processes are inhibited. On the other hand, Syt XI regulates functions that do not rely on calcium binding, such as phagosome and autophagosome maturation, and plasmalemma repair. The ribbon diagram of Syt XI was generated with the Swiss-Model PDB viewer (https://swissmodel.expasy.org/repository/uniprot/Q9R0N3). Serines and threonines are coloured in blue, and the position of the S247 residue is circled in red.

In addition to cytokine secretion and phagocytosis, we found that Syt XI KD impeded phagosome maturation, highlighting the important role of this protein in the response against infection (Arango Duque *et al.*, 2013). After our initial characterization of Syt XI, other scientists found that this Syt had similar inhibitory roles in the endocytosis and exocytosis of dopamine (Wang *et al.*, 2018). In that system, loss of Parkin leads to a pathogenic accumulation of Syt XI in the brain, eventually leading to the development of Parkinson's disease symptoms. Moreover, Syt XI was found to control autophagy in neuronal cells, where its absence results in defective autophagosome function. (Bento *et al.*, 2016) From these data, one can infer that excess of, or lack of Syt XI could both cause pathologies. Taking all of these studies into consideration, one can infer that Syt XI is a key regulator of immune and CNS function (Figure 1). Are the two linked? New data are demonstrating that chronic and sustained inflammation is involved in the development of many mental health problems such as depression and autism (Miller *et al.*, 2015). Hence, it will be important to employ pan- and macrophage-specific *SytXI*$^{-/-}$ mice to test whether the absence of Syt XI causes chronic inflammation in the CNS and other tissues.

1.3 How does Syt XI regulate vesicular traffic in macrophages?

The involvement of Syt XI in cellular process (Figure 1) raises many questions about how this protein regulates vesicle fusion. Since this Syt potentiates certain processes and inhibits others, multiple mechanisms of action are likely to exist. In regard to its inhibitory role in cytokine release and particle uptake (Arango Duque *et al.*, 2013, Du *et al.*, 2017), one hypothesis is that Syt XI could delay the untangling or recycling of SNARE complexes post-cytokine delivery. This can happen at the level of the Golgi, where the SNAREs Stx6/7 and Vtib promote the passage of cytokine-containing vesicles to VAMP3-positive REs (Murray *et al.*, 2014, Stow *et al.*, 2013). Alternatively, Syt XI may inhibit the recycling of the Stx4-SNAP23-VAMP3 SNARE complex at the plasmalemma. A temporary delay in the recycling of SNARE complexes would ensure that there is no excessive cytokine release, while allowing time for the recycling of

phospholipids and other proteins involved in vesicular traffic. In this line of thought, the Parkin ubiquitin ligase could target Syt XI for proteasome-mediated destruction (Huynh *et al.*, 2003, Wang *et al.*, 2018), an event that can relieve the break in vesicle delivery to the plasma membrane. Interestingly, co-IP data show that a Syt XI construct interacts with Stx4 and Vtib (Figure 2), supporting the argument that Syt XI modulates SNARE function at the Golgi and plasma membrane. How Syt XI regulates the accrual of LAMP-1- and gp91^{phox}-containing vesicles to the phagosome is also a matter that deserves further exploration (Arango Duque *et al.*, 2013). The importance of Syt XI to the accrual of those molecules to the phagosome was demonstrated via RNAi. On the other hand, silencing of LAMP-1 or gp91^{phox} ensues in immature phagosomes (Huynh *et al.*, 2007a, Levin *et al.*, 2016, Nauseef, 2004). The fact that Syt XI KD suppresses the microbicidal capacity of the phagosome suggests that Syt XI is a positive regulator of phagosomal maturation. Since the mechanism for this phenomenon is unknown, it will be important to use comparative proteomics to identify the proteins to which Syt XI binds to under different conditions (Arango Duque *et al.*, 2018, Arango Duque *et al.*, 2014a). For instance, LPS stimulation and *Leishmania* infection may induce certain Syt XI protein-protein interactions that are not observed in quiescent macrophages. We already possess macrophages stably transfected with a FLAG-Syt XI-GFP construction (Arango Duque *et al.*, 2013, Arango Duque *et al.*, 2014b) that will allow the immunopulldown of Syt XI partners. By using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), the identification of those interacting proteins is feasible. Once identified, the role of those proteins in Syt XI-regulated processes can be undertaken via RNAi and overexpression studies. Subsequently, mutational analysis of Syt XI's C2 domains will provide insight into how Syt XI interacts with its binding partners. If it is found that Syt XI modulates neuroinflammation, then Syt XI and its binding partners may become druggable targets.

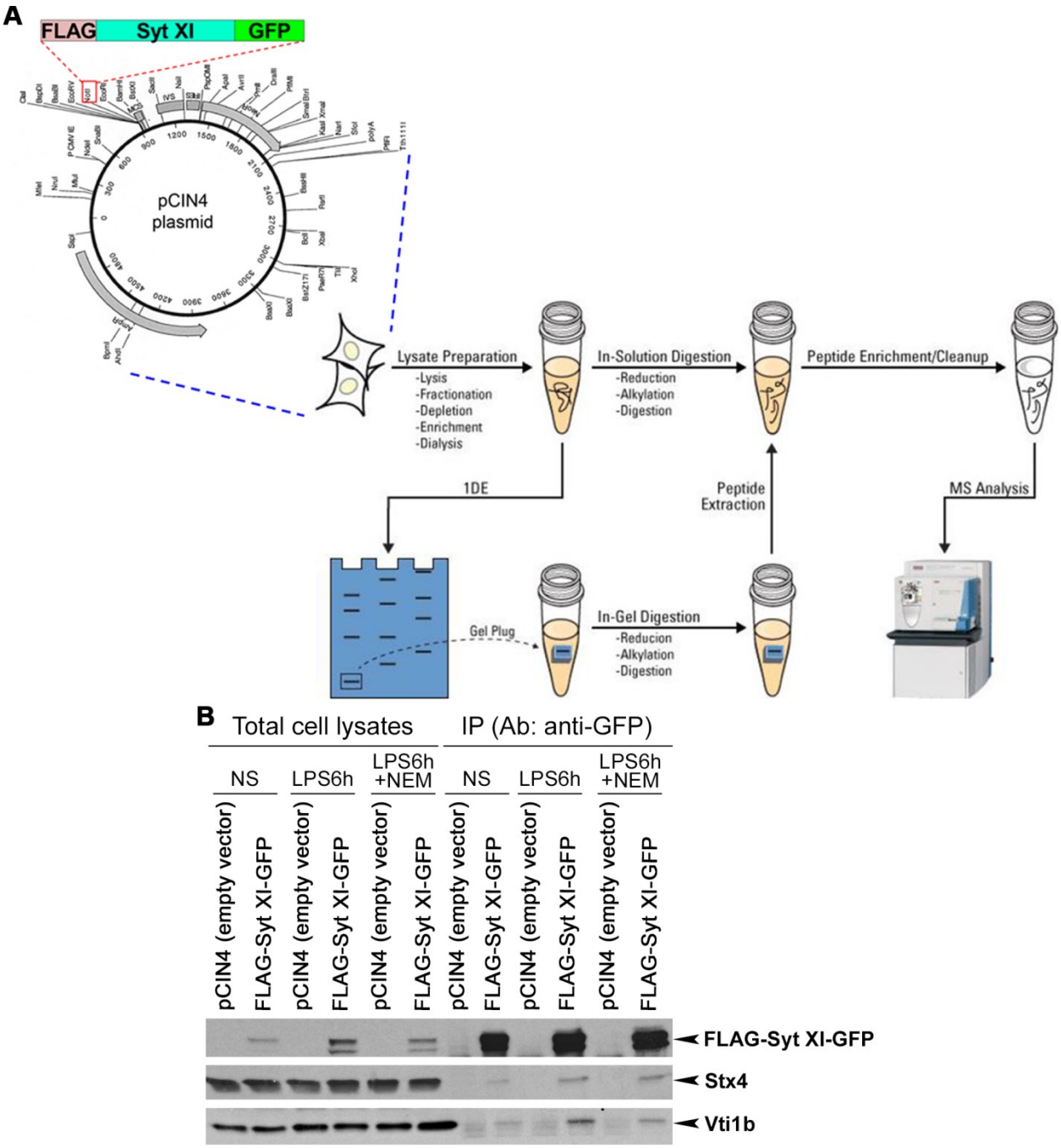

Figure 2 of discussion. The interacting partners of Syt XI will provide insight into its mechanism of action. (A) Schematic diagram depicting how RAW264.7 macrophages that are stably transfected with FLAG-Syt XI-GFP can be used to identify and characterize Syt XI's binding partners. In-gel and gel-free approaches combined with LC-MS/MS analysis can be used to identify protein-protein interactions in macrophages treated under different conditions. **(B)** Syt XI interacts with the SNAREs Stx4 and Vti1b. RAW264.7 macrophages stably transfected with pCIN4 or pCIN4(FLAG-Syt XI-GFP) were stimulated with LPS or LPS+*N*-ethylmaleimide (NEM) for 5h; SNARE complexes are stabilized by NEM. After pre-clearing of the lysates with normal rabbit serum, the FLAG-Syt XI-GFP protein was immunoprecipitated with an anti-GFP antibody. Western blots were used to test whether Syt XI coimmunoprecipitated SNAREs involved in cytokine secretion and phagocytosis.

Alternatively, a mechanism for Syt XI function on cytokine secretion could depend on the regulation of signalling pathways that modulate cytokine production and release. Upon macrophage stimulation with LPS, components of the Wnt signalling pathway were found to be involved in cytokine secretion. LPS stimulation promotes the stabilization of β-catenin, which exerts a negative regulatory role on IL-6 production (Lee *et al.*, 2012). Dvl, an important component of the Wnt signalling pathway, was found to interact with Syt XI (Kishida *et al.*, 2007). Though the functional significance of this interaction was not elucidated, the possibility exists that Syt XI could control delivery of Dvl to a membrane complex that is made up of Wnt, the low-density lipoprotein receptor-related protein, and frizzled (Nusse, 2012). It was found that in the presence of Dvl, the β-catenin protein is stabilized and translocates to the nucleus to modulate gene expression and control overproduction of cytokines such as IL-6 (Lee *et al.*, 2012). If Syt XI regulates Dvl delivery to the plasma membrane, Syt XI KD may promote β-catenin degradation and hyper secretion of IL-6 and other cytokines, as observed in our work (Arango Duque *et al.*, 2013).

2 THE IMPACT OF GP63 ON CYTOKINE RELEASE

2.1 The evolution of parasitic proteases

Secretion systems and toxins allow intracellular bacteria to subdue the inhospitable environment of the phagolysosome and promote their proliferation (Alix *et al.*, 2011, Weber *et al.*, 2018). In a similar manner, metalloproteases play a key role in parasite biology by cleaving host cell proteins involved in cellular defence (McKerrow *et al.*, 2006, Olivier *et al.*, 2012). This interaction is very relevant when the host's immune system has to combat a parasitic infection, or when the parasite must neutralize the host cell's innate defences. Indeed, metalloproteases are of great importance to the parasitic lifestyle and can be found in microscopic and macroscopic parasites of different phyla (Figure 3) (McKerrow *et al.*, 2006). Remarkably, a plethora of parasites developed the means to sabotage the process of proinflammatory cytokine secretion by macrophages. This highlights the fact that, although at a fitness cost to the host,

inflammation is beneficial to health; in the long term, the gains outweigh the costs. In the case of *Entamoeba histolytica* infection, inhibition of neutrophil recruitment and intestinal tissue damage are mediated by the direct cleavage of IL-1β and IL-18 by the EhCP1 and EhCP4 metalloproteases (Meléndez-López *et al.*, 2007, Serrano-Luna *et al.*, 2013). Another example is the small metalloproteases secreted by the parasitic worm *Necator americanus*, which inhibit eosinophil recruitment and TH1 inflammation by cleaving the CCL11 chemokine (Culley *et al.*, 2000). Moreover, the oral bacterium *Porphyromonas gingivalis* employs the gingipain protease to degrade IL-12 and hence inactivate the inflammatory response that would otherwise lead to the bacterium's demise (Yun *et al.*, 2001). In sum, metalloproteases cleave proinflammatory cytokines to break the intercellular communication that is necessary for the development of an effective immune response.

2.2 The pressure to inactivate is circumvented by the *Leishmania* GP63 metalloprotease

In contrast to the above-mentioned parasites, *Leishmania* uses the GP63 metalloprotease to induce the release of proinflammatory cytokines TNF and IL-6 (Figure 3). Indeed, GP63 is a multipronged *Leishmania* virulence molecule (Arango Duque *et al.*, 2014a, Arango Duque *et al.*, 2015) and is also one of the most abundant molecules on the promastigote plasmalemma (Joshi *et al.*, 2002, Moradin *et al.*, 2012, Yao, 2010). In infected macrophages, GP63 hijacks transcription and translation, deactivates several microbicidal pathways (Contreras *et al.*, 2010, Gómez *et al.*, 2009, Hallé *et al.*, 2009, Jaramillo *et al.*, 2011) and hampers antigen cross-presentation and LAP (Matheoud *et al.*, 2013, Matte *et al.*, 2016a). Of particular interest is the ability of GP63 to degrade SNAREs (Matheoud *et al.*, 2013, Matte *et al.*, 2016a, Matte *et al.*, 2016b), which raised the possibility that GP63 may cleave other membrane fusion regulators such as Syts.

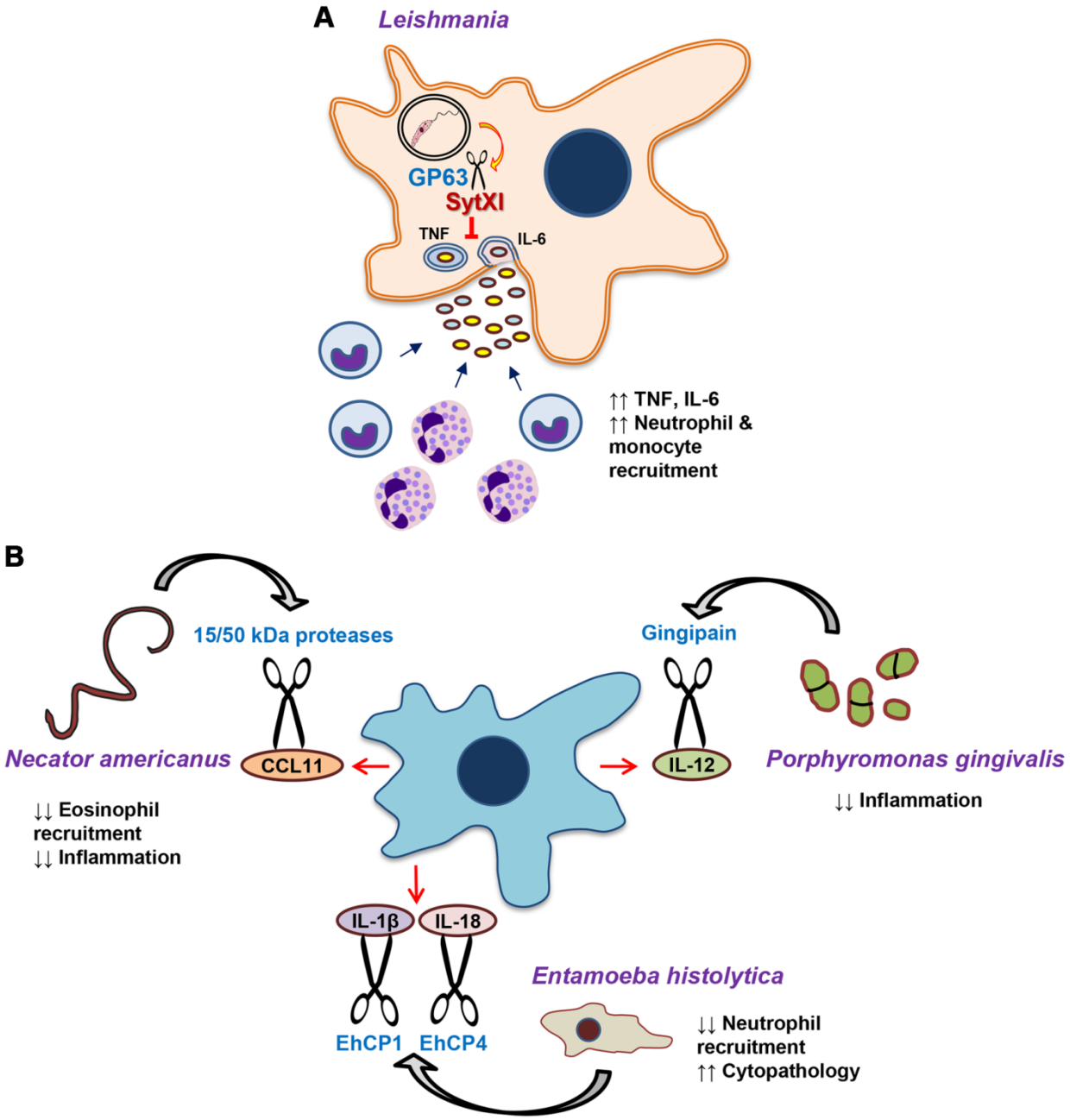

Figure 3 of discussion. Macrophage cytokines are targeted by metalloproteases of pathogen origin. (A) *Leishmania* employs the GP63 metalloprotease to target Syt XI and other substrates. Cleavage of Syt XI, a negative regulator of cytokine secretion, ensues in augmented release of TNF and IL-6. These cytokines may mediate the increase in neutrophil and monocyte accrual that is observed during early infection. **(B)** Schematic diagram depicting how microscopic and macroscopic pathogens employ metalloproteases to target macrophage cytokines. Proteolysis of these host molecules manipulates the immune response in favour of pathogen survival and dissemination.

Earlier observations found that *Leishmania* promastigotes elicit the secretion of TNF and IL-6 following their internalization by macrophages (Arena *et al.*, 1997, Karam *et al.*,

2006, Lapara *et al.*, 2010, Matte *et al.*, 2002, Wenzel *et al.*, 2012). Nonetheless, the mechanisms for this induction were not known. Hence, we hypothesized that Syt XI, a negative regulator of cytokine secretion (Arango Duque *et al.*, 2013), was targeted by *Leishmania* (Arango Duque *et al.*, 2014b). We observed that Syt XI is cleaved directly by GP63, ensuing in augmented release of TNF and IL-6 post-infection. Interestingly, this release correlated with the GP63 levels present in distinct *Leishmania* species. To validate these findings *in vivo*, GP63-expressing promastigotes were injected into mice. As predicted, TNF and IL-6 were released post-inoculation. These proinflammatory cytokines induce adhesion factor expression and chemokine release (Biswas *et al.*, 1998, Griffin *et al.*, 2012, Hurst *et al.*, 2001, Jones *et al.*, 2006, Vieira *et al.*, 2009). Importantly, we observed that GP63 promotes the influx of neutrophils and inflammatory monocytes in early infection, likely as a consequence of the initial induction in TNF and IL-6 release (Arango Duque *et al.*, 2014b).

In comparison to LPS, GP63 does not induce a massive release of TNF and IL-6 (Arango Duque *et al.*, 2014b), as such an event would severely activate the immune system, and that would not be beneficial for *Leishmania* or its host cell. Instead, the induced levels of these cytokines may be enough to trigger phagocyte migration to the infection site, while taking into account the fact that GP63 and LPG deactivate the antimicrobial mechanisms that could have been induced by those cytokines (Atayde *et al.*, 2016, Moradin *et al.*, 2012, Podinovskaia *et al.*, 2015). In sum, GP63 promotes parasite fitness by allowing *Leishmania* to evade the macrophage's microbicidal defences, and by facilitating parasite dissemination and establishment in a variety of phagocytes.

3 INVOLVEMENT OF THE HOST CELL SECRETORY PATHWAY ON THE TRAFFICKING OF *LEISHMANIA* VIRULENCE FACTORS

3.1 How do *Leishmania* virulence factors exit the PV?

Macrophages have an active secretory pathway that is necessary for the spatiotemporal delivery of proteins to extracellular milieu (Arango Duque *et al.*, 2014a, Weber *et al.*, 2018), and for phagosome biogenesis (Huynh *et al.*, 2007b, Murray *et al.*, 2005a). Once internalized, bacteria and apicomplexan parasites use specialized secretion systems to inject their virulence molecules into the host cell cytoplasm (Flannagan *et al.*, 2009, Poirier *et al.*, 2015, Sibley, 2011). However, no such system has been found in *Leishmania*. Hence, it is sensible to hypothesize that the host cell mediates the phagosomal egress of *Leishmania*'s virulence factors into the cytoplasm of the infected cell. We have provided evidence that GP63 and the PGs leave the phagosome in vesicles that cofractionate and colocalize with the ER and ERGIC. In contrast, colocalization with endosomal or lysosomal markers was nearly absent (not shown). Importantly, we showed that BFA-mediated disruption of these organelles abrogates the redistribution of GP63 and the PGs, thereby inhibiting cleavage of GP63 substrates.

The colocalization of GP63 and PGs with CRT could indicate a possible physical interaction. The ER chaperone CRT is a lectin that interacts with many proteins via their glycosylated $Glc_1Man_9GlcNAc_2$ moieties to promote proper protein folding (Scales *et al.*, 2000). Furthermore, the *Leishmania* phagosome has been reported to persistently accrue ER/ERGIC proteins (Ndjamen *et al.*, 2010), implying possible interaction between host cell chaperones and glycosylated molecules of *Leishmania* origin. In fact, the GP63 glycoprotein possesses the $GlcMan_6GlcNAc_2$ moiety (Funk *et al.*, 1997). To evaluate this possible interaction, one could attempt to immunoprecipitate GP63 with an antibody targeting CRT and vice-versa. Interactions of *Leishmania* molecules with ER chaperones could also be evaluated via the proximity ligation assay. In that technique, two primary antibodies recognize the target antigens in the infected macrophage. Two

secondary antibodies, containing a strand of DNA, will bind to the primary antibodies. If the two secondary antibodies are close, the DNA strands can pair. The hybridized DNA is amplified and imaged with fluorescent compounds (Andersen *et al.*, 2013). Preliminary co-IP data revealed a weak interaction between CRT and GP63 (Figure 4), raising the possibility that GP63 may undergo post-translational modifications in host organelles. It is likely that any such modification would not be required for protease activity, since coincubation of recombinant host proteins with GP63-containing parasite lysates results in the degradation of such proteins (Álvarez de Celis *et al.*, 2015, Arango Duque *et al.*, 2014b, Contreras *et al.*, 2010, Gómez *et al.*, 2009, Matheoud *et al.*, 2013). Instead, such modifications may be required for the protease to reach its substrates in the infected cell. The presence of ER protein folding chaperones in the PV may be beneficial to parasite survival in ways that have yet to be explored. Furthermore, mixing of ER contents with the PV may facilitate parasite access to MHC class I-associated proteins (Bertholet *et al.*, 2006, Ndjamen *et al.*, 2010). Studies with *L. major* revealed that priming of $CD8^+$ T cells occurs via a mechanism independent of the TAP transporter. Although we found that GP63 does not cleave ER chaperones CRT, CNX and PDI, it is possible that the protease may cleave other ER-bound proteins involved in antigen presentation, which may partly explain the priming delay observed in T cells (Bertholet *et al.*, 2006).

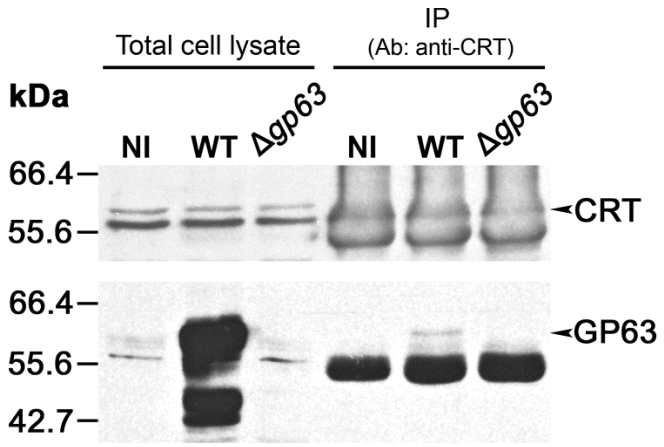

Figure 4 of discussion. CRT coimmunoprecipitates GP63. BMM were infected with opsonized *L. major* WT or Δ*gp63*, promastigotes for 6 h and lysed in RIPA buffer containing 1,10-phenanthroline and protease inhibitors. An antibody for CRT was used to immunoprecipitate this protein from lysates that were pre-cleared with normal rabbit serum. Immunoprecipitation of CRT and co-IP of GP63 were verified via Western blot.

Both GP63 and the PGs target vesicle fusion proteins in order to alter the maturation and functionality of the phagosome (Arango Duque *et al.*, 2015, Atayde *et al.*, 2016, Matte *et al.*, 2016b, Moradin *et al.*, 2012, Podinovskaia *et al.*, 2015). Via its GPI anchor, LPG – the most abundant PG – inserts itself in lipid rafts and hinders the accrual of Syt V, a recycling endosome-associated positive regulator of membrane delivery to the phagocytic cup and a recruiter of the V-ATPase to the maturing phagosome (Vinet *et al.*, 2009). Decreased Syt V recruitment ensues in decreased intraphagosomal acidification and increased parasite survival. GP63 cleaves the endosomal/lysosomal SNARE VAMP8 to inhibit NOX2 recruitment to the phagosome and inhibit antigen cross-presentation (Matheoud *et al.*, 2013) and LC3-associated phagocytosis (Matte *et al.*, 2016a), and Syt XI to deregulate cytokine release and phagocyte recruitment (Arango Duque *et al.*, 2014b). We and Gómez *et al.* reported that GP63 colocalizes with lipid rafts (Gómez *et al.*, 2009), which are found in many organelles including the ER. This indicates that GP63 and LPG are found in membrane microdomains in the ER/ERGIC. Nonetheless, it was found that their perturbation did not alter GP63 function (Gómez *et al.*, 2009). LPG is known to destabilize lipid microdomains (Dermine *et al.*, 2005), raising the possibility that perturbation of lipid rafts may be needed for trafficking of these virulence factors. These previous findings, along with the observation that the ER and the ERGIC are important for the trafficking of GP63 and PGs, prompted us to inquire whether vesicle fusion proteins of the ER/ERGIC were necessary for the trafficking and function of these virulence molecules.

3.2 *Leishmania* co-opts the host cell SNARE Sec22b

Sec22b is a SNARE in the ER-Golgi circuitry that regulates phagosomal functions such as the capacity to cross-present antigens (Alloatti *et al.*, 2017, Cebrian *et al.*, 2011, Nair-Gupta *et al.*, 2014). It is highly present in *Leishmania* PVs and siRNA-mediated knockdown reduces PV size and impairs parasite survival (Canton *et al.*, 2012a, Canton *et al.*, 2012b, Ndjamen *et al.*, 2010). The importance of this SNARE raised the

interesting possibility that it may be required for the egress and redistribution of *Leishmania* virulence factors from phagosomes to other cell compartments. Not only did we find that GP63 and PGs colocalize with Sec22b, but also that Sec22b knockdown leads to accumulation of virulence factors in the PV. We hypothesized that if GP63 and PGs do not leave the phagosome, they would not reach their substrates. The finding that Syt XI degradation is less prevalent in Sec22b KD cells supports this argument. There are many SNAREs present in phagocytes (Stow *et al.*, 2006), many of which are likely not degraded by GP63. It will therefore be interesting to find out whether there are other SNAREs involved in mediating the trafficking and function of *Leishmania* virulence factors.

In the parasite, GP63 is synthesized as a pro-form that undergoes self-cleavage in order to generate the mature form of the enzyme (McGwire *et al.*, 1996, Yao, 2010). Whether GP63 undergoes further cleavage in the host had not been reported until Matheoud and colleagues observed that infection induced the 63 kDa form of the enzyme to convert to a ~45 kDa form (Matheoud *et al.*, 2013). We expanded upon these observations and found that this conversion is also time-dependent and correlates with the loss of GP63 activity that occurs when promastigotes become amastigotes. Zymography revealed that the ~45 kDa fragment is not proteolytically active. This conversion is likely to be dependent on the ER/ERGIC, since BFA treatment and Sec22b KD inhibited the appearance of the 45 kDa form (not shown). Albeit the functional importance of this lighter fragment during infection is not apparent, it correlates with the egress of GP63 from the PV. The time-dependent and host cell-mediated conversion of GP63 into a catalytically inactive form supports the idea that GP63 activity is important in the early stages of infection.

