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# Trafficking of *Leishmania donovani* promastigotes in non-lytic compartments in neutrophils enables the subsequent transfer of parasites to macrophages

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## Summary

**Inoculation of *Leishmania* (*L.*) spp. promastigotes in the dermis of mammals by blood-feeding sand flies can be accompanied by the rapid recruitment of neutrophils, inflammatory monocytes and dendritic cells. Despite the presence of these lytic leucocytes, parasitism is efficiently established. We show here that *Leishmania donovani* promastigotes are targeted to two different compartments in neutrophils. The compartments harbouring either damaged or non-damaged parasites were characterized at the electron microscopy (EM) level using the glucose 6-phosphatase cytochemistry and endosome–phagosome fusion assays. One involves the contribution of lysosomes leading to the formation of highly lytic compartments where parasites are rapidly degraded. The other is lysosome-independent and involves the contribution of a compartment displaying some features of the endoplasmic reticulum (ER) where parasites are protected from degradation. Using genetically modified parasites, we show that the promastigote surface lipophosphoglycan (LPG) is required to inhibit lysosome fusion and maintain parasites in neutrophil compartments displaying ER features. *L. donovani*-harbouring neutrophils that eventually enter apoptosis can be phagocytosed by macrophages enabling the stealth entry of parasites into their final replicative host cells. Thus, the ability of *L. donovani* to avoid trafficking into lysosomes-derived compartments in short-lived neutrophils constitutes a key process for the subsequent establishment of long-term parasitism.**

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## Introduction

Leishmaniasis is a disease caused by intracellular protozoan parasites of the genus *Leishmania.* The most severe form of this disease, visceral leishmaniasis, is caused by *Leishmania donovani*, a parasite whose development occurs in tissues of mammalian hosts, distant from the cutaneous site of inoculation by the female sandfly carriers (Herwaldt, 1999; Murray *et al*., 2005; Wilson *et al*., 2005). Inoculation takes place when parasite-carrying sandflies try to locate blood vessels in order to blood-feed. During this process, both saliva and a low dose of the metacyclic promastigote developmental stage of *Leishmania* parasites are deposited into the upper dermis (Ribeiro, 1987; Schlein *et al*., 1992). In the case of mammals that were pre-exposed to a parasite-free sand fly saliva, an acute and transient inflammatory process is known to develop (Belkaid *et al*., 1998; 2000; Sacks and Kamhawi, 2001). This is accompanied by the rapid recruitment of neutrophils and monocytes at the site of parasite inoculation (Belkaid *et al*., 1998; 2000; Sacks and Kamhawi, 2001). Of note such a transient recruitment of neutrophils and monocytes is also observed after the inoculation of high dose of stationary-phase promastigotes (Wilson *et al*., 1987; Sunderkotter *et al*., 1993; Tacchini-Cottier *et al*., 2000; Muller *et al*., 2001; Laufs *et al*., 2002). Although unactivated/deactivated macrophages are recognized as the final host cells where *Leishmania* spp. promastigotes differentiate into amastigotes and replicate (Antoine *et al*., 2004), it has been proposed that the uptake of promastigotes by neutrophils might offer an indirect way of silent delivery to macrophages (Laskay *et al*., 2003).

Neutrophils are major leucocytic cell effectors of the innate immune response (Nathan, 2006; Urban *et al*., 2006). They have evolved various mechanisms allowing the rapid killing of microbes, and thus participate actively, at sites of inoculation, in the early clearance of microorganisms by phagocytosis. This process is often

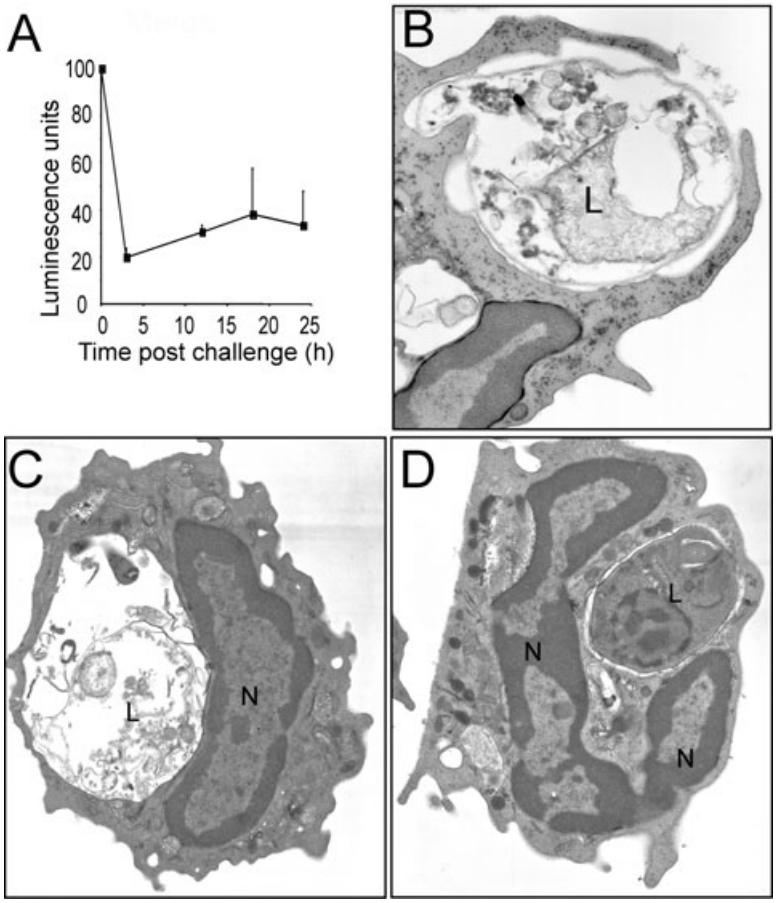


**Fig. 1.** Most *L. donovani* parasites are killed inside large phagosomes but some tight phagosomes harbour intact parasites. Phagosomes were formed in mouse neutrophils by the internalization of parasites (L) for 30 min.
A. Luciferase activity was measured after a 0, 3, 13, 18 or 24 h chase. Some parasites are able to survive in neutrophils. $n = 3$.
B. Nascent phagosomes containing degraded parasites in a G-6-Pase– compartment.
C. Degraded parasite (L) in a spacious phagosome.
D. Intact parasite in a tight phagosome.
N, nucleus.

accompanied by the simultaneous release of antimicrobial factors into the extracellular milieu, and, in human neutrophils, by the formation of extracellular fibres (or neutrophil extracellular traps; NETs), two related processes that may contribute to the killing of microorganisms before their complete engulfment (Henson *et al.*, 1992; Cougoule *et al.*, 2002; Brinkmann *et al.*, 2004; Nathan, 2006). Rapidly after their formation, phagosomes fuse with intracellular granules to form phagolysosomes where exposure to enzymes, antimicrobial peptides and reactive oxygen species (ROS) further contributes to the killing and degradation of microorganisms.

We show here that despite the impressive antimicrobial arsenal displayed by neutrophils, some *L. donovani* promastigotes still manage to survive in these otherwise known short-lived phagocytic leukocytes by promoting their targeting to non-lytic compartment unable to fuse with lysosomal organelles, and displaying well-defined ER markers. We also show that lipophosphoglycan (LPG), the major surface glycolipid of *Leishmania* promastigotes, is required for the maintenance of parasites in these compartments. This strategy may play a key role in the transfer of parasites to macrophages, and the establishment of long-term parasitism in mammalian hosts.

