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FUNGAL AND PARASITIC INFECTIONS

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

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

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

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

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

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

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

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

## RESULTS

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

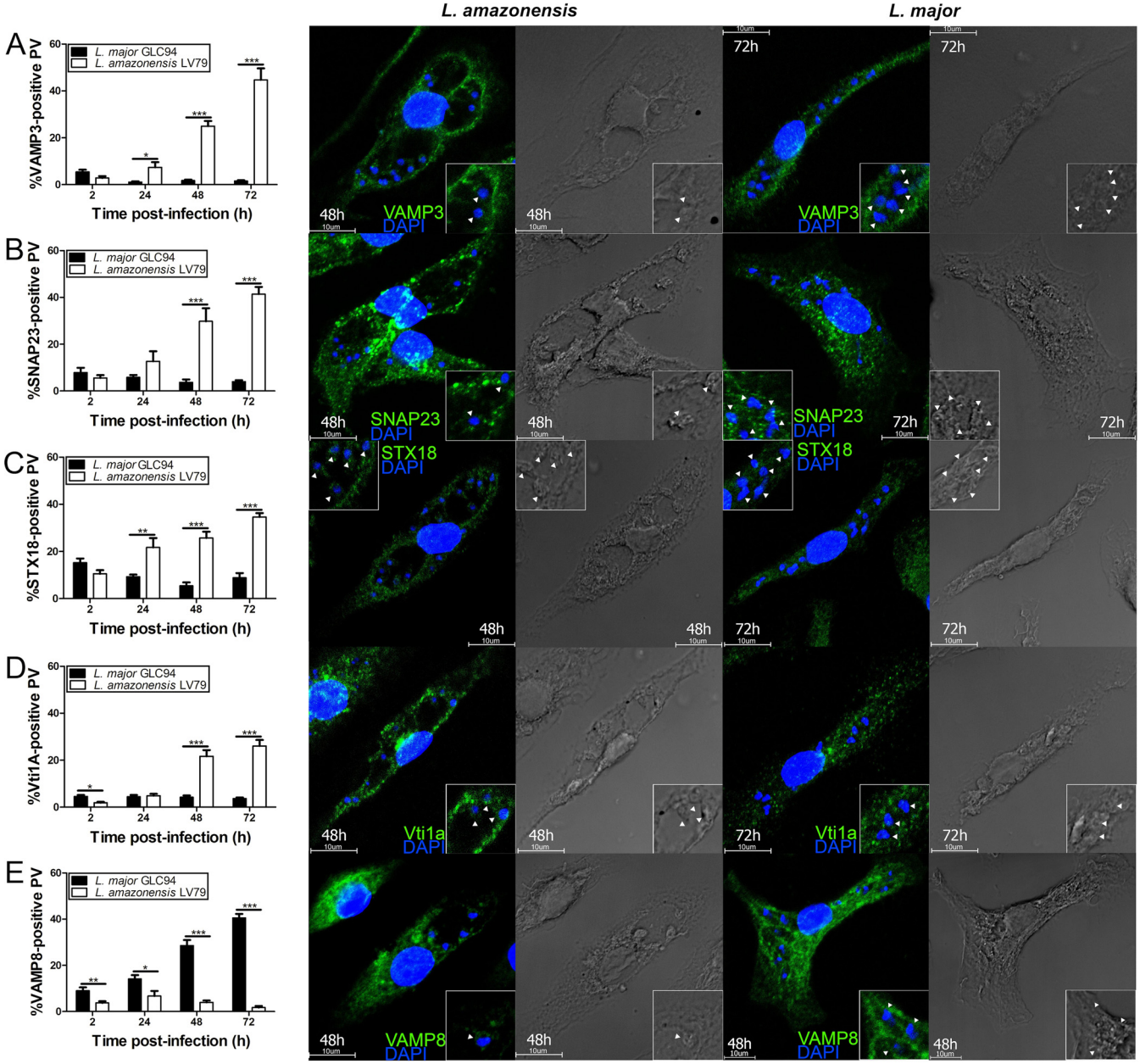


**FIG 1** Differential recruitment of SNAREs to *Leishmania*-harboring PVs. BMMs were infected with *L. major* GLC94 or *L. amazonensis* LV79 promastigotes, and the presence of VAMP3 (A), SNAP23 (B), STX18 (C), Vti1A (D), and VAMP8 (E) in PVs was assessed and quantified by confocal immunofluorescence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