Whether other host cell organelles or molecules are required by the parasite for the purpose of redistributing its virulence molecules in the infected cell is a possibility that deserves detailed study. We reported that the SNARE Sec22b colocalizes with and controls the redistribution of GP63 and PGs in infected phagocytes. The mechanism by

which these secretory pathway-derived vesicles migrate to the targets of these virulence molecules may involve the participation of other cell organelles. The ERGIC has recently been shown by Ge and colleagues to be critical for autophagosome biogenesis and is reported to be that organelle's primary membrane source (Ge *et al.*, 2013). Indeed, autophagy is a catabolic process of cellular homeostasis that involves the formation of double-membrane structures, or autophagosomes, around cellular structures that are to be digested (Deretic, 2011). The LC3B protein is necessary for the process of autophagosome generation; it undergoes a cleavage and palmitoylation process that anchors it to membranes. This lipidation occurs at the ERGIC and is dependent on the early autophagosome marker ATG14 (Ge *et al.*, 2013). *Leishmania* has been reported to induce LC3B conversion into LC3B-II (Matte *et al.*, 2016a, Mitroulis *et al.*, 2009). Moreover, cleavage of VAMP8 by GP63 inhibits LC3-mediated phagocytosis (Matte *et al.*, 2016a, Matte *et al.*, 2016b). Those observations imply that *Leishmania* subverts autophagy-associated proteins to the parasite's advantage. In addition, we observed that both GP63 and PGs colocalize with LC3B (not shown). This raises the possibility that GP63 may use autophagosomal membranes to migrate from the ERGIC to other cell compartments. This possibility can be assayed by testing whether the redistribution of *Leishmania* virulence molecules is hampered by knockdown of proteins that control autophagosome biogenesis.

In sum, our experiments provide biochemical and genetic evidence for the argument that the host cell SNARE Sec22b promotes the reproductive success of the *Leishmania* parasite. The newly discovered role of the secretory pathway on the intracellular transport of virulence molecules unveils a novel virulence mechanism. *Leishmania* parasite hijacks the vesicle fusion machinery of the host's secretory pathway, notably the ERGIC SNARE Sec22b, in order to promote the egress and redistribution of its virulence molecules. This trafficking then allows GP63 to access its substrates (Figure 5).

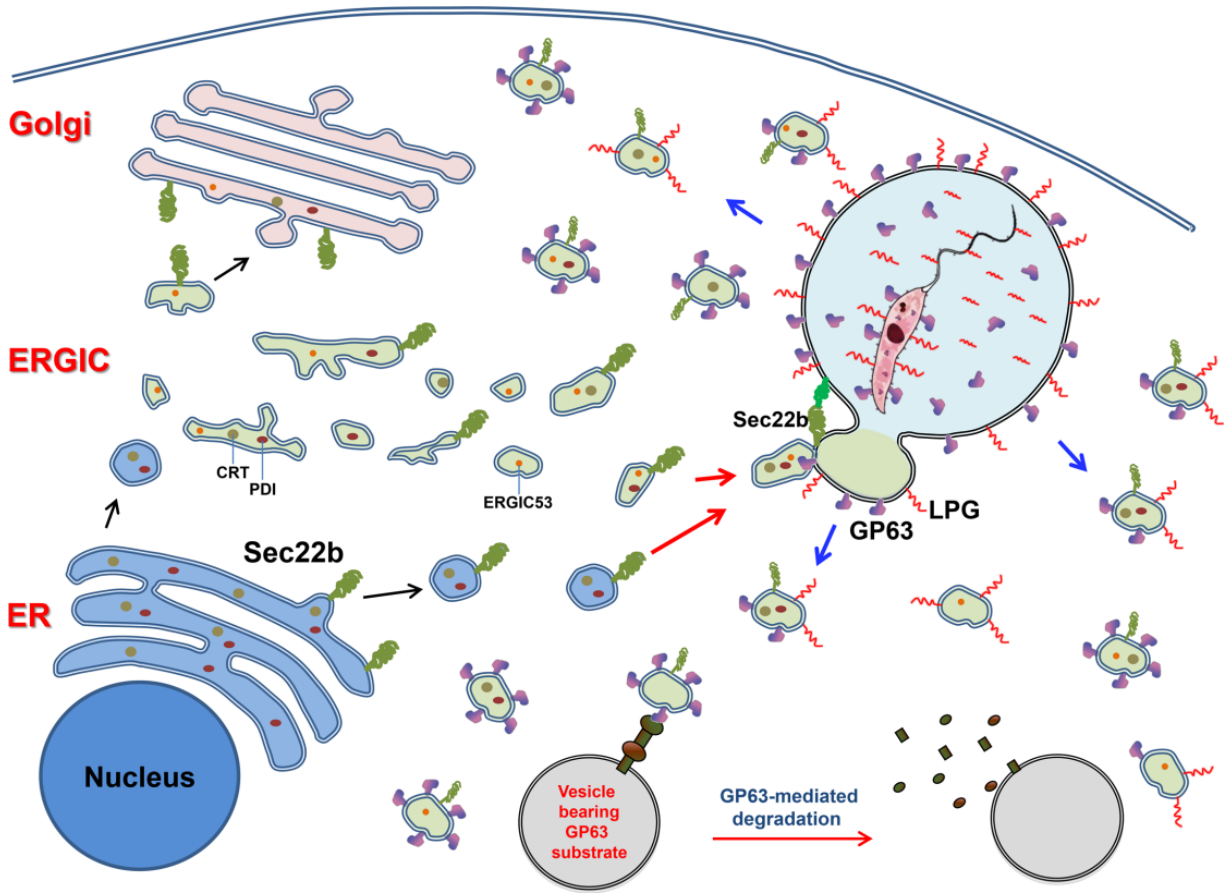

Figure 5 of discussion. The host cell provides the molecular machinery that promotes the intracellular spread of *Leishmania* virulence molecules. *Leishmania* co-opts the ER-ERGIC circuitry to facilitate the egress of its virulence molecules from the PV into the host cell cytoplasm. This redistribution is necessary for GP63 to access and cleave its substrates. Adapted from primary article no. 3.

3.3 How do *Leishmania* virulence factors reach host cell organelles?

The cell is an interconnected system of membrane organelles and cytoskeletal elements that mediate efficient intracellular communication. In fact, the ER network is apposed to endosomes, mitochondria, peroxisomes and lipid droplets via membrane contact sites (MCS) (Cohen *et al.*, 2018, Phillips *et al.*, 2015). These contacts are mediated by protein-membrane-cytoskeleton complexes that permit the exchange of metabolites, lipids, ions and proteins among organelles. MCSs are critical to the biogenesis and homeostasis of organelles such as mitochondria and the autophagosome (Phillips *et al.*, 2015). Once *Leishmania* virulence-associated

glycoconjugates transit through the ER/ERGIC network, many questions remain as to how they reach the host cell organelles that contain the targets of these parasitic molecules. The possible interaction of GP63 with CRT (Figure 4) indicates that this parasitic protease could interact with other glycan-binding proteins in the host cell. On the other hand, the significance of PG/LPG redistribution is not known. Due to the importance of MCSs in cellular function, it is possible for *Leishmania* virulence factors to hijack these structures to reach host cell organelles. Whether *Leishmania* exploits host MCSs by altering the integrity and function of the protein complexes associated with organelle contact is a question that deserves intense research.

4 SEC22B AND THE CONTRADICTORY ROLES OF THE ER/ERGIC DURING INFECTION

The survival of intracellular pathogens is contingent upon the complex interplay that microbial molecules have with host defence mechanisms (Arango Duque *et al.*, 2015, Olivier *et al.*, 2012, Sibley, 2011, Weber *et al.*, 2018). On the one hand, phagosomal interactions with the ER and ERGIC promote pathogen survival, whereas in other cases they promote their elimination. In the case of *Legionella* and *Leishmania*, we and others have observed that these organelles play a beneficial role (Canton *et al.*, 2012b, Cebrian *et al.*, 2011, Machner *et al.*, 2006). *Legionella* uses a type IV secretion system to accrue ERGIC membranes in a Sec22b-dependent manner, thence resulting in Golgi fragmentation (Machner *et al.*, 2006). Ablation of the secretion system impairs bacterial survival, which implies that the ERGIC may contain molecules that promote survival of that pathogen. Since lysosomes originate at the Golgi, its fragmentation may be an indirect mechanism used by *Legionella* to inhibit lysosome biogenesis. *Leishmania* and *Toxoplasma* also accrue ER and ERGIC membranes through a Sec22b-mediated mechanism (Canton *et al.*, 2012b, Cebrian *et al.*, 2011, Gagnon *et al.*, 2002, Magno Cardoso *et al.*, 2005). In the case of both parasites, inhibition of Sec22b expression leads to decreased antigen cross-presentation and T cell activation (Cebrian *et al.*, 2011). Therefore, Sec22b KD should promote parasite survival due to the decrease in T cell activation. However, it is known that *Leishmania* inhibits antigen cross-presentation

through the direct cleavage of the endosomal SNARE VAMP8 by GP63 (Matheoud *et al.*, 2013). Our data show that Sec22b is not cleaved by this protease, which signifies that the *Leishmania*-induced inhibition of antigen cross-presentation is independent of Sec22b. However, KD of Sec22b and its cognate ER/ERGIC SNAREs results in decreased parasite survival (Canton *et al.*, 2012a, Canton *et al.*, 2012b). Although the functional importance of Sec22b on parasite fitness was not known, we hypothesized that this ER/ERGIC SNARE shepherds *Leishmania*'s virulence molecules through the secretory pathway. The findings presented in article no. 3 revealed that Sec22b KD hampers the exit of GP63 and LPG from the PV, which hinders GP63 from accessing its cytoplasmic substrates. Those substrates are involved in antimicrobial control and their presence inhibits parasite survival (Matte *et al.*, 2016b). With the advent of *SEC22B*$^{-/-}$ mice (Alloatti *et al.*, 2017), it will be of great importance to investigate whether Sec22b promotes the formation of visceral and cutaneous lesions. Since we observed that Sec22b facilitates the intracellular spread of virulence factors, we infer that its absence precludes the establishment of chronic infections. The Sec22b-mediated recruitment of ER/ERGIC membranes to *Toxoplasma* PVs (Magno Cardoso *et al.*, 2005) implies that this SNARE plays a role in the survival of this parasite. In light of our recent findings on *Leishmania*, it will be interesting to test whether absence of Sec22b hinders *Toxoplasma* survival and the intracellular trafficking of its virulence molecules.

The beneficial role of Sec22b on *Leishmania* survival does not apply to other intracellular pathogens. The ER-ERGIC-Golgi circuitry is involved in processes ranging from antigen presentation to the quality control of misfolded proteins (Abuaita *et al.*, 2015, Appenzeller-Herzog *et al.*, 2006, Blander, 2018). Although the inositol-requiring enzyme 1α (IRE1α) senses unfolded proteins in the ER, it is also activated by TLR stimulation and promotes ROS production (Martinon *et al.*, 2010). Indeed, Abuaita and colleagues showed that IRE1α was necessary for the killing of MRSA bacteria (Abuaita *et al.*, 2015). Due to the residence of this protein in the ER, the authors tested whether the action of IRE1α was dependent on Sec22b-mediated trafficking events. Indeed, they found that KD of Sec22b and its cognate SNAREs Stx4 and Stx5 inhibits IRE1α activity and quells the production of ROS (Abuaita *et al.*, 2015). Although KD of these SNAREs

does not preclude total ROS production, it does inhibit intraphagosomal ROS formation, hence favouring MRSA survival. This study shows that the ER/ERGIC has a negative effect on bacterial growth (Abuaita *et al.*, 2015). This finding is important because it leads one to inquire on what the original purpose of the ER/ERGIC is in regards to antimicrobial defence. The Abuaita *et al.* study suggests that the ER/ERGIC and its associated SNAREs evolved to promote pathogen killing (Abuaita *et al.*, 2015). That study uses the MRSA bacterium, which is more primitive than the eukaryotic pathogen *Leishmania*. In contrast to *Leishmania,* MRSA may not possess the molecular weaponry to combat the intraphagosomal accumulation of ROS. If this line of thought is correct, it is worthwhile to test whether the ER/ERGIC and Sec22b promote the killing of attenuated laboratory strains of *E. coli*, which are rapidly eliminated by WT macrophages (Arango Duque *et al.*, 2013). It is known that the *Leishmania* parasite inhibits intraphagosomal killing via GP63 and LPG, which could effectively inhibit and resist the Sec22b- and IRE1α-dependent accumulation of ROS at the PV (Arango Duque *et al.*, 2015, Moradin *et al.*, 2012, Olivier *et al.*, 2012, Podinovskaia *et al.*, 2015). However, our data revealed that *Leishmania* hijacks the trafficking function of Sec22b, which allows the parasite's effectors to reach their host cell targets. In sum, although the ER/ERGIC may have originally evolved to combat intracellular infections, certain pathogens evolved counter-strategies to co-opt selected features of its function that promote their survival.

5 IS IT FEASIBLE TO TARGET VESICLE TRAFFICKING PATHWAYS DURING *LEISHMANIA* INFECTION?

The latest leishmaniasis report by the WHO highlights the rise of drug resistance, especially in geopolitical regions where treatment access and patient compliance are low (WHO, 2018, Zulfiqar *et al.*, 2017). The development of drug resistance in *Leishmania* is partly due to the genome plasticity of the parasite, which allows it to evolve resistance mechanisms very rapidly (Laffitte *et al.*, 2016). Current medicines target aspects of parasite physiology and metabolism, thereby providing the selecting pressure that is necessary for the development of drug resistance (Gupta *et al.*, 2017,

Laffitte *et al.*, 2016). Due to its neglected disease status, drug development proceeds slowly (WHO, 2018). Hence, there is a pressing need for new chemotherapies. Current efforts to find new antileishmanial drugs focus on the screening of millions of compounds for their potential to inhibit promastigote or intracellular amastigote growth (Yazdanparast *et al.*, 2014, Zulfiqar *et al.*, 2017). The intricate host-parasite interaction that exists between *Leishmania* and its host macrophage involve host organelles that the parasite ought to co-opt to ensure its survival. Therefore, targeting the host cell processes that facilitate parasite growth is a viable alternative to treat the leishmaniases (Matte *et al.*, 2016b). Indeed, this approach has been used with varying success in the treatment of bacterial and viral diseases (Gupta *et al.*, 2017). Since the pathogen is not directly targeted by the compound, drug resistance is less likely to develop (Bekerman *et al.*, 2015).

Due to the importance of the host cell ER/ERGIC and its resident SNAREs on parasite survival, compounds that target these organelles are being developed as potential leishmanicidals. This is this case of Retro-2, a non-toxic compound that selectively targets the retrograde pathway (Canton *et al.*, 2012a, Craig *et al.*, 2017). Although the mechanism is not clear, Retro-2 leads to the dispersal of Stx5, a partner SNARE of Sec22b (Canton *et al.*, 2012a). Interestingly, it also reduces the development of cutaneous and visceral lesions, and high doses are well tolerated by mice. Since this compound targets the retrograde pathway, we expected that it would hinder the redistribution of GP63 and PGs post-phagocytosis. Indeed, preliminary data indicate that this is the case (Figure 6).

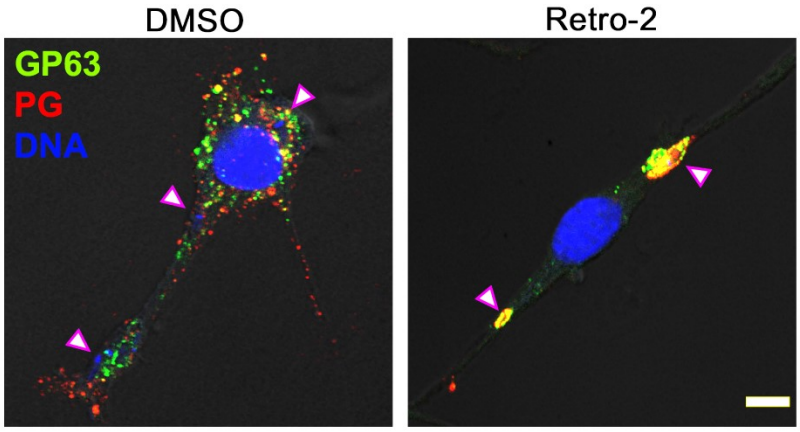

Figure 6 of discussion. Retro-2 is an inhibitor of the retrograde pathway that hinders the redistribution of GP63 and PGs. BMM were treated with DMSO or Retro-2 prior to infection with opsonized *L. major* Δ*gp63*+*gp63* metacyclic promastigotes for 6 h. GP63 is shown in green, PGs in red, and DNA in blue. White arrowheads denote internalized parasites; bar, 5 μm.

Since its discovery, Retro-2 has been structurally optimized into analogs that exert augmented leishmanicidal activity *in vitro* and *in vivo* (Craig *et al.*, 2017, Gupta *et al.*, 2017). The use of high-content screening (HCS) can be tailored to discover new compounds that selectively target the host's endomembrane system. In this regard, several compounds have already been discovered (Drakakaki *et al.*, 2011). With the development of microscopy-based HCS strategies in *Leishmania* (Yazdanparast *et al.*, 2014, Zulfiqar *et al.*, 2017), those compounds can be tested for their capacity to inhibit the redistribution of *Leishmania* virulence molecules, which can then be correlated with the compounds' leishmanicidal potential. Our work revealed a role for host molecules in the egress of virulence factors from the PV. However, much remains to be discovered about other host proteins that may regulate the spatiotemporal nature of this egress. The discovery of those host factors is a priority. Genome-wide RNAi screens have revealed several genes that affect protein trafficking in the secretory pathway (Simpson *et al.*, 2012). If silencing of those genes impacts the redistribution of *Leishmania* virulence molecules and intracellular survival, then said genes could become druggable targets. Due to vital importance of the host cell secretory pathway and its SNAREs in host cell physiology and immunity (Bonifacino *et al.*, 2004, Dingjan *et al.*, 2018), great caution must be exercised in the development of host-directed chemotherapy.

CHAPITRE 5: RÉSUMÉ EN FRANÇAIS

1 ABRÉGÉ

Les parasites du genre *Leishmania* causent une panoplie de maladies débilitantes dans le monde entier. Le cycle de vie de ce protozoaire est digénétique, démarrant avec des mouches phlébotomes qui inoculent des promastigotes métacycliques dans un hôte vertébré. Les promastigotes sont internalisés par des phagocytes tissulaires où ils se transforment en amastigotes. Les phagosomes se transforment en phagolysosomes, qui sont des organites hautement microbicides. Cette transformation est due à des échanges membranaires avec les lysosomes et avec des organites de la voie sécrétoire. Remarquablement, *Leishmania* remodèle les phagolysosomes en vacuoles parasitophores qui favorisent la croissance des parasites. Pour atteindre cet objectif, le parasite utilise des glycoconjugués liés à sa surface, dont la métalloprotéase GP63 et le lipophosphoglycane. La GP63 clive plusieurs substrats de la cellule hôte, permettant ainsi la subversion de la transcription, la traduction, le métabolisme des lipides, la sécrétion de cytokines, la phagocytose associée à LC3 et la présentation croisée des antigènes. Le lipophosphoglycane est un glycophospholipide qui favorise la survie du parasite en inhibant la maturation du phagolysosome, ce qui mitige sa capacité microbicide.

Les fonctions effectrices du macrophage reposent sur un système de trafic endomembranaire très actif qui régule la réponse de la cellule aux stimuli environnementaux. La fusion vésiculaire est régulée par des protéines de type SNARE et Syt. Nous avons caractérisé la Syt XI, un membre inhibiteur de la famille Syt dont sa fonction n'avait pas été rapportée. Nous avons découvert que la Syt XI s’associe aux endosomes de recyclage et aux lysosomes, et contrôle la phagocytose et la capacité microbicide du phagosome [article no. 1]. Nous avons trouvé que la Syt XI atténue la sécrétion de TNF et d'IL-6. Fait important, la Syt XI est dégradée par la GP63 de *Leishmania* et exclue des vacuoles parasitophores par les PGs. D’autre part, les macrophages infectés par *Leishmania* sécrètent du TNF et de l'IL-6 de manière GP63-dépendante. Pour démontrer que cette libération dépend de la dégradation de Syt XI, nous avons inhibé l’expression de Syt XI à l’aide des petits ARN interférents, ce qui a

été suivi par une infection aux promastigotes de *Leishmania*. Cela a révélé que l'effet des ARN interférents et du clivage par GP63 n'étaient pas cumulatifs. Pour démontrer la pertinence de ces résultats dans le contexte *in vivo*, nous avons montré que l'injection de parasites contenant GP63 chez la souris conduit également à une sécrétion accrue de TNF et d'IL-6 et à un afflux augmentée de neutrophiles et de monocytes inflammatoires au site d'infection [article no. 2].

Contrairement aux bactéries et d'autres parasites protozoaires, *Leishmania* n'a aucun système de sécrétion par lequel le parasite injecte ses facteurs de virulence dans le cytoplasme de la cellule infectée. Cela soulève la question sur comment les facteurs de virulence de *Leishmania* atteignent leurs cibles. En raison de l'importance précédemment rapportée de la voie sécrétoire de l'hôte sur la survie du parasite, nous avons postulé l'hypothèse que les facteurs de virulence de *Leishmania* détournent cette voie afin de se rendre dans le cytoplasme de la cellule hôte. À l'aide de techniques biochimiques et microscopiques, nous avons démontré que la GP63 et les PGs sont rapidement redistribués dans le cytoplasme dans des vésicules contenant des marqueurs du réticulum endoplasmique et de l'ERGIC. Il est important de noter que l'inhibition chimique de la voie sécrétoire a inhibé la redistribution de GP63 et des PGs, empêchant ainsi le clivage des cibles de GP63. Cela nous a incité à étudier le rôle de la SNARE Sec22b, qui régule le trafic entre le réticulum endoplasmique et le Golgi, sur le trafic intracellulaire de GP63 et des PGs. Nous avons trouvé que Sec22b favorise la redistribution de ces facteurs de virulence, permettant ainsi à GP63 d'accéder à ses substrats [article no. 3].

Somme toute, le travail présenté dans cette thèse éclaire comment les molécules de virulence de *Leishmania* se rendent dans la cellule hôte afin d'exercer leurs fonctions. Nous avons également révélé que GP63 induit la libération de TNF et d'IL-6 *in vitro* et *in vivo*, ce qui contribue à l'infiltration de phagocytes au site d'infection. Remarquablement, GP63 et les PGs accèdent à leurs cibles en s'appropriant des organites de la cellule hôte et de leurs molécules de fusion vésiculaire. Ces résultats

fournissent des informations importantes sur la façon dont le parasite *Leishmania* sabote la biologie des phagocytes.

2 SOMMAIRES DE PUBLICATIONS

2.1 Premier article : La Synaptotagmine XI régule la phagocytose et la sécrétion de cytokines dans les macrophages

Les Syts sont un groupe de protéines transmembranaires de type I qui régulent l'amarrage et la fusion des vésicules dans des processus tels que l'exocytose et la phagocytose (Arango Duque *et al.*, 2014a, Fukuda, 2007, Südhof, 2012). Toutes les Syts possèdent un seul domaine transmembranaire et deux domaines C2 en tandem qui permettent la liaison de cette protéine avec le Ca^{2+} (Fukuda, 2007, Südhof, 2012). Cependant, les Syts IV et XI possèdent une sérine conservée dans leur domaine C2A qui empêche ces Syts de se lier au Ca^{2+} et aux phospholipides, ce qui défavoriserait la fusion des vésicules (Dean *et al.*, 2009, von Poser *et al.*, 1997, Wang *et al.*, 2010). Tenant en compte l'importance du trafic vésiculaire dans la biologie des macrophages, nous avons étudié le rôle de la Syt XI dans la sécrétion de cytokines et la phagocytose (Arango Duque *et al.*, 2013). Nous avons démontré que la Syt XI est exprimée dans les macrophages murins, localisés dans les endosomes de recyclage, les lysosomes et recrutés aux phagosomes. La Syt XI exerce un effet direct sur la phagocytose et sur la sécrétion de TNF et d'IL-6. Alors que l'inhibition de son expression par des petits ARNs interférents potentialise la sécrétion des cytokines et la phagocytose, sa surexpression supprime ces processus. De plus, l'inhibition de l'expression de Syt XI a conduit à une diminution du recrutement de $gp91^{phox}$ et de LAMP-1 aux phagosomes, suggérant une activité microbicide atténuée. Remarquablement, une expression affaiblie de Syt XI permet la survie bactérienne dans le phagosome. En conclusion, ces données révèlent un nouveau rôle pour la Syt XI comme régulateur de l'exocytose et de l'activité microbicide des macrophages (Arango Duque *et al.*, 2014a, Arango Duque *et al.*, 2013).

2.2 Deuxième article : Les promastigotes de *Leishmania* induisent la sécrétion de cytokines dans les macrophages en dégradant la Synaptotagmine XI

Les Syts sont des protéines membranaires de type I qui régulent la fusion des vésicules dans des processus tels que l'exocytose et la phagocytose (Arango Duque *et al.*, 2014a, Fukuda, 2007, Südhof, 2012). À cause de l'importance de ces molécules dans les fonctions effectrices du macrophage, il y a des microbes qui ont évolué des stratégies pour détourner leurs fonctions (Matte *et al.*, 2016b). Par exemple, les trypanosomes exploitent le rôle de la Syt VII, qui contrôle la sécrétion de lysosomes à la membrane cellulaire, pour faciliter leur entrée dans la cellule hôte. De plus, le parasite *Leishmania* s'en sert de son lipophosphoglycane pour éviter le recrutement de la Syt V au phagosome, ce qui empêche l'acidification de cet organite (Caler *et al.*, 2001, Vinet *et al.*, 2009). Nous avons récemment découvert que la Syt XI est une protéine qui régule négativement la sécrétion de TNF et d'IL-6 (Arango Duque *et al.*, 2013). Dans cette étude, nous montrons que la Syt XI est directement dégradé par la métalloprotéase GP63 et exclue des vacuoles parasitophores de *Leishmania* par le lipophosphoglycane (Arango Duque *et al.*, 2014b). Les macrophages infectés libèrent du TNF et de l'IL-6 d'une manière dépendante de GP63. Pour démontrer que la libération de ces cytokines dépendait de la dégradation de Syt XI par la GP63, l'inhibition de l'expression de Syt XI médiée par le RNAi avant une infection a révélé que les effets du KD ne s'accumulent pas avec la dégradation de Syt XI. Chez la souris, l'injection intrapéritonéale de parasites exprimant GP63 a entraîné une augmentation de la sécrétion de TNF et d'IL-6 et un afflux accru de neutrophiles et de monocytes inflammatoires vers le site d'inoculation. Nous avons montré que ces deux types de cellules sont des cibles d'infection et aident à l'établissement d'une infection chronique. En résumé, ces données ont révélé que la GP63 induit une libération de cytokines pro-inflammatoires et augmente l'infiltration des phagocytes inflammatoires. Cette étude fournit des nouvelles informations sur la façon dont *Leishmania* exploite la réponse immunitaire pour établir une infection (Arango Duque *et al.*, 2015, Podinovskaia *et al.*, 2015).

2.3 Troisième article : La voie sécrétoire permet l'exportation des facteurs de virulence de *Leishmania* hors de la vacuole parasitophore

Pour se répliquer dans les phagocytes hôtes, le pathogène vacuolaire *Leishmania* subvertit les processus microbicides et immunitaires par l'action de composants de son revêtement de surface qui comprennent le lipophosphoglycane et la métalloprotéase GP63 (Arango Duque *et al.*, 2015, Moradin *et al.*, 2012, Olivier *et al.*, 2012). Alors que l'impact de ces glycoconjugués de virulence sur la fonction des cellules hôtes a été étudié en détail, il reste à élucider le mécanisme par lequel ils émigrent de la vacuole parasitophore au cytoplasme des cellules hôtes (Gómez *et al.*, 2009, Tolson *et al.*, 1990). Ceci est une question intrigante puisque *Leishmania* ne possède pas de système de sécrétion spécialisé analogue à ceux utilisés par les bactéries pathogènes pour injecter des effecteurs dans le cytosol de leurs cellules hôtes (Weber *et al.*, 2018). Dans cette étude, nous montrons qu'après la phagocytose du parasite par des phagocytes, le lipophosphoglycane et la GP63 sont redistribués du parasite au réticulum endoplasmique. La perturbation du transport qui a lieu entre le réticulum endoplasmique et le Golgi entrave la sortie de ces facteurs de virulence de la vacuole parasitophore. Cela mène à une atténuation du clivage des protéines de l'hôte par la GP63. De plus, l'inhibition de l'expression du SNARE Sec22b, qui régule le trafic entre le réticulum endoplasmique et le Golgi (Cebrian *et al.*, 2011), a identifié cette protéine hôte comme un composant de la machinerie responsable de la redistribution des effecteurs de *Leishmania* dans le cytoplasme des cellules hôtes. Nos résultats dévoilent un mécanisme par lequel un agent pathogène vacuolaire emploie la voie sécrétoire de la cellule hôte pour favoriser la livraison intracellulaire des facteurs de virulence.