## Results

### *Uptake of* L. donovani *promastigotes by neutrophils into two distinct phagosomes*

One of the key functions of neutrophils is to engulf and kill microorganisms at sites of infection. The ability of Gr1-positive murine neutrophils purified from C57BL/6 bone marrow to kill *Leishmania* parasites was studied using *L. donovani* expressing the reporter marker luciferase (Duclos *et al.*, 2000). Following the co-incubation of *Leishmania* parasites and neutrophils, a rapid decrease in luciferase activity (3 h) was observed, consistent with the rapid killing of the majority of parasites. However, a significant level of luciferase activity could still be observed up to 24 h after the initial contact between parasites and neutrophils (Fig. 1A). This result suggests that some of the parasites are able to survive in mouse neutrophils, despite the lytic arsenal displayed by these cells. Thus, we reasoned that *Leishmania* parasites might have evolved mechanisms to evade the lytic environment of phagolysosomes. In order to understand the mechanisms that might allow the survival of parasites in neutrophils, we performed ultrastructural analyses of the handling of *L. donovani* by neutrophils prepared from dog blood or

 

mouse bone marrow. In frequent occasions, we observed the presence of parasites displaying signs of lytic degradation within large/loose pseudopodia at the cell surface (Fig. 1B). The observation of intact parasites in the same thin section ruled out the possibility of an electron microscopy (EM) fixation artefact and rather suggested that some of the parasites could be degraded prior to their engulfment into phagosomes. Degraded parasites, which represented close to 80% of the parasite population, were observed in spacious phagosomes (Fig. 1C). In contrast, morphologically intact parasites (21.8%) were observed in tight compartments (Fig. 1D). These results suggest that the survival of *L. donovani* parasites in neutrophils is linked to their ability to be targeted to tight/non-cidal and non-degradative compartments.

### *Spacious and tight* L. donovani*-containing phagosomes display distinct properties*

Clearly, the two distinct types of phagosomes observed in neutrophils display different properties. The degradative nature of the spacious phagosomes indicates the possible involvement of lytic organelles like lysosomes in their formation. On the other hand, the presence of intact parasites within tight compartments was reminiscent of previous observations made in mouse J774 macrophages, where ER was shown to participate in phagosome formation (Gagnon *et al*., 2002). To determine whether the two distinct types of phagosomes can interact with lysosomes and/or ER, we used a high-resolution EM approach monitoring the transfer of tracers from these organelles to phagosomes. The tracers used were BSA-gold particles, pre-loaded to lysosomes by endocytosis, and the electron-dense product of the activity of the ER enzyme glucose 6-phosphatase (G-6-Pase). In addition, the immunolocalization of the ER protein calnexin was monitored. The high resolution of the EM approach enabled the quantitative analysis and the specific and simultaneous detection of both the lysosome and ER tracers in the same cells. When the distribution of BSA-gold particles and G-6-P labelling were looked at in parasite-free neutrophils, a strong enzymatic staining was observed in the ER throughout the cell and in the nuclear envelope (Fig. 2A), as well as in the foremost *cis*-cisterna of the Golgi complex (Fig. 2B), as reported in various studies (Griffiths *et al*., 1983; Melo and de Souza, 1997; Gagnon *et al*., 2002; Celli *et al*., 2003; Touret *et al*., 2005a). No G-6-Pase labelling was observed on other cellular organelles such as mitochondria and the plasma membrane (PM) (see Fig. 2A and C). Importantly, G-6-Pase activity was not detected in any of the BSA-gold-loaded endocytic organelles (Fig. 2C). It was argued recently that the G-6-Pase assay might reveal the presence of other phosphatases present at the cell surface or in lysosomes (Touret *et al*., 2005b). However, our results confirm that the only phosphatase-positive 'donor' membrane revealed by the G-6-Pase assay in our conditions is the ER. The high specificity of the G-6-P marker was taken advantage of in recent studies to confirm the well-established trafficking of both *Brucella* and *Legionella* bacteria to ER-like organelles (Celli and Gorvel, 2004; Robinson and Roy, 2006; Fortier *et al*., 2007).

In mouse neutrophils co-incubated with *L. donovani* promastigotes for 30 min, the vast majority of spacious compartments containing degraded parasites displayed numerous BSA-gold particles but no G-6-Pase labelling (Fig. 2D and E), as demonstrated by the quantitative analysis (Fig. 2F). In contrast, the vast majority of tight compartments displaying intact parasites was G-6-Pase+, and contained no BSA-gold particles (Fig. 2D–F). These distinct features were emphasized when both types of phagosomes were present in the same cell sections (Fig. 2D and E). Strikingly, the quantitative analyses indicated that BSA-gold particles and G-6-Pase labelling were never observed in the same compartments (Fig. 2F). Thirty minutes after contact, 23.4% of the parasite population was already observed inside G-6-Pase+ compartments. Further analyses indicated that none (0%) of the spacious phagosomes containing degraded parasites showed G-6-Pase labelling, while 77% of the tight phagosomes with intact parasites displayed this marker.

Altogether, these results suggest that specific organelles are involved in the formation of the two distinct types of phagosomes. Arguably, lysosomes participate in the formation of the spacious degradative compartments and do not contribute to the biogenesis of the tight phagosomes housing intact parasites. In contrast, tight phagosomes appear to derive some of their membranes from an interaction with ER, which do not seem to interact with the spacious compartments.

The contribution of ER in the formation of the tight compartments containing intact parasites is supported by the presence of a strong labelling for G-6-P within the lumen of phagosomes (Fig. 3A–D), and by immunofluorescence analyses showing the close association of a strong labelling for the ER protein calnexin with *Leishmania*-containing organelles (Fig. 3E). The presence of intact parasites in calnexin-positive (not shown) and tight G-6-Pase+ compartments for as long as 12–24 h after internalization (Fig. 3F and G) confirms the safe nature of this compartment.

### *The persistence of* L. donovani *in non-lytic compartments requires the* lpg1 *and* lpg2 *genes*

Clearly, the ability to invade and persist into non-lytic/calnexin+/G-6-Pase+ compartments constitutes a survival

