Degradation of host sphingomyelin is essential for Leishmania virulence

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Degradation of Host Sphingomyelin Is Essential for Leishmania Virulence

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Abstract

In eukaryotes, sphingolipids (SLs) are important membrane components and powerful signaling molecules. In Leishmania, the major group of SLs is inositol phosphorylceramide (IPC), which is common in yeast and Trypanosomatids but absent in mammals. In contrast, sphingomyelin is not synthesized by Leishmania but is abundant in mammals. In the promastigote stage in vitro, Leishmania use SL metabolism as a major pathway to produce ethanolamine (EtN), a metabolite essential for survival and differentiation from non-virulent procyclics to highly virulent metacyclics. To further probe SL metabolism, we identified a gene encoding a putative neutral sphingomyelinase (SMase) and/or IPC hydrolase (IPCase), designated ISCL (inositol phospho-Sphin golipid phospholipase C-Like). Despite the lack of sphingomyelin synthesis, L. major promastigotes exhibited a potent SMase activity which was abolished upon deletion of ISCL, and increased following over-expression by episomal complementation. ISCL-dependent activity with sphingomyelin was about 20 fold greater than that seen with IPC. Null mutants of ISCL (iscl−) showed modest accumulation of IPC, but grew and differentiated normally in vitro. Interestingly, iscl− mutants did not induce lesion pathology in the susceptible BALB/c mice, yet persisted indefinitely at low levels at the site of infection. Notably, the acute virulence of iscl− was completely restored by the expression of ISCL or heterologous mammalian or fungal SMases, but not by fungal proteins exhibiting only IPCase activity. Together, these findings strongly suggest that degradation of host-derived sphingomyelin plays a pivotal role in the proliferation of Leishmania in mammalian hosts and the manifestation of acute disease pathology.

Introduction

Leishmania parasites infect 10–12 million people worldwide, causing a spectrum of diseases known as leishmaniasis [1]. Transmitted by sandflies, these protozoan pathogens have tremendous negative impacts on public health worldwide, especially in developing countries [1]. During their life cycle, Leishmania parasites alternate between flagellate promastigotes which live in the midgut of sandfly and non-flagellate amastigotes which reside in the phagosome of mammalian macrophages. Control of Leishmaniasis has been hampered by the lack of a safe vaccine, limitation of frontline drugs, and the emergence of drug resistant strains [2]. To develop new and more cost effective drugs, it is necessary to understand the molecular mechanism of Leishmania pathogenesis. A number of surface molecules including lipophosphoglycan (LPG), glycosylinositolphospholipids (GIPLs), and the metalloprotease gp63 provide resistance to digestive enzymes, reactive oxygen species, and complement-mediated lysis [3,4]. Other virulence factors including the LACK antigen (Leishmania homologue of receptors for activated C kinase) [5] and cysteine proteases play important roles in modulating host immune response [6].

In addition to these well-studied virulence factors, recent work has provided insight into the function of sphingolipids (SLs) in Leishmania. SLs are ubiquitous membrane components in eukaryotes with well-documented functions in general membrane physiology, raft formation, cell-to-cell recognition, and signaling [7–10]. Unlike mammalian cells which synthesize sphingomyelin and glycosylphospholipids to high abundance, the majority of SLs in Leishmania are unmodified inositol phosphorylceramide (IPC), a class of lipids mainly found in fungi and plants [11,12]. The functions of SLs metabolism in L. major were recently probed using two deletion mutants: a spl2− strain that is SL-free due to the deletion of an essential subunit gene (SPT2) of serine palmitoyltransferase, the first enzyme in the de novo synthesis of SLs (Fig. S1) [12,13]; and a spl− strain that lacks the degradative enzyme.
Sphingolipid Degradation by Leishmania

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Author Summary

Leishmania are obligate intracellular parasites responsible for a spectrum of diseases in humans ranging from self-healing skin lesions to deadly visceral infections. To survive, they must downregulate the microbicidal activity and scavenger nutrients from the host. Although Leishmania parasites do not synthesize sphingomyelin, which is an abundant lipid in mammals, they do possess the ability to hydrolyze sphingomyelin. This neutral sphingomyelinase (SMase) activity is dependent on an ISCL gene, which is also required for the degradation of inositol phosphoceramide, the dominant sphingolipid in Leishmania. Deletion of ISCL in L. major led to a complete loss of SMase activity but no obvious defects in growth or differentiation in vitro. Remarkably, null mutants of ISCL failed to induce any detectable pathology in mammals yet were able to persist at low levels indefinitely. Such defect was completely reversed when a functional neutral SMase was introduced into the mutant. In total, our results suggest that degradation of host-derived sphingomyelin by Leishmania is essential for acute virulence. Further studies will elucidate the pivotal role of sphingolipid homeostasis in the molecular interaction between Leishmania parasites and their mammalian hosts.

Identification and targeted replacement of ISCL

Through a BLASTp search, ISCL (system ID: LmjF08.0200, 1962 bp) was identified from the L. major genome (www.genedb.org) as the sole homologue of human neutral SMase 1 (Genbank accession #NP_003071) and S. cerevisiae ISCL (Genbank accession #P40015). The predicted protein (ISCL, 653 aa) contains several conserved amino acids including Glu51, Asp116, Asp383, and His384 (Fig. S3). From studies of Saccharomyces cerevisiae, ScISC1 (which encodes the IPCase in C. neoformans or CnISC1p) was critical for the survival of this pathogen in macrophages and its dissemination to the brain [25]. Neither IPCase nor neutral SMase has been studied in protozoa.