APPENDIX 1: OTHER PRIMARY ARTICLES

1 ARTICLE NO. 4: *LEISHMANIA INFANTUM* LIPOPHOSPHOGLYCAN-DEFICIENT MUTANTS: A TOOL TO STUDY HOST CELL-PARASITE INTERPLAY

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Running title: Leishmania infantum Δ*lpg1* Mutants Characterization

Author contributions: conceived and designed the study, contributed to the data analysis, drafted and revised the manuscript: MLS, CM, JBL, GAD (5-10%), GQ-C, ACV, SMP, UGL, LPF, TA-S, AD and VMB. Performed the experiments: MLS, CM, JBL, GAD (5-10%, I helped prepare the Δ*lpg1* knockout and helped prepare the manuscript), GQ-C, ACV, SMP, CPF, FHJS and TA-S. Wrote and revised the manuscript: MLS, CM, JBL, LPF, TA-S, AD and VMB. All authors read and approved the final version of this manuscript.

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1.1 Abstract

Lipophosphoglycan (LPG) is the major surface glycoconjugate of metacyclic *Leishmania* promastigotes and is associated with virulence in various species of this parasite. Here, we generated a LPG-deficient mutant of *Leishmania infantum,* the foremost etiologic agent of visceral leishmaniasis in Brazil. The *L. infantum* LPG-deficient mutant (Δ*lpg1*) was obtained by homologous recombination and complemented via episomal expression of *LPG1* (Δ*lpg1* + *LPG1*). Deletion of *LPG1* had no observable effect on parasite morphology or on the presence of subcellular organelles, such as lipid droplets. While both wild-type and add-back parasites reached late phase in axenic cultures, the growth of Δ*lpg1* parasites was delayed. Additionally, the deletion of *LPG1* impaired the outcome of infection in murine bone marrow-derived macrophages. Although no significant differences were observed in parasite load after 4 h of infection, survival of Δ*lpg1* parasites was significantly reduced at 72 h post-infection. Interestingly, *L. infantum* LPG-deficient mutants induced a strong NF-κB-dependent activation of the inducible nitric oxide synthase (iNOS) promoter compared to wild type and Δ*lpg1* + *LPG1* parasites. In conclusion, the *L. infantum* Δ*lpg1* mutant constitutes a powerful tool to investigate the role(s) played by LPG in host cell-parasite interactions.

1.2 Introduction

Lipophosphoglycan (LPG) is one of the most abundant components of *Leishmania* membranes (Turco *et al.*, 1992). In the course of parasite interaction with invertebrate hosts, LPG binds to the midgut epithelium of specific species of the sandfly vectors (Sacks *et al.*, 2000), and protects parasites against the digestive enzymes present in the peritrophic matrix following blood feeding (Sacks *et al.*, 2001a). In vertebrate hosts, LPG contributes to virulence by shielding *Leishmania* against the complement system (Späth *et al.*, 2003) and by inhibiting phagolysosomal biogenesis (Desjardins *et al.*, 1997, Moradin *et al.*, 2012, Vinet *et al.*, 2009). Purified LPG has been considered as a pathogen-associated molecular pattern molecule (PAMP) that triggers TLRs, and is also known to interfere with pro-inflammatory and signaling pathways in host cells (Becker *et al.*, 2003, Descoteaux *et al.*, 1993, Descoteaux *et al.*, 1991, Kavoosi *et al.*, 2009, Lima *et al.*, 2017, Rojas-Bernabé *et al.*, 2014, Tavares *et al.*, 2014, Veer *et al.*, 2003)

This complex glycolipid is organized in four domains: a conserved 1-O-alkyl-2-lyso-phosphatidyl(myo)inositol membrane anchor, a conserved diphosphoheptasaccharide core structure, a polymer of repeating phosphodisaccharide units (phosphoglycan or PG) carrying species-specific side chains and variable, often mannose-rich cap structures (McConville *et al.*, 1993, Turco *et al.*, 1992). Although the biosynthesis of LPG has attracted considerable interest, to date only few enzymes and transporters involved in this process have been identified either biochemically, genetically, or both (Descoteaux *et al.*, 2002a, Descoteaux *et al.*, 1995, Descoteaux *et al.*, 1998, Ryan *et al.*, 1993).

One of the key enzymes in the biosynthesis of LPG is *LPG1*, a putative galactofuranosyl transferase specifically involved in the synthesis of the LPG glycan core (Ryan *et al.*, 1993). Consequently, parasites lacking the *LPG1* gene (Δ*lpg1*) express a truncated LPG without the PG domain; they nonetheless assemble and secrete other PG-containing molecules (Dermine *et al.*, 2000, Späth *et al.*, 2000). Both *L. major* and *L. donovani* require *LPG1* for the establishment of infection within macrophages, as

evidenced by the elimination of *LPG1*-null mutants following phagocytosis; yet, restoration of LPG expression by genetic complementation restored the capacity to replicate within macrophage (Lodge *et al.*, 2006b, Späth *et al.*, 2000). Interestingly, phosphoglycan synthesis does not seem to be an absolute requirement for virulence in all *Leishmania* species, since *L. mexicana* phosphoglycan-deficient parasites were found to be similarly virulent to their wild-type (WT) counterparts (Ilg, 2000, Ilg *et al.*, 2001, Ilg *et al.*, 1999). This difference in LPG requirement for the establishment of infection within macrophages may be related to the fact that *L. mexicana* resides in large fusogenic communal vacuoles, as opposed to the non-fusogenic, tight individual vacuoles in which *L. major* and *L. donovani* replicate. The role played by *LPG1* in *L. infantum* infectivity in mammals remains to be established.

This report describes the disruption of *LPG1* in *L. infantum*, the main etiological agent of visceral leishmaniasis in Brazil. While deletion of *LPG1* did not alter parasite morphology *in vitro* or the presence of subcellular organelles, e.g. lipid droplets (LD), Δ*lpg1* parasites experienced distinct infection outcomes in comparison to WT parasites. Hence, the *L. infantum LPG1*-null strain described in the present study constitutes a powerful tool to investigate the role of LPG in host-parasite interactions.

1.3 Methods

Ethics Statement

This study was carried out in accordance with the recommendations of Institutional Review Board for Animal Experimentation (CEUA), Gonçalo Moniz Institute, Fundação Oswaldo Cruz. The protocol was approved by the Institutional Review Board for Animal Experimentation (CEUA), Gonçalo Moniz Institute, Fundação Oswaldo Cruz (Protocol No. 021/2015).

Animals

Inbred male C57BL/6 mice, aged 6–8 weeks, were obtained from the animal care facility of the Gonçalo Moniz Institute, Fundação Oswaldo Cruz (IGM-FIOCRUZ, Bahia, Brazil).

Targeted deletion of the *LPG1* gene and complementation

The constructs for *LPG1* (beta galactofuranosyl transferase) gene targeting were designed based on the *L. infantum LPG1* gene sequence (GenBank accession No. GU233511). Homozygous *LPG1*-null mutants (Δ*lpg1*) were obtained using two targeting constructs (Figure 1A and B). For the *NEO* targeting construct, the entire *LPG1* gene was amplified by PCR from *L. infantum* BH46 (MCAN/BR/89/BH46) DNA using *Taq* DNA polymerase (New England Biolabs) and oligodeoxynucleotides AD-358 (forward) (5'-gtacaagcttccatATGGCGCCGCCTCGCTG-3') and AD-359 (reverse) (5'-gctactcgagTTAGCTGGGGTCAACAG-3'). This fragment was digested with *Hind*III and *Xho*I, and then ligated with the *Hind*III-*Xho*I-digested pBluescript II SK$^-$ vector, yielding pBS-*LPG1*. The *NEO* resistance cassette from pLeishNeo (unpublished) was extracted with *Not*I and *Eco*RV, blunted and inserted in the *Msc*I site of pBS-*LPG1*, within the *LPG1* gene, yielding pBS-*LPG1::NEO*. For the *HYG* targeting construct, nucleotides 1 to 437 of the *LPG1* gene were amplified by RT-PCR from *L. infantum* BH46 mRNA using oligodeoxynucleotides AD-53 (forward) (5'-cgggatccatATGGCGCCGCCTCGCTG-3') and AD-357 (reverse) (5'-ggaattcTCGGGGTGGTGAATG-3'). This fragment was digested with *Bam*HI and *Eco*RI, and then ligated with the *Bam*HI-*Eco*RI-digested pBluescript II SK$^-$ vector. A 467-bp fragment containing nucleotides 781 to 1247 of the

LPG1 ORF was amplified by PCR from *L. infantum* BH46 genomic DNA using oligodeoxynucleotides AD-355 (forward) (5'-gcaagcttGGCATCTATTACACAGACCACAAGG-3') and AD-356 (reverse) (5'-caggtcgacTGGCAGCGAATGTTTTCACC-3'). This fragment was digested with *Hind*III and *Sal*I, then ligated with the same vector, downstream of the first *LPG1* sequence, at the *Hind*III and *Sal*I restriction sites. The *HYG* resistance cassette from pX63-HYG was excised with *Sal*I and *Bam*HI, blunted and inserted between the two *LPG1* sequences, at the *Eco*RV restriction site, yielding pBS-*LPG1::HYG*. For genetic complementation of the ∆*lpg1* mutant, the entire *LPG1* ORF was amplified by PCR from *L. infantum* BH46 genomic DNA using Native *Pfu* polymerase (Stratagene, La Jolla, CA, USA) and oligodeoxynucleotides AD-358 (forward) (5'-gtacaagcttccatATGGCGCCGCCTCGCTG-3') and AD-359 (reverse) (5'-gctactcgagTTAGCTGGGGTCAACAG-3'). This fragment was digested with *Hind*III and *Xho*I, and then ligated with the *Hind*III-*Xho*I-digested pBluescript II SK$^-$ vector, yielding pBSII-*LPG1*. The absence of mutations in the amplified *LPG1* ORF was verified by Sanger sequencing (Génome Québec; GenBank accession No. GU233511). The *LPG1* gene was then excised from pBSII-*LPG1* with *Eco*RV and *Xho*I, blunted and ligated with the *Eco*RV-digested pLeishZeo vector (unpublished), yielding pLeishZeo-*LPG1*.

Transfection and selection of *L. infantum* ∆*lpg1* promastigotes

Log-phase WT *L. infantum* BA262 (MCAN/BR/89/BA262) promastigotes were first electroporated with the purified *LPG1::HYG* targeting construct (excised as a 2.9-kb *Xba*I-*Kpn*I fragment from pBS-*LPG1::HYG*) using 0.2 cm electroporation cuvettes, at 0.45 kV and a high capacitance of 500 µF as previously described (Turco *et al.*, 1994). Following electroporation, promastigotes were incubated for 24 h in drug-free, complete M199 medium and subsequently grown in the presence of 50 µg/mL Hygromycin B (Roche Diagnostics). To generate *LPG1*-null mutants, log-phase *lpg1*$^{+/HYG}$ heterozygous *L. infantum* BA262 promastigotes were electroporated with purified *LPG1::NEO* targeting construct (excised as a 3.8-kb *Hind*III-*Kpn*I fragment from pBS-*LPG1::NEO*) and grown after 24 h in the presence of both 50 µg/mL Hygromycin B and 70 µg/mL G418 (Life Technologies). Absence of LPG in the resulting double drug-resistant ∆*lpg1*

promastigotes was verified by Western blot analysis and confocal immunofluorescence. To restore *LPG1* expression, log-phase *L. infantum* BA262 Δ*lpg1* cells were electroporated with pLeishZeo-*LPG1*. Complemented mutants *(*Δ*lpg1* + *LPG1*) were selected with 80 μg/mL Zeocin (in addition to G418 and Hygromycin B at concentrations specified above) and verified by Western blotting and confocal immunofluorescence.

Parasite cultures

L. infantum promastigotes were cultured in HOMEM medium supplemented with 10% inactivated Fetal Bovine Serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine in 25 cm^2 flasks at 24°C until late log-phase. For *L. infantum* BA262 Δ*lpg1,* Hygromycin (50 μg/mL) and G418 (70 μg/mL) were added to the medium. For *LPG1*-complemented parasites (Δ*lpg1* + *LPG1*), Hygromycin (50 μg/mL), G418 (70 μg/mL) and Zeocin (100 μg/mL) were added to the medium.

Western blotting

Late log-phase promastigotes were washed with ice-cold PBS containing 1 mM Na_3VO_4, then lysed in 50 mM Tris-HCl pH 8, 150 mM NaCl and 1% Nonidet P-40, containing complete protease inhibitors (Roche Applied Science) and phosphatase inhibitors (1 mM Na_3VO_4, 50 mM NaF, 1.5 mM EGTA and 10 mM $Na_4P_2O_7$). Samples were sonicated briefly, and insoluble material was removed by centrifugation for 10 min at 4°C. Protein concentrations were determined using the Pierce BCA protein assay kit (Pierce). Proteins were separated by SDS-PAGE and then transferred to Hybond-LFP PVDF membranes (GE Healthcare Life Sciences) using a Trans-Blot SD Semi-Dry Transfer Cell apparatus (BioRad). Membranes were blocked with 5% BSA and incubated with the mouse monoclonal antibody CA7AE (MediMabs). For immunodetection, goat anti-mouse IgM Heavy Chain Secondary antibody conjugated with horseradish peroxidase (HRP), and enhanced chemiluminescence (ECL) detection reagents from GE Healthcare Life Sciences were used.

Confocal immunofluorescence microscopy

Late log-phase promastigotes were adhered on Poly-L-Lysine-coated glass coverslips (BD Biosciences, San Jose, CA) by centrifugation, fixed with 4% paraformaldehyde (Canemco and Marivac) for 20 min and simultaneously blocked and permeabilized with a solution of 0.1% Triton X-100, 1% BSA, 6% non fat dry milk, 20% goat serum and 50% FBS for 20 min. The distribution of LPG and other PGs containing the Gal(β1,4)Man(α1-PO_4) repeating unit epitope was visualized using the mouse monoclonal antibody CA7AE (MediMabs, 1:2000) after 2 h incubation followed by Alexa Fluor 568 goat anti-mouse IgM (Molecular Probes) at 1:500 for 30 min incubation. Parasite nuclei were stained with DAPI (Molecular Probes) at 1:17,000. All steps were performed at room temperature. Coverslips were then mounted in Fluoromount-G (Interscience) and sealed with nail polish. Promastigotes were observed with a Plan APOCHROMAT 63x oil-immersion DIC 1.4 NA objective on a Zeiss LSM780 confocal microscope equipped with a 30 mW 405 nm diode laser, 25mW 458/488/514 argon multiline laser, 20mW DPSS 561 nm laser and 5mW HeNe 633 nm laser, coupled to a Zeiss Axio Observer Z1. Images were acquired in plane scanning mode, and were minimally and equally processed using Carl Zeiss ZEN 2011 software.

Electron Microscopy

Late log-phase promastigotes were fixed with 2% paraformaldehyde plus 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) overnight at 4°C. Next, parasites were processed for Transmission Electron Microscopy (TEM) by post-fixing in 1% osmium tetroxide (OsO_4) plus 0.8% potassium ferricyanide in 0.1 M cacodylate buffer, pH 7.2, then dehydrated in acetone at increasing concentrations of 50, 70, 90, and 100% followed by processing for resin embedding (PolyBed 812, Polysciences). Sections were mounted on uncoated 200-mesh copper grids and viewed under a TEM microscope (JEOL JEM-1230). Alternatively, parasites were processed for Scanning Electron Microscopy (SEM) by first fixing as described above, then adhered on Poly-L-Lysine-coated glass coverslips and post-fixed as described above. Samples were then submitted to critical point-drying under CO_2, coated with a 20 nm-layer of gold particles and examined under SEM (JSM-6390LV, JEOL).

Parasite growth curves

Early log-phase promastigotes ($1x10^5$/ml) were cultured and the number of viable promastigotes was determined by daily direct counting performed in a Neubauer chamber.

Lipid droplets staining and quantification

Late log-phase promastigotes were fixed with 3.7% formaldehyde and stained with osmium tetroxide. Cell morphology was observed, and LD were counted by light microscopy using a 100X objective lens in 50 consecutively scanned parasites (Araújo-Santos *et al.*, 2014).

Bone marrow-derived macrophages (BMDM) macrophage and infection

Bone marrow-derived macrophages (BMDM) were obtained from C57BL/6 mice as previously described. Briefly, cells were collected from femurs and differentiated in RPMI 1640, 20% inactivated FBS, 30% L929 cell-conditioned media (LCCM), 2mM L-glutamine, 100 U/mL Penicillin, and 100 µg/mL Streptomycin at 36°C under 5% CO_2. BMDMs were collected after seven days and seeded on tissue culture plates in RPMI 1640 media, 10% inactivated FBS, 5% LCCM and 2 mM L-glutamine (Araújo-Santos *et al.*, 2014).

Cells ($2x10^5$) adhered on coverslips were infected with either WT, Δ*lpg1*, or Δ*lpg1*+*LPG1* parasites at a 10:1 multiplicity of infection (MOI). After 4 or 72 h of infection, coverslips were fixed and stained with DiffQuik (Wright-Giemsa). Intracellular parasites were counted under light microscopy to determine the infection index under each experimental condition (Araújo-Santos *et al.*, 2014).

RAW 264.7 cell line, culture, and infection

The mouse macrophage leukemia cell line RAW 264.7 (TIB-71; American Type Culture Collection (ATCC), Manassas, VA, USA) was maintained in DMEM medium with high glucose (Vitrocell Embriolife, Campinas, SP, Brazil) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin and 100 µg/ml streptomycin in an incubator at 37°C under 5% CO_2. RAW 264.7 cells

were infected with either WT, Δ*lpg1* or Δ*lpg1+LPG1* parasites at a 10:1 multiplicity of infection (MOI). After 4h or 8h of infection, cells were processed for quantitative RT-PCR. For the luciferase reporter assay, cultures were washed 2 h post-infection and analyzed 24 h later.

RNA extraction and RT-qPCR

For real time quantitative polymerase chain reaction analysis, total RNA of control and infected RAW 264.7 cells ($1x10^6$ cells) was extracted using an Invitrap® Spin Cell RNA mini kit (STRACTEC Molecular GmbH, Berlin, Germany). RNA extracts (2 µg) were reverse transcribed into first-strand cDNA with ImProm-II (Promega) and oligo(dT) primers in accordance with manufacturer instructions. The following primer DNA sequences were used to determine iNOS mRNA levels: Forward 5'-CAGCTGGGCTGTACAAACCTT-3' and Reverse: 5'-CATTGGAAGTGAAGCGTTTCG-3', while GAPDH mRNA levels were quantified using: Forward 5'-TGCACCACCAACTGCTTAGC-3' and Reverse 5'-GGCATGGACTGTGGTCATGAG-3'. Amplicon specificity was carefully verified by the presence of a single melting temperature peak in dissociation curves calculated following RT-qPCR, which was performed via the Applied Biosystems StepOne^{TM} detection system (Applied Biosystems) using GoTaq® qPCR Master Mix (Promega Corp., Madison, WI, USA). All RT-qPCR analyses were performed in triplicate. RT-qPCR data was normalized using GAPDH primers as an endogenous control. All gene expression ratios were calculated by the ΔΔCt method using StepOne software version 2.0 (Applied Biosystems).

Transient transfections and luciferase assays

To investigate NF-κB transcriptional activity, RAW 264.7 were plated in 48-well polystyrene plates ($1x10^5$ cells per well) and transfected with 1 µg of the p6kB-Luc luciferase reporter construct (kindly provided by Dr. Patrick Baeuerle, Munich University) in the presence of LIPOFECTAMINE 2000 reagent (Invitrogen, Carlsbad, CA, USA). pTK-3XNS luciferase reporter construct was used to measure iNOS promoter activity, provided by Dr. David Geller (University of Pittsburgh, Pennsylvania, EUA). Luciferase activity was normalized using 40 ng of pRL-CMV plasmid (Promega

Corp., Madison, WI, USA). Transfected cells were infected with either WT, Δ*lpg1* or Δ*lpg1+LPG1* parasites at a 10:1 MOI. After 24 h of infection, cells were washed with PBS, lysed according to the Dual Luciferase System protocol (Promega Corp.), and analyzed in a GloMax®-Multi detection system (Promega Corp.). Positive controls consisting of cells stimulated with 1ug/mL of LPS (Sigma-Aldrich) were used to induce the activation of iNOS gene expression.

Statistical analysis

BMDM and RAW 264.7 cell infection assays were performed in triplicate, and each experiment was repeated at least three times. Data are presented as the mean and SE (standard error) of representative experiments, and GraphPad Prism 5.0 software (GraphPad Software) was used for data analysis. Means from different groups were compared by One-way ANOVA and comparisons between two groups were performed using the Student Newman-Keuls post-test. Differences were considered statistically significant when $p \leq 0.05$.

1.4 Results

Generation of a *L. infantum LPG1*-null Δ*lpg1* mutant

To generate a *L. infantum* LPG-defective (Δ*lpg1*) mutant, WT *L. infantum* BA262 promastigotes were transfected with the *LPG1* targeting constructs (Figure 1A and 1B). The resulting *HYG*- and *NEO*-resistant Δ*lpg1* parasites were transfected with a *LPG1* expression vector to generate add-back LPG-expressing parasites (Δ*lpg1* + *LPG1*). Loss of LPG expression in the Δ*lpg1*, was determined by comparing LPG levels in WT, Δ*lpg1*, and Δ*lpg1* + *LPG1 L. infantum* promastigotes by Western blot promastigote lysates and by confocal immunofluorescence microscopy (Figure 1C and 1D). Together, these data indicate that the *LPG1* gene was successfully deleted in the Δ*lpg1* mutants, resulting in the generation of a LPG-defective *L. infantum* mutant.

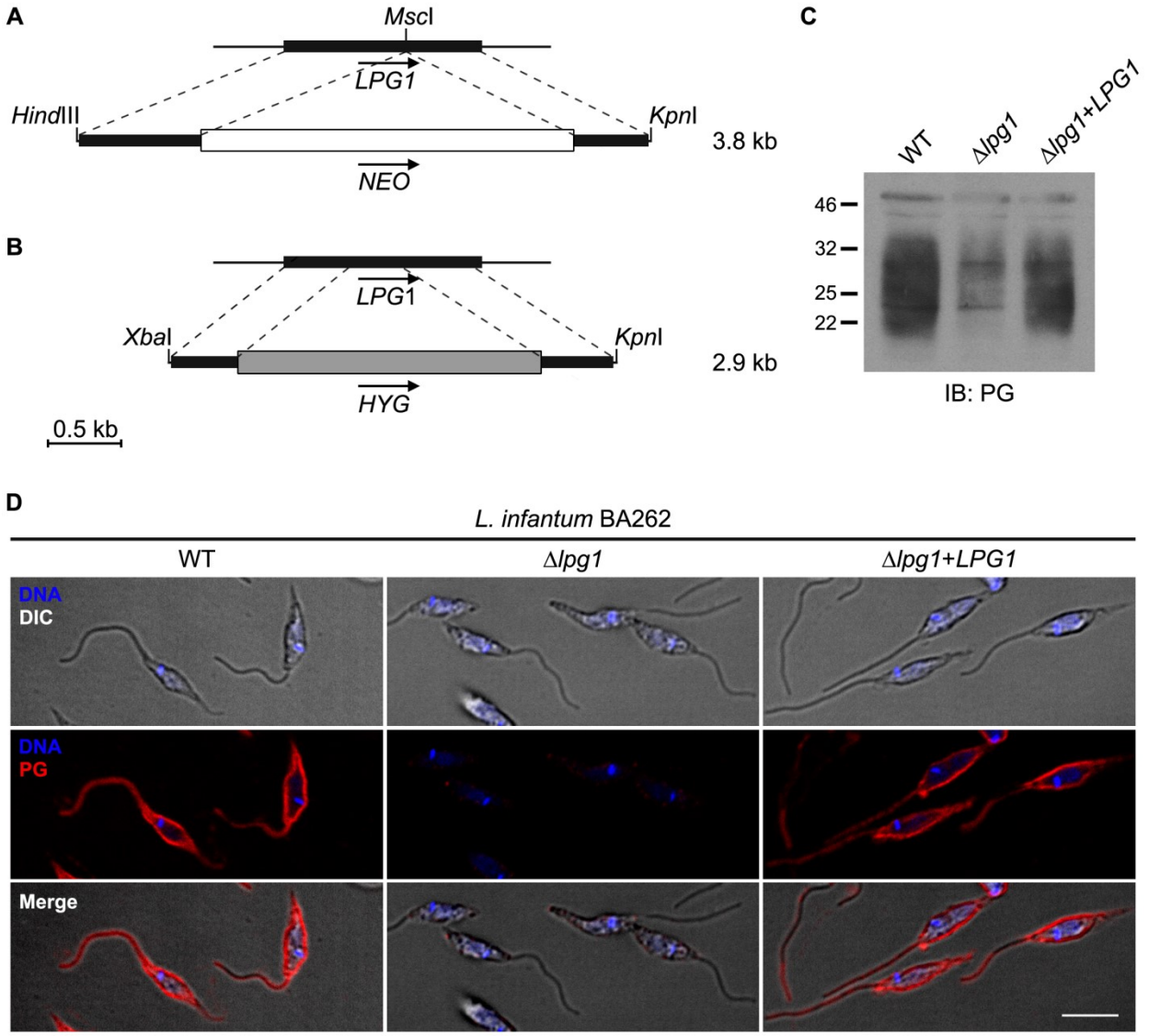

Figure 1 of article 4. Constructs for the targeted deletion and complementation of the *LPG1* gene in *Leishmania infantum*. (A, B) *LPG1*::*NEO* and *LPG1*::*HYG* targeting constructs for the disruption of *LPG1*. For the *LPG1*::*NEO* construct, the *NEO* resistance cassette (white box) was inserted in the *Msc*I site of the *LPG1* ORF (black rectangle). In the *LPG1*::*HYG* construct, portions of the *LPG1* ORF (black rectangles) corresponding to positions +1 to +437 and to positions +781 to +1247 downstream of the ATG translation initiation codon flank the HYG resistance cassette (shaded rectangle). Dashed lines delimit regions of recombination between the LPG1 gene and the targeting constructs. Arrows indicate gene orientation. (C) Western blot analysis of LPG expression in WT, Δ*lpg1*, and Δ*lpg1* + *LPG1* promastigotes. Parasite lysates were probed with the anti-phosphoglycan (PG) antibody CA7AE, as described in Materials and Methods. (D) Confocal immunofluorescence analysis of WT, Δ*lpg1* and Δ*lpg1* + *LPG1* parasites. Late log-phase promastigotes were adhered on Poly-L-Lysine-coated glass coverslips, fixed and incubated with DAPI to stain DNA (blue), and with the CA7AE antibody to visualize LPG and other Gal(β1,4)Man(α1-PO4) repeating unit-containing PGs (red), as described in Materials and Methods. Fluorescence staining images merged with differential interference contrast (DIC) are shown in the lower panels. Scale bar: 5 μm.