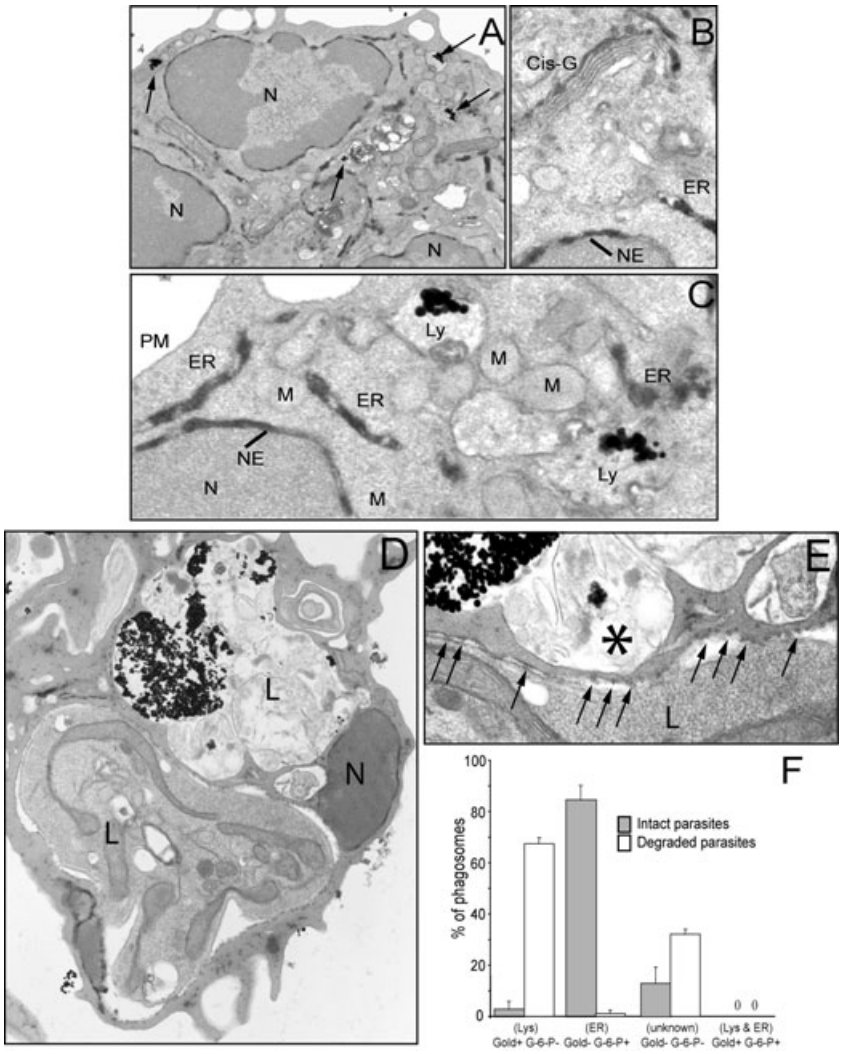


**Fig. 2.** Only the lysosomes contribute to the formation of the spacious phagosomes harbouring degraded *Leishmania*.
A–C. G-6-Pase labelling in BSA-gold pre-loaded neutrophils. (A) Arrows: BSA-gold loaded lysosomes. (B) G-6-Pase deposit on *cis*-cisterna of the Golgi complex (Cis-G). (C) Strong labelling of nuclear envelope (NE) and ER; G-6-Pase– labelling of BSA-gold loaded lysosomes (Ly), plasma membrane (PM) and mitochondria (M).
D. BSA-gold+ phagosome containing degraded parasites (L), absence of this tracer in a phagosome containing intact parasites. N, nucleus.
E. Inset: spacious phagosome (asterisk) G-6-Pase– and BSA-gold+ tight phagosome G-6-Pase+ (arrows) containing an intact parasite (L).
F. Quantitative analysis of the fusion events occurring between phagosomes and lysosomes: the morphological aspect (degraded or intact) of parasites was assessed for each phagosome. $n = 3$.

advantage for *Leishmania* promastigotes. In nature, the properties allowing *Leishmania* to effectively establish parasitism in mammals are acquired during a process called metacyclogenesis, occurring in the blood-feeding sand fly host and vector. During this process of differentiation, *Leishmania* promastigotes are remodelled, evolving from poorly invasive procyclics into highly invasive metacyclic promastigotes. Metacyclogenesis can also be reproduced in axenic cultures of promastigotes. Our results indicate that following *in vitro* differentiation, metacyclic promastigotes displayed a typical slender body and long flagellum (Fig. 4A). Furthermore, these elongated parasites were able to move very quickly, at the rate of $35.9 \pm 9.4\ \mu m\ s^{-1}$, versus $6.9 \pm 4.6\ \mu m\ s^{-1}$ for a mixture of parasites from stationary phase.

To test whether changes occurring during metacyclogenesis might influence the handling of parasites by mouse neutrophils, three promastigote populations were compared: (i) procyclic from early log-phase culture, (ii) a mixture of procyclic and metacyclic from stationary phase and (iii) enriched metacyclic from stationary phase. Our results indicate that only a small proportion of the log-phase parasites were detected into G-6-Pase+ compartments, while higher proportions of the stationary phase and purified metacyclic parasites were present in such compartments (Fig. 4B). This indicates that modifications occurring during metacyclogenesis influence the handling of parasites by neutrophils.

Phosphoglygans are among the known molecules remodelled during metacyclogenesis. In order to test whether some of these molecules might affect the handling and trafficking of parasites in neutrophils, series of experiments were performed with wild-type (WT) parasites, and two populations of *Leishmania* mutants lacking phosphoglycans: *lpg2*–/– mutants lacking LPG and other phosphoglycans, or *lpg1*–/– mutants lacking LPG only. First, we performed double immunofluorescence with calnexin (ER) and LAMP-1 (lysosomes) 4 h after the han-

 

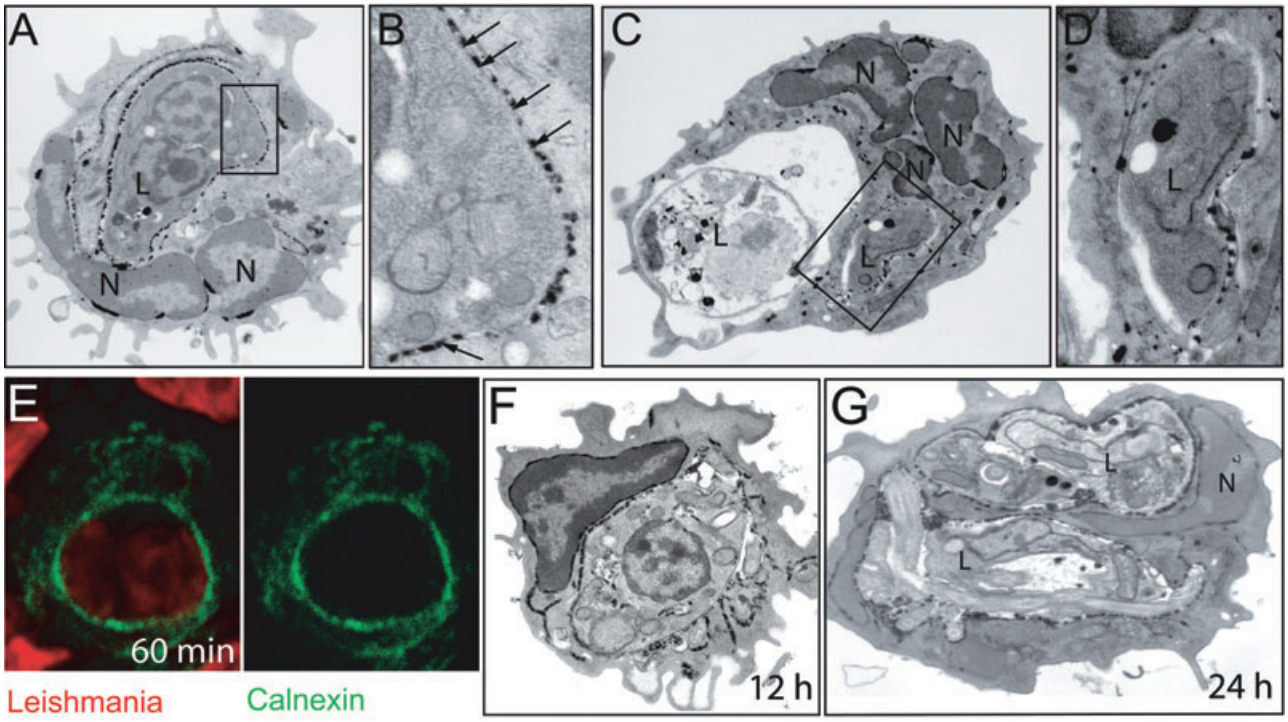


**Fig. 3.** Detection and maintenance of *L. donovani* parasites inside tight G-6-Pase+ calnexin+ compartments.
A. Parasite in a compartment strongly G-6-Pase+.
B. Inset: the phagosome membrane (arrows) is tightly apposed to the parasite surface with the G-6-Pase product present in the phagosome lumen.
C and D. Two distinct types of phagosomes in the same cell: a large spacious phagosome containing a degraded parasite (L) in a G-6-Pase– compartment (C), or a tight phagosome (boxed area) containing an intact parasite in a G-6-Pase+ compartment (D) (inset).
E. Presence of parasites (red) in calnexin+ compartments (green).
F and G. Intact parasites in tight compartments G-6-Pase+, 12 h (F) and 24 h (G) after internalization.
A–D. Dog neutrophils.
E–G. Bone marrow-purified neutrophils.

dling of WT or *lpg2*–/– parasites. Figure 4C shows an example of the labelling obtained with the WT parasite. Quantitative analysis revealed that a higher proportion of *lpg2*–/– mutants were present in calnexin– and LAMP-1+ compartments (lysosome-like), compared with the WT (Fig. 4D). These results illustrate that WT parasites are more suited to prevent their targeting to compartments displaying the lysosomal marker.