In this report, we identified a single ISCL gene in L. major homologous to the neutral SMase genes in mammals and the ISCL genes in fungi (Fig. S3). To study its role in SL metabolism and pathogenesis, null mutants of ISCL were generated through targeted gene deletion and results suggest the ability to degrade sphingomyelin is essential for acute virulence in Leishmania.

Results

In culture, iscl− promastigotes were fully viable in log phase but showed altered morphology and poor viability in late stationary phase. In culture, iscl− promastigotes grew normally with a doubling time of ~7.0 hours during logarithmic growth phase, indicating

sphingosine-1-phosphate lyase (SPL) (Fig. S1) [14]. Both mutants were fully viable and replicated normally during log phase growth; however, they died quickly in stationary phase and failed to differentiate from non-infective procyclics to infective metacyclics (metacyclogenesis) [14]. Remarkably, supplementation of ethanolamine (EtN) or phosphoethanolamine (a product of SPL, Fig S1) completely reversed the viability and differentiation defects of both mutants [14]. EtN is likely used to synthesize plasmenylethanolamine (PLE, the dominant phosphatidyethanolamine species in L. major), a class of plasmalogen lipid that is highly abundant in metacyclics [12,14]. These results indicate that a main function of SL synthesis and degradation is to generate EtN, a metabolite essential for survival and metacyclogenesis in Leishmania promastigotes (Fig. S1). The conversion from SLs to phosphoethanolamine could occur via two pathways: an IPC-independent route directly from sphingoid bases or ceramide, or an alternative route which requires the synthesis and degradation of IPC (Fig. S1).

Contrary to the problems encountered during metacyclogenesis, sp2− and sp1− amastigotes (the non-motile forms that reside in macrophages) were morphologically normal and fully infective [14]; in addition, expression of SPT2 (mRNAs and protein) was greatly downregulated in wild type (WT) amastigotes [12]; finally, amastigotes of WT and sp2− contained abundant amounts of IPC and PLE (similar to WT promastigotes), despite the loss of de novo SL synthesis and EtN production [15]. Since IPC is not found in mammals, these results imply: 1) amastigotes acquire SL metabolites from the mammalian host, either directly or through the hydrolysis of host SLs to generate metabolite; and 2) salvage of host lipids may play important roles for the survival and proliferation of Leishmania amastigotes. To explore the functions of SL metabolism that are independent of EtN production, it is necessary to study the enzymes that are directly involved in the uptake and degradation of host SLs in Leishmania.

In mammalian cells, the sphingomyelinase (SMase)-mediated sphingomyelin hydrolysis is the major pathway to produce stress-induced ceramide (Fig. S2) [16]. Ceramide is a well-studied bioactive molecule implicated in many signaling processes including apoptosis, inflammation, growth and differentiation [17]. Among the five known classes of SMases, neutral SMases have emerged as the main candidates for the production of stress-induced ceramide [16]. Although yeasts do not synthesize sphingomyelin, they produce abundant amount of glycosylated inositol phosphoceramide (glycosylated IPC) [18,19]. The functional homologue of mammalian neutral SMase in yeasts is inositol phosphosphingolipid-phospholipase C or IPCase (Fig. S2) [20]. In Saccharomyces cerevisiae, the activity and localization of IPCase (ScISC1p; encoded by ScISCL) were regulated in a growth-dependent manner: predominantly in the endoplasmic reticulum (ER) during early log stage but associated with mitochondria in late logarithmic growth, which might lead to the activation of this enzyme by the anionic phospholipids in the mitochondrial membrane [21,22]. Deletion of ScISCL resulted in a slow growth phenotype, suggesting it was required for the utilization of non-fermentable carbon sources and/or the respiration function of mitochondria [22]. The null mutant was also more sensitive to heat and high salt concentration, which seemed to be linked to the deregulation of several stress-response genes [23,24]. In the opportunistic fungal pathogen Cryptococcus neoformans, CnISC1 (which encodes the IPCase in C. neoformans or CnISC1p) was critical for the survival of this pathogen in macrophages and its dissemination to the brain [25]. Neither IPCase nor neutral SMase has been studied in protozoa.