LPG1-null mutants retain *L. infantum* viability and morphology

To determine the effect of deleting *LPG1* on parasite growth and morphology, axenic cultures of the three isolates were monitored and counted daily for 10 days until reaching late log phase. A delayed replication capability of the Δ*lpg1* mutant parasites was noted in comparison to the WT and Δ*lpg1* + *LPG1* parasites (Figure 2A). Wild-type *L*. *infantum* presented regular growth for seven days until reaching stationary phase, with a cell density of approximately 3-4x10^7 parasites/ml, while the Δ*lpg1* mutant reached the same phase approximately three days later, with a cell density of 1-2x10^7 parasites/ml (Figure 2A). The Δ*lpg1* + *LPG1* mutants presented an intermediate growth profile, reaching stationary phase shortly after the WT parasites. Area Under the Curve (AUC) analysis of the growth curve revealed a significant difference only when comparing WT and Δ*lpg1* parasites ($p < 0.05$), yet no differences were observed between WT and Δ*lpg1* + *LPG1* mutants (Figure 2B). Upon reaching stationary phase, parasites were examined by electron microscopy to assess the presence of morphological alterations. Under both SEM and TEM, no alterations in morphology (Figure 2C) or in ultrastructural characteristics (Figure 3A) were detected among WT, Δ*lpg1*, and Δ*lpg1* + *LPG1* promastigotes. In addition, the absence of *LPG1* had no impact on the number of lipid bodies present within the parasites (Figure 3A). Hence, whereas the *LPG1* gene had a limited impact on *L. infantum* promastigotes proliferation, it did not significantly alter morphological features of these parasites (Figure 3).

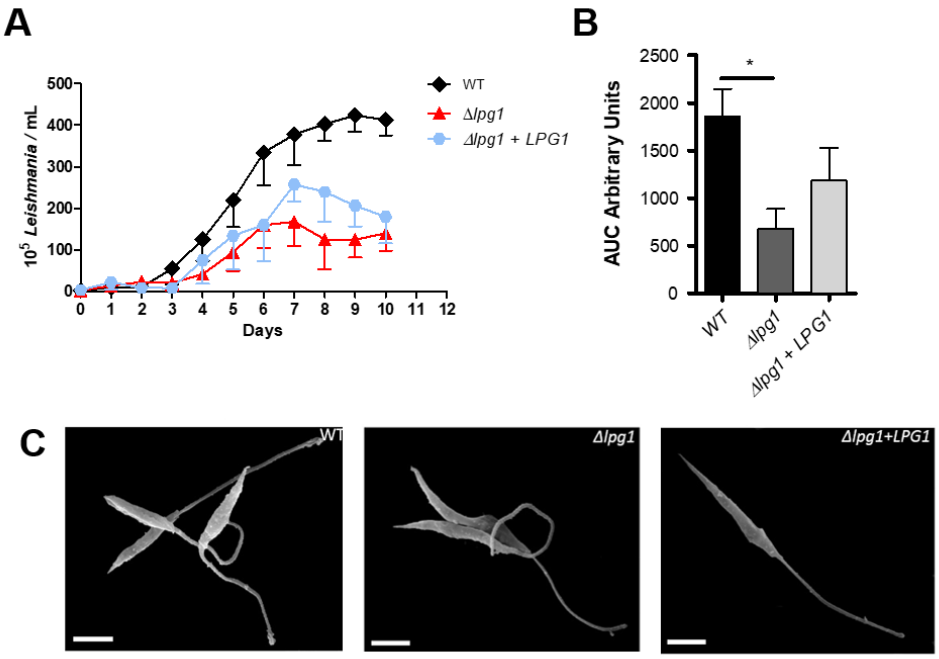

Figure 2 of article 4. Growth curve and morphology of the Δ*lpg1* mutant. WT, Δ*lpg1* and Δ*lpg1* + *LPG1* parasites were cultured at initial concentrations of 1x10^5/ml in HOMEM medium. (A) Axenic growth curve of late log-phase WT, Δ*lpg1* and Δ*lpg1* + *LPG1* parasites, as showed by the area under the curve (AUC) (B). The number of viable parasites was evaluated by direct counting. Each point represents mean and SE. Data are representative of at least three independent assays and were collected in triplicate for each condition. *p < 0.05. (C) Parasites were processed for scanning electron microscopy (SEM) and photographed under a JEOL JSM-6390LV microscope at 6000x magnification (C). Scale bar: 2μm.

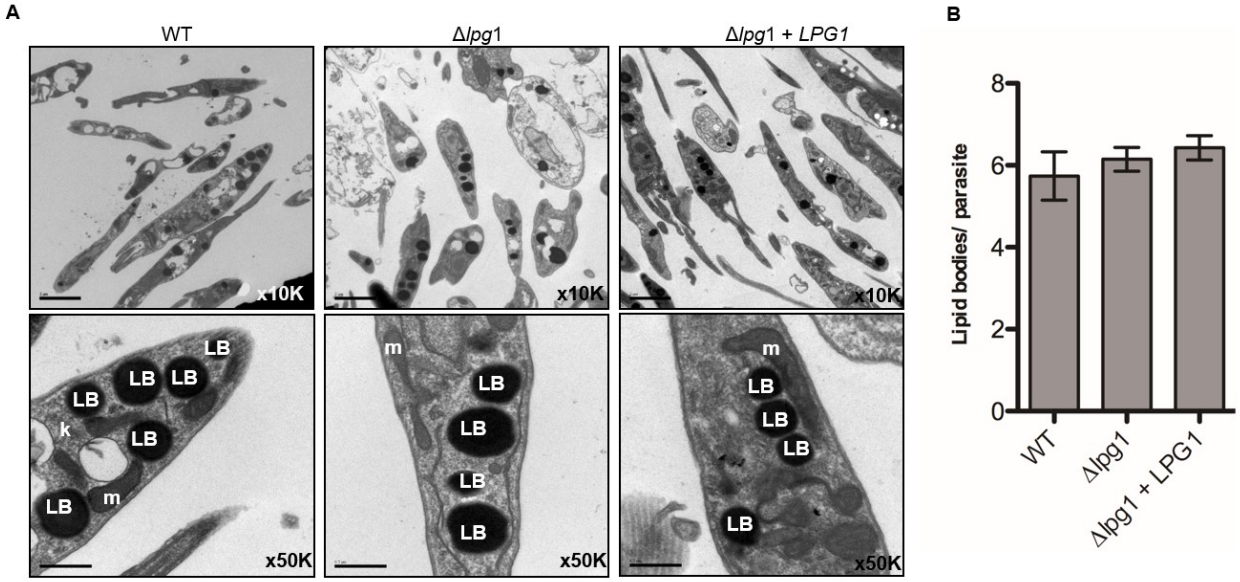

Figure 3 of article 4. Deletion of *LPG1* does not alter LD formation in *Leishmania infantum*. (A) Panels show stationary phase WT, Δ*lpg1* and Δ*lpg1* + *LPG1* promastigotes analyzed by transmission electron microscopy (TEM) and photographed under a JEOL 1230 microscope. (B) Bars represent the mean number of LD ± SE in WT, Δ*lpg1* or Δ*lpg1* + *LPG1* parasites stained with osmium tetroxide. k, kinetoplast; LD, lipid droplets; m, mitochondrion. Scale bar, 0.5μm.

LPG1-null mutants exhibit limited survival in macrophages

To evaluate differences in parasite survival among WT and transgenic parasites *in vitro*, BMDMs were infected for 4 h or 72 h. No differences were observed between the *Leishmania* parasites after 4 h of infection (Figure 4). However, at 72 h post-infection, WT parasites survived more efficiently than the Δ*lpg1* mutant. Expression of *LPG1* in the Δ*lpg1* mutant partially restored its capacity to survive and replicate within macrophages. These data reinforce the importance of LPG as a virulence factor in the successful maintenance of *Leishmania infantum* infection.

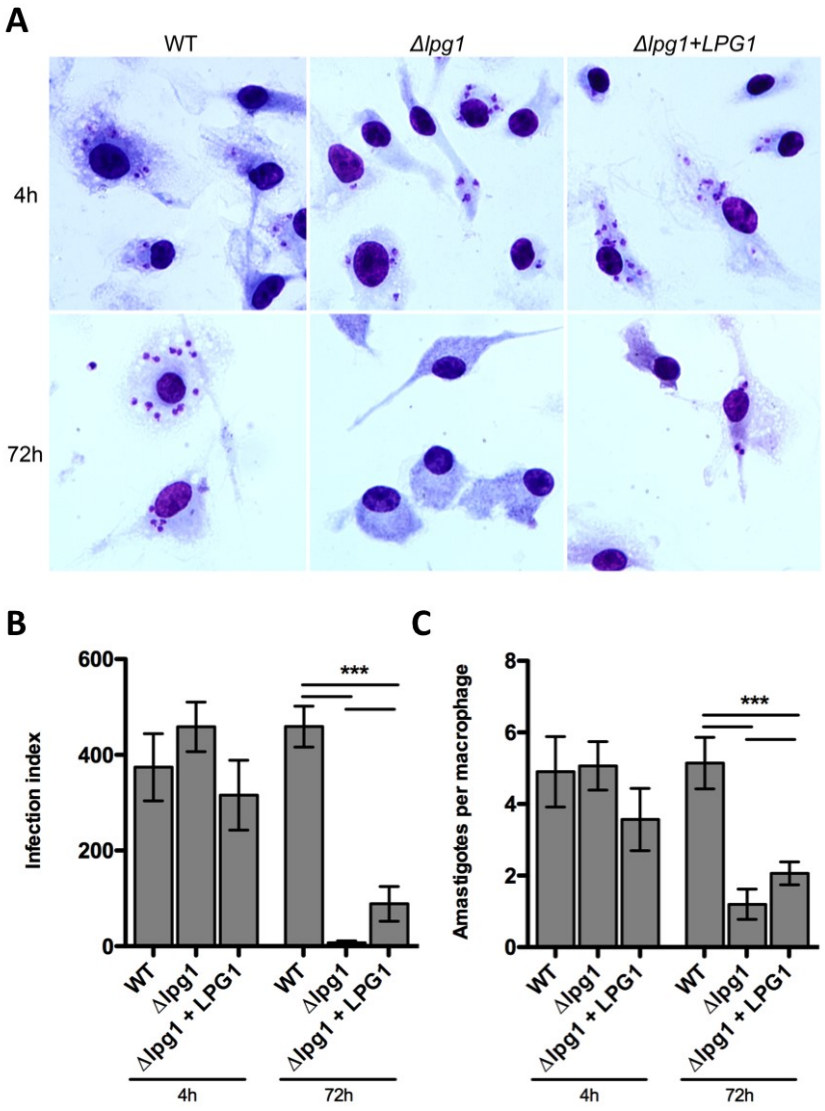

Figure 4 of article 4. *LPG1* promotes intraphagosomal survival in infected macrophages. C57BL/6 BMDMs were infected with WT, Δ*lpg1* and Δ*lpg1* + *LPG1* promastigotes as described under Materials and Methods. At 4 and 72 h post-infection, cells were fixed and Giemsa-stained. (A) Micrographs of infected BMDMs at 4 and 72 h post-infection. The infection index (B) and amastigotes per macrophage (C) were quantified by light microscopy. Arrows point to amastigotes inside a parasitophorous vacuole. Original magnification × 1000. Statistical differences were evaluated using the Student Newman-Keuls test.

LPG1-null mutants induce NF-κB-dependent iNOS expression in macrophages

To assess the impact of LPG on the expression of inducible nitric oxide synthase by host cells, we first analyzed iNOS transcript levels in RAW 264.7 cells infected with WT or transgenic parasites. The Δ*lpg1* mutant induced a robust (3.5-fold increase) expression of iNOS compared to WT and Δ*lpg1* + *LPG1* promastigotes (Figure 5A). We

next performed luciferase reporter assays to characterize the modulation of the iNOS promoter by *L. infantum* promastigotes. RAW 264.7 cells were transiently transfected with either the iNOS promoter reporter construct pTK-3XNS or the NF-κB consensus luciferase reporter construct (p6κB-Luc) prior to infection with either *L. infantum* WT, Δ*lpg1* or Δ*lpg1+LPG1*, or stimulation with LPS. As shown in Figures 5B and 5C, LPG-deficient promastigotes induced stronger activation of the iNOS promoter and of the NF-κB reporter. Collectively, these findings indicate that LPG contributes to the evasion of iNOS expression by *L. infantum* promastigotes.

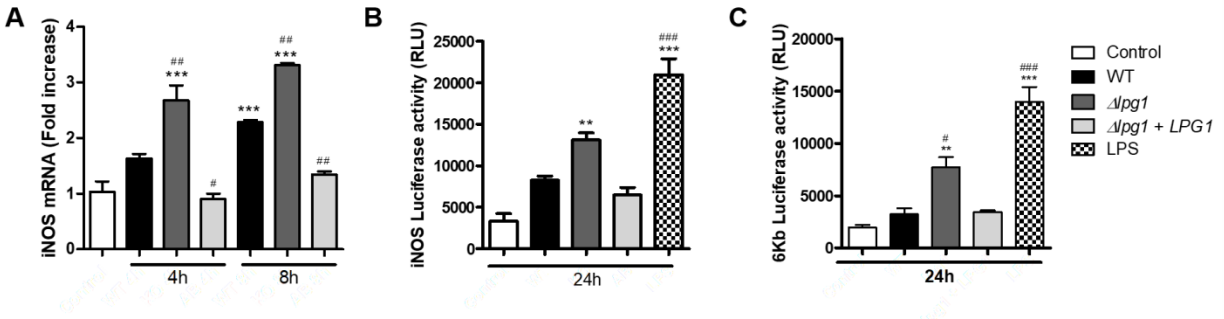

Figure 5 of article 4. *L. infantum* promastigotes evade NF-κB-dependent iNOS in an LPG-dependent manner in RAW 264.7 cells. RAW 264.7 cells were infected with either WT, Δ*lpg1* or Δ*lpg1* + *LPG1* promastigotes. After 4 h or 8 h of infection, iNOS expression was determined by qPCR (A). RAW 264.7 cells were transfected with either the iNOS promoter reporter construct pTK-3XNS, or the NF-κB consensus luciferase reporter construct (p6κB-Luc) prior to infection with either *L. infantum* WT, Δ*lpg1* or Δ*lpg1+LPG1*, or stimulation with LPS. At 24 h post-infection, activity of the iNOS promoter (B) and the NF-κB reporter (C) was measured by quantification of luciferase activity (B). Bars represent means ± SE of three representative experiments performed in triplicate for murine cells. P <0.0001 compared with control group Student Newman-Keuls post-test.

1.5 Discussion

Previous studies using purified LPG were important to unravel its impact on the activation of the immune system. Although purified LPG from different species can activate the release of inflammatory mediators, understanding the role of this response in the context of infection remains a challenge. While some groups have characterized Old World *LPG1*-defective *Leishmania* species, the behavior of these parasites when compared to their WT counterparts varies depending on the species under study (Capul *et al.*, 2007, Forestier *et al.*, 2015, Späth *et al.*, 2003). Here, we generated for the first time an LPG-deficient mutant of *L. infantum,* a New World species cluster.

A comparison of parasite growth between axenic cultures containing each of the three isolates showed that the deletion of *LPG1* resulted in a delayed capability of the ∆*lpg1* mutant parasites to replicate in comparison to cultures of WT and ∆*lpg1* + *LPG1* parasites. While the deletion of the *LPG1* gene had a limited impact on *L*. *infantum* promastigote proliferation, no significant morphological or ultrastructural alterations were seen in these parasites, indicating that targeting the *LPG1* gene does not interfere with the intrinsic cell biology of *L. infantum*.

Recently, we have demonstrated that intact LPG from *L. infantum* promastigotes, but not its glycan and lipid moieties, induced a range of pro-inflammatory responses, including prostaglandin E_2 (PGE_2) and nitric oxide (NO) release, increased LD formation, and inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 expression (Lima *et al.*, 2017). Consequently, a limitation of using purified LPG is that the physiological conditions present in host cell-parasite interactions are not accurately replicated. LDs are key cytoplasmic organelles involved in production of lipid mediators and pro-inflammatory cytokines in mammalian cells (Bozza *et al.*, 2011). Several intracellular pathogens, including *Leishmania*, take advantage of LD formation in host cells (Rabhi *et al.*, 2016). Moreover, LDs have also been described in trypanosomatids in association with arachidonic acid metabolism (Araújo-Santos *et al.*, 2014). Our group previously reported an increase in LD formation during *L. infantum* metacyclogenesis,

as well as in the intracellular amastigote form (Araújo-Santos *et al.*, 2014). Here, we showed that the absence of the *LPG1* gene in *L. infantum* did not alter the biogenesis of LDs. In addition, our previous findings showed that parasite-derived $PGF_{2\alpha}$ produced inside LDs plays a critical role during macrophage infection (Araújo-Santos *et al.*, 2014). We fully intend to comprehensively investigate the potential influence of *LPG1* on the release of $PGF_{2\alpha}$ in infected macrophages using this novel *L. infantum* Δ*lpg1* mutant.

In *L. major* and *L. donovani*, the specific loss of LPG through the ablation of LPG1 galactofuranosyl transferase strongly impairs the ability of parasites to survive within the sandfly host, as well as to establish infection in mammalian macrophages and in mice (Sacks *et al.*, 2000, Secundino *et al.*, 2010, Späth *et al.*, 2001). Hence, in these species, LPG impairs the microbicidal mechanisms associated with the biogenesis of phagolysosomes, including assembly of the NADPH oxidase and recruitment of the V-ATPase (Lodge *et al.*, 2006b, Vinet *et al.*, 2009). Our results indicate that, similarly to these species, *L. infantum LPG1* is required for replication within macrophages. Whether LPG contributes to the ability of *L. infantum* to successfully infect macrophages through the impairment of phagolysosomal biogenesis remains to be investigated. Interestingly, we observed that our Δ*lpg1* mutant parasites induced robust NF-κB-dependent iNOS expression compared to parental WT *L. infantum* promastigotes. This seems to suggest that the reduced survival of Δ*lpg1* mutants in mouse macrophages may be related to higher levels of iNOS, which is responsible for the generation of leishmanicidal nitric oxide (Coelho-Finamore *et al.*, 2011, Passero *et al.*, 2015). Further study will involve investigating the contribution of nitric oxide production with respect to the reduced ability of Δ*lpg1* mutants to survive within macrophages. The underlying mechanism by which Δ*lpg1* mutant parasites induce high levels of NF-κB activation remains unknown, and thus represents an additional aspect of host cell-parasite interplay that we intend to further investigate.

With regard to the partial restoration of the WT phenotype observed in Δ*lpg1* + *LPG1* parasites, it has been well-documented that complemented parasites commonly do not fully recover virulence. The inappropriate regulation of *LPG1* expression by the

episomal vector may be a possible explanation for this observation (Joshi *et al.*, 2002, Späth *et al.*, 2000, Späth *et al.*, 2003). A previous study demonstrated that *L. major LPG1*-deficient mutant promastigotes present an attenuated virulence phenotype, as evidenced by the delayed formation of lesions *in vivo* (Späth *et al.*, 2000). In addition, this delay was associated with a 100-fold decrease in parasite survival within macrophages *in vitro*. The data presented herein are consistent with these results, as well as with other reports in the literature (Privé *et al.*, 2000, Sacks *et al.*, 2000, Zhang *et al.*, 2004) propounding LPG as a virulence factor.

Taken together, the present findings support the importance of creating LPG-deficient mutants in various *Leishmania* spp. as a unique tool to investigate the specific impact and contribution of this abundant virulence factor in the complex host cell-*Leishmania* interplay. Hence, we are currently conducting studies to compare the responses of various immune cells to live *L. infantum* promastigotes in the presence or absence of surface-expressed LPG, since we feel it is important to thoroughly characterize these isolates to obtain a more comprehensive understanding regarding the role of *L. infantum LPG1* in future *in vitro* and *in vivo* studies.

1.6 Conflict of interest statement

The authors declare that they do not have a commercial association that might pose a conflict of interest.

1.7 Funding information

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APPENDIX 2: REVIEW ARTICLES

1 REVIEW ARTICLE NO. 1: *LEISHMANIA* SURVIVAL IN THE MACROPHAGE: WHERE THE ENDS JUSTIFY THE MEANS

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1.1 Abstract

Macrophages are cells of the immune system that mediate processes ranging from phagocytosis to tissue homeostasis. *Leishmania* has evolved ingenious ways to adapt to life in the macrophage. The GP63 metalloprotease, which disables key microbicidal pathways, has recently been found to disrupt processes ranging from antigen cross-presentation to nuclear pore dynamics. New studies have also revealed that *Leishmania* sabotages key metabolic and signalling pathways to fuel parasite growth. *Leishmania* has also been found to induce DNA methylation to turn off genes controlling microbicidal pathways. These novel findings highlight the multipronged attack employed by *Leishmania* to subvert macrophage function.

1.2 Highlights

- The GP63 metalloprotease selectively degrades molecules involved in membrane trafficking and nuclear physiology
- Cleavage of VAMP8 and Syt XI disrupts cross-presentation and cytokine secretion, respectively
- *Leishmania* sabotages cholesterol, iron and mitochondrial metabolism to fuel survival
- *Leishmania* alters the methylation of host genes controlling microbicidal pathways

1.3 Introduction

1.3.1 Macrophages: sentinels of the immune system

Macrophages are crucial to the immune response and their absence would allow infections to progress uncontrollably to the detriment and eventual demise of the host (Arango Duque *et al.*, 2014a). The importance of these phagocytic cells in antimicrobial defence and development was recognized and documented by Ilya Metchnikoff (Chernyak *et al.*, 1988), an achievement that merited him the 1908 Nobel Prize in Physiology or Medicine. Macrophages are found in every tissue, and most develop from bone marrow myeloid precursor cells. Phagocytosis is at the helm of macrophage biology (Flannagan *et al.*, 2012). This processes is essential for nutrient recycling, organismal homeostasis and defense from pathogens (Sieweke *et al.*, 2013). For example, macrophages phagocytose circulating erythrocytes in order to recycle iron back into circulation (Cairo *et al.*, 2011). Resting macrophages can be chemoattracted to the site of infection, and can be activated by cytokines such as interferon gamma (IFN-γ) and by microbial compounds (Nathan *et al.*, 1983, Tam *et al.*, 2014). In their first-of-the-line role in infections, macrophages are some of the first cells to come in contact with, recognize and kill microbes. Macrophages can also present antigens to lymphocytes (Pierce *et al.*, 1976) via major histocompatibility complex (MHC) molecules at the macrophage's plasmalemma.

Leishmania parasites have evolved to conquer macrophages

Pathogens have evolved to circumvent and conquer many of the antimicrobial strategies mounted by macrophages (Flannagan *et al.*, 2009, Sibley, 2011). Living inside of the macrophage is an ideal way to escape the immune system, obtain nutrients, and proliferate. *Leishmania* parasites, which cause the leishmaniases, constitute one such example of a microorganism that has successfully adapted to life in the macrophage. The leishmaniases constitute a spectrum of diseases caused by flagellate protozoa. These intracellular parasites cause cutaneous, mucocutaneous and visceral pathologies in humans and other animals (Hartley *et al.*). The leishamaniases

are prevalent in 98 countries spread over 5 continents (Alvar *et al.*, 2012). The parasite has a digenetic life cycle and is transmitted to humans by infected hematophagous female sand flies. Promastigotes, which are the extracellular forms of the parasite, are injected into the host via the proboscis of the sand fly (Killick-Kendrick *et al.*, 1981). Parasites in the skin are ingested primarily by macrophages, and also by neutrophils and dendritic cells. Although many promastigotes are destroyed by macrophages, some evade the microbicidal power of the phagolysosome, thereby transforming this powerful organelle into a parasitophorous vacuole (PV) that fosters parasite growth (Lodge *et al.*, 2008, Moradin *et al.*, 2012, Olivier *et al.*, 2012). Within PVs, promastigotes transform into amastigotes that multiply via binary fission. When the host cell becomes overwhelmed by parasites, it may either rupture and release amastigotes, or become apoptotic and pass the amastigote cargo to surrounding macrophages (Real *et al.*, 2014). Either way, amastigotes metastasize and cause pathology. The life cycle is completed when a previously uninfected sand fly takes a blood meal containing amastigotes or infected phagocytes.

Leishmania-macrophage interactions are multifaceted and involve a number of pathogenicity factors that allow parasites to use the phagolysosome to obtain nutrients, hijack antimicrobial pathways, and replicate (Podinovskaia *et al.*, 2015). The zinc metalloprotease GP63 is the most abundant molecule on the promastigote surface. It is a pathogenicity factor that cleaves host proteins, enabling *Leishmania* to subvert processes such as transcription and translation (Olivier *et al.*, 2012). In the process of conquering the macrophage, *Leishmania* has also been known to hijack signalling pathways (Shio *et al.*, 2012) that would otherwise kill the parasite in the phagolysosome and impede dissemination (Moradin *et al.*, 2012).

This review discusses recent studies (2013-2015) that cast light on how *Leishmania* sabotages macrophage functions for survival. It will focus on new discoveries concerning how the GP63 protease alters the macrophage's membrane fusion machinery to tamper with processes that are essential to macrophage physiology and function. Furthermore, it will discuss how *Leishmania* disrupts various host metabolic

and signalling pathways, as well as DNA accessibility, in order to promote intracellular survival.

1.4 The onslaught of the GP63 protease: from membrane trafficking to nuclear pore dynamics

1.4.1 Manipulation of membrane trafficking to subvert antigen presentation and cytokine secretion

Phagocytosis is governed by sequential interactions with cell organelles. These interactions are spatiotemporally regulated by a complex molecular machinery involving proteins that mediate vesicle fusion (Huynh *et al.*, 2007b). Proteins regulating neurotransmitter release, notably those of the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) family (Stow *et al.*, 2006, Weber *et al.*, 2018), are of salient importance in coordinating membrane trafficking in cells of the immune system. During vesicle fusion, SNAREs in vesicles and target membranes interact specifically to pull two membranes into close proximity (Stow *et al.*, 2006, Weber *et al.*, 2018). SNAREs are selectively distributed in different organelles. VAMP8, which forms complexes with syntaxin-7, syntaxin-8 and Vti1b, participates in homotypic fusion of late endosomes and is recruited to phagosomes (Antonin *et al.*, 2000, Pryor *et al.*, 2004). It also mediates the recruitment of $gp91^{phox}$ – a component of the NOX2 oxidase – to the phagosome (Matheoud *et al.*, 2013). This in turn implies that VAMP8 plays an important role in modulating intraphagosomal function and host defence. Antigen cross-presentation is an important function of phagosomes (Guermonprez *et al.*, 2003, Houde *et al.*, 2003). Cross-presentation of microbial peptides on major histocompatibility (MHC) I molecules is essential to activate $CD8^+$ T cells. Using cells from $Vamp8^{-/-}$ mice, Matheoud *et al.* discovered that VAMP8 plays an essential role in antigen cross-presentation in both bone marrow-derived macrophages (BMM) and dendritic cells (BMDC) (Matheoud *et al.*, 2013). Interestingly, infection of $Vamp8^{-/-}$ mice with *L. major* parasites results in larger footpad lesions (Matheoud *et al.*, 2013). Bacteria have evolved numerous ways to hamper SNARE function. For instance, clostridial toxins,

which block synaptic transmission in peripheral cholinergic synapses, cleave SNAREs (Montal, 2010). Using *gp63*-KO *L. major* parasites, Matheoud and colleagues found that VAMP8 and VAMP3 were directly cleaved by GP63 (Matheoud *et al.*, 2013). Given the importance of SNAREs in mediating trafficking to and from the phagosome, the authors sought to identify the consequences of SNARE cleavage on cross-presentation. Feeding *L. major*-infected macrophages with ovalbumin (OVA)-coated beads, the authors found that GP63 strongly inhibits OVA cross-presentation in both BMMs and BMDCs. Detailed analysis of these phagosomes revealed that cross-presentation is hindered due to decreased $gp91^{phox}$ recruitment, which leads to decreased intraphagosomal oxidation, increased proteolytic activity and altered pH (Figure 1A). In sum, by targeting SNAREs, *Leishmania* impairs crucial processes involved in phagolysosome biogenesis that are required for antigen cross-presentation.