Based on our results so far, we hypothesized that WT parasites should be able to evade trafficking to lytic compartments and establish a safe haven in compartments displaying ER-like features (non-degradative and G-6-P+). Accordingly, a quantitative analysis was performed to compare the handling of *lpg1*–/–, *lpg2*–/– mutants and WT promastigotes, at 1 and 16 h after internalization by neutrophils. The quantitative analysis showed that the proportion of WT parasites present in G-6-Pase+ compartments increased with time, while the proportion of degraded parasites decreased (Fig. 5A). These results are consistent with the proposal that a significant proportion of WT parasites are targeted to non-lytic compartments and able to persist in these compartments. A surprising profile was observed for the *lpg1*–/– mutant. Although an increase in the proportion of degraded parasites was observed with time, the proportion of parasites present in G-6-P+ phagosomes remained stable. This contrasts with the handling of the lpg2–/– mutant, which is characterized by a strong decrease of the proportion of parasites present in G-6-P+ compartments and a strong increase of their degradation with time. These results indicate that phosphoglycans are not significantly involved in determining the initial uptake of parasites, which are all present in the same proportion in G-6-P+ compartments after 60 min of internalization. However, phosphoglycans are clearly involved in the ability of parasites to persist in non-lytic compartments and avoid trafficking to degradative (G-6-P–) compartments, as shown by the strong increase in the proportion of degraded mutant parasites. Our results, especially those obtained with the lpg1–/– mutant, also indicate that a significant proportion of parasites are likely to be degraded in time in G-6-P+ compartments. Indeed, quantitative analyses indi-

 

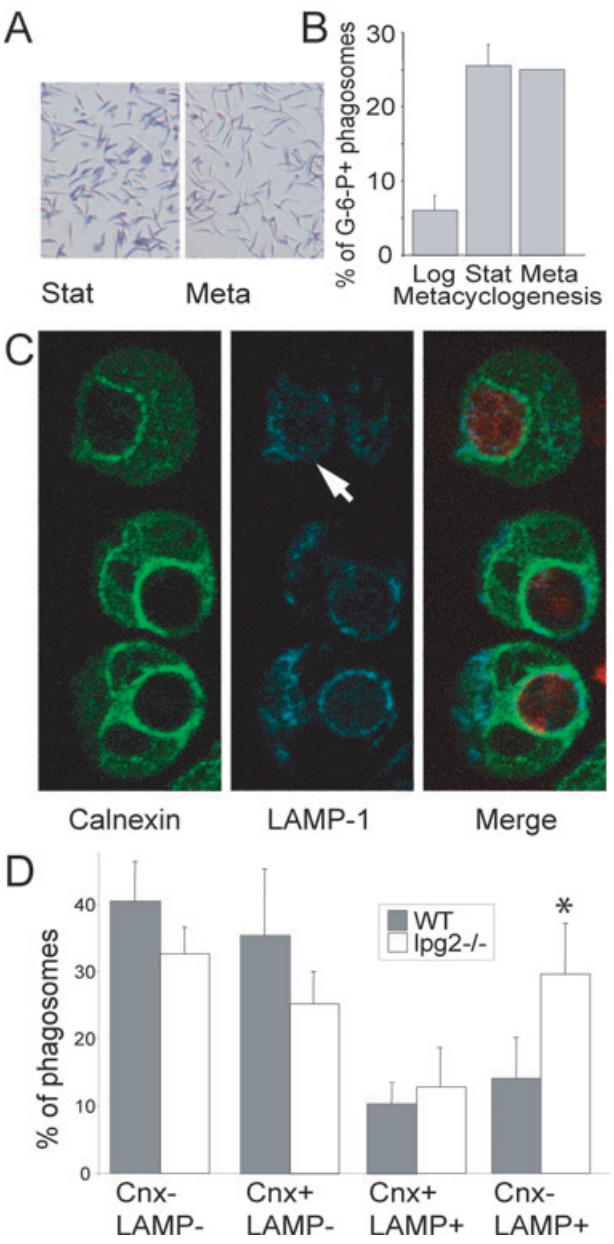


**Fig. 4.** Phagosomes acquire degradative properties of maturing organelles in the absence of LPG. Phagosomes were formed in mouse neutrophils by the internalization of parasites (L) for 30 min, followed by a 1 h (B) or a 4 h (C and D) chase.
A. Giemsa staining of axenic stationary (Stat), and metacyclic (Meta) WT promastigotes.
B. A small proportion of log phase parasites are detected within G-6-Pase+ compartments by EM compared with stationary (Stat) and metacyclic (Meta) growth-phase parasites. $n = 3$ for experiments on early log-phase and stationary-growth promastigotes; $n = 1$ for experiments on metacyclic promastigotes.
C. Confocal image showing an example of the presence of *lpg2–/–* parasites (red) in compartments both calnexin (green) and LAMP1 (blue) positive, 4 h after internalization.
D. The percentage of phagosomes positive for calnexin (Cnx+) or LAMP1 (LAMP+) was determined. $n = 3$; $^*P$-value of $< 0.05$ was considered significant.

cate that less than 20% of the WT parasites present in G-6-Pase+ compartments showed signs of degradation 16 h after internalization (Fig. 5B). In contrast, between 45% (*lpg2–/–*) and 70% (*lpg1–/–*) of the mutants promastigotes present in G-6-Pase+ compartments were degraded (Fig. 5B). This is in accordance with the proposal that although present in a non-lytic/G-6-Pase+ compartment initially, mutant parasites are unable to maintain the inhibitory pressure necessary to avoid interaction with lysosomes and further cidal and degradative processes. Micrograph presented in Fig. 5C illustrates the presence of degraded *lpg1–/–* mutants within tight/G-6-Pase+ phagosomes. Similar results were observed for the *lpg2–/–* mutant (data not shown).

### Leishmania donovani *parasites can delay mouse neutrophil apoptosis*

Our results showing that *L. donovani* can persist in neutrophils for up to 24–48 h, a period that largely exceeds the normal life span of neutrophils, indicate that the infectious process might alter the ability of neutrophils to initiate their programmed cell death. It has been shown that phagocytosis of *L. major* increases the lifetime of human neutrophils by delaying apoptosis (Aga *et al*., 2002). In our system, we observed a significant delay in the apoptosis of neutrophils loaded with WT *L. donovani* compared with parasite-free neutrophils. Interestingly, phagocytosis of the *lpg2–/–* mutant had no inhibitory effect on neutrophil apoptosis (Fig. 6A). Indeed, between 24 and 48 h after parasite addition, intact WT parasites were often observed in dying/dead neutrophils (Fig. 6B). When incubated with primary bone marrow-derived macrophages, the parasite-loaded neutrophils were engulfed (Fig. 6C and D). In the representative example showed in Fig. 6C, the *Leishmania* parasite displays an intact morphology within a tight G-6-Pase+ compartment.