To examine the role of ISCL in SL degradation and virulence, null mutants were generated in L. major through two rounds of targeted gene replacement as previously described, since Leishmania are predominantly diploid [30,31]. Southern-blot analysis confirmed the loss of both ISCL alleles in two candidate iscl− (ΔISCL::PAC/ΔISCL::BSD) clonal lines (Figs. 1A and S4). As a control, these mutants were complemented with an episome (pNX-ISCL) which drove the overexpression of ISCL (Fig. S4). The two clonal lines showed very similar phenotypes, and thus data for only clone #1-1 and its recombinant control (iscl−/+ISCL) are presented here.
**ISCL** is not required for survival or replication in the insect stage (Fig. 1B). The *iscl*^2^- mutants also attained similar densities as WT parasites in stationary phase (2.5–3.3 × 10^7 cells/ml) (Fig. 1B). Interestingly, although *iscl*^2^- promastigotes exhibited normal morphology in log phase and the first 1–2 days in stationary phase (Fig. 1C–D), they became progressively less elongated in late stationary phase (Fig. 1C–D). Microscopic evaluations revealed that after 3 days in stationary phase, 30–40% of *iscl*^2^- cells became round (round cells were defined as those with the length of the long axis less than twice the length of the short axis) whereas only 10–20% of WT cells were round (Fig. 1C–D). This difference became more pronounced as cultures continued to age, with 70–80% of *iscl*^2^- cells becoming round after 5 days in stationary phase (Fig. 1C–D). This round phenotype is characteristic of unhealthy promastigotes, consistent with the increased percentage of dead cells as 35–49% of *iscl*^2^- became permeable to propidium iodide versus 10–20% of WT after 3–4 days in stationary phase (Fig. 1E). These viability and shape defects did not arise from programmed cell death as *iscl*^-^ parasites showed no characteristics of apoptosis (such as the exposure of phosphatidylserine or PsS, data not shown). Importantly, stationary phase defects were solely due to the loss of **ISCL**, as the *iscl*^2^-/+ISCL and ISCL^+/^- (heterozygote) parasites showed normal viability and morphology (Fig. 1 C–E and data not shown). Finally, these viability and shape defects could not be reversed by EtN (Fig. 1D–E), which is different from the stationary phase defects exhibited by *spt2*^2^- and *spl*^2^- mutants [14].

**Iscl**^-^ mutants showed no apparent defects in differentiation to metacyclics

For *Leishmania* promastigotes, the cessation of growth in stationary phase coincides with the onset of metacyclogenesis, i.e. differentiation from non-infective procyclic forms to infective metacyclic forms [32]. Metacyclics can be distinguished from procyclics based on morphology, reactivity to lectins and monoclonal antibodies, and density gradient sedimentation.
ISCL is required for the degradation of sphingomyelin and IPC

Since ISCL is a homolog of mammalian neutral SMase and fungal ISCl1p, we tested whether it is required for the hydrolysis of sphingomyelin, IPC, or both. Briefly, Leishmania lysates were incubated with Triton X100/lipid mixed micelles prepared as described by Okamoto et al. [29] with minor modifications. When a NBD-labeled C6 sphingomyelin was used as substrate with whole cell extracts from WT log phase promastigotes, ceramide (one of the degradative products of SMase) was detected by thin layer chromatography (TLC) (Fig. 3A-B). This high level of SMase activity was comparable to what was observed from mammalian and yeast whole cell lysate [33,36]. In contrast, lysates from iscl−/− mutants did not induce ceramide production and were similar to the negative control made of boiled lysate from WT parasites (Fig. 3A-B), indicating ISCL is required for the hydrolysis of sphingomyelin in vivo. As expected, the complementation strain iscl+/+ISCL, predicted to have increased expression ISCL due to overexpression from a multicopy episomal vector, exhibited higher SMase activity (5–7 times more than WT, Fig. 3A-B; Table 1). Furthermore, we tested whether this ISCL-mediated SMase activity was sensitive to a specific inhibitor of mammalian neutral SMase 2 (IC50 around 1 μM) [37].

Next, we tested whether ISCL was required for the hydrolysis of sphingomyelin by intact promastigotes. To do so, parasites were metabolically labeled with NBD C6 sphingomyelin for 48 hours and cellular lipids were extracted and analyzed by TLC. As a control, we added NBD C6 sphingomyelin to growth medium without parasites for 48 hours and results showed very little spontaneous degradation (Fig. 3C). Both WT and iscl+/+ISCL parasites were capable of sphingomyelin uptake and degradation, whereas iscl−/− mutants only showed uptake without hydrolysis (Fig. 3C-D). Therefore, ISCL is essential for the SMase activity in vivo. Interestingly, iscl−/−/ISCL parasites only caused ~40% more degradation (based on quantitative analysis of the ceramide/ceramide + sphingomyelin ratio) than WT parasites (Fig. 3C-D). This differs from the SMase activity data acquired from cell lysate where iscl−/−ISCL showed 5–7 times more activity than WT (Fig. 3A-B).

We next examined whether Leishmania ISCL possessed IPCase activity. Assays were carried out similarly to those examining sphingomyelin hydrolysis, but using Triton X100/lipid mixed micelles containing PtS and NBD-labeled C12 IPC, followed by lipid extraction and TLC. As shown in Fig. 3E and 3F, WT parasites exhibited a detectable level of IPCase activity whereas iscl−/− mutants failed to degrade IPC. Episomal expression of ISCL in iscl−/−ISCL led to a marked increase of IPCase activity, suggesting ISCL is involved in the IPC degradation (Fig. 3E-F). Notably, the specific activity of IPCase in WT is 10–20 times lower than that of SMase (Table 1), suggesting sphingomyelin is the preferred substrate of ISCL.