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

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

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

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

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

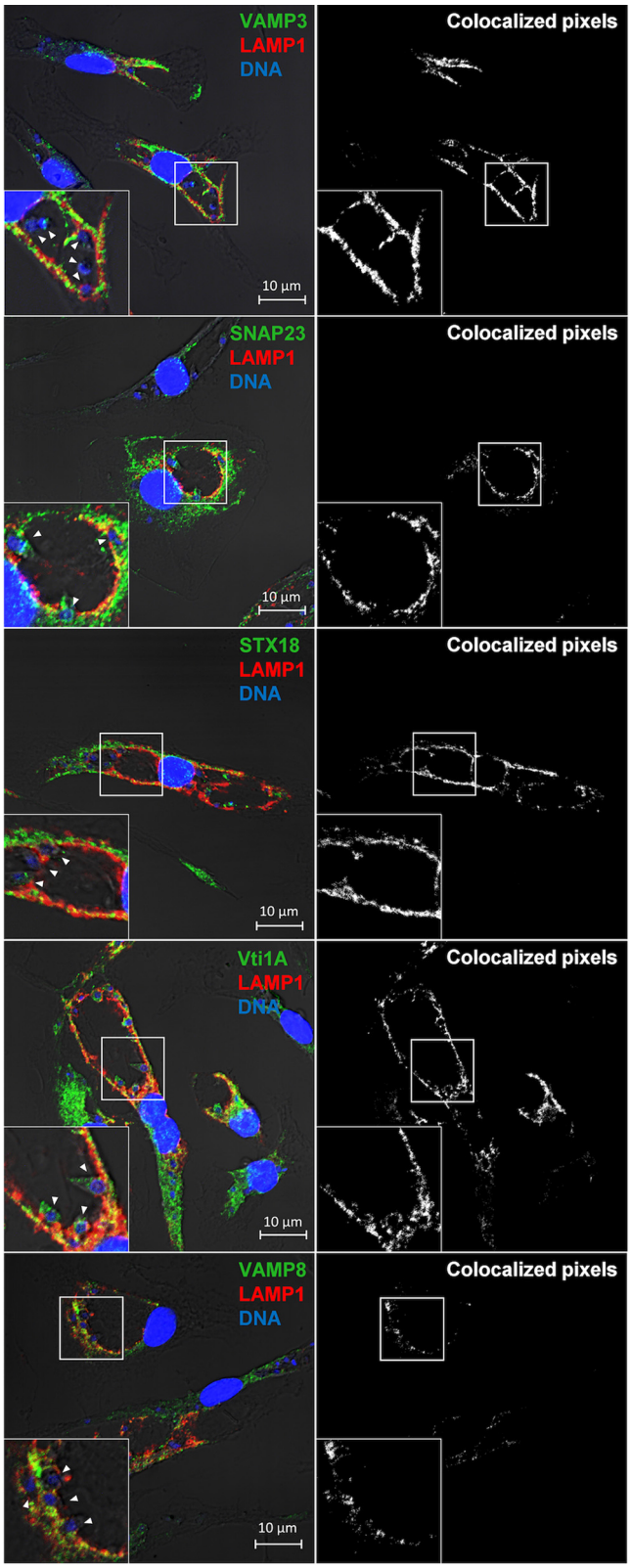


**FIG 2** Colocalization of SNAREs with LAMP1 on the membranes of communal PVs harboring *L. amazonensis*. BMMs were infected with *L. amazonensis* LV79 promastigotes, and at 72 h postphagocytosis, the colocalization (white pixels) of LAMP1 (red) with the SNAREs VAMP3, SNAP23, STX18, Vti1A, and VAMP8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