## Discussion

The natural mammalian hosts of *L. donovani* are macrophages. Yet, several parasites are rapidly internalized by neutrophils at sites of inoculation. It has been proposed that neutrophils may act as carriers of viable parasites, and participate in the transfer of parasites to macrophages when these cells engulf infected leucocytes (Laufs *et al*., 2002). The molecular mechanisms by which *Leishmania* evades the lytic arsenal of neutrophils during this process are still poorly understood. In the present study, we used quantitative high-resolution assays to show for the first time that at least two modes of internalization exist for the uptake of *Leishmania* parasites in neutrophils. The first clearly involves lysosomes and leads to the formation of spacious phagosomes where

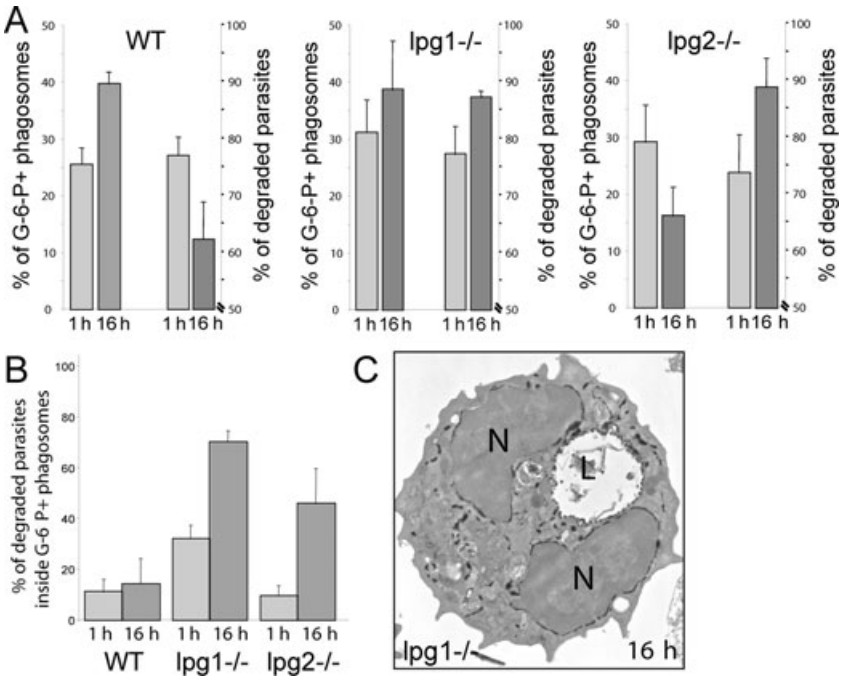


**Fig. 5.** LPG contributes to the maintenance of live parasites into non-lytic G-6-Pase+ compartments.
A. Phagosomes were formed in mouse neutrophils by the internalization of WT or LPG-defective mutants *lpg1*– or *lpg2*– for 30 min followed by a 1 h or 16 h chase. G-6-Pase activity was detected at the EM level. At each time point, counts were performed to evaluate the percentages of G-6-P+ phagosomes, and of degraded parasites.
B. After 16 h, the proportion of degraded *lpg1*–/– parasites inside G-6-Pase+ phagosomes was evaluated.
C. Degraded *lpg1*–/– parasites were observed in tight G-6-Pase+ compartments, 16 h after internalization. N, nucleus. L, *Leishmania*. $n = 3$.

degraded parasites are rapidly observed. These compartments do not display the ER marker G-6-P but contain BSA-gold particles pre-internalized to lysosomes by endocytosis. The presence of degraded parasites within phagocytic cups at the cell surface suggests that lytic granules and/or lysosomes contribute to phagosome formation by fusing directly with the PM, enabling the rapid killing of parasites. It is well documented that phagocytosis in neutrophils can be accompanied by the release of various hydrolases at the cell surface (Henson *et al*., 1992; Laufs *et al*., 2002; Nathan, 2006).

The second mode of entry, observed for around 20–25% of the parasites in the absence of serum, leads into phagosomes displaying completely different sets of features. These phagosomes are tight, with their membrane closely apposed to the parasite surface, and very

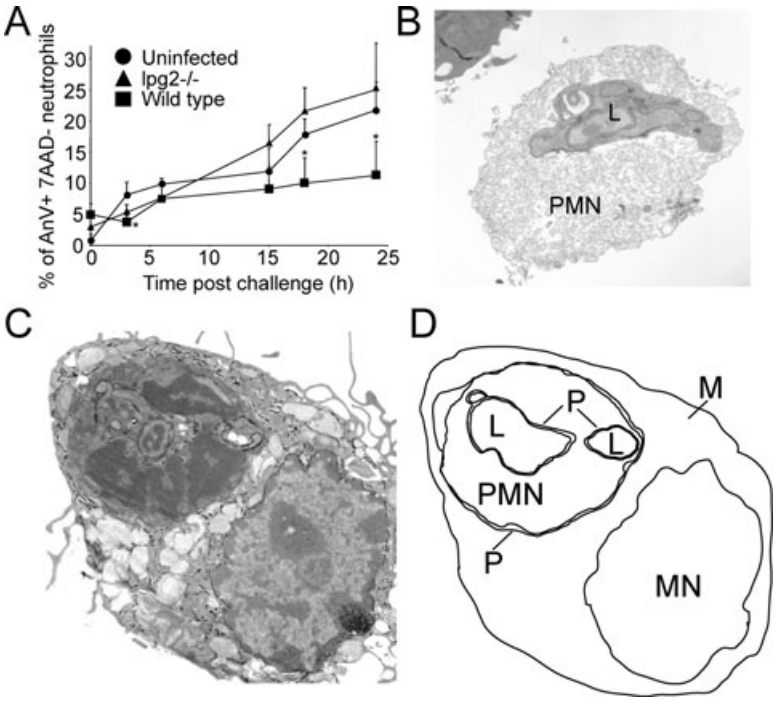


**Fig. 6.** Uptake of WT *L. donovani* parasites delays neutrophil apoptosis.
A. Sorted neutrophils were co-incubated with WT or *lpg2*–/–-defective mutant parasites for 45 min followed by a 24 h chase. Results are reported as the percentage of AnnexinV-APC-positive 7′AAD-negative (AnV+ 7ADD–) cells. $n = 3$; each experiment was performed in triplicate. *P*-values of $< 0.05$ were considered significant.
B. Dying neutrophil (PMN) containing an intact parasite after an 18 h chase.
C. Electron micrograph showing that an apoptotic neutrophil containing intact parasites is internalized by a primary macrophage.
D. Schematic representation of the image showed in (C). M, macrophage; MN, macrophage nucleus; P, phagosome; L, *Leishmania*; PMN, neutrophil.

strongly labelled for G-6-P and calnexin. None of the tight phagosomes displayed the lysosomal tracer BSA-gold. Furthermore, metabolically active *L. donovani*, expressing luciferase, survive in these compartments for up to 48 h. These results clearly indicate that the uptake of a significant proportion of the *Leishmania* parasites in neutrophils occurs via a lysosome-independent pathway, enabling the establishment of a safe niche in non-lytic compartments. The features displayed by these phagosomes supports the proposal that they may derive part of their membrane from interaction with the ER. Although concerns have been raised regarding the specificity of the G-6-Pase assay to localize ER (Touret *et al*., 2005b), this approach was used successfully to follow the known interaction of *Brucella* and *Legionella* bacteria with the ER (Celli and Gorvel, 2004; Robinson and Roy, 2006; Fortier *et al*., 2007). Our control experiments have clearly showed that, in our conditions, the only membrane compartments labelled following the G-6-Pase assay were the ER and the nuclear envelop. No labelling at all was observed on the plasma membrane or on any endocytic organelles containing BSA-gold particles pre-internalized by endocytosis. Interestingly, a recent study using proteomics and morphological approaches has shown that the vast majority of latex beads engulfed by human neutrophils (above 90%) reside in phagosomes displaying strong immunofluorescence labelling for several ER proteins (Burlak *et al*., 2006). This raises the interesting possibility that *L. donovani* may have evolved ways to exploit host processes accounting for the uptake of foreign inert particles, to establish a transient safe haven in ER-derived compartments in neutrophils.

