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1	Cysteine Peptidase B Regulates <i>Leishmania mexicana</i> Virulence
2	through the Modulation of GP63 Expression
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ABSTRACT

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Cysteine peptidases play a central role in the biology of *Leishmania*. In this work, we sought to 28 further elucidate the mechanism(s) by which the cysteine peptidase CPB contributes to L. 29 mexicana virulence and whether CPB participates in the formation of large communal 30 parasitophorous vacuoles induced by these parasites. We initially examined the impact of L. 31 mexicana infection on the trafficking of VAMP3 and VAMP8, two endocytic SNARE proteins 32 associated with phagolysosome biogenesis and function. Using a CPB-deficient mutant, we 33 found that both VAMP3 and VAMP8 were down-modulated in a CPB-dependent manner. We 34 also discovered that expression of the virulence-associated GPI-anchored metalloprotease GP63 35 was inhibited in the absence of CPB. Expression of GP63 in the CPB-deficient mutant was 36 sufficient to down-modulate VAMP3 and VAMP8. Similarly, episomal expression of GP63 37 38 enabled the CPB-deficient mutant to establish infection in macrophages, induce the formation of large communal parasitophorous vacuoles, and cause lesions in mice. These findings implicate 39 CPB in the regulation of GP63 expression and provide evidence that both GP63 and CPB are key 40 41 virulence factors in L. mexicana.

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AUTHOR SUMMARY

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The parasite Leishmania mexicana expresses several cysteine peptidases of the papain family that 46 are involved in processes such as virulence and evasion of host immune responses. The cysteine 47 peptidase CPB is required for survival within macrophages and for lesion formation in 48 susceptible mice. Upon their internalization by macrophages, parasites of the L. mexicana 49 complex induce the formation of large communal parasitophorous vacuoles in which they 50 51 replicate, and expansion of those large vacuoles correlates with the ability of the parasites to survive inside macrophages. Here, we found that CPB contributes to L. mexicana virulence 52 (macrophage survival, formation and expansion of communal parasitophorous vacuoles, lesion 53 formation in mice) through the regulation of the virulence factor GP63, a Leishmania zinc-54 metalloprotease that acts by cleaving key host cell proteins. This work thus elucidates a novel 55 Leishmania virulence regulatory mechanism whereby CPB controls the expression of GP63. 56

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INTRODUCTION

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The protozoan Leishmania parasitizes macrophages and causes a spectrum of human 62 diseases ranging from self-healing cutaneous lesions to a progressive visceral infection that can 63 be fatal if left untreated. Infection is initiated when promastigote forms of the parasite are 64 inoculated into the mammalian host by infected sand flies and are internalized by phagocytes. 65 Inside macrophages, promastigotes differentiate into amastigotes to replicate within 66 phagolysosomal compartments also known as parasitophorous vacuoles (PVs). Upon their 67 internalization, L. donovani and L. major promastigotes arrest phagolysosomal biogenesis and 68 69 create an intracellular niche favorable to the establishment of infection and to the evasion of the 70 immune system [1, 2]. Disruption of the macrophage membrane fusion machinery through the action of virulence factors plays an critical role in this PV remodeling. Hence, insertion of the 71 72 promastigote surface glycolipid lipophosphoglycan (LPG) into the PV membrane destabilizes lipid microdomains and causes exclusion of the membrane fusion regulator synaptotagmin V 73 from the PV [2-4]. Similarly, the parasite GPI-anchored metalloprotease GP63 [5, 6] 74 redistributes within the infected cells and cleaves key Soluble NSF Attachment Protein Receptors 75 (SNAREs) and synaptotagmins to impair phagosome functions [1, 7]. 76

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Whereas *L. major* and *L. donovani* multiply in tight individual PVs, parasites of the *L. mexicana* complex (*L. mexicana, L. amazonensis*) replicate within large communal PVs. Relatively little is known about the host and parasite factors involved in the biogenesis and expansion of those communal PVs. Studies with *L. amazonensis* revealed that phagosomes containing promastigotes fuse extensively with late endosomes/lysosomes within 30 minutes post-infection [8]. At that stage, parasites are located within small individual compartments and

by 18 to 24 hours large PVs containing several parasites are observed. The rapid increase in the 84 size of those PVs requires extensive fusion with secondary lysosomes and correlates with the 85 depletion of those organelles in infected cells [9-11]. Homotypic fusion between L. 86 amazonensis-containing PVs also occurs, but its contribution to PV enlargement remains to be 87 further investigated [12]. These studies highlighted the contribution of the host cell membrane 88 fusion machinery in the biogenesis and expansion of large communal PVs and are consistent with 89 a role for endocytic SNAREs in this process [13]. Interestingly, communal PVs interact with the 90 host cell's endoplasmic reticulum (ER) and disruption of the fusion machinery associated with 91 92 the ER and Golgi inhibits parasite replication and PV enlargement [14-16].

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94 The Leishmania-derived molecules involved in the expansion of the communal PVs 95 remains to be identified. LPG and other phosphoglycans do not play a significant role in L. 96 mexicana promastigote virulence and PV formation [17], in contrast to L. major and L. donovani 97 [2]. Cysteine peptidases (CP) are a large family of papain-like enzymes that play important roles 98 in the biology of Leishmania [18]. Three members of these papain-like proteases are expressed 99 by L. mexicana and the generation of CP-deficient mutants revealed that CPB contributes to the ability to infect macrophages and to induce lesions in BALB/c mice [19-21]. The precise 100 101 mechanism(s) by which CPB participates in the virulence of L. mexicana is poorly understood. Previous studies revealed that CPB traffics within and outside infected macrophages [18]. In 102 103 infected macrophages, CPB alters signal transduction and gene expression through the activation 104 of the protein tyrosine phosphatase PTP-1B and the cleavage of transcription factors responsible for the expression of genes involved in host defense and immunity [20, 22]. The observation that 105 CPs interfere with the host immune response through the degradation of MHC class II molecules 106

107	and invariant chains present in PVs housing L. amazonensis [23], raises the possibility that CPB
108	participates in the modulation of PV composition and function.