Synaptotagmins (Syts) are membrane proteins that regulate vesicle docking and fusion in processes such as exocytosis (Arango Duque *et al.*, 2013, Baram *et al.*, 1999) and phagocytosis (Arango Duque *et al.*, 2013, Czibener *et al.*, 2006, Vinet *et al.*, 2008). They regulate SNARE activity by mediating membrane fusion in a Ca^{2+}-dependent manner (Südhof, 2012). All Syts possess two conserved tandem Ca^{2+}-binding C2 domains. However, Syt XI contains a conserved serine in its C2A domain that precludes this Syt from mediating vesicle fusion (Wang *et al.*, 2010). In macrophages, Syt XI is a recycling endosome- and lysosome-associated protein that negatively regulates the secretion of tumour necrosis factor (TNF) and IL-6 (Arango Duque *et al.*, 2014a, Arango Duque *et al.*, 2013, Weber *et al.*, 2018). The roles of Syts in vesicle trafficking make these proteins great targets for attack by intracellular pathogens. For instance, the parasite *Trypanosoma cruzi* uses Syt VII, a Ca^{2+}-dependent regulator of lysosome exocytosis, to invade target cells (Caler *et al.*, 2001). It had previously been observed that *Leishmania* promastigotes of certain species are able to trigger TNF and IL-6 secretion post-infection (Arena *et al.*, 1997, Karam *et al.*, 2006, Lapara *et al.*, 2010, Matte *et al.*, 2002, Wenzel *et al.*, 2012). Although the parasite signal that induces TNF and IL-6 secretion is not known, synthesis and secretion of these cytokines may be

initially triggered by engagement of TLR2 and TLR3 with parasite molecules (Flandin *et al.*, 2006). Given the role of Syt XI in cytokine secretion, it was hypothesized that *Leishmania* could affect Syt XI function to deregulate cytokine release. Using *L. major* strains that express or lack GP63, Arango Duque *et al.* found that the GP63 protease degrades Syt XI and positively regulates the post-infection release of TNF and IL-6 (Arango Duque *et al.*, 2014b). Using RNA interference, it was shown that cytokine release was induced by GP63-mediated degradation of Syt XI. At the forefront of these findings is the observation that, early during infection, GP63 induces the release of TNF and IL-6 *in vivo*. Importantly, injection of GP63-expressing parasites induces the influx of neutrophils and inflammatory monocytes to the inoculation site. This is likely to be a consequence of increased TNF and IL-6 release. Infection of recruited inflammatory monocytes (Ribeiro-Gomes *et al.*, 2014) can trigger IL-10 secretion (Belkaid *et al.*, 2001, Kane *et al.*, 2001), and induce their differentiation into immunosuppressive, arginase-expressing alternatively activated macrophages (Gundra *et al.*, 2014). The role of Syt XI in the recruitment of these phagocyte populations is to be elucidated. This new role for GP63 implicates this protease in the manipulation of cytokine trafficking, which may play a pivotal role in the phagocyte accrual observed at the early stages of infection (Arango Duque *et al.*, 2014b, Ribeiro-Gomes *et al.*, 2014) (Figure 1B).

Dok proteins are important for dampening the signaling program induced by microbial molecules and cytokines on macrophages. This is achieved by recruiting inhibitory molecules following receptor activation (Mashima *et al.*, 2009). These important adaptor proteins are recruited to latex bead and zymosan phagosomes, and may be important in phagolysosomal function. Similar to VAMP8 and Syt XI, the levels of Dok proteins are diminished in macrophages infected with GP63-expressing promastigotes (Álvarez de Celis *et al.*, 2015). Interestingly, infected IFN-γ-primed *Dok-1/Dok-2*$^{-/-}$ macrophages release less NO and TNF in comparison to WT macrophages; this effect is independent of GP63. Although the functions of Dok proteins in the context of phagosome maturation and cytokine secretion are not yet known, the findings by Álvarez *et al.* suggest that Dok-1 and Dok-2 regulate the secretion of immune effectors triggered by *Leishmania* infection. The role of these proteins in an *in vivo* infection model is yet to be investigated.

Manipulating membrane trafficking thus emerges as an efficient strategy to curb the microbicidal power of the macrophage. Another way to manipulate this process is during parasite egress and transmission to uninfected cells. Using live cell imaging, Real *et al.* discovered that after long-term infections, zeiotic macrophages extrude amastigote-containing PVs that can be ingested by other macrophages (Real *et al.*, 2014). These extruded PVs were found to be strongly associated with LAMP1. Extruded PVs were found to induce IL-10 secretion from the ingesting macrophages, and this was found to be dependent on the presence of LAMP1. In the *in vivo* setting, this mode of transmission – and LAMP1-induced IL-10 secretion – is likely to play a key role in the immunosuppression of the host and in the maintenance of chronic infection.

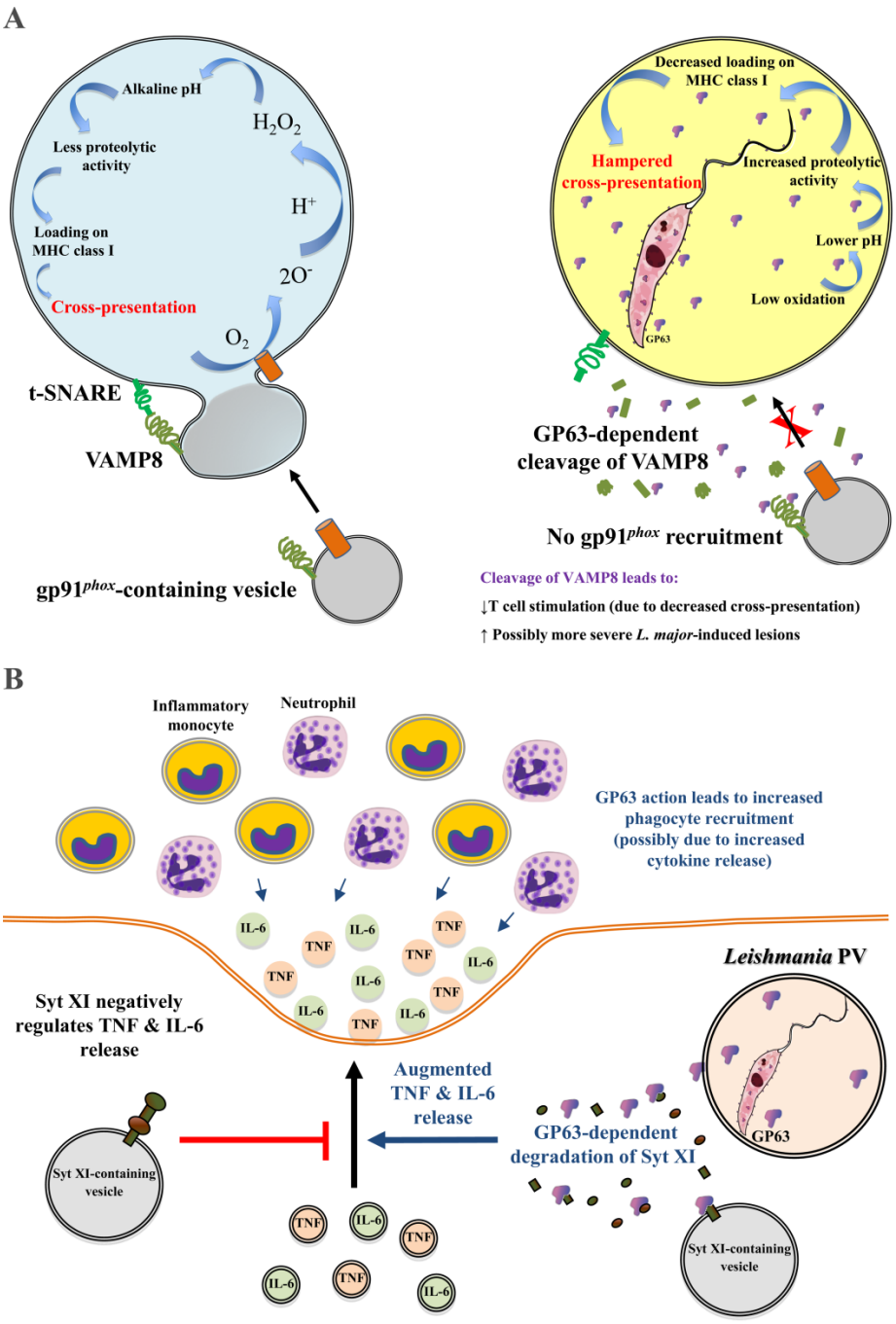

Figure 1 of review 1. *Leishmania* disrupts antigen cross-presentation and cytokine secretion. The GP63 protease degrades key proteins that mediate vesicle trafficking. **(A)** Cleavage of VAMP8 hinders antigen cross-presentation. Phagosomes (left panel) recruit the gp91^{phox} via VAMP8. The action of gp91^{phox} results in increased intraphagosomal oxidation, which helps keep a more alkaline pH in the phagosome. The consequent decrease in proteolytic activity allows processed antigens to be loaded onto MHC class I molecules. Cross-presentation plays an important role in the stimulation of T cells and is essential in the immune response. When macrophages and dendritic cells are infected with GP63-expressing *L. major* (right panel), VAMP8 is degraded and recruitment of gp91^{phox} to the phagosome is hindered. This results in a lower pH that fosters increased proteolytic activity, but leads to decreased cross-presentation of antigens. **(B)** GP63 degrades Syt XI, a negative regulator of TNF and IL-6 secretion. The ensuing post-infection release of TNF and IL-6 can help recruit inflammatory monocytes and neutrophils to the infection site. Infection of these inflammatory phagocytes promotes the establishment of chronic infections.

1.4.2 Impact of GP63 on nuclear integrity and physiology

Recent studies have revealed that GP63 is found in the vicinity of the nucleus (Gómez *et al.*, 2009), raising the possibility that GP63 could be interfering with nuclear dynamics. This is supported by the fact that cleavage of the AP-1 transcription factor occurs at the nucleus (Contreras *et al.*, 2010), suggesting that GP63 enters and disrupts this compartment. Isnard and colleagues provided evidence that *L. major* GP63 localizes to the nuclear envelope of infected macrophages and is active in this compartment and in the nucleoplasm (Isnard *et al.*, 2015). This nuclear confinement is nuclear localization signal (NLS)-independent, and occurs instead via interactions with Nup members of the nuclear pore complex. Indeed, proteins such as Nup62 and Nup368 were found to be cleaved by GP63, and mass spectrometry analyses revealed that the levels of several nuclear pore complex-associated proteins are altered. Importantly, GP63 was also found to alter the nucleoplasm proteome, with proteins involved in translational regulation and DNA accessibility severely diminished in the presence of GP63. It was also found that *L. mexicana*, a species that also causes cutaneous disease, alters nuclear physiology more severely than *L. major*. Further work will be needed to elucidate the consequences of GP63-induced changes in proteins associated with the nucleus. To further support the argument that *Leishmania* infection manipulates the expression of proteins found in the nucleus, Singh *et al.* also used proteomics to gain a global understanding of how *L. donovani* infection reprograms cells (Singh *et al.*, 2015). It was observed that histone proteins such as H2A, H2B and H4 were upregulated in infected cells, indicating that infection impacts chromatin remodelling and host gene regulation. Moreover, the expression of several proteins involved in DNA repair and replication, translation and RNA splicing were also found to be deregulated post-*L. donovani* infection.

1.5 *Leishmania* hijacks metabolic pathways in the macrophage to promote survival

1.5.1 Retention of intracellular iron fuels amastigote survival

Iron metabolism encompasses processes including intestinal iron absorption, iron transport and storage into cells, and macrophage-mediated iron recycling from erythrocytes (Hentze *et al.*, 2010). A dynamic balance for iron in the circulation among the different compartments is maintained, with most iron released by the decomposition of hemoglobin from senescent erythrocytes. At the end of their lives, senescent erythrocytes show a series of biochemical changes that trigger their phagocytosis by macrophages, mainly in the spleen (Hentze *et al.*, 2010, Nairz *et al.*, 2014). During this process, hemoglobin is degraded and Fe^{2+} is released. Recycled Fe^{2+} is then transported back to circulation via ferroportin, oxidized by ceruloplasmin to Fe^{3+}, bound to transferrin, and reused for hemoglobin synthesis. The main regulator for iron metabolism is the hormone hepcidin, which exerts its function by triggering the endocytosis and subsequent intralysosomal degradation of ferroportin. The bioactive form is produced mainly by the liver, but also by macrophages where it acts in an autocrine fashion. Hepcidin action causes iron trapping in macrophages, enterocytes, and hepatocytes (Hentze *et al.*, 2010, Nairz *et al.*, 2014). Intracellular pathogens have evolved approaches to manipulate iron recycling to fit their needs (Nairz *et al.*, 2014). *Leishmania* is an example of a pathogen that requires iron for survival and replication (Flannery *et al.*, 2013). A number of studies have started to elucidate how iron is transported from the phagolysosome into amastigotes. Three channels known as LFR1, LIT1 and LABCG5 have been implicated in this process (Flannery *et al.*, 2013). However, whether *Leishmania* modulates iron dynamics in macrophages was not known. A study by Ben-Othman *et al.* demonstrated that *L. amazonensis* is capable of inhibiting iron efflux (Ben-Othman *et al.*, 2014) (Figure 2).

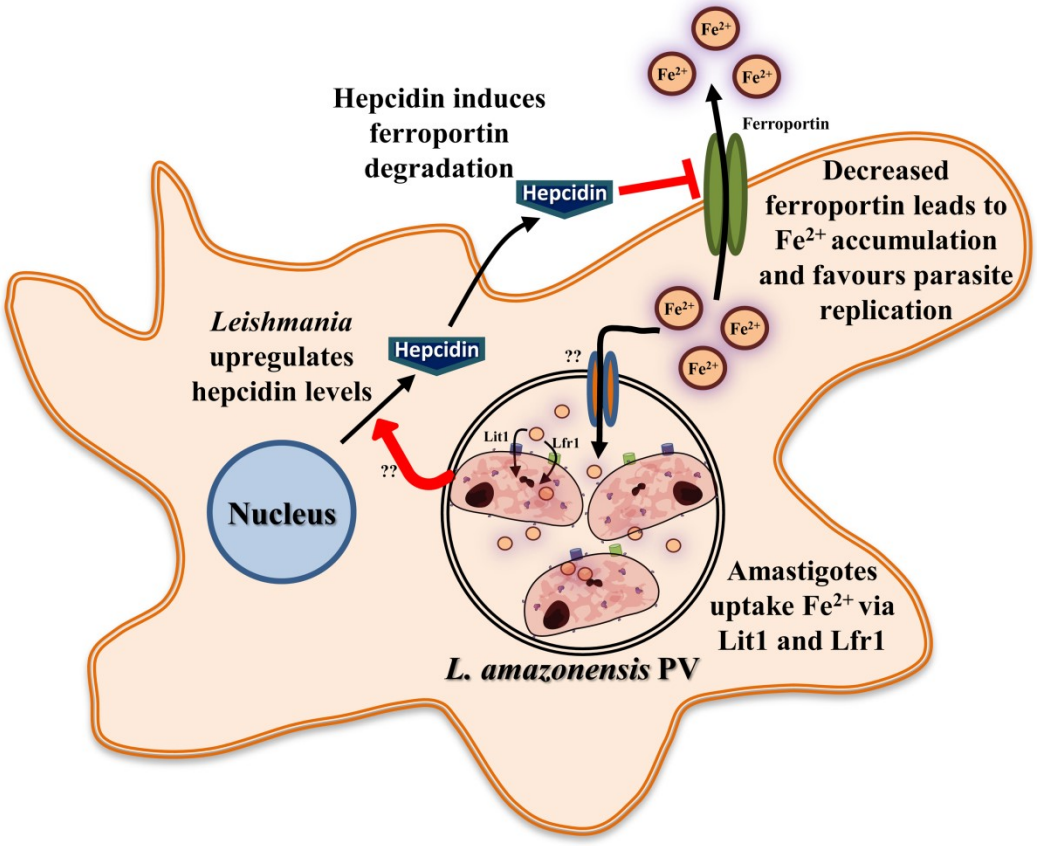

Figure 2 of review 1. *Leishmania* prevents iron efflux via hepcidin upregulation. Infection with *L. amazonensis* induces hepcidin levels. Hepcidin, a peptide hormone that binds to ferroportin outside of the cell, induces ferroportin degradation. The consequent increase in intracellular iron promotes parasite growth.

The authors observed that amastigote infection lowered ferroportin at the transcriptional and protein level. Knowing that hepcidin plays a crucial role in ferroportin turnover, the authors found that hepcidin levels were higher in infected macrophages and in the livers and footpads of infected mice. The effects of infection on ferroportin and hepcidin levels were found to be TLR4-dependent. The increase in hepcidin was correlated with higher intracellular iron levels. Importantly, overexpression of a dominant negative ferroportin mutant that fails to go to the macrophage plasmalemma resulted in increased parasite survival. The consequent increase in intracytoplasmic iron also allowed the proliferation of parasites that uptake iron poorly. Conversely, the authors showed that overexpression of hepcidin-insensitive ferroportin resulted in decreased parasite survival. This study is the first to show how an intracellular protozoan manipulates the

recycling of iron of the host cell. The mechanism by which *Leishmania* augments hepcidin levels is to be elucidated. Increased expression of hepcidin is observed under inflammatory conditions and can be triggered by IL-6 (Nemeth *et al.*, 2004). This raises the possibility that the GP63-mediated increase in IL-6 secretion (Arango Duque *et al.*, 2013) could increase hepcidin expression in infected macrophages. How iron availability and recycling influences the various leishmanial pathologies also remains to be explored (Ben-Othman *et al.*, 2014).

1.5.2 Disruption of cholesterol dynamics favours *Leishmania* growth

Cholesterol is an essential lipid for membrane biogenesis and cellular physiology. *Leishmania* promastigotes use LPG to disrupt cholesterol-rich lipid rafts to gain access to the cell, disrupt signaling pathways, and weaken the phagolysosome (Dermine *et al.*, 2005, Vinet *et al.*, 2009, Winberg *et al.*, 2009b). It had been observed that patients afflicted with visceral leishmaniasis have lower levels of circulating cholesterol and other lipids (Ghosh *et al.*, 2011). The importance of cholesterol during *Leishmania* infection was highlighted by a study that showed that GP63 cleaves DICER1, thence lowering miR-122 levels in hepatocytes of infected mice (Ghosh *et al.*, 2013). Diminished levels of miR-122, an miRNA and master regulator of lipid synthesis in the liver, ensues in lower cholesterol levels and parasite survival. Restitution of miR-122 rescues cholesterol levels and inhibits parasite growth. Whether miR-122 controls cholesterol metabolism in macrophages remains to be investigated (Descoteaux *et al.*, 2013, Ghosh *et al.*, 2013). Infected macrophages are defective in stimulating T cells and in assembling IFN receptor subunits at the surface (Sen *et al.*, 2011). T cell stimulation and proper IFN-γ signaling are restored when infected macrophages are supplied with liposomal cholesterol. This treatment also activates infected macrophages and promotes parasite killing (Ghosh *et al.*, 2014a). These findings raised the possibility that receptor movement at the cell surface is disrupted by *L. donovani* infection. To address this question, Ghosh *et al.* transfected macrophages with a PLCδ1-GFP fusion protein and monitored the protein's distribution and lateral movement using quantitative live cell

imaging (Ghosh *et al.*, 2014b). PLCδ1 is found in lipid rafts. Albeit infection does not affect the expression or plasmalemmal localization of PLCδ1-GFP, its motion in the plasma membrane was augmented. Recovery after photobleaching was also faster in infected cells. Normal PLCδ1-GFP motion is restored after macrophages are given liposomal cholesterol. This finding could be explained by the observation that *L. donovani* infection disrupts actin filaments and lowers F-actin protein levels; this phenomenon is also reversible by liposomal cholesterol (Ghosh *et al.*, 2014b). Increased receptor movement and altered actin dynamics may all lead to a disruption in signal transduction from the cell surface and the cytoskeleton. Improved understanding on the interplay between host actin and cholesterol will shed further light on how *Leishmania* survives in the macrophage. These studies may also foreshadow the use of liposomal cholesterol for the treatment of the leishmaniases.

1.5.3 Manipulation of the host's energy resources promotes parasite survival

Nutrient acquisition and conversion into energy is essential to the life every cell, and organisms have evolved diverse mechanisms to utilize what is available in their environment. Hence, it comes as no surprise that eukaryotic parasites compete with their hosts for some of the same nutrients required for the survival of both entities. Moreira and colleagues elegantly demonstrated that *Leishmania* hijacks mitochondrial pathways required for glucose metabolism (Moreira *et al.*, 2015). Early during infection, *L. infantum* was found to augment aerobic glycolysis, which was followed by a switch to mitochondrial metabolism and oxidative phosphorylation later during infection; infection progression was accompanied by increased mitochondria biogenesis. This claim is supported by proteomic mapping of *L. donovani*-infected cells, which revealed that several enzymes involved in beta-oxidation, the tricarboxylic acid cycle and mitochondrial respiration are upregulated (Singh *et al.*, 2015). To explain the drastic shift from glycolysis to mitochondrial metabolism, the Moreira *et al.* focused on the AMP-activated protein kinase (AMPK), a master regulator of energy homeostasis in the cell (Viollet *et al.*, 2010). AMPK is activated with rising AMP levels. Indeed, infection

augmented the AMP/ATP ratio and induced the phosphorylation and subsequent activation of AMPK. This activation leads to increased levels of glucose transporters (GLUTs) and increased glucose uptake. LKB1 mediates phosphorylation of AMPK, and is required for *Leishmania*-induced activation of AMPK. The SIRT1 protein, a sensor of NAD^+/NADH levels in cell, was found to regulate AMPK activation by *L. infantum*. At the helm of this research is the finding that genetic ablation of AMPK, LKB1 or SIRT1 promotes parasite clearance in *in vivo* and *in vitro* models of infection, hence highlighting the importance of these key metabolic regulators in the progression of visceral leishmaniasis (Moreira *et al.*, 2015).

1.6 DNA methylation is a strategy to shut down genes involved in host defence

Epigenetic regulation is central for the proper spatiotemporal regulation of gene expression in most vertebrates. DNA methylation is the addition of a methyl group to cytosine molecules, and is associated with gene silencing. DNA methylation can prevent promoter recognition by polymerases, and is associated with decreased nuclear clustering of chromatin (Gilbert *et al.*, 2007). Base methylation results in the silencing of a particular gene or set of genes. Microorganisms can alter the epigenome of host cells (Gómez-Díaz *et al.*, 2012). For instance, hepatitis B infection ensues in the hypermethylation of the urokinase-type plasminogen activator promoter, which is essential for hepatocyte growth and tissue repair (Park *et al.*, 2013). A study by Marr and colleagues (Marr *et al.*, 2014) found that *L. donovani* induces changes in DNA methylation to modulate genes involved in antimicrobial pathways (Figure 3). Using macrophages infected with either live or heat-killed *L. donovani*, it was found that live parasites induced significant DNA methylation changes at 443 CpG sites. Affected sites were located at proximal-promoter and at non-promoter regions. The affected genes were found to participate in processes such as calcium, chemokine and Notch signaling, and actin cytoskeleton assembly. Genes such as IRAK2, which encodes the interleukin-1 receptor-associated kinase 2 and promotes upregulation of NF-κB, was found to be

methylated and its transcription was decreased. This in turn contributes to decreased production of microbicidal molecules, and complements other *Leishmania*-mediated mechanisms that result in hindered NF-κB signaling (Olivier *et al.*, 2012, Shio *et al.*, 2012). How the parasite affects host methylation remains to be investigated. Given the evidence presented by Isnard *et al.* (Isnard *et al.*, 2015), it is possible for nuclear GP63 to indirectly affect the availability of DNA to methylating enzymes, or the enzymes themselves. It is also probable that parasites export DNA methyltransferases or methylation inhibitors from the PV into the cytoplasm, or via parasite-derived exosomes (Marr *et al.*, 2014).

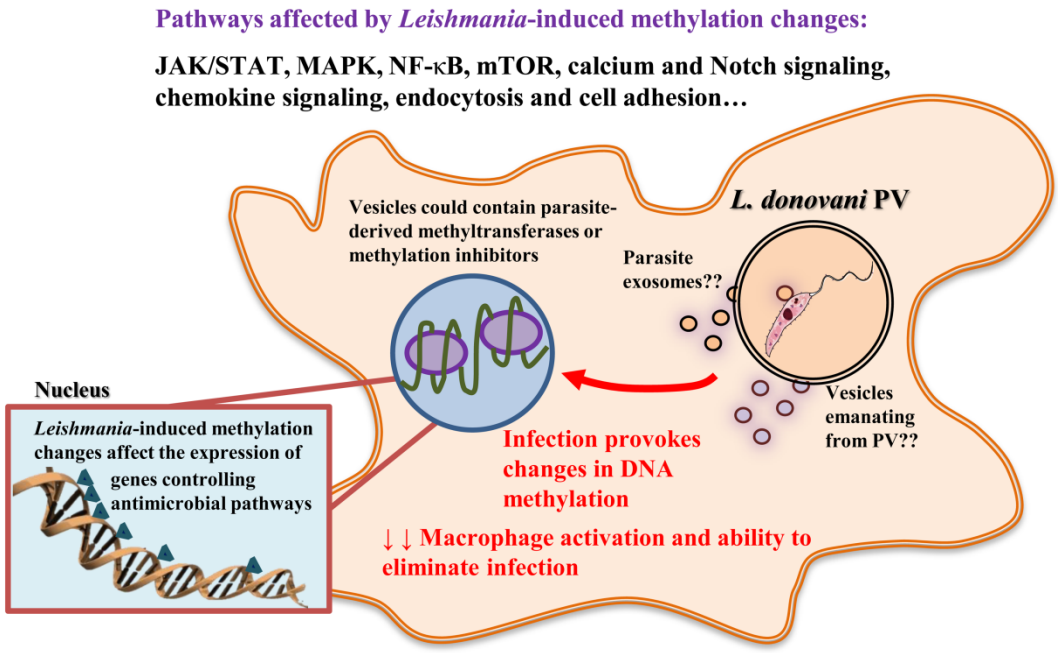

Figure 3 of review 1. *Leishmania* induces changes in the host macrophage methylome for survival. Infection with *L. donovani* alters the DNA methylation status of many host genes. Several of these genes, which partake in important antimicrobial functions, are shut down as a result.

1.7 Conclusion

Elucidating the mechanisms by which *Leishmania* alters the function of its host cell is essential for the design of novel and effective approaches to prevent or treat leishmaniases. In particular, future studies will be required to understand how

Leishmania effectors exit the PV and traffic within the host cell. Further understanding on how *Leishmania* reprograms key metabolic pathways is required in order to identify new targets for the treatment of the leishmaniases.

1.8 Acknowledgements

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2 REVIEW ARTICLE NO. 2: MACROCHAGE CYTOKINES: INVOLVEMENT IN IMMUNITY AND INFECTIOUS DISEASES

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2.1 Abstract

The evolution of macrophages has made them primordial for both development and immunity. Their functions range from the shaping of body plans to the ingestion and elimination of apoptotic cells and pathogens. Cytokines are small soluble proteins that confer instructions and mediate communication among immune and non-immune cells. A portfolio of cytokines is central to the role of macrophages as sentries of the innate immune system that mediate the transition from innate to adaptive immunity. In concert with other mediators, cytokines bias the fate of macrophages into a spectrum of inflammation-promoting 'classically activated', to anti-inflammatory or 'alternatively activated' macrophages. Deregulated cytokine secretion is implicated in several disease states ranging from chronic inflammation to allergy. Macrophages release cytokines via a series of beautifully orchestrated pathways that are spatiotemporally regulated. At the molecular level, these exocytic cytokine secretion pathways are coordinated by multi-protein complexes that guide cytokines from their point of synthesis to their ports of exit into the extracellular milieu. These trafficking proteins, many of which were discovered in yeast and commemorated in the 2013 Nobel Prize in Physiology or Medicine, coordinate the organelle fusion steps that are responsible for cytokine release.

This review discusses the functions of cytokines secreted by macrophages, and summarizes what is known about their release mechanisms. This information will be used to delve into how selected pathogens subvert cytokine release for their own survival.