Our data clearly indicate that the targeting of *L. donovani* to ER-derived compartments does not involve surface phosphoglycans, nor LPG. Indeed, a similar proportion of the population of WT parasites and LPG-deficient mutants is observed in G-6-P+ compartments, at 60 min after internalization. However, phosphoglycans are clearly required for the maintenance of parasites in non-lytic/ER-derived compartments. LPG, the major surface phosphoglycan of *Leishmania*, has been involved in the parasite ability to inhibit the interaction of phagosomes with lysosomes and late endocytic organelles (Desjardins and Descoteaux, 1997; Scianimanico *et al*., 1999). LPG was also shown to inhibit the association and retention of the NADPH oxidase cytosolic components $p47^{phox}$ and $p67^{phox}$ to the phagosome membrane, preventing the generation of superoxide at the phagosome (Lodge and Descoteaux, 2005; Lodge *et al*., 2006). Recent results indicate that the modulation of phagosomal properties by *L. donovani* LPG is linked to the ability of this molecule to disorganize and inhibit the formation of membrane microdomains on phagosomes (Dermine *et al*., 2005). Interestingly, *Brucella* is also able to disorganize phagosome membrane microdomains and inhibit phagosome–lysosome fusion (Arellano-Reynoso *et al*., 2005), raising the possibility that this common aspect of their survival strategies might play a key role in the early establishment of both microorganisms in their hosts.

*Leishmania* and *Brucella* also establish parasitism by delaying entry of their host cells into apoptosis (Gross *et al*., 2000; Aga *et al*., 2002). In our study, only WT *L. donovani* parasites were able to induce a delay of apoptosis. The lpg2–/– mutant, which is unable to persist in non-lytic/ER-derived compartments, had no such effect on neutrophil apoptosis. We have shown previously that molecules involved in apoptotic and antiapoptotic signalling are present on latex bead-containing phagosomes isolated from J774 macrophages, and proposed that microorganisms might have evolved ways to use these molecules to prevent apoptosis and maintain the integrity of their replicative niches (Garin *et al*., 2001). Interestingly, several molecules known to be involved in apoptotic signalling accumulate in phagosome membrane microdomains (G. Goyette and M. Desjardins, unpub. data), suggesting that this location might be important to their specific function. As *L. donovani* was shown to use its LPG to disrupt phagosome microdomains, it may well be that this disruptive process is involved in the ability of *Leishmania* to inhibit neutrophil apoptosis.

After a delayed period of time, *L. donovani*-loaded neutrophils entered apoptosis. Despite the fact that some of these neutrophils were highly degraded, they, nevertheless, contained intact parasites. In mammals, dying neutrophils are cleared mainly by macrophages (Savill *et al*., 1992). In our system, we observed that apoptotic neutrophils loaded with parasites could be engulfed by primary macrophages. Senescent neutrophils and inflammatory monocytes have been shown to home to the bone marrow (Martin *et al*., 2003; Varol *et al*., 2007). Thus, phagocytosis of parasite-loaded neutrophils, conceived as a shuttling process, would enable the transfer of live parasites to macrophages where subsequent differentiation and replication would occur (Laskay *et al*., 2003; van Zandbergen *et al*., 2004). This may contribute to the ability of *L. donovani* to disseminate from the skin to distant tissues, such as the bone marrow, where stromal macrophages intimately associate with mature neutrophils (Cotterell *et al*., 2000).

Altogether, our results support a model according to which *Leishmania* parasites are able to avoid trafficking to lysosome-derived organelles in neutrophils and actively establish a safe niche in a compartment displaying some features of the ER (non-lytic, G-6-Pase+ and calnexin+). The extent to which ER membranes contribute to this compartment remains to be established. Although the contribution of ER to phagosomes is a controversial issue

 

(Touret *et al*., 2005b), recent reports have clearly demonstrated the presence of key ER fusion molecules on phagosomes, and their involvement in phagocytosis (Hatsuzawa *et al*., 2006), as well as the involvement of the ER retrotranslocation machinery in the ability of phagosomes to participate in the cross-presentation of exogenous antigens (Ackerman *et al*., 2006). Hence, promastigote *Leishmania* parasites may have co-evolved with their mammalian hosts to take advantage of phagosomes–ER interaction to establish a privileged niche for the transient parasitism of leukocytes and their subsequent invasion of macrophages.

## Experimental procedures

### *Cells*

Dog neutrophils were isolated from peripheral blood using standard protocols (Gagnon *et al*., 2002). Mouse neutrophils were purified from the bone marrow of C57BL6 mice, as described previously (Cowland and Borregaard, 1999). The mature Neutrophils Ly-6G (Gr-1)$^{high}$ positive (PE-conjugated monoclonal antibody clone RB6-8C5, BD Biosciences) were isolated by cell sorting on a BD FACS Aria cell sorter (BD Biosciences, San Jose, CA). The purity and the viability of neutrophils after sorting was greater than 99%. Bone marrow-derived mouse macrophages were prepared using 8- to 12-week-old C57BL/6 mice, as described previously (Flamant *et al*., 2003).

### *Parasites*

Wild-type Sudanese strain 1S *L. donovani* promastigotes and LPG-defective mutants *lpg1–/–* and *lpg2–/–* (Ryan *et al*., 1993) were grown at 26°C in modified M199 medium, as described previously (Desjardins and Descoteaux, 1997). Promastigotes were grown to stationary phase unless otherwise noted. Early log growth promastigotes were harvested at concentrations of $5 \times 10^6$ parasites ml$^{-1}$. Enriched metacyclic promastigotes suspensions were obtained using a Ficoll gradient centrifugation-based approach (Spath and Beverley, 2001). The morphology of purified parasites was analysed after Giemsa staining. The moving rate of parasites was measured using an inverted Leica microscope and Northern Eclipse software. For the neutrophil apoptosis experiments, promastigotes from stationary growth phase were stained with the intracytoplasmic dye 5-6-carboxyfluorescein diacetate succinimidyl ester, using established protocols (Kamau *et al*., 2000).

### *Addition of parasites to neutrophils*

Purified neutrophils ($4 \times 10^6$ ml$^{-1}$) were co-incubated with parasites at 37°C (parasite-to-PMN ratio of 6:1) in RPMI 1640 medium (Gibco Laboratories) supplemented with 2 mM L-glutamine, 100 U ml$^{-1}$ penicillin and 100 μg ml$^{-1}$ streptomycin. After three washes and centrifugation at 200 *g* to remove extracellular parasites, cells were incubated in complete medium supplemented with G-CSF (20 ng ml$^{-1}$) for the assessment of parasite survival. In designated survival experiments, neutrophils exposed to parasites were sorted a second time to retrieve all the non-internalized parasites.

### *Neutrophil apoptosis monitoring*

The percentage of cells in the early stage of apoptosis based on AnnexinV-APC-positive staining and 7-Amino-Actinomycin D-negative incorporation was evaluated. Labelled cells were analysed by flow cytometry using a BD LSRII with Diva software.

### *Phagocytosis of parasite-loaded neutrophils by autologous macrophages*

Neutrophils were co-incubated with parasites for 30 min followed by several washes and an 18 h chase. Primary mouse macrophages were co-incubated with parasite-loaded neutrophils for 1 h at 37°C (neutrophil-to-macrophage ratio of 10:1). The uptake of parasite-loaded apoptotic neutrophils by macrophages was visualized by EM.

### *Immunofluorescence analyses*

Mouse neutrophils were co-incubated with WT parasites or *lpg2–/–*-defective mutant in various conditions. The following primary antibodies were used: a hamster polyclonal antibody raised against *L. donovani* (a kind gift of Albert Descoteaux, INRS Institut Armand Frappier, Canada), a rabbit polyclonal antibody raised against calnexin (a kind gift of John J.M. Bergeron, Mc Gill University, Canada), a monoclonal rat anti-LAMP1 (Developmental Studies Hybridoma Bank, Department of Pharmacology and Molecular Sciences, the Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biological Sciences, University of Iowa, Iowa City, IA, under contract N01-HD-6-2915 from the NICHD). The percentage of phagosomes positive for calnexin or LAMP1 was determined after counting 200 phagosomes in randomly selected fields, using methods previously described (Scianimanico *et al*., 1999; Dermine *et al*., 2001).