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110	In this study, we sought to gain insight into the mechanism by which CPB contributes to
111	L. mexicana virulence, with a focus on the PV. We provide evidence that CPB participates in PV
112	biogenesis and virulence through the regulation of GP63 expression.

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RESULTS

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CPB enables L. mexicana to down-modulate VAMP3 and VAMP8. Formation and expansion 116 of communal PVs hosting L. mexicana involve fusion between PVs and endocytic organelles, as 117 118 well as homotypic fusion among PVs [10-12]. To identify the host and parasite factors involved in this process, we embarked on a study to elucidate the fate of endosomal SNAREs during 119 infection of macrophages with L. mexicana. Given the requirement of CPB for L. mexicana to 120 replicate normally inside macrophages [19], we included a L. mexicana CPB-deficient mutant 121 (Δcpb) in our investigation. We infected BMM with either WT or Δcpb L. mexicana 122 123 promastigotes for 2 h and we assessed the distribution of the endosomal SNAREs VAMP3 and VAMP8 by confocal immunofluorescence microscopy. As previously observed during infection 124 with L. major promastigotes [1], we found a notable reduction in the staining intensity for both 125 126 VAMP3 (Figure 1A) and VAMP8 (Figure 1B) in BMM infected with WT L. mexicana, but this was not observed with Δcpb . This reduction in staining intensity correlated with a down-127 modulation of VAMP3 and VAMP8 proteins in BMM infected with WT L. mexicana, compared 128 to cells infected with $\triangle cpb$ (Figure 1C). These results suggested that L. mexicana causes the 129 reduction of VAMP3 and VAMP8 levels in infected BMM through the action of CPB. However, 130 we considered the possibility that CPB acted indirectly on VAMP3 and VAMP8 because we 131 previously found that GP63 targets those SNAREs in L. major-infected BMM [1]. We therefore 132 ensured that similar levels of GP63 were present in lysates of BMM infected with WT and Δcpb 133 L. mexicana promastigotes. As shown in Figure 2, GP63 was detected in lysates of BMM 134 infected with WT L. mexicana up to 72 h post-infection, when the parasites replicate as 135 amastigotes. The important reduction in GP63 levels at this time point is consistent with 136 previously published data showing a 90% reduction in the amount of GP63 detected in 137

amastigotes with respect to promastigotes [24, 25]. Surprisingly, we found that GP63 was barely detectable in BMM infected with Δcpb at all time points tested. This observation raised the possibility that the lack of VAMP3 and VAMP8 down-regulation in Δcpb -infected BMM was due to defective expression of GP63.

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Figure 1. Down-modulation of VAMP3 and VAMP8 by L. mexicana. BMM were infected 143 with serum-opsonized stationary phase L. mexicana (WT and Δcpb) promastigotes for 2 h. 144 VAMP3 (A) and VAMP8 (B) levels (green) were then visualized by confocal microscopy. 145 Macrophage and parasite nuclei are shown in blue (DAPI). Internalized parasites are denoted by 146 147 white arrowheads. In (C), VAMP3 and VAMP8 levels in total cell extracts were assessed by Western blot analysis. Each immunofluorescence assay was done on 300 phagosomes on 148 triplicate coverslips in two independent experiments and Western blot analyses were performed 149 150 twice in three independent experiments. VAMP3 and VAMP8/β-actin ratios were determined by densitometry. Original magnification X63. 151

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Figure 2. GP63 is down-modulated in the *L. mexicana* Δcpb mutant. BMM were infected with serum-opsonized stationary phase *L. mexicana* (WT and Δcpb) promastigotes for 2 h, 24 h, 48 h and 72 h. Total cell extracts were assayed for GP63 levels by Western blot analysis. GP63/ β -actin ratios were determined by densitometry. Similar results were obtained in three independent experiments.

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159 *CPB is required for GP63 expression.* To address the issue of GP63 down-regulation in *L*. 160 *mexicana* Δcpb , we first determined whether complementation of Δcpb with the *CPB* gene array

 $(\Delta cpb+CPB)$ restores wild type GP63 levels. As shown in Figure 3A, GP63 levels and activity 161 162 are down-modulated in the Δcpb mutant, and complementation with the CPB array restored GP63 levels and activity similar to those observed in WT parasites. It was previously reported 163 that expression of the cell surface glycolipid LPG and of GP63 may share common biosynthetic 164 steps [26-29]. We therefore evaluated the levels of LPG in lysates of WT, Δcpb , $\Delta cpb+CPB$, and 165 $\Delta cpb+GP63$ parasites by Western blot analysis. Strikingly, similar to GP63, LPG levels were 166 also down-modulated in the $\triangle cpb$ mutant and complementation with either the CPB array or 167 GP63 restored wild type LPG levels. To further investigate the possible role of CPB in the 168 regulation of GP63 expression, we determined the levels of GP63 mRNA in WT, Δcpb , 169 $\Delta cpb+CPB$, and $\Delta cpb+GP63$ parasites by RT-PCR. As shown in Figure 3B, GP63 mRNA levels 170 were highly down-regulated in $\triangle cpb$ and complementation with the CPB array restored wild type 171 levels of GP63 mRNA. Interestingly, complementation of $\triangle cpb$ with L. major GP63 did not 172 173 increase endogenous GP63 mRNAs. RT-PCR using L. major GP63-specific primers showed that this gene is expressed only in $\Delta cpb+GP63$. Together, these results suggest that CPB controls 174 GP63 mRNA levels at the post-transcriptional level. Clearly, additional studies will be required 175 to elucidate the underlying mechanism(s). Our results also raised the possibility that down-176 modulation of GP63 in the Δcpb mutant may have accounted for the inability of Δcpb to down-177 regulate VAMP3 and VAMP8. The finding that expression of GP63 in $\triangle cpb$ restored LPG levels 178 was unexpected and suggested a role for GP63 in the expression of LPG in L. mexicana. As it is 179 estimated that at least 25 genes are required for the synthesis, assembly, and transport of the 180 181 various components of LPG [30], it may be difficult to determine whether GP63 acts on the expression of a LPG biosynthetic gene or on a biosynthetic step. Assessment of LPG2 gene 182 expression revealed that it was equally expressed WT, Δcpb , $\Delta cpb+CPB$, and $\Delta cpb+GP63$ 183 184 parasites. Further studies will be necessary to understand how GP63 expression restores LPG 185 synthesis in Δcpb . Since LPG does not play a major role in the virulence of *L. mexicana* [17], the 186 Δcpb mutant expressing exogenous GP63 provides a unique opportunity to address the impact of 187 GP63 on SNARE cleavage, as well as on the *in vitro* and *in vivo* virulence of *L. mexicana*.