We then examined the cellular level of IPC, ceramide, and PLE (which is synthesized from SL-derived EtN) in WT, iscl−/−, and iscl+/+ISCL promastigotes by electrospray ionization mass spectrometry (ESI/MS) in the negative ion mode (Table 2). Abundances of IPC (composed of d16:1/18:0:Pi-Cer, d18:1/18:0:Pi-Cer, and d16:1/18:0:Pi-PhytoCer [12]) and PLE (composed of p18:0/18:2-PtE and p18:0/18:1-PtE [12]) were estimated through comparison with appropriate internal standards (d18:1/18:0-ceramide phosphate for IPC, d18:1/18:0-ceramide for ceramide, and p18:0/20:4-PtE for PLE). As summarized in Table 2, iscl−/− mutants contained 53–59% more IPC and 30–52% less ceramide than WT and iscl+/+ISCL parasites, consistent with a role for ISCL in IPC degradation. The cellular level of PLE in iscl−/− mutants was very similar to WT and iscl+/+ISCL parasites (Table 2). As described before [12], L. major promastigotes did not contain sphingomyelin.

iscl−/− mutants failed to induce pathology in susceptible mice but persisted for at least 7 months

Next, we assessed the virulence of iscl−/− mutants in BALB/c mice, which are highly susceptible to L. major. In footpad and ear infections, WT, ISCL+/+ (heterozygote), and iscl−+/+ISCL parasites caused rapid progression of lesion which correlated with the increasing number of parasites in the infected tissue (Fig. 4A-D and data not shown). Remarkably, neither stationary phase promastigotes nor purified metacyclics of iscl−/− mutants induced detectable lesions in the footpads or ears of BALB/c mice (Fig. 4A-C). This virulence defect was not reversed by EtN (Fig. 4A), which makes iscl−/− clearly different from the spt2+ and spl−/− mutants [14]. The defect in cell viability was not sufficient to cause a complete loss of virulence in iscl−/− because we used 3-day old stationary phase promastigotes of which 85–95% of cells were healthy as judged by PI-
Figure 3. ISCL is required for the hydrolysis of sphingomyelin and IPC. (A)–(B) In vitro neutral SMase assay. Whole cell lysates from log phase promastigotes (~40 µg of protein each) were incubated with TX100-based micelles containing NBD-labeled sphingomyelin as described in Materials and Methods. Neutral SMase inhibitor GW4869 was provided as indicated (1 µM final concentration). Lipids were extracted and separated by TLC. Plates were scanned using a Storm 860 phosphoimager and the activity of SMase was normalized to nmol/µg/hour. Positive control (+): 0.1 unit of B. cereus SMase; negative control (−): boiled WT lysate. (C)–(D) Degradation of sphingomyelin by promastigotes in vivo. Parasites were cultured in the presence of NBD-labeled sphingomyelin for 48 hours and lipids were extracted and separated by TLC (“medium only” means NBD-labeled sphingomyelin was incubated in the absence of parasites for 48 hours). Ratio of ceramide/ceramide + sphingomyelin (SM) was determined using an Image Analysis Software (ImageQuant TL 7.0) and used as an indicator of sphingomyelin hydrolysis. (E)–(F) ISCL is required for the degradation of IPC. Whole cell lysates from log phase promastigotes (~40 µg of protein each) were incubated with TX100-based micelles containing NBD-labeled IPC as described in Materials and Methods. Lipids were extracted and separated by TLC. Plates were scanned using a Storm 860 phosphoimager and the activity of IPCase was normalized to pmol/µg/hour. Experiments were performed in duplicates and error bars represent standard deviations. O, origin of TLC.

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exclusion (Figs. 1E, 4A–B). In addition, metacyclic forms of \textit{isl} \textsuperscript{−} that were morphologically normal and impermeable to propidium iodide also failed to cause pathology (Fig. 3C–D and data not shown). Despite the lack of pathology, limiting dilution assays showed \textit{isl} \textsuperscript{−} mutants were able to persist at the site of infection at very low levels (50–120 parasites/footpad) for at least 7 months post infection (Fig. 4D). Therefore, \textit{ISCL} was essential for acute virulence and pathology but not long-term persistence.

To corroborate the results obtained in mouse infections, we examined the virulence of \textit{isl} \textsuperscript{−} mutants in an \textit{in vitro} macrophage infection assay using peritoneal macrophages isolated from BALB/c mice [38]. When the infection was performed at 37°C, \textit{isl} \textsuperscript{−} mutants were able to bind and enter macrophages efficiently (as shown by the 2-hour time points in Fig. 4E–F) but these parasites failed to replicate thereafter and were quickly eliminated (Fig. 4E–F). In contrast, when the infection was done at 33°C, \textit{isl} \textsuperscript{−} mutants were able to survive in macrophages, albeit at a lower level than WT and \textit{isl} \textsuperscript{−}/+\textit{ISCL} parasites (2–3 times lower in both infection rate and the number of parasites/100 macrophages, Fig. 3G–H). The ability to survive better at lower temperatures could be related to the ability to survive for long periods in peripheral mouse tissues like the footpad or ear. Our results also imply \textit{ISCL} is involved in heat tolerance as the \textit{isl} \textsuperscript{−}/+\textit{ISCL} parasites, which overproduced ISCL from a multicopy episome (pXG–\textit{ISCL}), survived better than WT at 37°C in macrophages (Fig. 4E–F). The ability to survive better at a lower temperature could be related to the ability to survive for long periods in peripheral mouse tissues like the footpad or ear.