### *EM analyses*

Neutrophils co-incubated with parasites were pelleted and G-6-Pase activity was detected using EM cytochemistry (Griffiths *et al*., 1983), as described previously (Gagnon *et al*., 2002). A phagosome was considered G-6-Pase+ when parasites were surrounded by membrane displaying one or more large dark deposit(s) of G-6-Pase activity reaction product. Only neutrophils displaying a strong labelling of the nuclear envelope were analysed. A phagosome was described as 'tight' when the membrane was directly in contact with the parasite surface. Quantitative analyses were performed. For each experiment, at least 100 phagosomes were counted.

### *Endosome/phagosome fusion assay*

To label lysosomes, sorted neutrophils were incubated with 16 nm BSA-gold particles for 15 min followed by a 60 min chase

at 37°C (Dermine *et al*., 2000; Duclos *et al*., 2003). BSA-gold-loaded neutrophils were co-incubated with parasites for 30 min. Phagosomes were analysed for the presence of G-6-Pase activity by EM. A fusion event between lysosomes and phagosomes was based on the presence of one or more gold particles inside phagosomes. At least 100 phagosomes were analysed for each sample.

### *Assessment of parasite survival in neutrophils using luciferase-expressing* L. donovani *parasites*

Sorted neutrophils were co-incubated with luciferase-expressing parasites for 30 min and washed six times, as described (Duclos *et al*., 2000). Survival rates were determined 0, 3, 12, 18 and 24 h post parasite addition by measuring luciferase activity in total-cell extracts. Results are expressed as percentages of relative luminescence units compared with the maximum level of luminescence observed after the initial 30 min of contact between neutrophils and parasites.

### *Statistics*

Statistical analysis was performed using the impaired Student's *t*-test.

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## References

Ackerman, A.L., Giodini, A., and Cresswell, P. (2006) A role for the endoplasmic reticulum protein retrotranslocation machinery during crosspresentation by dendritic cells. *Immunity* **25:** 607–617.

Aga, E., Katschinski, D.M., van Zandbergen, G., Laufs, H., Hansen, B., Muller, K., *et al.* (2002) Inhibition of the spontaneous apoptosis of neutrophil granulocytes by the intracellular parasite *Leishmania major*. *J Immunol* **169:** 898–905.

Antoine, J.C., Prina, E., Courret, N., and Lang, T. (2004) *Leishmania* spp. on the interactions they establish with antigen-presenting cells of their mammalian hosts. *Adv Parasitol* **58:** 1–68.

Arellano-Reynoso, B., Lapaque, N., Salcedo, S., Briones, G., Ciocchini, A.E., Ugalde, R., *et al.* (2005) Cyclic beta-1,2-glucan is a *Brucella* virulence factor required for intracellular survival. *Nat Immunol* **6:** 618–625.

Belkaid, Y., Kamhawi, S., Modi, G., Valenzuela, J., Noben-Trauth, N., Rowton, E., *et al.* (1998) Development of a natural model of cutaneous leishmaniasis: powerful effects of vector saliva and saliva preexposure on the long-term outcome of *Leishmania major* infection in the mouse ear dermis. *J Exp Med* **188:** 1941–1953.

Belkaid, Y., Valenzuela, J.G., Kamhawi, S., Rowton, E., Sacks, D.L., Ribeiro, J.M., *et al.* (2000) Delayed-type hypersensitivity to *Phlebotomus papatasi* sand fly bite: an adaptive response induced by the fly? *Proc Natl Acad Sci USA* **97:** 6704–6709.

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D.S., *et al.* (2004) Neutrophil extracellular traps kill bacteria. *Science* **303:** 1532–1535.

Burlak, C., Whitney, A.R., Mead, D.J., Hackstadt, T., and Deleo, F.R. (2006) Maturation of human neutrophil phagosomes includes incorporation of molecular chaperones and endoplasmic reticulum quality control machinery. *Mol Cell Proteomics* **5:** 620–634.

Celli, J., and Gorvel, J.P. (2004) Organelle robbery: *Brucella* interactions with the endoplasmic reticulum. *Curr Opin Microbiol* **7:** 93–97.

Celli, J., de Chastellier, C., Franchini, D.M., Pizarro-Cerda, J., Moreno, E., and Gorvel, J.P. (2003) *Brucella* evades macrophage killing via VirB-dependent sustained interactions with the endoplasmic reticulum. *J Exp Med* **198:** 545–556.

Cotterell, S.E., Engwerda, C.R., and Kaye, P.M. (2000) *Leishmania donovani* infection of bone marrow stromal macrophages selectively enhances myelopoiesis, by a mechanism involving GM-CSF and TNF-alpha. *Blood* **95:** 1642–1651.

Cougoule, C., Constant, P., Etienne, G., Daffe, M., and Maridonneau-Parini, I. (2002) Lack of fusion of azurophil granules with phagosomes during phagocytosis of *Mycobacterium smegmatis* by human neutrophils is not actively controlled by the bacterium. *Infect Immun* **70:** 1591–1598.

Cowland, J.B., and Borregaard, N. (1999) Isolation of neutrophil precursors from bone marrow for biochemical and transcriptional analysis. *J Immunol Methods* **232:** 191–200.

Dermine, J.F., Scianimanico, S., Prive, C., Descoteaux, A., and Desjardins, M. (2000) *Leishmania* promastigotes require lipophosphoglycan to actively modulate the fusion properties of phagosomes at an early step of phagocytosis. *Cell Microbiol* **2:** 115–126.

Dermine, J.F., Duclos, S., Garin, J., St Louis, F., Rea, S., Parton, R.G., *et al.* (2001) Flotillin-1-enriched lipid raft domains accumulate on maturing phagosomes. *J Biol Chem* **276:** 18507–18512.

Dermine, J.F., Goyette, G., Houde, M., Turco, S.J., and Desjardins, M. (2005) *Leishmania donovani* lipophosphoglycan disrupts phagosome microdomains in J774 macrophages. *Cell Microbiol* **7:** 1263–1270.

Desjardins, M., and Descoteaux, A. (1997) Inhibition of phagolysosomal biogenesis by the *Leishmania* lipophosphoglycan. *J Exp Med* **185:** 2061–2068.

Duclos, S., Diez, R., Garin, J., Papadopoulou, B., Descoteaux, A., Stenmark, H., and Desjardins, M. (2000) Rab5 regulates the kiss and run fusion between phagosomes and endosomes and the acquisition of phagosome

 

leishmanicidal properties in RAW 264.7 macrophages. *J Cell Sci* **113** (Part 19)**:** 3531–3541.

Duclos, S., Corsini, R., and Desjardins, M. (2003) Remodeling of endosomes during lysosome biogenesis involves 'kiss and run' fusion events regulated by rab5. *J Cell Sci* **116:** 907–918.

Flamant, S., Lebastard, M., Pescher, P., Besmond, C., Milon, G., and Marchal, G. (2003) Enhanced cloning efficiency of mouse bone marrow macrophage progenitors correlates with increased content of CSF-1 receptor of their progeny at low oxygen tension. *Microbes Infect* **5:** 1064–1069.

Fortier, A., de Chastellier, C., Balor, S., and Gros, P. (2007) Birc1e/Naip5 rapidly antagonizes modulation of phagosome maturation by *Legionella pneumophila*. *Cell Microbiol* **9:** 910–923.

Gagnon, E., Duclos, S., Rondeau, C., Chevet, E., Cameron, P.H., Steele-Mortimer, O., *et al.* (2002) Endoplasmic reticulum-mediated phagocytosis is a mechanism of entry into macrophages. *Cell* **110:** 119–131.

Garin, J., Diez, R., Kieffer, S., Dermine, J.F., Duclos, S., Gagnon, E., *et al.* (2001) The phagosome proteome: insight into phagosome functions. *J Cell Biol* **152:** 165–180.