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Figure 3. Expression of GP63 and LPG is impaired in the absence of CPB. (A) Stationary phase promastigotes were lysed and total cell extracts were analysed by Western blotting and zymography for GP63 levels and activity and for LPG levels. Aldolase was used as a loading control. GP63 and LPG/aldolase ratios were determined by densitometry. (B) Promastigote total RNA was extracted and reverse transcription followed by PCR was performed to assess mRNA levels for *L. mexicana GP63-C1, LPG2*, and α -tubulin, and *L. major GP63-1*. Similar results were obtained in three independent experiments.

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GP63 is responsible for the cleavage of VAMP3 and VAMP8 by L. mexicana. We next 197 198 assessed the impact of GP63 on VAMP3 and VAMP8 during L. mexicana infection. To this end, we infected BMM with either WT, Δcpb , $\Delta cpb+CPB$, or $\Delta cpb+GP63$ L. mexicana promastigotes 199 for various time points, and we assessed VAMP3 and VAMP8 levels and intracellular 200 201 distribution. Figure 4A shows that GP63 is present at high levels in lysates of BMM infected for 2 h with WT, $\Delta cpb+CPB$, and $\Delta cpb+GP63$ promastigotes (compared to lysates of BMM infected 202 with Δcpb). At 72 h post-infection, GP63 levels are strongly reduced in BMM infected with WT 203 and $\triangle cpb+CPB$, whereas they remain elevated in BMM infected with the $\triangle cpb+GP63$ (Figure 204 205 4A) [25]. The high levels of GP63 present in BMM infected with $\Delta cpb+GP63$ for 72 h may be 206 related to the fact that expression of the L. major GP63 gene from the pLEXNeo episomal vector [31] is not under the control of endogenous GP63 3' untranslated regions. Western blot analyses 207

revealed that down-regulation of VAMP3 and VAMP8 correlated with GP63 levels expressed by 208 209 the parasites. Consistently, the staining intensity of VAMP3 and VAMP8 was reduced in BMM 210 infected with GP63-expressing parasites, as assessed by confocal immunofluorescence microscopy (Figure 4D and E). These results suggest that GP63 is responsible for the down-211 212 modulation of the endosomal SNAREs VAMP3 and VAMP8 in L. mexicana-infected BMM. Interestingly, we observed recruitment of VAMP3 to PVs containing L. mexicana parasites at 213 later time points, when promastigotes have differentiated into amastigotes, with the exception of 214 215 $\Delta cpb+GP63$ L. mexicana promastigotes (Figure 4B). In contrast, we found that VAMP8 is excluded from L. mexicana-containing PVs both at early and later time points post-infection, 216 independently of GP63 levels, suggesting that additional mechanisms regulate VAMP8 217 recruitment to L. mexicana PVs. 218

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220 Figure 4. GP63 is responsible for the down-modulation of VAMP3 and VAMP8 in L. mexicana-infected macrophages. BMM were infected with serum-opsonized stationary phase 221 L. mexicana (WT, Δcpb , $\Delta cpb+CPB$ and $\Delta cpb+GP63$) promastigotes for 2 h and 72 h. Total cell 222 extracts were analysed by Western blot (A). Similar results were obtained in three independent 223 experiments. VAMP3 and VAMP8 recruitment to the phagosome was visualized by 224 immunofluorescence microscopy and quantified for 300 phagosomes on triplicate coverslips (B 225 and C). Representative pictures from each condition are shown (D and E). Immunofluorescence 226 assays were performed on 300 phagosomes on triplicate coverslips for three independent 227 228 experiments. *p<0.0001. Original magnification X63.

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230 *GP63 expression restores virulence of* Δcpb . Since GP63 was shown to contribute to *L. major* 231 virulence [32], we next sought to determine whether expression of GP63 is sufficient to restore

the ability of Δcpb to replicate inside macrophages and to cause lesions in mice [19]. To this 232 233 end, we first infected BMM with either WT, Δcpb , $\Delta cpb+CPB$, or $\Delta cpb+GP63$ stationary phase promastigotes and we assessed parasite burden and PV surface area at various time points post-234 infection. We found that Δcpb had an impaired capacity to replicate inside macrophages and to 235 236 induce the formation of large communal PVs compared to WT and $\Delta cpb+CPB$ parasites (Figure 5A, B, C). Strikingly, expression of GP63 in Δcpb restored its ability to replicate in macrophages 237 and to induce large communal PVs up to 72 h post-infection. These results underline the role of 238 GP63 in the ability of *L. mexicana* to infect and replicate in macrophages, even in the absence of 239 CPB. Following inoculation inside the mammalian host, promastigotes are exposed to 240 241 complement and both GP63 and LPG confer resistance to complement-mediated lysis [32, 33]. L. mexicana promastigotes were therefore analyzed for their sensitivity to complement-mediated 242 lysis in the presence of fresh human serum. As shown in Figure 6A, over 40% of Δcpb was 243 244 killed after 30 min in the presence of 20% serum, whereas $\Delta cpb+CPB$, and $\Delta cpb+GP63$ were more resistant to serum lysis at 14% and 10%, respectively. Absence of both GP63 and LPG 245 may be responsible for the serum sensitivity of Δcpb . Finally, to assess the impact of GP63 on 246 the ability of Δcpb to cause lesions, we used a mouse model of cutaneous leishmaniasis. 247 Susceptible BALB/c mice were infected in the hind footpad with either WT, Δcpb , $\Delta cpb+CPB$, 248 or $\Delta cpb+GP63$ promastigotes and disease progression was monitored for 9 weeks. Consistent 249 with its reduced capacity to replicate inside macrophages, Δcpb failed to cause significant lesions 250 compared to WT parasites [19] and Δcpb complemented with CPB (Figure 6B). Remarkably, 251 expression of GP63 in Δcpb restored its capacity to cause lesions, albeit to a lower level than 252 Δcpb complemented with CPB. Lesion size correlated with parasite burden, as measured at 9 253 weeks post-infection (Figure 6C). Collectively, these results indicate that expression of GP63 is 254 255 sufficient to restore virulence of Δcpb .