Heterologous expression of mammalian neutral SMases and fungal \textit{ISC1}ps in \textit{isl} \textsuperscript{−}

To probe the importance of SMase vs. IPCase in \textit{Leishmania}, we expressed SL hydrolases of known specificity in the \textit{isl} \textsuperscript{−} mutant. These included human neutral SMase 1 (NP_003071), murine neutral SMase 2, and the \textit{S. cerevisiae} \textit{ISC1}p, as this latter enzyme exhibits both SMase and IPCase activity [39]. Human and murine ORFs were cloned into the pIR1SAT vector and integrated into the RNA locus to generate \textit{isl} \textsuperscript{−} \textit{SSU::hNSM1} or \textit{isl} \textsuperscript{−} \textit{SSU::mNSM2}, respectively which results in high levels of expression [40]. Similarly, \textit{ScISC1} was inserted into the multicopy episomal vector pXG and transfected into the \textit{isl} \textsuperscript{−} mutant yielding \textit{isl} \textsuperscript{−}/+\textit{ScISC1}, which also yields high levels of expression.

We examined the ability of these transgenic parasites to hydrolyze sphingomyelin \textit{in vivo} following provision of NBD C6 sphingomyelin as described above. Both human neutral SMase 1 and murine neutral SMase 2 possessed the ability to break down sphingomyelin when expressed in \textit{isl} \textsuperscript{−} promastigotes (Fig. 5A and 5B); in contrast, control parasites transfected with the pIR vector alone (\textit{isl} \textsuperscript{−} \textit{SSU::pIR}) did not exhibit SMase activity despite maintaining sphingomyelin uptake (Fig. 5A and 5B). Similarly, expression of \textit{ScISC1} restored the ability to degrade sphingomyelin in \textit{isl} \textsuperscript{−} (Fig. 5C–D). Consistent with these \textit{in vivo} labeling results, we were able to detect strong SMase activity in the \textit{in vitro} assay with whole cell lysates from \textit{isl} \textsuperscript{−} \textit{SSU::hNSM1}, \textit{isl} \textsuperscript{−} \textit{SSU::mNSM2}, and \textit{isl} \textsuperscript{−}/+\textit{ScISC1}, but not from \textit{isl} \textsuperscript{−} \textit{SSU::pIR} (data not shown). Similar \textit{in vitro} experiments were performed to assess whether these SMases could degrade IPC (using NBD C12 IPC as described above) when expressed heterologously in \textit{isl} \textsuperscript{−} mutants. As shown in Fig. 5E–F, \textit{ScISC1} and murine neutral SMase 2 induced modest IPC degradation, whereas the activity from human neutral SMase 1 was close to background. Similar to \textit{L. major} \textit{ISCL}, the IPCase activity exhibited by these enzymes were much lower compared to SMase activity (Table 2).

Complementation of \textit{isl} \textsuperscript{−} mutants by heterologous SMases

Next we examined the effects of mammalian neutral SMases and \textit{ScISC1} on cell morphology and virulence in \textit{isl} \textsuperscript{−}. As shown in Fig. 6A and 6B, both human neutral SMase 1 and murine neutral SMase 2 reversed the cell shape defects in \textit{isl} \textsuperscript{−} during stationary phase and restored the ability to elicit lesion pathology in BALB/c mice (lesion sizes correlated with parasite numbers in the footpads, Table S1), whereas control parasites with the empty vector (\textit{isl} \textsuperscript{−} \textit{SSU::pIR}) behaved similarly to the parental \textit{isl} \textsuperscript{−} mutants. Further analyses confirmed that \textit{isl} \textsuperscript{−} \textit{SSU::hNSM1} and \textit{isl} \textsuperscript{−} \textit{SSU::mNSM2} also had improved viability in late stationary phase (by propidium exclusion flow cytometry) and increased virulence in macrophage infections (data not shown). Therefore, defects in \textit{isl} \textsuperscript{−} can be complemented by mammalian neutral SMases. Similar to mammalian enzymes, \textit{ScISC1} completely restored morphology, viability, and virulence in \textit{isl} \textsuperscript{−} parasites (Fig. 6C and 6D), indicating it could functionally substitute \textit{ISCL}. Again, lesion pathology induced by \textit{isl} \textsuperscript{−}/+\textit{ScISC1} was consistent with parasite numbers over time (Table S2). Together, our complementation study strongly suggests that SMase activity is required for acute virulence in \textit{L. major}.

**Table 1.** Summary of neutral SMase and IPCase activity in \textit{L. major} promastigotes lysates.

<table>
<thead>
<tr>
<th>Molecules</th>
<th>WT</th>
<th>\textit{isl} \textsuperscript{−}</th>
<th>\textit{isl} \textsuperscript{−}/+\textit{ISCL}</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMase</td>
<td>7.9</td>
<td>&lt;0.1</td>
<td>42</td>
</tr>
<tr>
<td>IPCase</td>
<td>0.39</td>
<td>&lt;0.1</td>
<td>4.4</td>
</tr>
</tbody>
</table>

Normalized activities of neutral SMase and IPCase (average values) in log phase promastigotes were determined from whole cell extracts (Figs. 3, 5, and data not shown).

doi:10.1371/journal.ppat.1000692.t002

**Table 2.** Abundance of IPC, PLE, and ceramide in promastigotes.

<table>
<thead>
<tr>
<th>Molecules/cell (x10^6)</th>
<th>WT</th>
<th>\textit{isl} \textsuperscript{−}</th>
<th>\textit{isl} \textsuperscript{−}/+\textit{ISCL}</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPC</td>
<td>2.52 ± 0.52</td>
<td>3.55 ± 0.36</td>
<td>2.41 ± 0.47</td>
</tr>
<tr>
<td>PLE</td>
<td>1.57 ± 0.33</td>
<td>1.72 ± 0.23</td>
<td>1.47 ± 0.42</td>
</tr>
<tr>
<td>Ceramide</td>
<td>2.61 ± 0.20</td>
<td>1.91 ± 0.07</td>
<td>3.41 ± 0.47</td>
</tr>
</tbody>
</table>

Abundances of IPC, PLE, and ceramide in stationary phase promastigotes (3 days after reaching maximal density) were determined by quantitative mass spectrometry (with internal standards) as described in Materials and Methods. Average values from two independent experiments are summarized with standard deviations.