Griffiths, G., Quinn, P., and Warren, G. (1983) Dissection of the Golgi complex. I. Monensin inhibits the transport of viral membrane proteins from medial to trans Golgi cisternae in baby hamster kidney cells infected with Semliki Forest virus. *J Cell Biol* **96:** 835–850.

Gross, A., Terraza, A., Ouahrani-Bettache, S., Liautard, J.P., and Dornand, J. (2000) *In vitro Brucella suis* infection prevents the programmed cell death of human monocytic cells. *Infect Immun* **68:** 342–351.

Hatsuzawa, K., Tamura, T., Hashimoto, H., Hashimoto, H., Yokoya, S., Miura, M., *et al.* (2006) Involvement of syntaxin 18, an endoplasmic reticulum (ER)-localized SNARE protein, in ER-mediated phagocytosis. *Mol Biol Cell* **17:** 3964–3977.

Henson, P.E., Henson, J.E., Fittschen, C., Bratton, D.L., and Riches, D.W.H. (1992) Degranulation and secretion by phagocytic cells. In *Inflammation: Basic Principles and Clinical Correlates*, 2nd edn. Gallin, J.I., and Snyderman, R. (eds). New York: Raven Press, pp. 511–539.

Herwaldt, B.L. (1999) Leishmaniasis. *Lancet* **354:** 1191–1199.

Kamau, S.W., Hurtado, M., Muller-Doblies, U.U., Grimm, F., and Nunez, R. (2000) Flow cytometric assessment of allopurinol susceptibility in *Leishmania infantum* promastigote. *Cytometry* **40:** 353–360.

Laskay, T., van Zandbergen, G., and Solbach, W. (2003) Neutrophil granulocytes – Trojan horses for *Leishmania major* and other intracellular microbes? *Trends Microbiol* **11:** 210–214.

Laufs, H., Muller, K., Fleischer, J., Reiling, N., Jahnke, N., Jensenius, J.C., *et al.* (2002) Intracellular survival of *Leishmania major* in neutrophil granulocytes after uptake in the absence of heat-labile serum factors. *Infect Immun* **70:** 826–835.

Lodge, R., and Descoteaux, A. (2005) Modulation of phagolysosome biogenesis by the lipophosphoglycan of *Leishmania*. *Clin Immunol* **114:** 256–265.

Lodge, R., Diallo, T.O., and Descoteaux, A. (2006) *Leishmania donovani* lipophosphoglycan blocks NADPH oxidase assembly at the phagosome membrane. *Cell Microbiol* **8:** 1922–1931.

Martin, C., Burdon, P.C., Bridger, G., Gutierrez-Ramos, J.C., Williams, T.J., and Rankin, S.M. (2003) Chemokines acting via CXCR2 and CXCR4 control the release of neutrophils from the bone marrow and their return following senescence. *Immunity* **19:** 583–593.

Melo, E.J., and de Souza, W. (1997) Relationship between the host cell endoplasmic reticulum and the parasitophorous vacuole containing *Toxoplasma gondii*. *Cell Struct Funct* **22:** 317–323.

Muller, K., van Zandbergen, G., Hansen, B., Laufs, H., Jahnke, N., Solbach, W., and Laskay, T. (2001) Chemokines, natural killer cells and granulocytes in the early course of *Leishmania major* infection in mice. *Med Microbiol Immunol (Berl)* **190:** 73–76.

Murray, H.W., Berman, J.D., Davies, C.R., and Saravia, N.G. (2005) Advances in leishmaniasis. *Lancet* **366:** 1561–1577.

Nathan, C. (2006) Neutrophils and immunity: challenges and opportunities. *Nat Rev Immunol* **6:** 173–182.

Ribeiro, J.M. (1987) Role of saliva in blood-feeding by arthropods. *Annu Rev Entomol* **32:** 463–478.

Robinson, C.G., and Roy, C.R. (2006) Attachment and fusion of endoplasmic reticulum with vacuoles containing *Legionella pneumophila*. *Cell Microbiol* **8:** 793–805.

Ryan, K.A., Garraway, L.A., Descoteaux, A., Turco, S.J., and Beverley, S.M. (1993) Isolation of virulence genes directing surface glycosylphosphatidylinositol synthesis by functional complementation of Leishmania. *Proc Natl Acad Sci USA* **90:** 8609–8613.

Sacks, D., and Kamhawi, S. (2001) Molecular aspects of parasite–vector and vector–host interactions in leishmaniasis. *Annu Rev Microbiol* **55:** 453–483.

Savill, J., Hogg, N., Ren, Y., and Haslett, C. (1992) Thrombospondin cooperates with CD36 and the vitronectin receptor in macrophage recognition of neutrophils undergoing apoptosis. *J Clin Invest* **90:** 1513–1522.

Schlein, Y., Jacobson, R., and Messer, G. (1992) *Leishmania* infections damage the feeding mechanism of the sandfly vector and implement parasite transmission by bite. *Proc Natl Acad Sci USA* **89:** 9944–9948.

Scianimanico, S., Desrosiers, M., Dermine, J.F., Meresse, S., Descoteaux, A., and Desjardins, M. (1999) Impaired recruitment of the small GTPase rab7 correlates with the inhibition of phagosome maturation by *Leishmania donovani* promastigotes. *Cell Microbiol* **1:** 19–32.

Spath, G.F., and Beverley, S.M. (2001) A lipophosphoglycan-independent method for isolation of infective *Leishmania* metacyclic promastigotes by density gradient centrifugation. *Exp Parasitol* **99:** 97–103.

Sunderkotter, C., Kunz, M., Steinbrink, K., Meinardus-Hager, G., Goebeler, M., Bildau, H., *et al.* (1993) Resistance of mice to experimental leishmaniasis is associated with more rapid appearance of mature macrophages *in vitro* and *in vivo*. *J Immunol* **151:** 4891–4901.

Tacchini-Cottier, F., Zweifel, C., Belkaid, Y., Mukankundiye, C., Vasei, M., Launois, P., *et al.* (2000) An immunomodulatory function for neutrophils during the induction of a CD4+ Th2 response in BALB/c mice infected with *Leishmania major*. *J Immunol* **165:** 2628–2636.

Touret, N., Paroutis, P., and Grinstein, S. (2005a) The nature of the phagosomal membrane: endoplasmic reticulum versus plasmalemma. *J Leukoc Biol* **77:** 878–885.

Touret, N., Paroutis, P., Terebiznik, M., Harrison, R.E., Trombetta, S., Pypaert, M., *et al.* (2005b) Quantitative and dynamic assessment of the contribution of the ER to phagosome formation. *Cell* **123:** 157–170.

Urban, C.F., Lourido, S., and Zychlinsky, A. (2006) How do microbes evade neutrophil killing? *Cell Microbiol* **8:** 1687–1696.

Varol, C., Landsman, L., Fogg, D.K., Greenshtein, L., Gildor, B., Margalit, R., *et al.* (2007) Monocytes give rise to mucosal, but not splenic, conventional dendritic cells. *J Exp Med* **204:** 171–180.

Wilson, M.E., Innes, D.J., Sousa, A.D., and Pearson, R.D. (1987) Early histopathology of experimental infection with *Leishmania donovani* in hamsters. *J Parasitol* **73:** 55–63.

Wilson, M.E., Jeronimo, S.M., and Pearson, R.D. (2005) Immunopathogenesis of infection with the visceralizing *Leishmania* species. *Microb Pathog* **38:** 147–160.

van Zandbergen, G., Klinger, M., Mueller, A., Dannenberg, S., Gebert, A., Solbach, W., *et al.* (2004) Cutting edge: neutrophil granulocyte serves as a vector for *Leishmania* entry into macrophages. *J Immunol* **173:** 6521–6525.

 