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257 Figure 5. GP63 enables L. mexicana $\triangle cpb$ to infect macrophages and induce large PVs. 258 BMM were infected with stationary phase serum-opsonized L. mexicana (WT, Δcpb , $\Delta cpb+cpb$ and $\Delta cpb+GP63$) promastigotes for 2 h, 24 h, 48 h and 72 h. Macrophages were stained with the 259 HEMA 3^{TM} kit. Representative pictures from each condition are shown (A) Parasites were 260 counted in 300 macrophages on triplicate coverslips (B). Macrophages were stained with the 261 LAMP1 antibody and vacuole sizes were measured with the ZEN 2012 software (C). Parasitemia 262 and vacuole size was determined on 300 phagosomes in triplicate in three independent 263 experiments. *p<0.0001. 264

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Figure 6. GP63 confers virulence to L. mexicana $\triangle cpb$. Stationary phase L. mexicana (WT, 266 Δcpb , $\Delta cpb+cpb$ and $\Delta cpb+GP63$) promastigotes were incubated in the presence of 20% human 267 serum for 30 min, stained with a fixable viability dve, and then subjected to flow cytometry (A). 268 Mice were challenged with 5×10^6 late-stationary phase L. mexicana (WT, Δcpb , $\Delta cpb+cpb$ and 269 $\Delta cpb+GP63$) promastigotes that were injected subcutaneously into the hind footpad. Disease 270 progression was monitored at weekly intervals, by measuring the thickness of the infected 271 footpad and the contralateral uninfected footpad. (B). Parasite burden was obtained by limiting 272 dilution assay from infected homogenized footpads 9 weeks after inoculation (C). Human serum 273 lyses were performed in two independent experiments and six mice per group were used for the 274 determination of lesion formation and parasite burden. Each data point represents the average + 275 SEM of 6 mice per group, and statistical significance was denoted by * p≤0.01, and *** 276 p≤0.0001. 277

DISCUSSION

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This study aimed at investigating the mechanism(s) by which CBP contributes to L. 280 mexicana virulence. To this end, we initially examined PV biogenesis by assessing the impact of 281 L. mexicana infection on the trafficking of VAMP3 and VAMP8, two endocytic SNAREs 282 associated with phagosome biogenesis and function [1, 34]. We found that both SNAREs were 283 down-modulated in a CPB-dependent manner, which hampered VAMP3 recruitment to PVs. We 284 also discovered that expression of GP63, which we previously showed to be responsible for 285 cleaving SNAREs in L. major-infected macrophages [1], was down-modulated in the L. 286 287 *mexicana* Δcpb . Strikingly, restoration of GP63 expression in Δcpb bypassed the need for CPB for SNARE cleavage. Similarly, episomal expression of GP63 enabled the Δcpb mutant to 288 establish infection in macrophages, induce larger PVs and cause lesions in mice. These findings 289 290 imply that CPB contributes to L. mexicana virulence in part through the regulation of GP63 expression, and provide evidence that GP63 is a key virulence factor for L. mexicana. 291

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The observation that CPB regulates GP63 expression at the mRNA levels was both 293 unexpected and intriguing. Insight into the possible mechanism(s) may be deduced from a recent 294 study on the role of cathepsin B in L. donovani, which is homologous to the L. mexicana CPC 295 Similar to L. mexicana Δcpb , L. donovani $\Delta catB$ displays reduced virulence in 296 [35]. macrophages. To investigate the role of cathepsin B in virulence, the authors performed 297 298 quantitative proteome profiling of WT and $\Delta catB$ parasites and identified 83 proteins whose expression is altered in the absence of cathepsin B, with the majority being down-modulated [35]. 299 Among those were a group of proteins involved in post-transcriptional regulation of gene 300 301 expression (RNA stability, processing, translation) [35]. Whether this is the case in Δcpb deserves further investigation. Clearly, a detailed analysis of wild-type and Δcpb parasites may provide the information required to understand the extent of the impact of CBP on the expression and synthesis of virulence factors and the exact role of CPB in *L. mexicana* virulence. The observation that episomal expression of GP63 in Δcpb restored LPG synthesis is an intriguing issue, as it suggests that GP63 acts on a LPG biosynthetic step. This role for GP63 is likely redundant, since *L. major* $\Delta gp63$ promastigotes express LPG levels similar to that of wild type parasites (S1 Fig).

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It has been proposed that expansion of the PVs hosting parasites of the L. mexicana 310 311 complex leads to the dilution of the microbicidal effectors to which the parasites are exposed, thereby contributing to parasite survival [36]. Both host and parasite factors may be involved in 312 the control of PV enlargement. On the host side, it has been previously reported that L. 313 314 amazonensis cannot survive in cells overexpressing LYST, a host gene that restricts Leishmania 315 growth by counteracting PV expansion [37]. Similarly, disrupting the fusion between PVs housing L. amazonensis and the endoplasmic reticulum resulted in limited PV expansion and 316 inhibition of parasite replication [15, 16]. On the parasite side, virulence of L. amazonensis 317 isolates was shown to correlate with the ability to induce larger PVs [38]. Our results indicate 318 that the inability of Δcpb to multiply inside macrophages is associated with smaller PV size, and 319 that expression of GP63 is sufficient to restore the capacity of Δcpb to survive within 320 macrophages and to induce PV expansion. How does GP63 modulate L. mexicana virulence and 321 322 PV expansion? In addition to the numerous macrophage proteins known to be targeted by GP63, it is possible that SNARE cleavage is one of the factors associated with L. mexicana virulence 323 and PV expansion. For instance, we previously reported that VAMP8 is required for phagosomal 324 325 oxidative activity [1]. One may envision that its degradation by GP63 is part of a strategy used

by L. mexicana to establish infection in an environment devoid of oxidants, thereby contributing 326 327 to parasite survival. The LYST protein is a regulator of lysosome size and its absence leads to further PV expansion and enhanced L. amazonensis replication [37]. It is interesting to note that 328 LYST was proposed to function as an adaptor protein that juxtaposes proteins such as SNAREs 329 330 that mediate intracellular membrane fusion reactions [39]. In this context, cleavage of SNAREs that interact with LYST may interfere with its function and promote PV expansion. Further 331 studies will be necessary to clarify these issues, including the potential role of VAMP3 and 332 VAMP8 in PV biogenesis and expansion. 333