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Expression of a specific IPCase fails to rescue iscl

Recently, it was shown by the group of Maurizio Del Poeta (Medical University of South Carolina, Charleston) that unlike the ScISC1p, the ISC1 of Cryptococcus neoformans lacked significant activity with sphingomyelin while retaining activity with IPC (M. Del Poeta, personal communication). Heterologous expression of an enzyme lacking SMase activity could thus serve as a probe to test the relative importance of SMase vs. IPCase activity. The C. neo ISC1 ORF was inserted into pXG and introduced into the iscl−/− mutant, yielding iscl−/+CnISC1. Consistent with Del Poeta's

Figure 4. ISCL is essential for acute virulence but not persistence. (A)–(B) iscl− mutants failed to induce pathology in mice. BALB/c mice were infected in the footpads (A) or ears (B) with stationary phase promastigotes and lesion sizes were measured weekly with a caliper. (C)–(D) iscl− mutants were able to persist at low levels. BALB/c mice were infected in the footpads with purified metacyclics at 2×10⁶ parasites per mouse. Lesion sizes were measured with a caliper (C) and parasite numbers in infected footpads were determined by the limiting dilution assays (D). (E)–(H) iscl− infection of peritoneal macrophages (Mφs). Stationary phase promastigotes were used to infect peritoneal Mφs from BALB/c mice as described [38]. Percentages of infected Mφs (E, G) and the number of parasites per 100 Mφs were recorded (F, H). As a control, WT parasites were also used to infect activated Mφs (Δ: stimulated with 100 ng/ml of LPS and 100 ng/ml of IFN-γ) to ensure these Mφs possessed microbicidal activities. Error bars represent standard deviations.

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findings, lysate from this line showed a high level of IPCase activity but little if any SMase activity (Fig. 5C–F; Table 1).

In contrast to the ability of the SL hydrolases with significant SMase activity, expression of \textit{CnISC1} in \textit{iscl} \textsuperscript{2} did not restore their ability to induce lesion pathology in susceptible BALB/c mice infection (Fig. 6C–D). Instead, these \textit{iscl} \textsuperscript{2} /+\textit{CnISC1} parasites persisted at low levels in BALB/c mice, similar to those \textit{iscl} \textsuperscript{2} mutants transfected with pIR1SAT vector only (Fig. 6C–D) (Table S2). Interestingly, the \textit{iscl} \textsuperscript{2} /+\textit{CnISC1} parasites exhibited normal morphology (Fig. 6C) and viability (data not shown) in stationary phase. Therefore, the cell shape defect appears to be separate from the virulence defect in \textit{iscl} \textsuperscript{2}. In total, our complementation experiments with SL hydrolases suggest the degradation of host sphingomyelin is essential for acute pathology in \textit{L. major}, whereas the activity of IPCase is not required for virulence (Table 1 and Fig. 6).

Expression of surface virulence factors is unaltered in \textit{iscl} \textsuperscript{2} mutants

Surface glycoconjugates including LPG, GP63, and GIPLs are important virulence factors in \textit{Leishmania}. Because the degradation of IPC generates phosphoinositol (Fig. S2), which could be used to synthesize GPI-anchored molecules, we tested whether the deletion of ISCL affects the production of LPG or GP63. Whole cell extracts from log phase promastigotes were subjected to western-blot analysis using monoclonal antibody WIC79.3 [41,42] and a rabbit anti-GP63 antiserum to detect LPG and GP63, respectively. As illustrated in Fig. 7A, cellular levels of LPG and GP63 were not significantly altered in \textit{iscl} \textsuperscript{2} mutants or the \textit{iscl} \textsuperscript{2} /+\textit{ISCL} parasites; in addition, both LPG and GP63 showed normal surface (cell membrane) localization in log and stationary phases (Fig. 7B and 7C; data not shown). Together, these results indicate the loss of ISCL has no adverse effects on the expression or localization of GPI-anchored molecules. Therefore, the loss of acute virulence in \textit{iscl} \textsuperscript{2} is not due to defects in surface virulent factors.

Localization of ISCL

To determine the cellular localization of ISCL, a GFP-ISCL fusion protein was introduced into the \textit{iscl} \textsuperscript{2} mutant. Fluorescence microscopy revealed the distribution of GFP-ISCL to be similar to the staining pattern of the mitochondrial marker MitoTracker, in
Figure 6. Restoration of stationary phase morphology and acute virulence in *isc*− mutants by mammalian neutral SMases and fungal ISC1ps. (A)−(B) Morphology and virulence of *isc*− parasites with human neutral SMase 1 (isc− SSU::hNSM1), murine neutral SMase 2 (isc− SSU::mNSM2), or pIR1SAT vector only (isc− SSU::pIR) were examined as described. (C)−(D) Similar experiments were performed using *isc*− parasites transfected with pIR1SAT-ScISC1 (isc−/+ScISC1), pIR1SAT-CnISC1 (isc−/+CnISC1), and pIR1SAT vector only (isc−/+pIR). In (A) and (C), promastigotes were grown to stationary phase and the percentage of round cells (day 1 through day 5 in stationary phase) was determined. In (B) and (D), infectivity of stationary phase promastigotes was examined by footpad infection assay using BALB/c mice (2×10^7 cells/mouse in B and 1×10^6 cells in D) and the progression of lesions was monitored weekly. Error bars represent standard deviations.