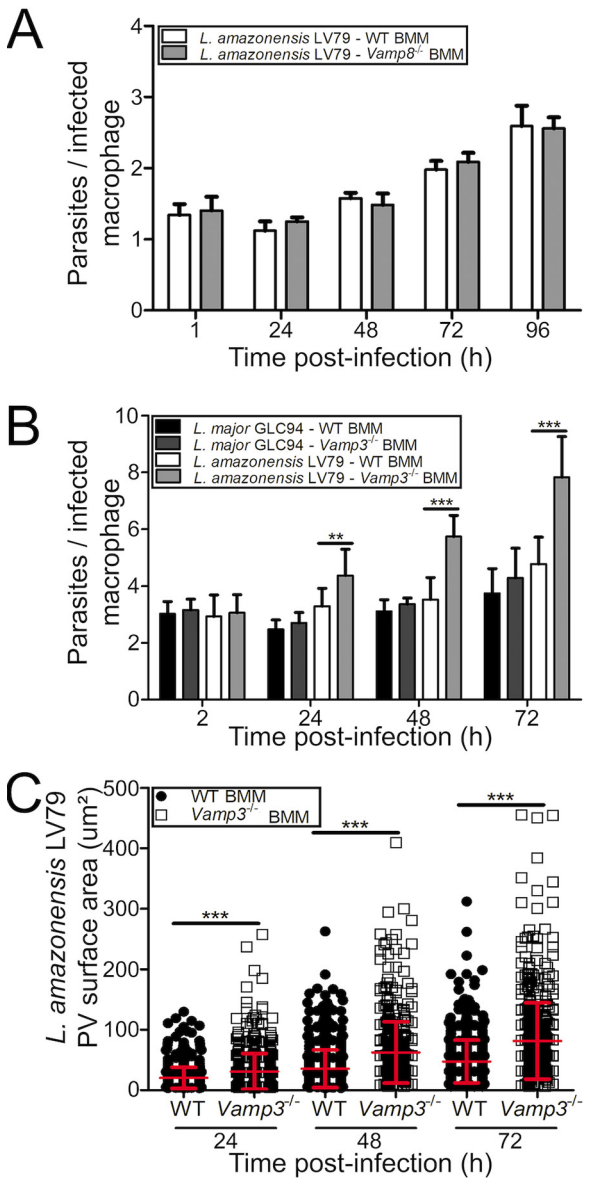


**FIG 3** VAMP3 negatively regulates the replication of *L. amazonensis* LV79 and PV expansion. WT, *Vamp8*$^{-/-}$, and *Vamp3*$^{-/-}$ BMMs were infected with *L. major* GLC94 or *L. amazonensis* LV79 promastigotes, and at various time points after phagocytosis, parasite replication and PV size were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