2.2 Introduction: cytokines and macrophages

Macrophages are phagocytic cells of the innate immune system that are located in various tissues. The Russian scientist Elie Metchnikoff received the 1908 Nobel Prize in Physiology or Medicine for his work on immunity when he observed that when he punctured starfish larvae, a population of cells migrated to the wound. He also observed cells that were able to uptake particles that had been placed in the digestive tracts of the larvae. Elie Metchnikoff coined these cells as phagocytes and later called them white blood cells for their first-line-of-defense role against infection in living organisms (Tauber, 2003). Later, the term macrophage was introduced by Aschoff in 1924 to designate a set of cells of the reticuloendothelial system formed not only by monocytes, macrophages and histiocytes, but also by fibroblasts, endothelial and reticular cells. After 1969, the concept of the mononuclear phagocyte system – formed by a variety of macrophages derived from monocytes from the bone marrow – was introduced to replace the concept of the reticuloendothelial system, which is constituted of functionally and immunologically distinct cells. Most macrophages are derived from bone marrow precursor cells that develop into monocytes. These are formed in the bone marrow from stem cells of the granulocytic-monocytic lineage that are exposed to cytokines such as the granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin-3 (IL-3). Differentiation from stem cells is associated with the expression of specific membrane receptors for cytokines. Monocytes remain in the bone marrow less than 24 h and they move into the bloodstream and circulate throughout the body. In normal healthy adults, the half-life of a circulating monocyte is estimated at 70 h. Monocytes constitute 1% to 6% of total leukocytes in healthy periphery blood. After crossing the walls of capillaries into connective tissue, monocytes turn into macrophages. This differentiation process involves many changes as the cell increases in size from 5 to 10 times, its organelles increase both in number and complexity, phagocytic capacity increases, etc. It is important to note that not all macrophages, such as Langerhans cells and brain microglia, develop from monocytes (Sieweke *et al.*, 2013).

The main function of macrophages is to engulf foreign agents that enter the body. These include microbes and other particulate matter. In addition, they eliminate apoptotic cells and recycle nutrients by digesting waste products from tissues. Macrophages are hence essential not only for immunity, but also for development and tissue homeostasis (Sieweke *et al.*, 2013). These cells are normally at rest, but can be activated by a variety of stimuli during the immune response (Huber *et al.*, 1981, Unanue *et al.*, 1976). Albeit phagocytosis may provide the initial antigen stimulus, the activity of macrophages can be increased by cytokines secreted by helper T cells, with interferon gamma (IFN-γ) being one of the most potent macrophage activators. In addition, these multifaceted cells are also capable of chemotaxis, namely the process of being attracted and displaced to a particular location by specific molecules. Besides phagocytosis, macrophages play a central role in inflammation. They initiate the immune response against microorganisms, since macrophages are some of the first cells to come in contact with these invaders. This is in part due to their toll-like and scavenger receptors, which have broad ligand specificity for lectins, lipoproteins, proteins, oligonucleotides, polysaccharides, and other molecules. In addition to these functions, macrophages express major histocompatibility complex (MHC) class II molecules on their membranes, and as such, also present antigens to lymphocytes. When macrophages engulf a microbe, its antigens are processed and situated on the outer surface of the plasmalemma, where they will be recognized by T helper cells. Following this recognition, T lymphocytes release cytokines that activate B cells, and activated B lymphocytes then secrete antibodies specific to the antigens presented by the macrophage. These antibodies attach to antigens on microbes, or to cells invaded by microbes; in turn, these antibody-bound complexes are phagocytosed more avidly by macrophages.

Cytokines and chemokines are potent signalling molecules that are as important to life as hormones or neurotransmitters. They are low molecular weight proteins that mediate intercellular communication and are produced by many cell types, primarily those of the immune system. They were discovered in the early 60s-70s, and today, over 100

different proteins are known as cytokines (Dinarello, 2007). These molecules orchestrate a variety of processes ranging from the regulation of local and systemic inflammation to cellular proliferation, metabolism, chemotaxis, and tissue repair. In other organisms such as fruit flies and lizards (Bernheim *et al.*, 1976), cytokine-like molecules are known to regulate host defense and temperature homeostasis. The primary function of cytokines is to regulate inflammation, and as such, play a vital role in regulating the immune response in health and disease. There are proinflammatory and anti-inflammatory cytokines.

Each cytokine binds to a specific cell surface receptor to generate a cell signaling cascade that affects cell function. This includes the positive or negative regulation of several genes and their transcription factors. This may ensue in the production of other cytokines, in an increase in the number of surface receptors for other molecules, or eventually in the suppression of the cytokine's own effect. Each cytokine is produced by a cell population in response to different stimuli; they induce an array of agonist, synergistic or antagonistic effects that functionally alter target cells. A primary feature of cytokine biology is that of functional redundancy: different cytokines share similar functions. Furthermore, cytokines are pleiotropic since they act on many different cell types and cells may express more than one receptor for a given cytokine. To generalize the effect of a particular cytokine is virtually impossible. Cytokines are classified as paracrine if the action in the vicinity of the place of release is restricted, autocrine if the cytokine acts on the cell that secretes it, and endocrine if the cytokine reaches remote regions of the body. Most cytokines are short-lived and act locally in an autocrine and paracrine fashion. Only some cytokines present in the blood, such as erythropoietin (EPO), transforming growth factor beta (TGF-β), and monocyte colony stimulating factor (M-CSF), are capable of acting at a distance.

Cytokines are mainly produced by macrophages and lymphocytes, although they can also be produced by polymorphonuclear leukocytes (PMN), endothelial and epithelial cells, adipocytes and connective tissue. Cytokines are essential to the functions of macrophages. They mediate the unleashing of an effective immune response, link

innate and adaptive immunity and influence the macrophage's microenvironment (Huynh *et al.*, 2007b, Unanue *et al.*, 1976). Multiple subsets of macrophages have been characterized depending on the origin and microenvironment in which the macrophage is found. Contingent on activation status, macrophages have been classified as classically and alternatively activated. In turn, these different macrophages types drastically differ in the cytokines that they secrete, and consequently, their functions (Biswas *et al.*, 2010). The process of cytokine secretion is masterfully regulated by a series of interorganellar exchanges that rely on vesicular trafficking and cytoskeletal remodeling (Stow *et al.*, 2013). Proteins regulating neurotransmitter release, notably members of the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) (Stow *et al.*, 2006, Stow *et al.*, 2013), and more recently synaptotagmins (Syt) (Arango Duque *et al.*, 2013), are pivotal for the spatiotemporal regulation of cytokine secretion. In immune cells, SNAREs and Syts have been found to regulate processes ranging from cytokine trafficking to cell migration and phagocytosis.

This review will present the functions of macrophage cytokines and, where known, summarize findings on how these cytokines are released. The types of macrophages that secrete these cytokines will also be depicted. To illustrate the importance of macrophage cytokines in health and disease, we will describe selected examples of how pathogens use cytokines to their advantage.

2.3 The macrophage cytokine portfolio

2.3.1 Proinflammatory cytokines

When macrophages are exposed to inflammatory stimuli, they secrete cytokines such as TNF, IL-1, IL-6, IL-8, and IL-12. Although monocytes and macrophages are the main sources of these cytokines, they are also produced by activated lymphocytes, endothelial cells and fibroblasts. Additionally, macrophages release chemokines, leukotrienes, prostaglandins, and complement. All of these molecules, in concert, may induce increased vascular permeability and recruitment of inflammatory cells. Aside

from local effects, these mediators also produce systemic effects such as the induction of fever and the production of acute inflammatory response proteins. The inflammatory response is beneficial for the host when the aforementioned cytokines are produced in appropriate amounts, but toxic when produced in a deregulated fashion. For example, excessive production of IL-1β and TNF triggers an acute generalized inflammatory response characteristic of septic shock and multi-organ failure (Beutler, 1999).

2.3.1.1 Tumour Necrosis Factor (TNF)

TNF (formerly known as TNF-α) is a 185-aminoacid glycoprotein that was initially described for its ability to induce necrosis in certain tumours (Carswell *et al.*, 1975). It stimulates the acute phase of the immune response. This potent pyrogenic cytokine is one of the first to be released in response to a pathogen, and is able to exert its effects in many organs (Beutler, 1999). As such, TNF is one of the main cytokines responsible for septic shock. In the hypothalamus, TNF stimulates the release of corticotropic releasing hormone, suppresses appetite and induces fever. In liver, it stimulates the acute inflammatory response by elevating the synthesis of C-reactive protein and other mediators. TNF induces vasodilation and loss of vascular permeability, which is propitious for lymphocyte, neutrophil and monocyte infiltration. It helps recruit these cells to the inflammation site by regulating chemokine release. TNF, in concert with IL-17, triggers the expression of neutrophil-attracting chemokines CXCL1, CXCL2 and CXCL5 (Griffin *et al.*, 2012) and can also augment the expression of cell adhesion molecules (Vieira *et al.*, 2009) that facilitate diapedisis. This in turn increases CXCR2-dependent neutrophil migration to the inflammation site. Being an inducer of the inflammatory response, excess amounts of TNF have been found to play pathological roles in ailments such as inflammatory bowel disease, psoriasis, rheumatoid arthritis, asthma, cancer, infectious diseases, and other autoimmune pathologies. Some of these conditions are currently co-treated with monoclonal antibodies that neutralize this cytokine (Sozzani *et al.*, 2014).

In macrophages, TNF is released to the extracellular milieu via the constitutive secretion pathway, and its trafficking is the best understood of all cytokines (Murray *et al.*, 2005a, Pagan *et al.*, 2003, Stow *et al.*, 2013). Details on TNF trafficking will be discussed in another article of this issue. After synthesis in the ER, the SNARE proteins Stx6, Stx7, Vtib mediate the fusion of TNF-containing vesicles from the Golgi complex with $VAMP3^{+}$-recycling endosomes (Murray *et al.*, 2005a, Murray *et al.*, 2005b). Thence, the Stx4/SNAP23/VAMP3 complex facilitates the passage of TNF from recycling endosomes to the cell membrane (Murray *et al.*, 2005a, Pagan *et al.*, 2003). Rho1 and Cdc42, two proteins that govern cell shape via actin remodeling, also regulate the post-recycling endosome trafficking of TNF to the plasmalemma (Stanley *et al.*, 2014). Moreover, LPS was found to increase the expression of vesicle trafficking proteins that regulate TNF trafficking (Murray *et al.*, 2005a, Pagan *et al.*, 2003). Finally, release of mature TNF from the plasmalemma requires cleavage of the membrane-bound precursor by the TNF-α-converting enzyme (TACE) (Black *et al.*, 1997). The process of phagocytosis also requires extensive membrane exocytosis from several organelles that also partake in TNF secretion (Huynh *et al.*, 2007b). Interestingly, it was found that TNF is not only secreted to the extracellular milieu at the plasma membrane, but also in a polarized manner at the phagocytic cup (Murray *et al.*, 2005a). This highlights an efficient and elegant strategy where macrophages can promptly release cytokines at the same time that they phagocytose microbial invaders. The importance of regulating TNF secretion implies that there exist negative regulators for its secretion. One such regulator is the recently characterized protein Syt XI, which associates to recycling endosomes and lysosomes in macrophages (Arango Duque *et al.*, 2013, Arango Duque *et al.*, 2014b). Syts constitute a group of membrane proteins that regulate vesicle docking and fusion in processes such as exocytosis (Arango Duque *et al.*, 2013, Baram *et al.*, 1999) and phagocytosis (Arango Duque *et al.*, 2013, Czibener *et al.*, 2006, Vinet *et al.*, 2008). Syts control vesicle fusion by virtue of their Ca^{2+}-binding C2 domains (Südhof, 2012). However, Syt XI cannot bind calcium and inhibits vesicle fusion (Wang *et al.*, 2010). Upon LPS stimulation of macrophages treated with siRNA to Syt XI, more TNF and IL-6 are released. The inverse is true when Syt XI is overexpressed (Arango Duque *et al.*, 2013). Though the mechanism for this finding is not yet known, Syt XI

likely regulates cytokine release by interacting with members of the SNARE complex. Indeed, Syt XI was found to interact with the Golgi SNARE Vti1a (Milochau *et al.*, 2014), raising the question of whether Syt XI regulates SNARE complex formation at the Golgi.

2.3.1.2 IL-1

Three forms of IL-1 are known: IL-1α and IL-1β and IL-1Ra. Although both IL-1α and IL-1β are strongly proinflammatory, perform many of the same functions and bind to the IL-1 receptor (IL-1R), there is only 25% aminoacid homology between them. Similarly to TNF, IL-1β is also an endogenous pyrogen that is produced and released at the early stages of the immune response to infections, lesions and stress. Although monocytes and macrophages are the main sources of IL-1β, it is also released by NK cells, B cells, dendritic cells, fibroblasts and epithelial cells. During inflammation, IL-1β stimulates the production of acute phase proteins from the liver and acts on the central nervous system to induce fever and prostaglandin secretion. In mast cells, IL-1β induces the release of histamine, which in turn elicits vasodilation and localized inflammation. It is also a chemoattractant for granulocytes, enhances the expansion and differentiation of CD4 T cells (Ben-Sasson *et al.*, 2009), and increases the expression of cell adhesion molecules on leukocytes and endothelial cells. Additionally, IL-1β augments the expression of the genes that produce it (Carmi *et al.*, 2009). To quell the proinflammatory action of IL-1α and IL-1β, IL-1Ra competes for the same receptor. IL-1Ra is secreted via the classical secretory, though the exact mechanism is not well known. Its binding to the IL-1R does not induce the signalling program induced by IL-1α and IL-1β.

In stimulated macrophages, IL-1α is synthesized *de novo* and can be actively secreted (Yazdi *et al.*, 2010) or passively released from apoptotic cells (Chen *et al.*, 2007). It can also exert its effects in an intracrine fashion and act as a transcription factor (Ben-Sasson *et al.*, 2009, Carmi *et al.*, 2009). IL-1β is synthesized as a leaderless precursor that must be cleaved by inflammasome-activated caspase-1. After activation, autophagy plays a major role in the release of this cytokine. Autophagy is a highly conserved

process in eukaryotes in which the cytoplasm, aberrant or damaged organelles are sequestered in double-membrane vesicles and released into the lysosome for breakdown and eventual recycling of resulting macromolecules (Ravikumar *et al.*, 2009). This process plays a crucial role in adaptation to changing environmental conditions, starvation, cellular remodeling during development, and senescence. Autophagy is characterized by the formation of double membrane vesicles, called autophagosomes, which capture and transport cytoplasmic material to acidic compartments where material is degraded by hydrolytic enzymes (Ravikumar *et al.*, 2009). Autophagy has also been recognized to mediate the secretion of proteins (Duran *et al.*, 2010) – such as IL-1β and IL-18 (Dupont *et al.*, 2011, Jiang *et al.*, 2013) – that would otherwise not enter the classical secretory pathway due to lack of a leader peptide. In the case of IL-1β, the autophagic protein Atg5, the Golgi protein GRASP55 and Rab8a are essential for translocating IL-1β-containing cargo to the outside of the cell. In peritoneal macrophages, it has been shown that IL-1β is transported to the extracellular milieu via membrane transporters (Brough *et al.*, 2007); knockdown of ABC transporters inhibits IL-1β secretion (Marty *et al.*, 2005). Additionally, exocytosis of P2X7R-positive multivesicular bodies containing exosomes has also been reported to play an important role in the release of this cytokine (Qu *et al.*, 2007). The various modes of IL-1 secretion highlight the exquisite machinery that macrophages have evolved as a means for rapidly responding to inflammatory stimuli.

2.3.1.3 IL-6

IL-6 is a pleiotropic cytokine that has both proinflammatory and anti-inflammatory functions that affect processes ranging from immunity to tissue repair and metabolism. It promotes the differentiation of B cells into plasma cells, activates cytotoxic T cells, and regulates bone homeostasis. As with other proinflammatory cytokines, IL-6 is has been implicated in Crohn's disease and rheumatoid arthritis (Nishimoto *et al.*, 2004). Similar to TNF and IL-1β, IL-6 is an endogenous pyrogen that promotes fever and the production of acute phase proteins from liver. Proinflammatory properties are elicited when IL-6 signals in *trans* via soluble IL-6 receptors binding to gp130, which is

ubiquitous in all cells. Inhibition of *trans* signalling via gp130 blockade in murine sepsis models rescues mice from widespread inflammation and death (Barkhausen *et al.*, 2011). IL-6 *trans* signalling also leads to recruitment of monocytes to the inflammation site (Hurst *et al.*, 2001), promotes the maintenance of Th17 cells, and inhibits of T cell apoptosis and the development of Tregs (Scheller *et al.*, 2011). In contrast, anti-inflammatory properties are elicited when IL-6 signals through the classical pathway, which occurs via the IL-6 receptor that only few cells express. The anti-inflammatory properties of IL- are illustrated by IL-$6^{-/-}$ mice, which exhibit hepatosteatosis, insulin resistance and liver inflammation (Matthews *et al.*, 2010). IL-6 classic signalling also mediates apoptosis inhibition and the regeneration of intestinal epithelial cells (Scheller *et al.*, 2011).

IL-6 is a soluble cytokine that is synthesized in the ER and, unlike TNF, is not processed as a membrane-bound precursor. Upon stimulation of macrophages with LPS, IL-6 starts accumulating in the Golgi after 4 h of stimulation (Manderson *et al.*, 2007). From the Golgi, IL-6 exits in tubulovesicular carriers that may also contain TNF. Golgi-derived vesicles then fuse with VAMP3-positive recycling endosomes. Three-dimensional reconstruction of fluorescence images showed that recycling endosomes can harbour both TNF and IL-6, albeit both occupy different subcompartments (Manderson *et al.*, 2007). The post-Golgi trafficking of IL-6 follows a route that is also dependent on Stx6 and Vti1b, which form a complex with cognate SNARE VAMP3 at recycling endosomes (Murray *et al.*, 2005a, Pagan *et al.*, 2003). Knockdown and overexpression of these SNAREs decreases and augments IL-6 release, respectively (Manderson *et al.*, 2007). Syt XI may be negatively modulating the secretion of this cytokine by regulating the formation of these SNARE complexes (Arango Duque *et al.*, 2013, Milochau *et al.*, 2014). Unlike TNF, IL-6 is not secreted at the phagocytic cup (Manderson *et al.*, 2007).

2.3.1.4 IL-12

IL-12 is produced primarily by monocytes, macrophages and other antigen-presenting cells; it is essential for fighting infectious diseases and cancer. IL-12 is a heterodimeric cytokine comprised of the p35 and p40 subunits, which come together after their synthesis. Deletions within the p40 gene have been observed in patients suffering from concurrent multiple bacterial infections (Dorman *et al.*, Xia *et al.*, 2017b). IL-12 promotes cell-mediated immunity via stimulation of Th1 cells. It synergizes with TNF and other proinflammatory cytokines in stimulating IFN-γ production, as well as the cytotoxicity of NK and CD8 T cells (Wang *et al.*, 2000). IL-12 can also inhibit angiogenesis through IFN-γ-mediated upregulation of the anti-angiogenic chemokine CXCL10. The involvement of this cytokine in these processes has made it a target in both auto-immune pathologies and cancer (Dorman *et al.*, Xia *et al.*, 2017b). After protein synthesis, both p40 and p35 subunits associate at the ER, where they undergo subsequent glycosylation steps prior to being released at the cell membrane (Duitman *et al.*, 2011). Although the precise post-Golgi trafficking mechanisms in macrophages are not known, the release route is likely to resemble that of TNF and IL-6 (Stow *et al.*, 2013). Data from neutrophils localized the SNAREs VAMP2, VAMP7, Stx2, Stx6 and SNAP23 in the granules that contain and secrete IL-12 (Logan *et al.*, 2006, Mollinedo *et al.*, 2006). Although macrophages do not possess secretory granules, IL-12 release from these cells may involve some of the same SNARE complexes. Furthermore, IL-12 is secreted in a polarized manner from lymphocytes; this process is dependent on Cdc42 (Pulecio *et al.*, 2010), which also regulates release of TNF to the plasma membrane. This raises the interesting prospect that IL-12 may be released in a polarized fashion, along with TNF (Murray *et al.*, 2005a), at nascent macrophage phagosomes.

2.3.1.5 IL-18

IL-18 is a member of the IL-1 family and also an inducer IFN-γ production. It synergizes with IL-12 to activate T cells and NK cells. Albeit the fact that IL-18 signals similarly to IL-1β, IL-18 is not a pyrogen, and can even attenuate IL-1β-induced fever (Gatti *et al.*, 2002). Lack of fever induction may be explained by the fact that IL-18 signals through

the MAPK p38 pathway instead of the NF-κB pathway, which is used by IL-1β (Lee *et al.*, 2004). IL-18 trafficking is similar to that of IL-1β, with secretory autophagy also playing a major role in its release (Dupont *et al.*, 2011, Jiang *et al.*, 2013).

2.3.1.6 IL-23

IL-23 is also an IFN-γ inducer and T cell activator that is involved in a variety of diseases ranging from psoriasis to schizophrenia (Xia *et al.*, 2017b). It is similar to IL-12 in that both induce inflammation. Moreover, both IL-12 and IL-23 share the IL-12p40 subunit and thus have similar signalling pathways. In contrast to IL-12, IL-23 augments IL-10 release and induces IL-17 synthesis by activated naïve T cells (Vanden Eijnden *et al.*, 2005).

2.3.1.7 IL-27

IL-27 is a member of the IL-12 family, and is composed of subunits p28 and Epstein-Barr virus-induced gene 3. Similar to TNF, it is produced early in monocytes and macrophages stimulated with LPS and IFN-γ. Knockout of its receptor ensues in increased susceptibility of mice to bacterial and parasitic infections due to impaired IFN-γ production (Yoshida *et al.*, 2001). In addition to favouring the differentiation of naïve T cells to Th1 cells via IFN-γ induction, IL-27 can also inhibit the differentiation of Th17 cells (Stumhofer *et al.*, 2006). IL-27 also has anti-inflammatory properties, which are exemplified by the fact that IL-27 receptor-deficient mice are more susceptible to autoimmune encephalomyelitis, which correlates with increased levels of Th17 cells (Vanden Eijnden *et al.*, 2005). The fact that this cytokine has selective inflammatory and anti-inflammatory properties supports the concept that the inflammatory response is prompt, but also carefully calibrated to avoid damage to the host.

2.3.2 Anti-inflammatory cytokines

2.3.2.1 IL-10

Inflammation is tightly regulated by multiple inhibitors and antagonists. IL-10 is a 35 kD cytokine identified in 1989, and is produced by activated macrophages, B and T cells (Mosser *et al.*, 2008). Its main activities concern the suppression of macrophage activation and production of TNF, IL-1β, IL-6, IL-8, IL-12 and GM-CSF (Fiorentino *et al.*, 1991). IL-10 suppresses MHC-II expression in activated macrophages and is thus a potent inhibitor of antigen presentation (Chadban *et al.*, 1998). Of particular interest is that IL-10 inhibits the production of IFN-γ by Th1 and NK cells, and induces the growth, differentiation and secretion of IgGs by B cells (Defrance *et al.*, 1992, Rousset *et al.*, 1992). Macrophages themselves are affected by IL-10 in that exposure to this cytokine lowers their microbicidal activity and diminishes their capacity to respond to IFN-γ (Cunha *et al.*, 1992, Oswald *et al.*, 1992). Experiments in murine models have shown that blocking or neutralizing IL-10 leads to increased levels of TNF and IL-6; on the contrary, exogenous IL-10 improves survival and reduces the levels of inflammatory cytokines (Varzaneh *et al.*, 2014). It has been observed that reduced levels of IL-10 favour the development of gastrointestinal pathologies such as inflammatory bowel disease (Varzaneh *et al.*, 2014). Recombinant IL-10 has indeed been effective in the treatment of some of these diseases.

The mechanism of IL-10 trafficking and release resembles that of TNF and IL-6 (Stanley *et al.*, 2012). IL-10 traffics from Golgi tubular carriers associated with p230/golgin-245 along with TNF-containing vesicles, or in golgin-97-associated tubules. The Golgi-associated p110δ isoform of PI3K was also found to be a positive regulator of IL-10 release. From the Golgi, IL-10-containing vesicles move to recycling endosomes, where VAMP3 and Rab11 then modulate the transit of this cytokine – and of TNF and IL-6 – to the cell surface. Independent of recycling endosomes, IL-10 was also observed to exit directly from the Golgi to the cell surface in apoE-labelled vesicles (Stanley *et al.*, 2012).

2.3.2.2 TGF-β

Together with IL-10, TGF-β is another powerful anti-inflammatory cytokine that acts on many target cells and tones down the inflammatory effects of TNF, IL-1β, IL-2, and IL-12, etc. (Becker *et al.*, 2004, Defrance *et al.*, 1992, Travis *et al.*, 2014). TGF-β is a potent suppressor of both Th1 and Th2 cells, but foments the maintenance and function of Tregs (Josefowicz *et al.*, 2012, Travis *et al.*, 2014). The importance of TGF-β in the immune system is highlighted by the fact that mice lacking the TGF-β1 isoform, which is predominant in cells of the immune system, develop severe multiorgan inflammation and die by week 4 (Gleizes *et al.*, 1997). TGF-β is also implicated in hematopoiesis and has a crucial role in embryogenesis, tissue regeneration, and cell proliferation and differentiation.

TGF-β is synthesized as a precursor and is directed to the ER by virtue of its signal peptide. Cleavage by the endoprotease furin, which can happen at the ER or in the extracellular environment, is required for activation of this cytokine (Gleizes *et al.*, 1997). Although the secretory mechanism of this cytokine has not been explored, it is possible that it follows a post-Golgi pathway similar to that of TNF, IL-6 or IL-10.

2.3.3 Chemokines

Chemokines are a special family of heparin-binding cytokines that are able to guide cellular migration in a process known as chemotaxis. Cells that are attracted by chemokines migrate towards the source of that chemokine. During immune surveillance, chemokines play a crucial role in guiding cells of the immune system to where they are needed (Comerford *et al.*, 2011). Some chemokines also play a role during development by promoting angiogenesis, or by guiding cells to tissues that provide critical signals for the cell's differentiation. In the inflammatory response, chemokines are released by a wide variety of cells involved in both innate and adaptive immunity (Comerford *et al.*, 2011). As already mentioned, chemokine release is often

induced by proinflammatory cytokines such as TNF, IL-6 and IL-1β. Below is a description of the main chemokines released by macrophages.

2.3.3.1 CXCL1 and CXCL2 (MIP-2α)

CXCL1 and CXCL2 (also known as macrophage inflammatory protein 2-α, MIP) share 90% amino acid similarity and are secreted by monocytes and macrophages to recruit neutrophils and hematopoietic stem cells (Moser *et al.*, 1990, Pelus *et al.*, 2006). Both chemokines are angiogenic and may promote the development of tumours such as melanomas (Addison *et al.*, 2000).

2.3.3.2 CCL5 (RANTES)

CCL5, or the regulated upon activation normal T cell expressed and secreted (RANTES), is an inflammatory chemoattractant for T cells, basophils, eosinophils and dendritic cells to the site of inflammation (Donlon *et al.*, 1990). Aside from this role, it can also mediate the activation of NK cells into chemokine-activated killers (CHAK) (Maghazachi *et al.*, 1996). Similar to CXCL1 and 2, it promotes tumorigenesis and metastasis (Addison *et al.*, 2000). CCL5 is synthesized in the ER and traffics to the Golgi complex before being exported outside of the cell. The secretory carrier membrane protein (SCAMP) 5, a recycling endosome-associated protein, governs post-Golgi trafficking of CCL5 to the plasmalemma. Stimulation of macrophages with ionomycin induces SCAMP5 translocation to the plasma membrane, where it colocalizes and interacts with Syt I and II, which in turn mediate interactions with various SNAREs (Han *et al.*, 2009).

2.3.3.3 CXCL8 (IL-8)

CXCL8 is a potent chemoattractant for neutrophils, in which it also induces degranulation and morphological changes (Gouwy *et al.*, 2004, Starckx *et al.*, 2002). Since macrophages are some of the first cells to respond to an antigen, they are likely the first cells to release CXCL8. Other cells such as keratinocytes, endothelial cells,

eosinophils and basophils also respond to this chemokine. The importance of IL-8 has made this chemokine important in inflammatory diseases such as psoriasis, Crohn's disease and cancer (Gijsbers *et al.*, 2004, Van Damme *et al.*, 2004).

2.3.3.4 CXCL9 (MIG)

CXCL9, also known as monokine induced by gamma interferon (MIG), is a strong T cell chemoattractant to the site of inflammation (Comerford *et al.*, 2011, Rosenblum *et al.*, 2010). It mediates cell recruitment necessary for inflammation and repair of tissue damage. CXCL9 also inhibits neovascularization (Keeley *et al.*, 2008) and has anti-tumour and anti-metastatic effects (Addison *et al.*, 2000).

2.3.3.5 CXCL10 (IP-10)

CXCL10, or interferon gamma-induced protein 10, is secreted not only by monocytes and macrophages, but also by fibroblasts and endothelial cells (Keeley *et al.*, 2008). It serves to attract T cells, NK cells, dendritic cells (Dufour *et al.*, 2002), and also has potent anti-cancer activity.