334

Previous studies using Δcpb parasites led to the conclusion that CPB enables L. mexicana 335 to alter host cell signaling and gene expression through the cleavage of various host proteins [20, 336 22]. Hence, CPB-dependent cleavage of PTP-1B, NF-kB, STAT1, and AP1 in L. mexicana-337 338 infected macrophages was associated with the inhibition of IL-12 expression and generation of nitric oxide, both of which are important for initiation of a host immune response and parasite 339 killing, respectively. Our finding that GP63 expression is down-modulated in the Δcpb mutant 340 341 raises the possibility that cleavage of those transcription factors may actually be mediated by GP63. Indeed, GP63 cleaves numerous host macrophage effectors, including PTP-1B, NF-kB, 342 STAT1, and AP1 [40]. Revisiting the role of CPB in the context of GP63 expression will be 343 necessary to elucidate whether, and to which extent, CPB is acting directly on the host cell 344 proteome. 345

346

In sum, we discovered that CPB contributes to *L. mexicana* virulence in part through the regulation of GP63 expression. Complementation of $\triangle cpb$ revealed the importance of GP63 for

the virulence of *L. mexicana*, as it participates in the survival of intracellular parasites, PV
expansion, and the formation of cutaneous lesions.

352

MATERIALS AND METHODS

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354 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 INRS-Institut Armand-Frappier. *In vivo* infections were performed as per Animal Use Protocol #4859, which was approved by the Institutional Animal Care and Use Committees at McGill University. These protocols respect procedures on good animal practice provided by the Canadian Council on Animal Care (CCAC).

360

361 Antibodies

The mouse anti-GP63 monoclonal antibody was previously described [41]. The mouse antiphosphoglycans CA7AE monoclonal antibody [42] was from Cedarlane and the rabbit polyclonal anti-aldolase was a gift from Dr. A. Jardim (McGill University). Rabbit polyclonal antibodies for VAMP3 and VAMP8 were obtained from Synaptic Systems.

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367 Cell culture

Bone marrow-derived macrophages (BMM) were differentiated from the bone marrow of 6- to 8-368 week-old female 129XB6 mice (Charles River Laboratories) as previously described [43]. Cells 369 were cultured for 7 days in complete medium (DMEM [Life Technologies] supplemented with L-370 glutamine [Life Technologies], 10% heat-inactivated FBS [PAA Laboratories], 10 mM HEPES at 371 pH 7.4, and antibiotics) containing 15% v/v L929 cell-conditioned medium as a source of M-372 CSF. Macrophages were kept at 37°C in a humidified incubator with 5% CO₂. To render BMM 373 quiescent prior to experiments, cells were transferred to 6- or 24-well tissue culture microplates 374 375 (TrueLine) and kept for 16 h in complete DMEM without L929 cell-conditioned medium.

Promastigotes of L. mexicana wild-type strain (MNYC/BZ/62/M379) and of L. major NIH S 376 377 (MHOM/SN/74/Seidman) clone A2 were grown at 26°C in Leishmania medium (Medium 199 supplemented with 10% heat-inactivated FBS, 40 mM HEPES pH 7.4, 100 µM hypoxanthine, 5 378 uM hemin, 3 uM biopterin, 1 uM biotin, and antibiotics). The isogenic L. mexicana CPB-379 deficient mutant Δcpb^{pac} (thereafter referred to as Δcpb) and its complemented counterpart 380 Δcpb^{pac} [pGL263] (thereafter referred to as $\Delta cpb+CPB$) were described previously [21]. L. 381 *mexicana* Δcpb promastigotes were electroporated as described [44] with the pLEXNeoGP63.1 382 plasmid [32] to generate $\triangle cpb+GP63$ parasites. L. mexicana $\triangle cpb+CPB$ and $\triangle cpb+GP63$ 383 promastigotes were grown in the presence of 50 µg/ml hygromycin or 50 µg/ml G418, 384 respectively. The L. major NIH clone A2 isogenic $\Delta gp63$ mutant and its complemented 385 386 counterpart $\Delta gp63+gp63$ have been previously described [32]. Cultures of $\Delta gp63+gp63$ promastigotes were supplemented with 50 µg/ml G418. 387

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389 Synchronized phagocytosis assays

Prior to macrophage infections, promastigotes in late stationary phase were opsonized with DBA/2 mouse serum. For synchronized phagocytosis using parasites, macrophages and parasites were incubated at 4°C for 10 min and spun at 167 g for 1 min, and internalization was triggered by transferring cells to 34°C. Macrophages were washed twice after 2h with complete DMEM to remove the non-internalized parasites and were further incubated at 34°C for the required times. Cells were then washed with PBS and stained using the Hema 3TM Fixative and Solutions kit (Fisher HealthCare), or prepared for confocal immunofluorescence microscopy.