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

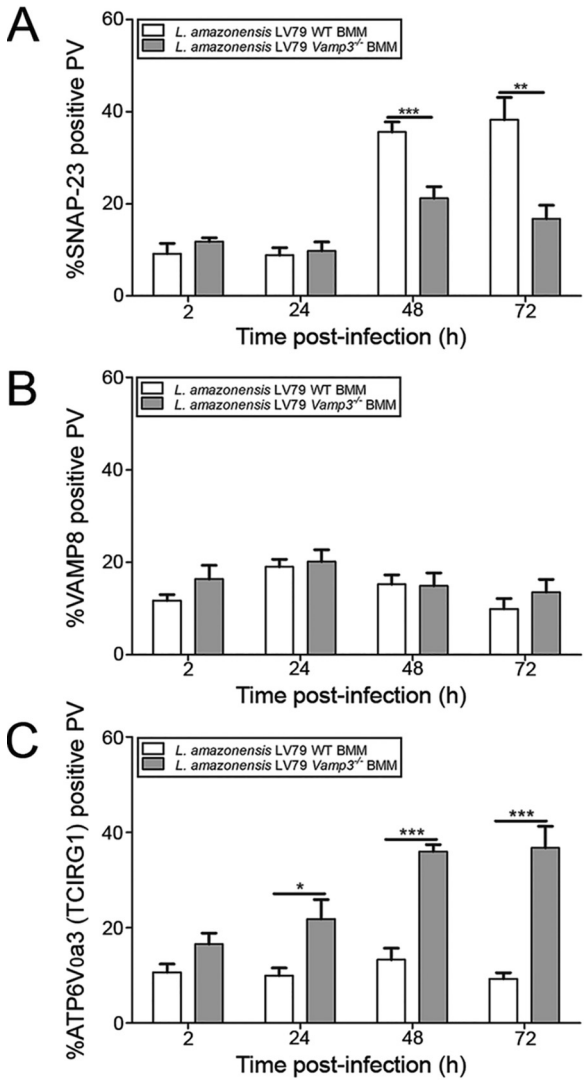


**FIG 4** The absence of VAMP3 alters the recruitment of SNAP23 and TCIRG1 to PVs harboring *L. amazonensis* LV79. WT and *Vamp3*$^{-/-}$ BMMs were infected with *L. amazonensis* LV79 promastigotes, and the presence of SNAP23 (A), VAMP8 (B), and ATP6V0a3 (C) in PVs was assessed and quantified by confocal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

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

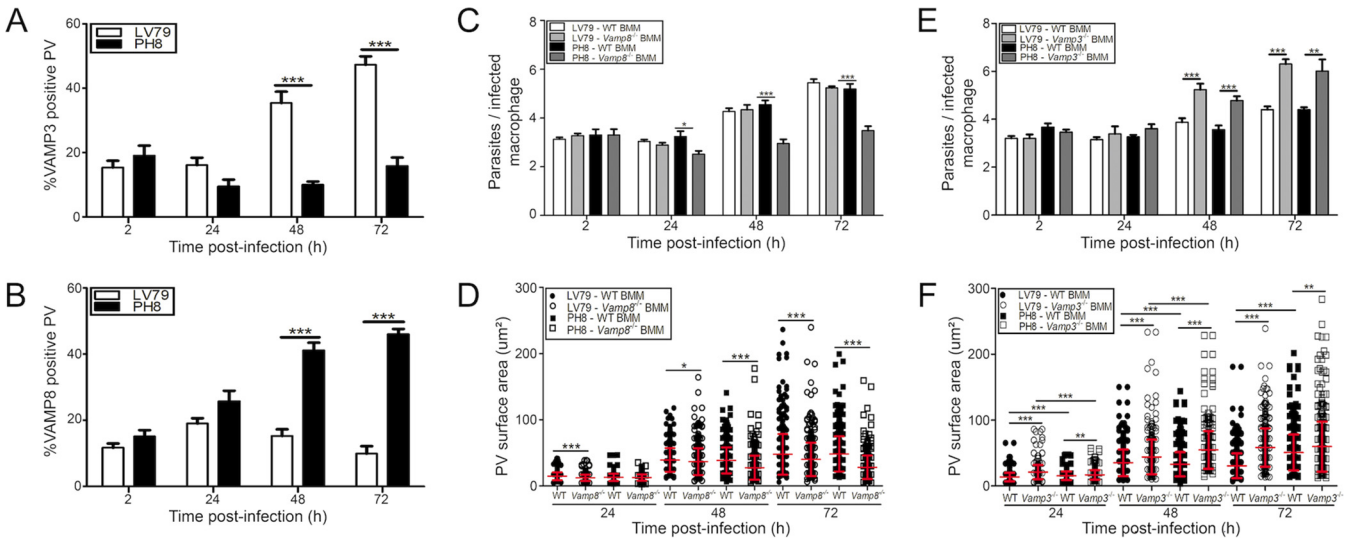


**FIG 5** Strain-specific differences in the recruitment of VAMP3 and VAMP8 to PVs harboring *L. amazonensis*. (A and B) WT BMMs were infected with either *L. amazonensis* LV79 or *L. amazonensis* PH8 promastigotes, and the presence of VAMP3 (A) and VAMP8 (B) was assessed and quantified by confocal immunofluorescence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