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Figure 7. Expression of LPG and GP63 is not altered in *isc*− mutants. (A) Whole cell lysates from log phase promastigotes were resolved by SDS/PAGE and blotted to PVDF membranes. Monoclonal antibody WIC79.3 was used to detect LPG; rabbit anti-*L. major* GP63 polyclonal antibody was used to detect GP63; and monoclonal anti-α-tubulin antibody (Sigma) was used as a loading control [41]. Localizations of LPG (B; primary antibody: WIC79.3; secondary antibody: goat anti-mouse IgG-Texas Red) and GP63 (C; primary antibody: a monoclonal anti-*L. major* GP63 antibody from Cedarlane Inc.; secondary antibody: goat anti-mouse IgG-FITC) were determined by indirect immuno-fluorescence microscopy as we previously described [12].

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both log and stationary phase (Fig. 8A–E and data not shown), suggesting this protein is mostly localized in the mitochondria. The GFP-ISCL was functional, as iscl\(^{-}\)/+GFP-ISCL parasites were able to degrade sphingomyelin (Fig. 8F) and showed normal morphology and virulence (data not shown). GFP-ISCL did not overlap with the plasma membrane, as revealed using an anti-LPG monoclonal antibody WIC 79.3 or the endoplasmic reticulum (ER) revealed by fluorescence microscopy with an anti-T. brucei Bip antiserum (data not shown).

Next, we examined whether ISCL could be secreted. Promastigotes of iscl\(^{-}\}/+ISCL were grown from log to stationary phase and culture supernatant was examined for neutral SMase activity. As shown in Fig. S5, contrary to the robust SMase activity from iscl\(^{-}\}/+ISCL cells, no activity was detected in the supernatant. Adding sphingomyelin to the culture medium did not induce any ISCL secretion (Fig. S5). Similar results were observed with concentrated supernatant (using filtration-based concentrators) (data not shown). Therefore, consistent with the localization study and the predicted ISCL sequence which lacks an obvious signal peptide, promastigotes do not secrete ISCL.

Neutral SMase activity in L. amazonensis amastigotes

The lack of acute virulence in iscl\(^{-}\) mutants suggests SMase activity is essential for amastigote survival and replication in the mammalian host. However, methods for the generation of axenic L. major parasites amastigotes are not available, and lesion-derived or macrophage-derived amastigotes typically are contaminated with host material likely including neutral SMase. To circumvent this problem, we used L. amazonensis with host material likely including neutral SMase. To circumvent or macrophage-derived amastigotes typically are contaminated or contamination with host material likely including neutral SMase. To circumvent this problem, we used L. amazonensis, a new world Leishmania species able to form axenic amastigotes that resemble closely to lesion-derived amastigotes in morphology, virulence, expression of stage-specific genes, and interaction with mammalian cells [43,44]. As shown in Fig. 9, lysates from both promastigotes and amastigotes of L. amazonensis were able to hydrolyze sphingomyelin. Similar to L. major, the neutral SMase activity in L. amazonensis promastigotes and amastigotes was sensitive to GW4869 (data not shown). After normalizing to protein levels, a two-fold higher level of activity was seen in amastigotes than promastigotes (Fig. 9) (p<0.05). Thus L. amazonensis amastigotes express SMase which was shown genetically in L. major to be required for parasite survival and growth in the mammalian host.

Discussion

ISCL is responsible for the degradation of host-derived sphingomyelin and Leishmania-derived IPC, but is not required for the production of EtN

In L. major, ISCL is the sole homologue of the neutral SMase genes in mammals and the ISCL genes (which encode inositol phosphosphingolipid phospholipase C or IPCase) in fungi (Figs. S2 and S3). The predicted ISCL contains several well-conserved regions that are essential for the catalytic activity of SMase and IPCase, e.g. a P-loop motif which may be involved in Mg\(^{2+}\) binding, and two transmembrane helices near the C-terminus, which may anchor the protein to mitochondrial membrane for activation [20,27]. Despite the lack of sphingomyelin synthesis, L. major parasites can actively take up and hydrolyze sphingomyelin and the neutral SMase activity clearly requires ISCL (Fig. 3A–D). In addition to sphingomyelin, ISCL also contributes to the turnover of endogenous IPC as the iscl\(^{-}\)/+ISCL parasites exhibited elevated IPCase activity whereas the activity in iscl\(^{-}\) mutants was close to background (Fig. 3E–F). Consistent with this result, iscl\(^{-}\) mutants showed modest accumulation of IPC and less ceramide compared to WT and iscl\(^{-}\)/+ISCL parasites (Table 2). Similar to mammalian neutral SMases, L. major ISCL exhibited much stronger activity with sphingomyelin than IPC (10–20 times higher, Table 1), suggesting sphingomyelin is the preferred substrate.