397

399 Confocal immunofluorescence microscopy

Macrophages on coverslips were fixed with 2% paraformaldehyde (Canemco and Mirvac) for 40 400 min and blocked/permeabilized for 17 min with a solution of 0.05% saponin, 1% BSA, 6% skim 401 milk, 2% goat serum, and 50% FBS. This was followed by a 2 h incubation with primary 402 antibodies diluted in PBS. Macrophages were then incubated with a suitable combination of 403 secondary antibodies (anti-rabbit Alexa Fluor 488 and anti-rat 568; Molecular Probes) and DAPI 404 (Life technologies). Coverslips were washed three times with PBS after every step. After the final 405 washes, Fluoromount-G (Southern Biotechnology Associates) was used to mount coverslips on 406 glass slides, and coverslips were sealed with nail polish (Sally Hansen). Macrophages were 407 408 imaged with the 63X objective of an LSM780 microscope (Carl Zeiss Microimaging), and images were taken in sequential scanning mode. Image analysis and vacuole size measurements 409 were performed with the ZEN 2012 software. 410

411

412 Electrophoresis, Western blotting, and zymography

Prior to lysis, macrophages were placed on ice and washed with PBS containing 1 mM sodium 413 414 orthovanadate and 5 mM 1,10-phenanthroline (Roche). Cells were scraped in the presence of lysis buffer containing 1% Nonidet P-40 (Caledon), 50 mM Tris-HCl (pH 7.5) (Bioshop), 150 415 mM NaCl, 1 mM EDTA (pH 8), 5 mM 1,10-phenanthroline, and phosphatase and protease 416 inhibitors (Roche). Parasites were washed twice with PBS and lysed in the presence of lysis 417 buffer containing 0.5% Nonidet P-40 (Caledon), 100mM Tris-HCl (Bioshop) and 150 mM NaCl 418 at -70° C. Lysates were thawed on ice and centrifuged for 10 min to remove insoluble matter. 419 After protein quantification, protein samples were boiled (100°C) for 6 min in SDS sample buffer 420 and migrated in 12% or 15% SDS-PAGE gels. Three micrograms and 15 µg of protein were 421 422 loaded for parasite and infected macrophage lysates, respectively. Proteins were transferred onto

Hybond-ECL membranes (Amersham Biosciences), blocked for 1 h in TBS 1X-0.1% Tween 423 424 containing 5% skim milk, incubated with primary antibodies (diluted in TBS 1X-0.1% Tween 425 containing 5% BSA) overnight at 4°C, and thence with appropriate HRP-conjugated secondary antibodies for 1 h at room temperature. Then, membranes were incubated in ECL (GE 426 427 Healthcare), and immunodetection was achieved via chemiluminescence. Membranes were washed 3 times between each step. For zymography, 2 µg of lysate were incubated at RT for 6 428 min in sample buffer without DTT and then migrated in 12% SDS-PAGE gels with 0.2% gelatin 429 (Sigma). Gels were incubated for 1 h in the presence of 50 mM Tris pH 7.4, 2,5% Triton X-100, 430 5 mM CaCl₂ and 1 µM ZnCl₂ and incubated overnight in the presence of 50 mM Tris pH 7.4, 5 431 432 mM CaCl₂, 1µM ZnCl₂ and 0,01% NaN₃ at 37°C [45].

433

434 FACS analysis

Late stationary phase promastigotes were incubated for 30 min in complete DMEM medium with 20% human serum from healthy donors. Parasites were then incubated in LIVE/DEAD® Fixable Violet Dead Cell Stain Kit (Life technologies) and fixed in 2% paraformaldehyde. Flow cytometric analysis was carried out using the LSRFortessa cytometer (Special Order Research Product; BD Biosciences), and the BD FACSDiva Software (version 6.2) was used for data acquisition and analysis.

441

442 Mouse infections

443 Male BALB/c mice (6 to 8 weeks old) were purchased from Charles River Laboratories and 444 infected in the right hind footpad with 5×10^6 stationary phase *L. mexicana* promastigotes as 445 described [46]. Disease progression was monitored by measuring footpad swelling weekly with a

446 metric caliper, for up to 9 weeks post-infection. Footpads were then processed to calculate447 parasite burden using the limiting dilution assay.

448

449 Limiting dilution assay

After 9 weeks of infection, mice were euthanized under CO₂ asphyxiation and subsequently by 450 cervical dislocation. The infected footpads were surface-sterilized with a chlorine dioxide based 451 disinfectant followed by ethanol 70% for 5 min. Footpads were washed in PBS, lightly sliced, 452 transferred to a glass tissue homogenizer containing 6 ml of PBS, and manually homogenized. 453 The last step was repeated two to three times, until complete tissue disruption was achieved. 454 Final homogenate was then centrifuged at 3,000 x g for 5 min and resuspended in the appropriate 455 456 volume of PBS. 100 µl of homogenate were added in duplicates to 96-well plates containing 100 µl of complete Schneider's medium in each well (twenty-four 2-fold dilutions for each duplicate). 457 458 Plates were incubated at 28°C. After 7-10 days, the number of viable parasites was determined from the highest dilutions at which promastigotes were observed using an inverted microscope 459 [47]. 460

461

462 **Reverse transcription-PCR (RT-PCR)**

Total RNA was extracted from promastigotes using the TRIzol reagent (Invitrogen Life 463 Technology, Carlsbad, CA) and reverse transcribed. One-tenth of the resulting cDNA was 464 amplified by PCR on a DNA thermal cycler, version 2.3 (Perkin-Elmer Corporation, Norwalk, 465 for 466 CT). with the following primer pairs: the L. mexicana GP63 C-1 5'-ACCGTCTGAGAGTCGGAACT-3' (forward), 5'-GTAGTCCAGGAATGGCGAGT-3' 467 (reverse); the L. major GP63-1 5'-TCTGAGGCACATGCTTCGTT-3' (forward), 5'-468 GTCAGTTGCCTTCGGTCTGA-3' (reverse), the L. mexicana LPG2 469

470 5'CATTTGGTATCCTGGTGCTG-3' (forward), 5'-GAGGAAGCCACTGTTAGCC-3' (reverse),
471 and the *L. mexicana* α-tubulin 5'-CTATCTGCATCCACATTGGC-3' (forward), 5'472 ACTTGTCAGAGGGCATGGA-3' (reverse). The PCR products were analyzed by
473 electrophoresis on a 3% (wt/vol) agarose gel, and the pictures were taken using AlphaImager
474 3400 imaging software (Alpha Innotech Corporation, San Leandro, CA).