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

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

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

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

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

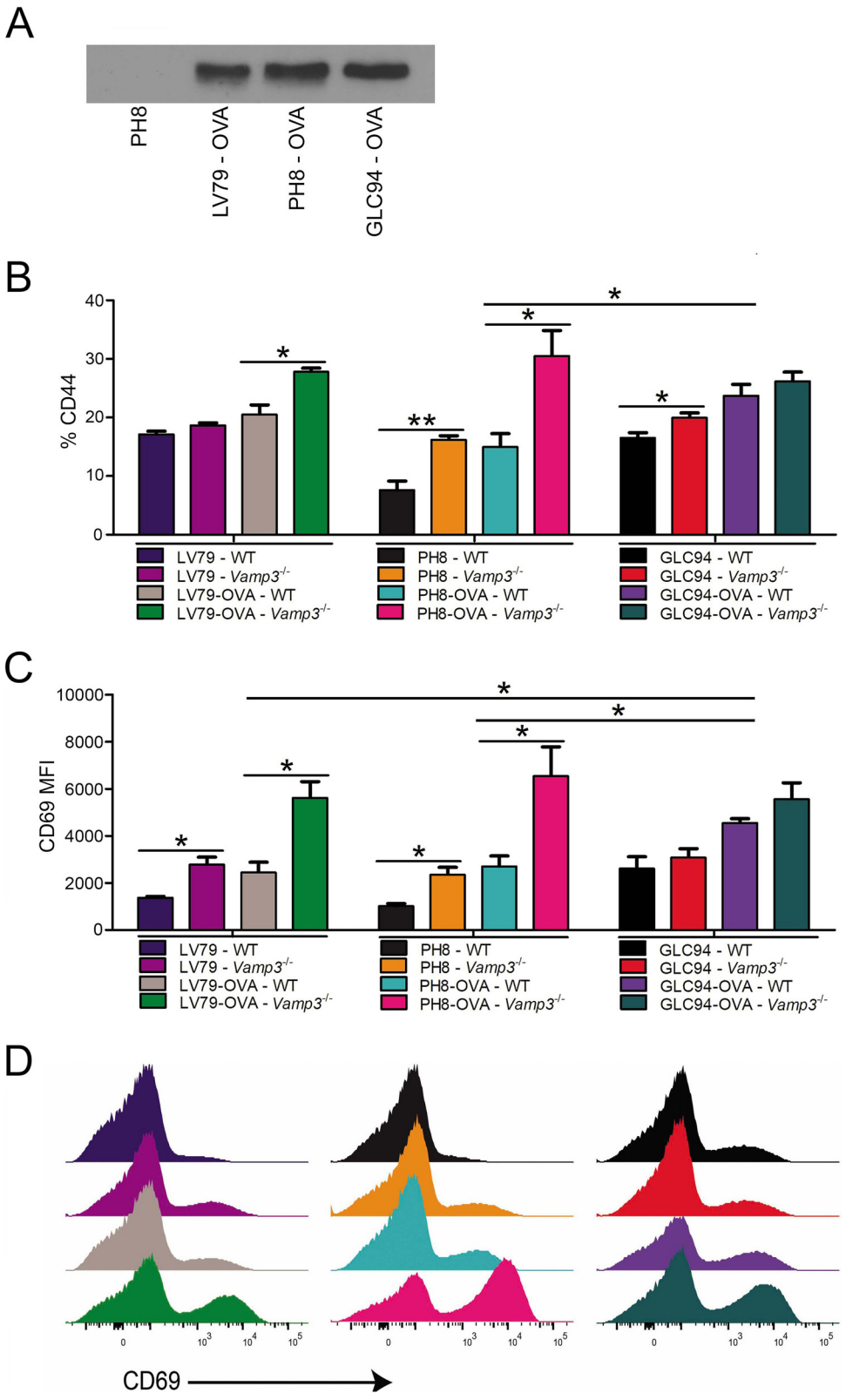


**FIG 6** VAMP3 negatively regulates antigen cross-presentation from communal PVs harboring *L. amazonensis*. (A) The expression of ovalbumin by *L. amazonensis*-OVA LV79, *L. amazonensis*-OVA PH8, and *L. major*-OVA GLC94 was assessed by Western blotting. A blot representative of results from two experiments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

(Continued on next page)

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

## DISCUSSION

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

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

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

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

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

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

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

## MATERIALS AND METHODS

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

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

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

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

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

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

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

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

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 3 MB.

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We declare no conflicts of interest.

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