2.3.3.6 CXCL11 (IP-9)

Similar to CXCL9 and CXCL10, CXCL11 is interferon-inducible and also mediates T cell recruitment, although more potently than CXCL9 and CXCL10 (Cole *et al.*, 1998). It also inhibits angiogenesis and tumour formation (Addison *et al.*, 2000).

2.4 Alternatively activated macrophages and their cytokines

The microenvironment in which a macrophage is found provides it with diverse signals that divergently bias the macrophage's phenotype towards 'classically activated' (M1) or 'alternatively activated' (M2a, M2b or M2c) (Figure 1) (Vanden Eijnden *et al.*, 2005).

Polarization signals may be apoptotic cells, hormones, immune complexes or cytokines provided by lymphocytes or other cells. Exposure of naïve monocytes or recruited macrophages to the Th1 cytokine IFN-γ, TNF, or LPS, promotes M1 development. Those macrophages in turn secrete proinflammatory cytokines TNF, IL-1β, IL-6, IL-12, IL-23, and promote the development of Th1 lymphocytes. In addition, M1 macrophages secrete high levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS), produce and secrete iNOS, and promote the metabolism of arginine into nitric oxide and citrulline. As a result, M1 macrophages foster a highly microbicidal environment, and have a role in mediating the destruction of pathogens and tumour cells. M1-derived chemokines help recruit NK and Th1 cells. In stark contrast, exposure or treatment of monocytes with IL-4 and IL-13 polarizes these cells towards an M2a phenotype (Biswas *et al.*, 2010, Gundra *et al.*, 2014). Those macrophages express a series of chemokines that promote the accrual of Th2 cells, eosinophils, and basophils. M2b macrophages are induced by a combination of LPS, immune complexes, apoptotic cells and IL-1Ra. They secrete high levels of IL-10, but also proinflammatory cytokines TNF and IL-6 and express iNOS. Through chemokine production, M2b also promote recruitment of eosinophils and Tregs that foster a Th2 response. M2c macrophages are induced by a combination of IL-10, TGF-β and glucocorticoids. In turn, those macrophages secrete IL-10 and TGF-β, both of which are immunosuppressive cytokines that promote the development of Th2 lymphocytes and Tregs. They also express high levels of arginase and promote tissue regeneration and angiogenesis (Biswas *et al.*, 2010, Zhang *et al.*, 2010). The capacity of M2c macrophages to induce Tregs makes them more effective than M2a macrophages at protecting organs from injury caused by inflammatory infiltrates (Lu *et al.*, 2013). Macrophage bias is reversible. For example, if an M1 macrophage is given apoptotic cells, it may transform into an M2 macrophage.

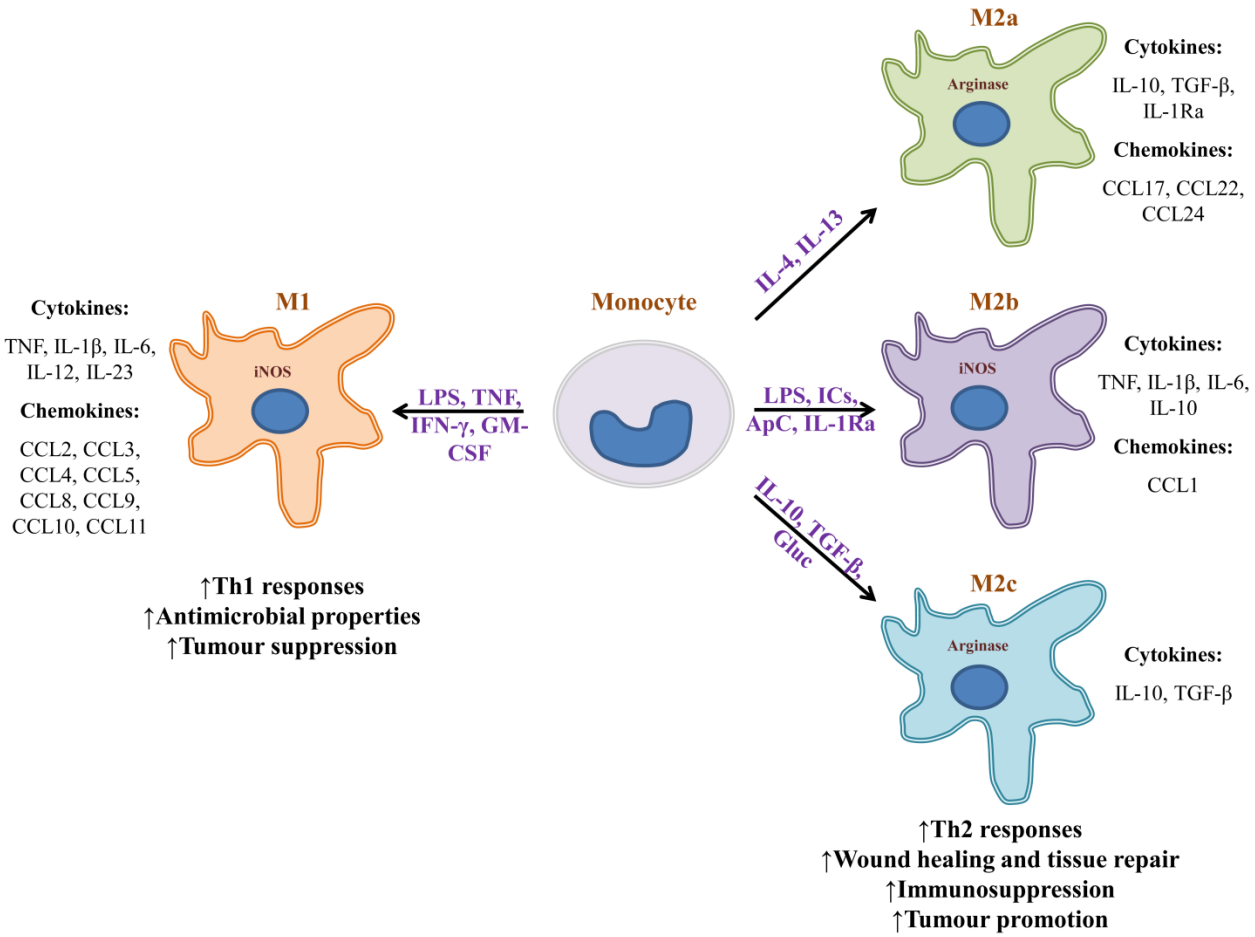

Figure 1 of review 2. Monocytes can become phenotypically distinct macrophages. Upon encountering different stimuli, monocytes turn into highly microbicidal (M1), or into immunosuppressive macrophages (M2). Stimuli can range from microbial substances to biochemical signals provided by the microenvironment of a given tissue. Many of the cytokines that bias macrophage phenotype are provided by surrounding lymphocytes or other non-immune cells. Macrophage subtypes release a vastly different array of cytokines and chemokines that can either promote inflammation and sometimes tissue destruction, or wound healing and tissue repair. M1 macrophages are known to be tumour suppressive whereas M2 macrophages generally promote tumorigenesis. It is important to note that macrophage bias is a spectrum and is reversible. IC, immune complexes; ApC, apoptotic cells; Gluc, glucocorticoids.

The characteristics of M1 and M2 macrophages have implicated them in the development of infectious disease and cancer. For example, helminth-derived molecules can strongly bias macrophages towards an M2 phenotype. The cytokines and associated Th2 response that ensues promote immunosuppression and parasite survival (Weng *et al.*, 2007). In cancer, tumour-associated macrophages (TAMs) have been known to either promote or hinder neoplasia (Biswas *et al.*, 2010, Hao *et al.*, 2012). In colorectal cancer, TAMs are inflammatory and promote the development of a

Th1 response (Ong *et al.*, 2012). In contrast, many other neoplasms are associated with M2-like TAMs that secrete immunosuppressive cytokines that promote tumour growth and metastasis (Biswas *et al.*, 2010, Hao *et al.*, 2012). TAMs may facilitate tumour growth by facilitating the chemotaxis of Th2 and Treg cells, promoting angiogenesis and lymphoangiogenesis via production of VEGF, VEGF-C and -D, PDGF and TGF-β (Schoppmann *et al.*, 2002). Additionally, TAMs secrete MMP9, a matrix metalloprotease that promotes tumour growth and spread. Importantly, TAMs induce immunosuppression via release of IL-10 and TGF-β, both of which inhibit the development of cytotoxic T cells and NK cells, and may fuel the appearance of more M2-like TAMs at the tumour site (Biswas *et al.*, 2010, Hao *et al.*, 2012, Travis *et al.*, 2014). The contribution of alternatively activated macrophages and their cytokines to disease has made them a target for immunotherapies that seek to alter the phenotypic bias of macrophage populations. For instance, helminth-derived molecules could be used to alter the proinflammatory cytokine profile of colitis-associated macrophages (Weng *et al.*, 2007).

2.5 How pathogens disrupt cytokine secretion from macrophages

The evolutionary race that has taken place over millions of years among pathogens and their hosts has given rise to a multitude of adaptations that have allowed these pathogens to resist the defenses mounted by their hosts. Several of these adaptations endow pathogens to evade the immune system in order to survive destruction and thrive. Both intracellular and extracellular parasites have evolved mechanisms to not only avoid or survive the immune response, but also to use it for their own benefit (Flannagan *et al.*, 2009, Sibley, 2011). Upregulating or downregulating the production and release of macrophage cytokines can have profound effects on the immune response. A variety of pathogenicity factors target these important molecules of the immune system. The following examples describe how certain pathogens, depending on their needs, deregulate cytokine secretion to aid in their survival and dissemination.

2.5.1 *Mycobacterium ulcerans* uses mycolactone to inhibit cytokine production

Mycobacteria are intracellular pathogens that cause a variety of human diseases that are difficult to treat. Due to their particular cell wall, these bacteria are very resistant to antibiotics and innate host defences. *M. ulcerans*, the causative agent of the Buruli ulcer, induces deep necrotizing ulcers that are often ironically painless (Walsh *et al.*, 2011). Lesions can cause incapacitation, disfigurement and severe deformities (Walsh *et al.*, 2011). The disease is the third-most common mycobacterial infection and affects areas of the world with hot and humid climates. *M. ulcerans* produces a macrolide toxin called mycolactone that is highly cytotoxic and immunosuppressive (George *et al.*, 1999, George *et al.*, 2000). It causes broad tissue damage in the absence of an acute inflammatory response. Injection of mycolactone alone can induce lesions similar to those caused by infection (George *et al.*, 2000). In contrast to other mycobacterial infections, *M. ulcerans* is found mostly extracellularly. This may be explained by the fact that mycolactone inhibits phagocytosis and hampers phagolysosomal maturation in macrophages (Demangel *et al.*, 2009, Hall *et al.*, 2014a). In addition, mycolactone contributes to immunosuppression by hampering the production of several cytokines and chemokines from macrophages (Figure 2A) (Hall *et al.*, 2014a, Simmonds *et al.*, 2009, Torrado *et al.*, 2007); mycolactone is effective at dampening the production of LPS-induced mediators. Although the mechanism for these findings was unknown, data from multiple studies suggested that inhibition was at the post-transcriptional level. Indeed, Hall *et al.* found that mycolactone does not cause gross changes in translation, with proinflammatory mRNAs being actively translated (Hall *et al.*, 2014b). That finding prompted the investigators to check whether TNF was being translocated to the ER for processing. Interestingly, inhibiting the 29S proteasome showed that non-glycosylated TNF accumulates in the cytoplasm of mycolactone-treated macrophages, indicating that this causes the failure in TNF secretion. To show that TNF was not being translocated into the ER lumen, Hall *et al.* performed *in vitro* translation assays with ER-containing membranes to study whether TNF was being protected from proteinase K degradation (Hall *et al.*, 2014b). In the presence of mycolactone TNF is not protected from

proteinase K digestion, indicating that this cytokine does not translocate into the ER under these conditions. These effects were found not to be due to mycolactone disrupting ER membrane integrity or due to induction of ER-associated degradation pathways. It would be interesting to investigate whether mycolactone can physically block the channel activity of the Sec61, or that of other, ER translocons. These findings were made more general by showing that – in many cell types – mycolactone was inhibiting the translocation of several secreted and membrane proteins into the ER. Importantly, mycolactone blocked the release of several cytokines, chemokines, and other inflammatory mediators from LPS-activated macrophages (Hall *et al.*, 2014b). Quenching cytokine production in this way can thus severely obstruct the development of the immune response and promote the survival of *M. ulcerans*.

2.5.2 *Leishmania* promastigotes employ GP63 to augment TNF and IL-6 release

Protozoa of the *Leishmania* genus are parasites of phagocytic cells, especially macrophages. Depending on the species, *Leishmania* can cause self-healing cutaneous lesions (eg. *L. tropica*, *L. major*, *L. mexicana*, *L. pifanoi*), disfiguring mucocutaneous disease (eg. *L. braziliensis*, *L. guyanensis*), or severe visceral illness (eg. *L. donovani*, *L. Infantum chagasi*). Mucocutaneous and visceral disease can be lethal if untreated, but most deaths are attributable to visceral leishmaniasis (Alvar *et al.*, 2012). *Leishmania* has a digenetic lifecycle. Promastigotes are elongated and have a flagellum that allows them to move in extracellular environments. Dividing procyclic promastigotes develop in the gut of infected sandflies, where they transform into infectious non-dividing metacyclic promastigotes that can be ejected upon the sandfly's next blood meal (Kedzierski, 2010). Once inside the host, metacyclic promastigotes are phagocytosed by neutrophils or by macrophages. *Leishmania* promastigotes are able to cripple the microbicidal power of the phagosome, rendering it a propitious parasitophorous vacuole (PV) for the parasite (Lodge *et al.*, 2008, Moradin *et al.*, 2012). Within PVs, promastigotes differentiate into amastigotes, which are the non-flagellated intracellular form of the parasite. Amastigotes replicate inside macrophages, and when these apoptose, surrounding macrophages uptake the amastigote cargo (Real *et al.*,

2014), eventually propagating the infection. The *Leishmania* lifecycle is perpetuated when free amastigotes and amastigote-containing phagocytes are taken up by sandflies that bite infected hosts. The GP63 zinc metalloprotease is a multifaceted *Leishmania* pathogenicity factor and is also one of the most abundant molecules on the surface of promastigotes (Joshi *et al.*, 2002, Moradin *et al.*, 2012, Yao, 2010). In infected macrophages, GP63 impairs antigen cross-presentation (Matheoud *et al.*, 2013), stalls transcription and translation, and deactivates several microbicidal pathways (Contreras *et al.*, 2010, Gómez *et al.*, 2009, Hallé *et al.*, 2009, Jaramillo *et al.*, 2011). Additionally, GP63 hampers lipid metabolism in liver, and helps the parasite evade complement-mediated lysis and avoid killing by NK cells (Descoteaux *et al.*, 2013, Ghosh *et al.*, 2013, Yao, 2010). Of particular note is the capacity of GP63 to cleave members of the SNARE complex (Moradin *et al.*, 2012), which raises the possibility that GP63 may cleave other membrane fusion regulators. Earlier studies found that *Leishmania* promastigotes of certain species were able to induce the release of TNF and IL-6 (Arena *et al.*, 1997, Karam *et al.*, 2006, Lapara *et al.*, 2010, Matte *et al.*, 2002, Wenzel *et al.*, 2012) following their engulfment by macrophages. However, the mechanisms for this induction were not known. Hence, Arango Duque *et al.* hypothesized that Syt XI, a negative regulator of cytokine secretion (Arango Duque *et al.*, 2013), was targeted by *Leishmania* (Figure 2B) (Arango Duque *et al.*, 2014b). Infection of macrophages with $GP63^{+/+}$ or $GP63^{-/-}$ parasites revealed that Syt XI is degraded by GP63, leading to the release of TNF and IL-6. Moreover, cytokine release by infected macrophages positively correlated with the GP63 content of different *Leishmania* species. To highlight the relevance of these findings in an *in vivo* setting, it was demonstrated that intraperitoneal injection of GP63-expressing promastigotes induces TNF and IL-6 release 4 h after inoculation. As already described, these cytokines induce adhesion factor expression and chemokine release (Biswas *et al.*, 1998, Griffin *et al.*, 2012, Hurst *et al.*, 2001, Jones *et al.*, 2006, Vieira *et al.*, 2009). Interestingly, it was observed that GP63 also promotes the infiltration of neutrophils and inflammatory monocytes early during infection.

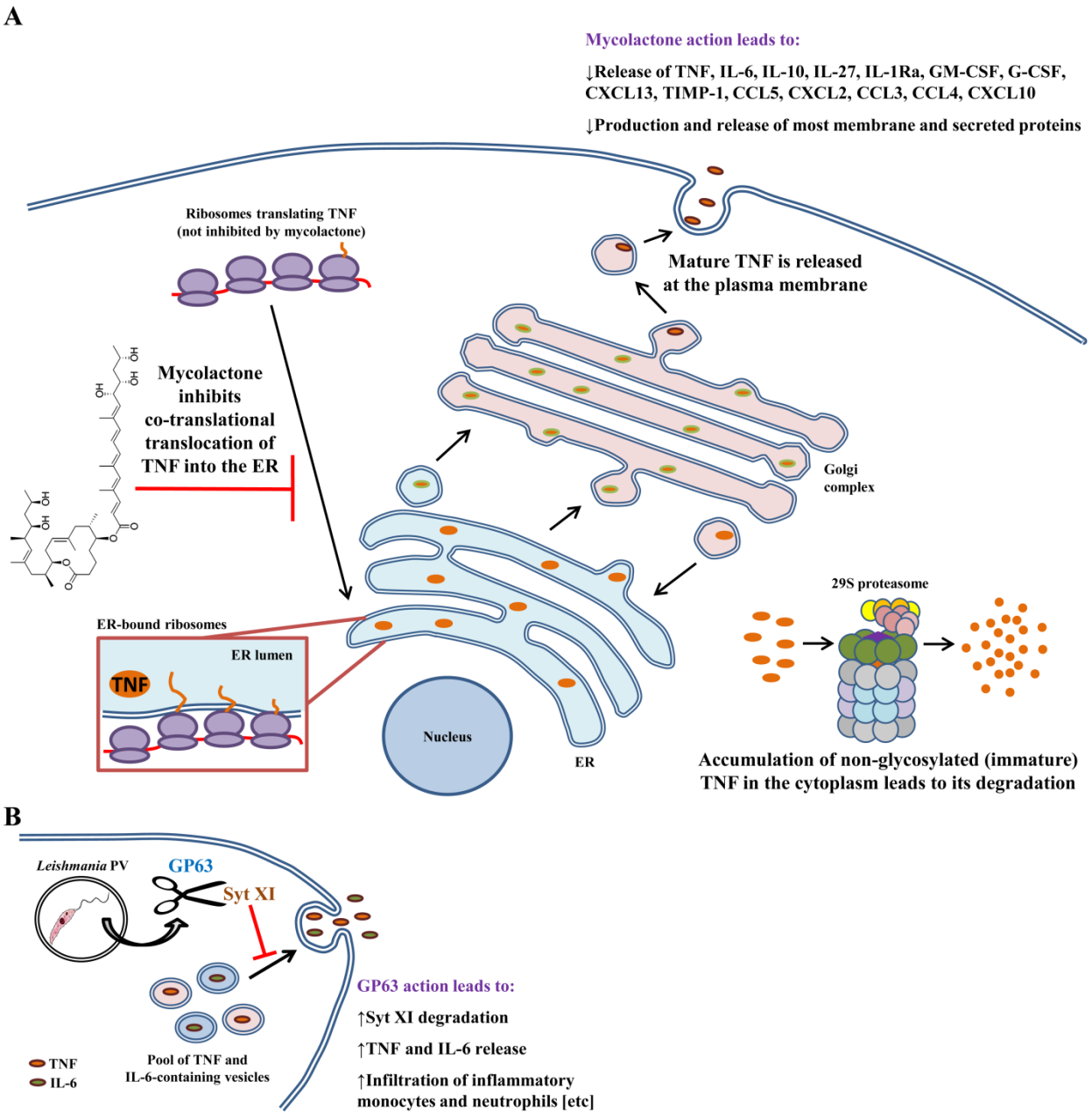

Figure 2 of review 2. Modulation of macrophage cytokine secretion by *Mycobacterium ulcerans* bacteria and *Leishmania* promastigotes. Disruption of cytokine release has evolved as an effective means by which several pathogens contravene the immune response. **(A)** *M. ulcerans* employs mycolactone to sabotage the immune response by inhibiting the secretion of more than 17 cytokines, chemokines, and inflammatory mediators. TNF, as well as other cytokines and chemokines, undergo post-translational modifications in the ER and Golgi prior to being shepherded outside of the macrophage. Mycolactone hampers delivery of TNF into the ER. As a consequence, immature protein that accumulates in the cytoplasm is eventually degraded by the proteasome. **(B)** Unlike *M. ulcerans*, *Leishmania* promastigotes trigger the release of TNF and IL-6 from infected macrophages via GP63-mediated degradation of Syt XI (a negative regulator of cytokine release). *In vivo*, GP63 also facilitates the infiltration of inflammatory monocytes and neutrophils to the infection site. Both of these phagocytes are infection targets for *Leishmania* and aid in establishing infection. These findings can be explained by the fact that TNF and IL-6 mediate phagocyte infiltration by upregulating the expression of adhesion molecules and chemokines. Arrows indicate multiple steps and drawings are not to scale.

Future research will reveal whether phagocyte recruitment is dependent on GP63-mediated cleavage of Syt XI *in vivo*. It will also be interesting to research whether Syt XI is targeted by other pathogens. The involvement of GP63 in cytokine secretion and phagocyte recruitment can aid in the establishment of infection. Infection of recruited inflammatory monocytes and resident macrophages can induce IL-10 secretion, which fosters the immunosuppressive environment observed in chronic infection (Belkaid *et al.*, 2001, Kane *et al.*, 2001). Infection of inflammatory monocytes (Ribeiro-Gomes *et al.*, 2014) may also turn them into arginase-expressing alternatively activated macrophages that trigger the differentiation of naïve CD4 T cells into $FoxP3^+$ cells (Gundra *et al.*, 2014), which are immunosuppressive in leishmaniasis (Silva *et al.*, 2014, Tiwananthagorn *et al.*, 2012). Overall, those findings underline the importance of proinflammatory cytokines and phagocytes at the early stages of *Leishmania* infection (Arango Duque *et al.*, 2014b).

2.6 Acknowledgements

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3 REVIEW ARTICLE NO. 3: UNDERSTANDING TGEV-ETEC COINFECTION THROUGH THE LENS OF PROTEOMICS: A TALE OF PORCINE DIARRHEA

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3.1 Abstract

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

3.2 Main text

Enteric infections affecting pigs are widespread, and cause a decrease in feed conversion and performance that ultimately result in high financial losses. Among these ailments, diarrhea and gastroenteritis are some of the most important as they cause high morbidity and mortality (Lee *et al.*, 2016). The small intestine of a pig is a major site of nutrient absorption. Similar to the colon, this organ harbours a diverse microbiota that is pivotal to digestion and nutrient absorption. Although most of these microbes have a symbiotic relationship with the host, some can cause extensive harm (Fouhse *et al.*, 2016, Lee *et al.*, 2016).

Escherichia coli is a Gram-negative, facultatively anaerobic bacterium that is usually harmless and aids digestion (Fairbrother *et al.*, 2005, Fouhse *et al.*, 2016). However, several *E. coli* strains have evolved toxins that cause extensive pathology in the gut. Indeed, *E. coli* is the most important cause of diarrhea in young swine (Fairbrother *et al.*, 2005). Of particular interest is ETEC, a non-invasive type that adheres to the microvilli of intestinal epithelial cells. These bacteria release toxins that evoke gastrointestinal hypersecretion of electrolytes and water. The diarrhea and vomiting that ensues causes dehydration, stunted growth and death. Profuse diarrhea and gastroenteritis are also caused by the TGEV, which is a coronavirus that survives the acidic pH of the stomach and the proteolytic enzymes of the duodenum (Kim *et al.*, 2000). It multiplies in the cell lining of the small intestine resulting in the loss of absorptive cells and villous atrophy (Kim *et al.*, 2000, Morin *et al.*, 1974). TGEV spreads rapidly, causes outbreaks involving large numbers of pigs, and often leads to 100% mortality in young piglets. Diarrhea is often caused by coinfecting pathogens that synergize to cause severe disease. For instance, humans with diarrhea have been found to be coinfected with rotaviruses and *E. coli* or *Giardia* (Bhavnani *et al.*, 2012). Furthermore, infection by multiple microbes has also been reported to cause purulent diarrhea in swine (de la Fé Rodríguez *et al.*, 2013). This raises many questions concerning the pathogenesis of coinfections. In a recent issue of *Proteomics Clinical Applications*, Xia and colleagues (Xia *et al.*, 2017b) unveiled that TGEV infection

augments the attachment of ETEC to intestinal cells, thence altering host cell homeostasis and the production of inflammatory cytokines (Figure 1).

Since both TGEV and ETEC infect and attach to a pig's intestinal epithelium, a suitable enterocytic cell line of porcine origin is required to study coinfection in the laboratory. In this regard, the IPEC-J2 cell line is an appropriate model since they are morphologically differentiated cells derived from the small intestine of young piglets (Nossol *et al.*, 2015). Using these cells, Xia and colleagues investigated the dynamics of coinfection by TGEV and ETEC (Xia *et al.*, 2017a, Xia *et al.*, 2017b). The authors showed that TGEV is able to grow (Xia *et al.*, 2017b) and persist (Xia *et al.*, 2017a) in IPEC-J2 cells. Importantly, the authors found that a pre-existing TGEV infection augmented ETEC attachment to these cells. This observation raised the possibility that there might be a survival advantage for either pathogen during coinfection. To that effect, Xia and colleagues examined viral mRNA and protein expression and found that both decrease when ETEC is present. Why is coinfection beneficial for the bacterium but seemingly unfavourable for the virus? To gain important insight into how TGEV promotes ETEC attachment, Xia et al. undertook a comparative proteomics approach where they employed *LC-MS/MS* coupled to iTRAQ to study cells infected with TGEV, ETEC or both (Xia *et al.*, 2017b). Relative to non-infected cells, TGEV infection modulated the expression of 77 proteins. Of particular interest was integrin α5, a matrix macromolecule known for binding fibronectin and stimulating angiogenesis. Adhesion molecules such as integrins, cadherins and selectins have been found to facilitate bacterial attachment and invasion (Boyle *et al.*, 2003). Xia and colleagues demonstrated that integrin α5 mRNA and protein expression increased upon TGEV infection, and even more saliently so upon coinfection with ETEC. These findings were validated by flow cytometry, which revealed increased cell surface expression of integrin α5 on infected cells. In order to show a causal link between integrin α5 and increased ETEC adhesion to TGEV-infected cells, the authors employed peptide agonists or inhibitors of integrin α5. When integrin α5 is inhibited on TGEV-infected IPEC-J2 cells, ETEC attachment decreases significantly; the opposite is observed upon treatment with an integrin α5 agonist (Xia *et al.*, 2017b). In an accompanying study, Xia and colleagues further elucidated how TGEV induces

integrin α5 (Xia *et al.*, 2017a). There, the authors established that TGEV evoked the production of TGF-β, which in turn induced EMT through the PI3K/Akt pathway. This TGEV-induced EMT was found to augment the expression of vimentin, fibronectin and integrin α5 in IPEC-J2 cells and pig intestines (Xia *et al.*, 2017a) (Figure 1). Additionally, the proteomes of TGEV-ETEC coinfected cells revealed higher expression of ECM-related proteins HSPG2 and LAMC2.