475

476 Statistical analyses

477 Statistically significant differences were analyzed by ANOVA followed by the Tukey post-hoc 478 test using the Graphpad Prism program (version 5.0). For the limiting dilution assay, the non-479 parametric Mann-Whitney or Kruskal-Wallis test was used. Values starting at P<0.05 were 480 considered statistically significant. All data are presented as mean \pm SEM.

481

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488 REFERENCES 489 Matheoud D, Moradin N, Bellemare-Pelletier A, Shio MT, Hong WJ, Olivier M, et al. 490 1. Leishmania evades host immunity by inhibiting antigen cross-presentation through direct 491 cleavage of the SNARE VAMP8. Cell Host Microbe. 2013;14: 15-25. 492 2. 493 Moradin N, Descoteaux A. Leishmania promastigotes: building a safe niche within 494 macrophages. Front Cell Infect Microbiol. 2012;2: 121. 3. Desjardins M, Descoteaux A. Inhibition of phagolysosomal biogenesis by the Leishmania 495 lipophosphoglycan. J Exp Med. 1997;185: 2061-2068. 496 497 4. Vinet AF, Fukuda M, Turco SJ, Descoteaux A. The Leishmania donovani lipophosphoglycan excludes the vesicular proton-ATPase from phagosomes by impairing the 498 recruitment of synaptotagmin V. PLoS Pathog. 2009;5: e1000628. 499 500 5. Olivier M, Atayde VD, Isnard A, Hassani K, Shio MT. Leishmania virulence factors: focus on the metalloprotease GP63. Microbes Infect. 2012;14: 1377-1389. 501 6. Arango Duque G, Descoteaux A. *Leishmania* survival in the macrophage: where the ends 502 justify the means. Curr Opin Microbiol. 2015;26: 32-40. 503 Arango Duque G, Fukuda M, Turco SJ, Stäger S, Descoteaux A. Leishmania 7. 504 promastigotes induce cytokine secretion in macrophages through the degradation of 505 synaptotagmin XI. J Immunol. 2014;193: 2363-2372. 506 Courret N, Frehel C, Gouhier N, Pouchelet M, Prina E, Roux P, et al. Biogenesis of 507 8. 508 Leishmania-harbouring parasitophorous vacuoles following phagocytosis of the metacyclic

25

promastigote or amastigote stages of the parasites. J Cell Sci. 2002;115: 2303-2316.

9. Alexander J, Vickerman K. Fusion of host cell secondary lysosomes with the
parasitophorous vacuoles of *Leishmania mexicana*-infected macrophages. J Protozool. 1975;22:
502-508.

513 10. Barbieri CL, Brown K, Rabinovitch M. Depletion of secondary lysosomes in mouse
514 macrophages infected with *Leishmania mexicana amazonensis*: a cytochemical study. Z
515 Parasitenkd. 1985;71: 159-168.

11. Real F, Pouchelet M, Rabinovitch M. *Leishmania* (*L.*) *amazonensis*: fusion between
parasitophorous vacuoles in infected bone-marrow derived mouse macrophages. Exp Parasitol.
2008;119: 15-23.

12. Real F, Mortara RA. The diverse and dynamic nature of *Leishmania* parasitophorous
vacuoles studied by multidimensional imaging. PLoS Negl Trop Dis. 2012;6: e1518.

521 13. Stow JL, Manderson AP, Murray RZ. SNAREing immunity: the role of SNAREs in the
522 immune system. Nat Rev Immunol. 2006;6: 919-929.

14. Ndjamen B, Kang BH, Hatsuzawa K, Kima PE. *Leishmania* parasitophorous vacuoles
interact continuously with the host cell's endoplasmic reticulum; parasitophorous vacuoles are
hybrid compartments. Cell Microbiol. 2010;12: 1480-1494.

526 15. Canton J, Ndjamen B, Hatsuzawa K, Kima PE. Disruption of the fusion of *Leishmania*527 parasitophorous vacuoles with ER vesicles results in the control of the infection. Cell Microbiol.
528 2012.

16. Canton J, Kima PE. Targeting host syntaxin-5 preferentially blocks *Leishmania*parasitophorous vacuole development in infected cells and limits experimental *Leishmania*infections. Am J Pathol. 2012;181: 1348-1355.

532 17. Ilg T. Lipophosphoglycan is not required for infection of macrophages or mice by
533 *Leishmania mexicana*. EMBO J. 2000;19: 1953-1962.

18. Mottram JC, Coombs GH, Alexander J. Cysteine peptidases as virulence factors of *Leishmania*. Curr Opin Microbiol. 2004;7: 375-381.

Mottram JC, Souza AE, Hutchison JE, Carter R, Frame MJ, Coombs GH. Evidence from
disruption of the lmcpb gene array of *Leishmania mexicana* that cysteine proteinases are
virulence factors. Proc Natl Acad Sci USA. 1996;93: 6008-6013.

- Cameron P, McGachy A, Anderson M, Paul A, Coombs GH, Mottram JC, et al. Inhibition
 of lipopolysaccharide-induced macrophage IL-12 production by *Leishmania mexicana*amastigotes: the role of cysteine peptidases and the NF-kappaB signaling pathway. J Immunol.
 2004;173: 3297-3304.
- 543 21. Denise H, McNeil K, Brooks DR, Alexander J, Coombs GH, Mottram JC. Expression of
 544 multiple CPB genes encoding cysteine proteases is required for *Leishmania mexicana* virulence
 545 in vivo. Infect Immun. 2003;71: 3190-3195.
- Abu-Dayyeh I, Hassani K, Westra ER, Mottram JC, Olivier M. Comparative study of the
 ability of *Leishmania mexicana* promastigotes and amastigotes to alter macrophage signaling and
 functions. Infect Immun. 2010;78: 2438-2445.
- 549 23. De Souza Leao S, Lang T, Prina E, Hellio R, Antoine JC. Intracellular *Leishmania*550 *amazonensis* amastigotes internalize and degrade MHC class II molecules of their host cells. J
 551 Cell Sci. 1995;108: 3219-3231.
- Bahr V, Stierhof YD, Ilg T, Demar M, Quinten M, Overath P. Expression of
 lipophosphoglycan, high-molecular weight phosphoglycan and glycoprotein 63 in promastigotes
 and amastigotes of *Leishmania mexicana*. Mol Biochem Parasitol. 1993;58: 107-121.
- 555 25. Medina-Acosta E, Karess RE, Schwartz H, Russell DG. The promastigote surface 556 protease (gp63) of *Leishmania* is expressed but differentially processed and localized in the 557 amastigote stage. Mol Biochem Parasitol. 1989;37: 263-273.
 - 27

Garami A, Ilg T. Disruption of mannose activation in *Leishmania mexicana*: GDPmannose pyrophosphorylase is required for virulence, but not for viability. EMBO J. 2001;20:
3657-3666.