TGEV causes severe intestinal inflammation (Kim *et al.*, 2000, Morin *et al.*, 1974). Xia and colleagues expanded upon this by reporting that this viral infection induces production of proinflammatory cytokines TNF, IL-1β, IL-6 and IL-8 in IPEC-J2 cells (Xia *et al.*, 2017a, Xia *et al.*, 2017b) (Figure 1). Those cytokines contribute to immune cell infiltration at the infection site (Arango Duque *et al.*, 2014a) and may result to viral clearance. Upon coinfection with ETEC, the authors found that the bacterium lowered cytokine levels by at least 40% (Xia *et al.*, 2017b). This finding can be explained by the fact that ETEC lowers TGEV replication (Xia *et al.*, 2017b), and may – by itself – induce apoptosis (Johnson *et al.*, 2009). As observed with other pathogens (Arango Duque *et al.*, 2014a), one can hypothesize that a decreased inflammatory response might prevent removal of TGEV and ETEC from the gut. The question still remains on whether ETEC promotes the long-term survival of TGEV. From the proteome of TGEV-ETEC coinfected cells reported by the authors, one can see an increase in FAM129B (Xia *et al.*, 2017b), a negative regulator of apoptosis that is overexpressed in cancer cells (Ito *et al.*, 2010). Also exclusive to TGEV-ETEC coinfection was a decrease in GABARAPL2 (Xia *et al.*, 2017b), a protein that is essential to autophagy (Weidberg *et al.*, 2010). Decreased autophagy could prevent the elimination of TGEV from the intestine. Since increased viral persistence could translate into augmented transmission and greater financial losses, future experiments may investigate whether the presence of ETEC improves the long-term fitness of TGEV *in vitro* and *in vivo*. Future investigations may also evaluate the roles of FAM129B and GABARAPL2 in TGEV-ETEC infection; their knockdown could hamper the survival of TGEV and ETEC in the porcine gut.

In sum, Xia and colleagues convincingly show that TGEV promotes ETEC adhesion to intestinal epithelial cells (Xia *et al.*, 2017a, Xia *et al.*, 2017b). The coinfection that ensues lowers the production of proinflammatory molecules and may modulate – to the pathogens' advantage – the expression of host proteins involved in homeostasis and microbial clearance (Figure 1).

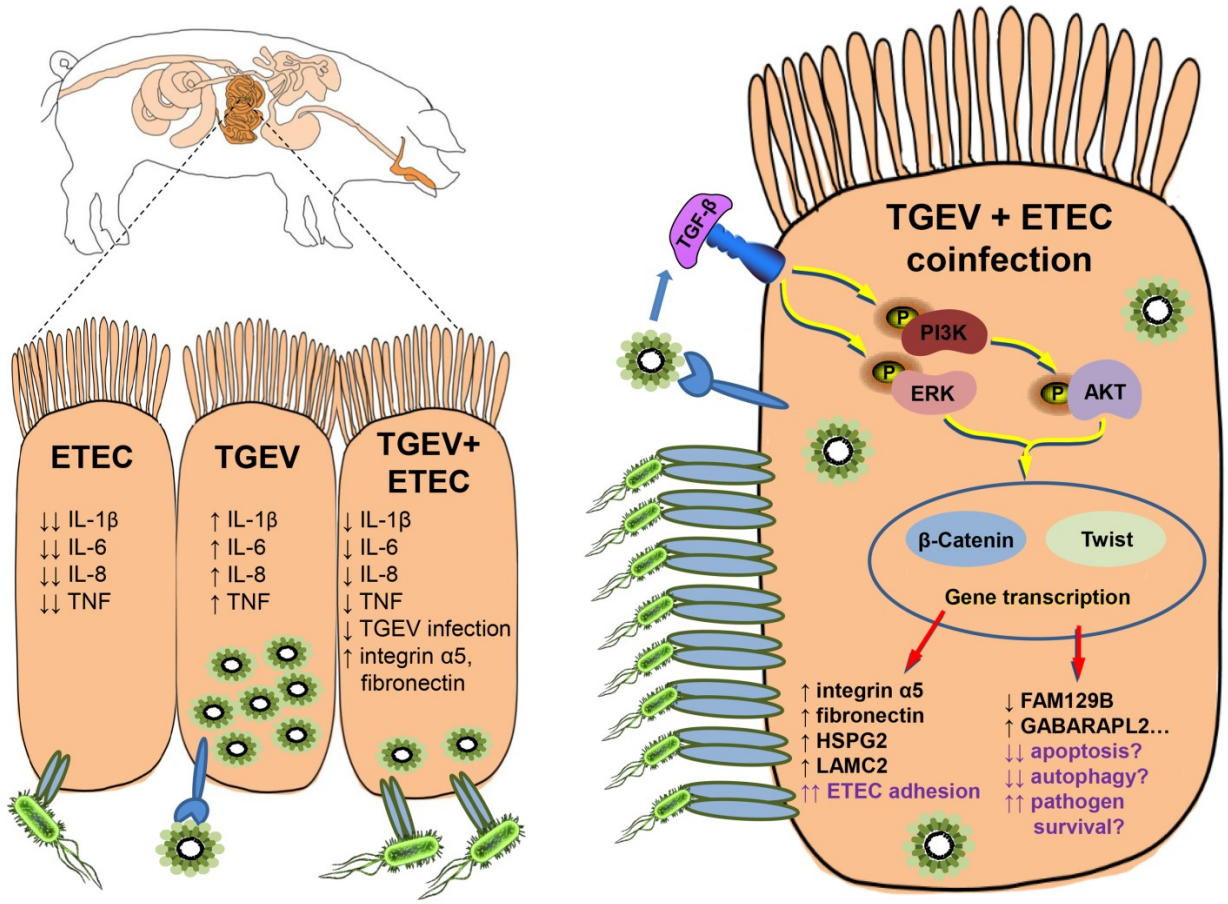

Figure 1 of review 3. TGEV and ETEC elicit context-dependent host cell responses. Infection of intestinal epithelial cells by TGEV induces a PI3K/Akt-dependent EMT phenotype that elicits plasmalemmal expression of proteins such as integrin α5. Contrary to ETEC, TGEV augments the production of inflammatory molecules. Increased levels of integrin α5 promote the attachment of ETEC to epithelial cells, which quells the TGEV-induced production of inflammatory cytokines. Coinfection by TGEV and ETEC also modulates the expression of many proteins involved in homeostasis and host defence, which could ultimately worsen disease severity by enhancing long-term pathogen survival.

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3.4 Conflict of interest statement

The authors declare no conflict of interest.

4 REVIEW ARTICLE NO. 4: MACROPHAGES TELL THE NON-PROFESSIONALS WHAT TO DO

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4.1 Abstract

Phagocytosis by professional and non-professional phagocytes plays a critical role in tissue homeostasis and the immune response. Using an airway inflammation model, Han and colleagues (Han *et al.*, 2016) report in *Nature* that macrophages secrete IGF-1 to signal epithelial cells to stop ingesting apoptotic cells while increasing the uptake of anti-inflammatory macrophage-derived microvesicles.

4.2 Main text

Phagocytosis is the process by which cells ingest a diverse array of large particles (>0.5 μm) within a plasmalemma-derived envelope. This process was initially recognized by Ilya Metchnikoff, whose pioneering work in embryology and immunity led to the identification of phagocytes as pivotal players in nutrient acquisition, tissue homeostasis and host defense (Gordon, 2016, Tauber, 2003). Contingent upon cell origin and phagocytic efficiency, phagocytes can be classified as professional or non-professional (Rabinovitch, 1995). Professional phagocytes are myeloid cells including macrophages, dendritic cells, neutrophils, monocytes, osteoclasts and eosinophils. These phagocytes possess specialized cell-surface receptors that allow them to efficiently recognize and degrade particles such as microbes and senescent cells. Professional phagocytes can be rapidly recruited to an injury or infection site via chemotaxis. In contrast, non-professional phagocytes are usually sessile, do not perform phagocytosis as a primary function and are limited in the range of particles they can ingest (Parnaik *et al.*, 2000, Rabinovitch, 1995). These cells include fibroblasts, epithelial and endothelial cells that are located in various organs such as the lung and skin. Moreover, non-professional phagocytes are crucial in clearing apoptotic cells from organs such as lung alveoli, where macrophages are less abundant (Arandjelovic *et al.*, 2015). The type of ingested particle determines whether macrophages unleash an inflammatory or anti-inflammatory response (Gordon, 2016). This is achieved through the secretion of soluble mediators such as growth factors and cytokines (Arango Duque *et al.*, 2014a). Additionally, professional phagocytes use these molecules to influence the development and function of lymphocytes. It is therefore tempting to speculate that professional phagocytes also release molecules to communicate with and alter the function of their non-professional counterparts. In a recent issue of *Nature*, Han and colleagues (Han *et al.*, 2016) unveiled a mechanism by which macrophages secrete insulin-like growth factor 1 (IGF-1) to modulate phagocytosis by non-professional phagocytes (Figure 1).

IGF-1 is an insulin-like hormone that is primarily produced by the liver and at the local level in the lung, heart and kidney. Similar to human growth hormone, IGF-1 promotes

musculoskeletal growth (Delafontaine *et al.*, 2004). Known for its anabolic functions, its effects on the immune system are only starting to be uncovered. Given the importance of apoptotic cell clearance in tissue remodeling and inflammation, Han *et al.* (2016) tested whether IGF-1, and ten other growth factors known to be involved in tissue growth and repair, affected the phagocytosis of apoptotic cells by professional and non-professional phagocytes. In the authors' screen, IGF-1 was found to diminish the phagocytosis of dead thymocytes by fibroblasts and lung epithelial cells. The IGF-1-mediated decrease in the phagocytosis of apoptotic cells was inhibited by antibodies targeting the IGF-1 receptor (IGF-1R), IGF-1 binding protein and by an inhibitor of IGF-1R. Importantly, the IGF-1-mediated decrease in phagocytosis was concomitant with an increase in the uptake of liposomes by non-professional phagocytes. These IGF-1-induced effects did not apply to macrophages. These results suggested that IGF-1 influences the type of particle that non-professional phagocytes ingest.

Based on the finding that macrophages secrete IGF-1 after IL-4 stimulation, the authors hypothesized that macrophages act as an IGF-1 source. To test this idea, Han et al. (2016) first confirmed that recombinant IL-4 and apoptotic cells elicited IGF-1 release from macrophages. Subsequently, non-professional phagocytes were incubated with the supernatants derived from these treated macrophages. IGF-1-contaning supernatants were found to suppress the phagocytosis of apoptotic cells by non-professional phagocytes. To bring the authors' findings to the *in vivo* context, mice were given intranasal IGF-1 followed by apoptotic cells or liposomes. As expected, IGF-1 suppressed the uptake of apoptotic cells and augmented the uptake of liposomes by alveolar epithelial cells. In addition, when mice lacking IGF-1 in macrophages were given intranasal IL-4 or IL-13, IGF-1 concentrations were found to be diminished in the lungs. These results show that macrophages and other myeloid cells are the primary producers of IGF-1 in the lungs.

Inflammation in the airways is a hallmark of diseases such as asthma and allergic rhinitis. Using house dust mite (HDM) to induce allergic airway inflammation, Han and colleagues elegantly demonstrated that IGF-1 is a major modulator of inflammation in

the lung (Han *et al.*, 2016). Using mice that can be induced to delete IGF-1 in bronchial epithelial cells, the authors induced IGF-1R deletion followed by HDM challenge. In mice lacking IGF-1R, the challenge elicited greater inflammation in the airways, as evidenced by increased bronchial infiltration of eosinophils and $CD4^+$ T cells. If IGF-1 limits the removal of apoptotic cells, why does IGF-1R deletion worsen disease severity? To solve this question, the authors studied the temporal requirement of IGF-1R in their HDM sensitization-challenge model. Only when IGF-1R was deleted prior to sensitization was it found to affect disease outcome. Moreover, when epithelial cells in the airways are exposed to allergens, they produce cytokines such as CSF-2, TSLP, IL-6, and IL-33, which ultimately cause innate lymphoid cells to produce IL-13. Alveolar macrophages from lungs exposed to cigarette smoke release microvesicles (MVs) containing anti-inflammatory mediators (Bourdonnay *et al.*, 2015). Since IL-13 induces IGF-1 release by alveolar macrophages, the authors speculated that alveolar macrophages from HDM-sensitized mice secrete these MVs, and thereby impact inflammatory responses by epithelial cells. Indeed, alveolar macrophages were observed to release such MVs, which in turn thwart production of inflammatory molecules by epithelial cells (Figure 1).

How IGF-1 instructs non-professional phagocytes to ingest MVs rather than apoptotic cells is an issue that deserves future investigation. Phagocytosis involves extensive membrane donations from lysosomes and organelles in the secretory pathway (Gordon, 2016). Since MV uptake does not place such a high membrane demand, it is possible that IGF-1 modulates membrane trafficking and vesicle fusion molecules in non-professional phagocytes. MVs have been found to play a vital role in the interactions that occur among cells of the immune system and beyond (Bourdonnay *et al.*, 2015). Is the cargo of macrophage MVs influenced by IGF-1 or other secreted mediators? The cell biological mechanisms by which these MVs get formed, sorted, secreted and delivered to target cells will need to be characterized in prospective studies.

The roles of IGF-1 in immunity are only starting to emerge. Recently, it was discovered that *Mycobacterium leprae* (Batista-Silva *et al.*, 2016) induces an increase in

macrophage IGF-1 production, which then attenuates bacterial killing. Given the role of IGF-1 in modulating particle uptake by lung epithelial cells (Han *et al.*, 2016), it is possible that excess IGF-1 continuously instructs epithelial cells to ingest anti-inflammatory MVs, thus inhibiting the normal inflammatory process that is required to fight bacterial infections. Future experiments may investigate whether pathogens promote their own survival by hijacking communications between professional and non-professional phagocytes.

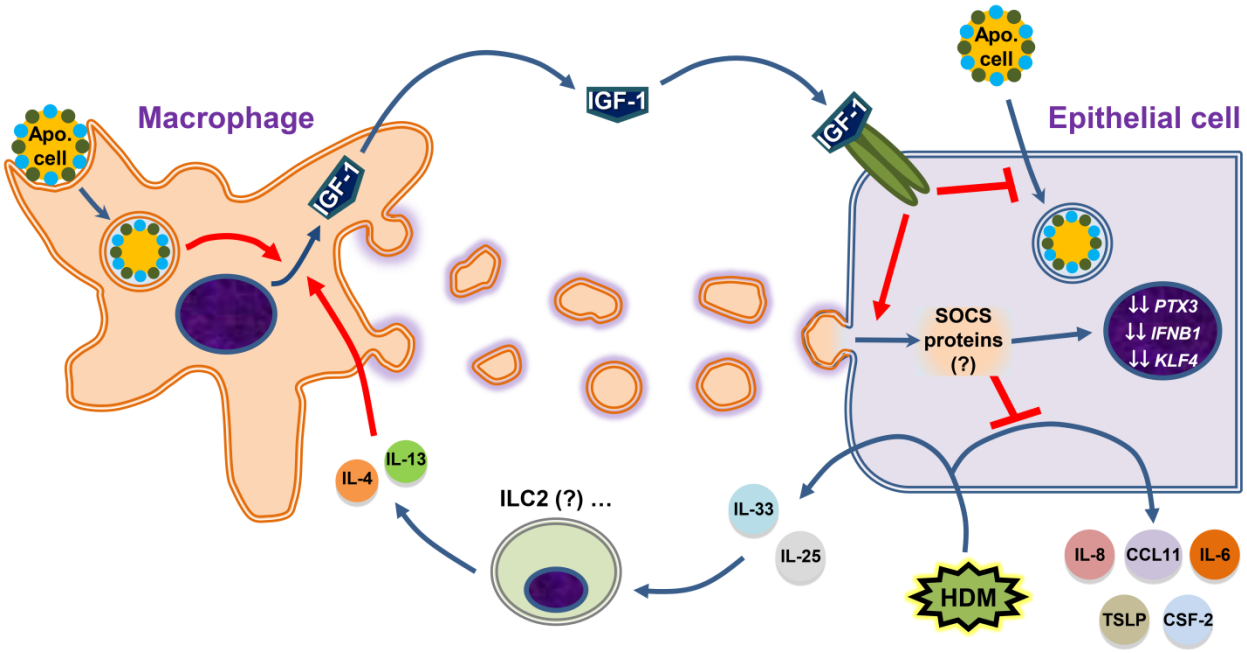

Figure 1 of review 4. IGF-1 modulates inflammation and phagocytosis by non-professional phagocytes. Engulfment of apoptotic cells, or stimulation with IL-4 and IL-13, induces macrophages to release IGF-1. Binding of IGF-1 to IGF-1R in non-professional phagocytes instructs these cells to stop ingesting apoptotic cells and to increase microvesicle (MV) uptake. When lung epithelial cells encounter an allergen such as HDM, an inflammatory response ensues. Secretion of IL-25 and IL-33 induces certain immune cells to secrete IL-4 and IL-13, which in turn makes alveolar macrophages secrete IGF-1. Consequently, epithelial cells increase their uptake of anti-inflammatory macrophage-derived MVs that diminish the expression of genes such as *PTX3*, *IFNB1* and *KLF4*. These MVs ultimately allow epithelial cells to control inflammation.

In sum, Han and colleagues convincingly show that macrophages secrete IGF-1 to control the phagocytic activity and inflammatory phenotype of non-professional phagocytes (Figure 1) (Han *et al.*, 2016). The effect of IGF-1 in diminishing inflammation opens very exciting possibilities for investigating how this growth factor modulates the

intracellular trafficking of professional and non-professional phagocytes in health and disease.

5 REVIEW ARTICLE NO. 5: *LEISHMANIA* DICES AWAY CHOLESTEROL FOR SURVIVAL

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5.1 Abstract

Host lipid alterations are centrally involved in *Leishmania donovani* infection, and infected patients exhibit hypocholesterolemia. In this issue of Cell Host & Microbe, Ghosh *et al.* (2013) show that the metalloprotease GP63 released by *L. donovani* in the liver cleaves DICER1, inhibiting miR-122 maturation, which regulates cholesterol metabolism. These events decrease serum cholesterol and promote parasite growth (Ghosh *et al.*, 2013).

5.2 Main text

Protozoan parasites of the genus *Leishmania* are responsible for a spectrum of human diseases ranging from self-healing ulcers to potentially fatal visceral leishmaniasis (VL), which affect millions of people worldwide. The parasites are transmitted to mammals under the promastigote form during the blood meal of infected sand flies. Following phagocytosis by macrophages, promastigotes differentiate into amastigotes, the mammalian stage of the parasite, and replicate inside phagolysosomal compartments. *Leishmania donovani*, the causative agent of VL, disseminates and multiplies within mononuclear phagocytes of the reticuloendothelial system, including the liver, spleen, lymph nodes, and bone marrow. VL can remain asymptomatic or subclinical, or may take an acute or chronic course. Heavily infected patients may develop disease after an incubation period varying from weeks to months. Fever and hepatosplenomegaly are common symptoms. In addition, VL patients suffer from progressive weight loss, cachexia, and pancytopenia. Recent clinical evidence pointed towards an alteration of lipid metabolism in VL patients, characterized by a marked hypocholesterolemia and reduced LDL-cholesterol (Lal *et al.*, 2007).

In this issue, Ghosh and colleagues (Ghosh *et al.*, 2013) unveil the mechanism(s) responsible for alteration of cholesterol metabolism in experimental VL. Cholesterol is an essential lipid for the life of prokaryotic cells. It is a major component of biomembranes as it regulates their fluidity and structural stability and maintenance. Cholesterol plays central roles in processes ranging from signal transduction to hormone synthesis (Simons *et al.*, 2000). In addition, it is involved in inflammation and infectious disease. Cholesterol biogenesis occurs in the cytoplasm of liver and intestinal cells. Its synthesis starts with the condensation of three acetate molecules to hydroxy-methyl-glutarate by the action of the rate-limiting enzyme hydroxymethylglutaryl-coenzyme A (HMGCoA) reductase. Hydroxymethyl-glutarate is then reduced to mevalonate, which in turn is decarboxylated to a 5-carbon isoprenoid. Condensation of six molecules of isoprenoids leads to squalene, which is cyclised into lanosterol

byoxidosqualene cyclase. Through a 19-step process, lanosterol is finally processed into cholesterol.

Cholesterol metabolizing enzymes are stringently regulated at many stages. At the post-transcriptional level, micro RNAs (miRNA) regulate genes involved in cholesterol synthesis, efflux and catabolism. miRNA genes are transcribed as long 'pri-miRNA' precursors (Rotllan *et al.*, 2012). In animals, these precursors are cleaved in the nucleus by a complex called Microprocessor, which is formed by enzymes Drosha and DGCR8. The intermediate product, or 'pre-mRNA', is a molecule of approximately 70 nucleotides long that is bent into an imperfect stem-loop by means of complementary base pairing. pre-miRNAs are then transported to the cytosol via exportin 5. In the cytosol, pre-miRNAs are cleaved by an enzyme of the Dicer family (e.g. DICER1) allowing the hydrolysis of the loop structure to release a small dsRNA strand called 'duplex miRNA/miRNA'. These in turn interact with Argonaute family proteins (AGO1 or AGO2) to form the RNA-induced silencing (RISC) complex, a type of miRNA ribonucleoprotein (miRNP). miRNPs then repress protein expression by impeding translation or by mediating degradation of target mRNAs. Each cell type has a different miRNA pool. In liver, miR-122 accounts for 70% of miRNAs, and is a key player in cholesterol metabolism, liver inflammation, and carcinogenesis (Wen *et al.*, 2012). An accumulating body of evidence has implicated cholesterol in the host-pathogen interaction; lipid rafts, which are signaling clusters enriched in cholesterol, are used by a myriad of pathogens to gain entry into host cells. For instance, intact lipid rafts are required for *Leishmania*-mediated phagolysosome attenuation (Winberg *et al.*, 2009b). Amazingly, *Leishmania* also extracts cholesterol from macrophage membranes, resulting in decreased capacity to stimulate T cells (Chakraborty *et al.*, 2005). It is therefore tempting to infer that *Leishmania* could also tamper with cholesterol biosynthesis to improve its own survival.

Ghosh and colleagues elegantly show that *Leishmania* sabotages cholesterol metabolism through GP63-mediated cleavage of DICER1. This in turn hinders miR-122

maturation, therefore ensuing in decreased serum cholesterol and augmented parasitemia in liver (Figure 1). Interestingly, the authors observed that mice also suffered from a gradual post-infection decrease in cholesterol and other blood lipids. To find the cause of this phenotype, whole genome microarray analyses of infected liver were performed. The resulting data revealed clusters of upregulated and downregulated genes, many of which were involved in lipid metabolism. After validation, the authors found that genes encoding for proteins such as HMGCoA were severely downregulated. Of utmost importance was the fact that the presence of mature miR-122 was inhibited by infection. In addition, the authors found that exogenous expression of miR-122 reduced parasite burden in liver.

The authors discovered that the factor responsible for miR-122 inhibition lied in *Leishmania*-derived exosomes, which are known to be enriched in GP63 (Silverman *et al.*, 2011). This GPI-anchored zinc-dependent metalloprotease is a pathogenesis factor that promastigotes use to modulate macrophage responses to infection (Isnard *et al.*, 2012). Upon incubating these exosomes with anti-GP63 antibodies or with phenanthroline (an inhibitor of zinc-dependent metalloproteases), the authors found that miR-122 inhibition was abolished. Ghosh and colleagues also noticed accumulation in miR-122 precursors, and found that mature miR-122 failed to get loaded onto AGO2, which is necessary for miRNP formation. These results prompted the examination of DICER1, which was found to be cleaved by GP63 *in vitro*. Such cleavage was also abolished by GP63 inhibition. To further support their argument, exogenous expression of DICER1 in infected mice also abolished parasitemia and reconstituted serum cholesterol to near-normal levels. Although these results strongly support a role for GP63 in the cleavage of DICER1 and the inhibition of miR-122, they remain indirect evidence. It will thus be crucial to generate *L. donovani gp63* knockout mutants to directly ascertain the importance of GP63 in the alteration of serum cholesterol in experimental VL. This genetic approach was instrumental in assessing the role of GP63 in the pathogenesis of *L. major* (Isnard *et al.*, 2012, Joshi *et al.*, 2002). How GP63 exits infected liver macrophages and enters neighboring hepatocytes to cleave DICER1 is another issue that will clearly deserve further investigation. The fact that amastigotes

are present within phagolysosomes suggests that GP63 must be released from the parasite, cross the membrane of the parasitophorous vacuole, get out of the infected cells, enter hepatocytes, and finally access DICER1. Elucidating this process is critical to our understanding of the pathology associated to visceral leishmaniasis.

It is important to point out that, albeit absence of miRNP-122 in liver results in diminished serum cholesterol due to decreased synthesis of HMGCoA reductase, this also leads to augmented cytoplasmic triglyceride accumulation through upregulation of FSP27 (Wen *et al.*, 2012). This protein, a direct target of miR-122, is a negative regulator of lipolysis and promotes triglyceride build up. Hence, it is possible that increased FSP27 in *Leishmania*-infected liver could promote triglyceride accumulation and gradual steatohepatitis. Furthermore, miR-122 absence results in liver inflammation, increased cytokine secretion and carcinogenesis. This raises the intriguing possibility that GP63-mediated cleavage of DICER1 could play a primordial role in hepatocyte-mediated liver inflammation.

Though the authors clearly showed that reconstitution of miR-122 or DICER1 vastly decreases parasite burden, cleavage of DICER1 certainly has a more profound impact on liver pathology. DICER1 regulates the maturation of a panoply of miRNAs that also modulate liver homeostasis and function. For example, it would be of interest to elucidate the effect of *Leishmania* on miR-33, which regulates cholesterol efflux and fatty acid oxidation (Rotllan *et al.*, 2012). Moreover, whether DICER1 degradation affects the pathology of cutaneous and mucocutaneous leishmaniasis by tampering with miRNA pools in dermal cells and macrophages is an attractive hypothesis that deserves attention.

In sum, Ghosh and colleagues convincingly show that *Leishmania donovani* lowers serum cholesterol by hindering the maturation of miR-122 via cleavage of DICER1. This

GP63-mediated effect opens very exciting possibilities for investigating how *Leishmania* affects the host's miRNA pool to cause disease and promote the parasite's success.

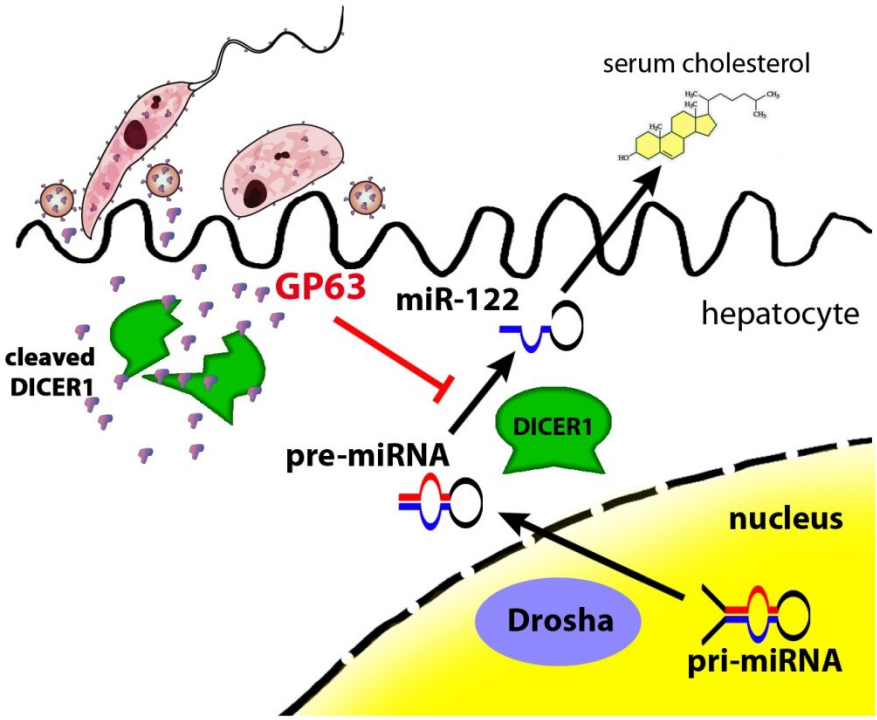

Figure 1 of review 5. GP63 disrupts cholesterol metabolism through DICER1 cleavage. In hepatocytes of non-infected liver, pri-miR-122 in nucleus is processed by Drosha into pre-miR-122, which is then transported to the cytoplasm. Pre-miR-122 is further processed by DICER1 and AGO2 into mature miR-122, thence forming miRNP-122. This ribonucleoprotein regulates many enzymes that modulate cholesterol metabolism. In the other hand, hepatocytes in *Leishmania*-infected liver internalize GP63-containing exosomes. Thereafter, DICER1 is cleaved, leading to an accumulation of pre-miR-122. The consequent disruption in cholesterol metabolism ensues in hypocholesterolemia.

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