561 27. Garami A, Ilg T. The role of phosphomannose isomerase in *Leishmania mexicana* 562 glycoconjugate synthesis and virulence. J Biol Chem. 2001;276: 6566-6575.

563 28. Garami A, Mehlert A, Ilg T. Glycosylation defects and virulence phenotypes of
564 *Leishmania mexicana* phosphomannomutase and dolicholphosphate-mannose synthase gene
565 deletion mutants. Mol Cell Biol. 2001;21: 8168-8183.

Descoteaux A, Avila HA, Zhang K, Turco SJ, Beverley SM. *Leishmania* LPG3 encodes a
GRP94 homolog required for phosphoglycan synthesis implicated in parasite virulence but not
viability. EMBO J. 2002;21: 4458-4469.

30. Beverley SM, Turco SJ. Lipophosphoglycan (LPG) and the identification of virulence
genes in the protozoan parasite *Leishmania*. Trends Microbiol. 1998;6: 35-40.

Joshi PB, Webb JR, Davies JE, McMaster WR. The gene encoding streptothricin
acetyltransferase (sat) as a selectable marker for *Leishmania* expression vectors. Gene. 1995;156:
145-149.

Joshi PB, Kelly BL, Kamhawi S, Sacks DL, McMaster WR. Targeted gene deletion in *Leishmania major* identifies leishmanolysin (GP63) as a virulence factor. Mol Biochem Parasitol.
2002;120: 33-40.

577 33. Spath GF, Garraway LA, Turco SJ, Beverley SM. The role(s) of lipophosphoglycan
578 (LPG) in the establishment of *Leishmania major* infections in mammalian hosts. Proc Natl Acad
579 Sci USA. 2003;100: 9536-9541.

34. Murray RZ, Kay JG, Sangermani DG, Stow JL. A role for the phagosome in cytokine
secretion. Science. 2005;310: 1492-1495.

35. Gerbaba TK, Gedamu L. Cathepsin B gene disruption induced *Leishmania donovani*proteome remodeling implies cathepsin B role in secretome regulation. PLoS One. 2013;8:
e79951.

- 585 36. Sacks D, Sher A. Evasion of innate immunity by parasitic protozoa. Nat Immunol.
 586 2002;3: 1041-1047.
- 37. Wilson J, Huynh C, Kennedy KA, Ward DM, Kaplan J, Aderem A, et al. Control of
 parasitophorous vacuole expansion by LYST/Beige restricts the intracellular growth of *Leishmania amazonensis*. PLoS Pathog. 2008;4: e1000179.
- 590 38. Franca-Costa J, Wanderley JL, Deolindo P, Zarattini JB, Costa J, Soong L, et al. Exposure
 591 of phosphatidylserine on *Leishmania amazonensis* isolates is associated with diffuse cutaneous
 592 leishmaniasis and parasite infectivity. PLoS One. 2012;7: e36595.
- 39. Tchernev VT, Mansfield TA, Giot L, Kumar AM, Nandabalan K, Li Y, et al. The
 Chediak-Higashi protein interacts with SNARE complex and signal transduction proteins. Mol
 Med. 2002;8: 56-64.
- 40. Isnard A, Shio MT, Olivier M. Impact of *Leishmania* metalloprotease GP63 on
 macrophage signaling. Front Cell Infect Microbiol. 2012;2: 72.
- 598 41. Button LL, Wilson G, Astell CR, McMaster WR. Recombinant *Leishmania* surface
 599 glycoprotein GP63 is secreted in the baculovirus expression system as a latent metalloproteinase.
 600 Gene. 1993;134: 75-81.
- 42. Tolson DL, Turco SJ, Beecroft RP, Pearson TW. The immunochemical structure and
 surface arrangement of *Leishmania donovani* lipophosphoglycan determined using monoclonal
 antibodies. Mol Biochem Parasitol. 1989;35: 109-118.
- 43. Descoteaux A, Matlashewski G. c-*fos* and tumor necrosis factor gene expression in *Leishmania donovani* infected macrophages. Mol Cell Biol. 1989;9: 5223-5227.

606	44. Descoteaux A, Garraway LA, Ryan KA, Garrity LK, Turco SJ, Beverley SM.
607	Identification of genes by functional complementation in protozoan parasite Leishmania. In:
608	Adolph KW, editor. Methods in Molecular Genetics (Molecular Microbiology Techniques).
609	Molecular Biology Techniques. 3. San Diego: Academic Press; 1994. p. 22-48.
610	45. Hassani K, Shio MT, Martel C, Faubert D, Olivier M. Absence of metalloprotease GP63
611	alters the protein content of Leishmania exosomes. PLoS One. 2014;9: e95007.
612	46. Gomez MA, Contreras I, Halle M, Tremblay ML, McMaster RW, Olivier M. Leishmania
613	GP63 alters host signaling through cleavage-activated protein tyrosine phosphatases. Sci Signal.
614	2009;2: ra58.
615	47. Lima HC, Bleyenberg JA, Titus RG. A simple method for quantifying Leishmania in
616	tissues of infected animals. Parasitol Today. 1997;13: 80-82.

618 Supporting Information

619

S1 Fig. *L. major* Δ*gp63* promastigotes express normal levels of LPG. Stationary phase
promastigotes were lysed and total cell extracts were analysed by Western blotting for LPG
levels. Similar results were obtained in two separate experiments.









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