For promastigotes, the synthesis and degradation of sphingoid base is a major pathway to generate EtN [14] (Fig. S1). Although Leishmania do possess other pathways to generate EtN (such as salvage from the medium), they are not sufficient to support growth and metacyclogenesis in the absence of SM metabolism as manifested by the spt2\(^{-}\) and spl\(^{-}\) mutants [14]. As illustrated in Fig. S1, the production of phosphoethanolamine from SL metabolites could occur via two routes: an IPC independent route from sphingoid bases and ceramide; and an alternative route which requires the synthesis and degradation of IPC (Fig. S1). The latter is considered a reasonable pathway because SL metabolites such as sphingoid bases and ceramides are toxic and labile with low steady state concentrations, whereas IPC is highly abundant and could serve as a reservoir for EtN production [14]. However, our results clearly indicate although ISCL is responsible for the degradation of IPC, it is not essential for EtN production. First, iscl\(^{-}\) mutants are fundamentally different from spt2\(^{-}\) and spl\(^{-}\) mutants: iscl\(^{-}\) parasites grow like WT in vitro and form apparently normal metacyclics yet fail to induce pathology in mice, whereas spt2\(^{-}\) and spl\(^{-}\) mutants grow well but upon entry into stationary phase die rapidly and fail to differentiate; however, they retain the ability to yield acute disease pathology, as some surviving parasites are able to differentiate into amastigotes, which are able to acquire ethanolamine by savage [14]; second, defects in iscl\(^{-}\) mutants cannot be reversed by exogenous EtN at all (Figs. 1–2, 4); third, the abundance of PLE was similar between iscl\(^{-}\) mutants and WT parasites (Table 2). Therefore, there is no shortage of EtN in iscl\(^{-}\) mutants, suggesting the IPC-independent pathway is sufficient for EtN/PLE biosynthesis in the absence of ISCL (Fig. S1).
The activity of neutral SMase but not IPCase is essential for parasite proliferation and pathology in mammals

The most striking defect of iscl mutants is their complete lack of acute virulence in susceptible BALB/c mice, as stationary phase promastigotes or purified metacyclics yielded no pathology even after 7–8 months (Fig. 4A–D). In vitro macrophage infection indicated that iscl were able to enter host cells but did not survive well, especially at 37 °C (Fig. 4E–H). This virulence defect was completely reversed by the neutral SMases from human, mouse, and yeast (Table 1) (Fig. 6). We confirmed the finding from Del Poeta’s group that the C. neo ISCL1 lacked significant SMase activity in our studies, when it was expressed heterologously in Leishmania (Fig. 5). Despite its strong IPCase activity, heterologous expression of CnISC1p failed to reverse the virulence defect (Figs. 5–6). Together, these complementation results strongly suggest the degradation of host-derived sphingomyelin is necessary and sufficient for parasite survival and replication in mammals, whereas the degradation of endogenous IPC by itself is not essential for Leishmania virulence. In agreement with this conclusion, axenic amastigotes of L. amazonensis showed strong SMase activity, about 2-fold higher than promastigotes (Fig. 9).

Interestingly, most GFP-tagged ISCL is localized in the mitochondria during the promastigote stage (Fig. 8) when cells can take in sphingomyelin for hydrolysis (Fig. 3E–F). In Leishmania, lipid vesicles and lipid-protein complexes can be incorporated into the parasitic plasma membrane through fusion or be taken up through an endocytic pathway including the flagellar pocket and endosomes [45,46]. Although mitochondria are not directly connected to the vesicular pathways, phospholipids such as PSs are synthesized in the ER and transported to the mitochondria transported in mammalian cells and yeast [47,48]. In S. cerevisiae, an ER-mitochondria tethering complex has been identified composed of proteins resident of both ER and mitochondria [49]. Consistent with these findings, our data imply lipids from the plasma membrane and/or endocytic compartments may contribute to the homeostasis of mitochondrial membrane (where GFP-ISC1 resides and the hydrolysis of SLs occurs). Localization of ISCL in amastigotes is currently under investigation.

In S. cerevisiae, ScISC1p generates phytoceramide in the mitochondrial membrane and such activity may be important for the respiratory function of mitochondria and the metabolism of non-fermentable carbon sources [36]. As promastigotes, Leishmania acquire energy through the metabolism of sugars, amino acids and fatty acids [50]. It is possible that the mitochondrial-localized ISCL has similar functions as the S. cerevisiae ISC1 enzyme in regulating respiration and plays an important role in parasite growth in low sugar conditions. Consistent with this hypothesis, iscl mutants showed poor viability and were more round in shape (signs of cells under stress) in late stationary phase when glucose became depleted (Fig. 1). Interestingly, although CnISC1p failed to complement the virulence defect in iscl, it did restore the mutants’ morphology and viability in late stationary phase, implying that while SMase activity is required for the survival and proliferation of amastigotes, IPC hydrolysis, however, may be involved in the maintenance of mitochondrial function in promastigotes.

In addition to its potential role in mitochondria, we could envision several other mechanisms by which SMase activity may contribute to virulence; notably these mechanisms are not mutually exclusively. First, amastigotes of most Leishmania species reside within mature phagolysosomes which are enriched in amino acids and lipids but poor in carbohydrates [51]. To survive, these amastigotes need to salvage lipids (including SLs) from the host [15] and SMase (along with other lipases) may be required for the acquisition of nutrients. In addition, the degradation of host SLs by amastigotes could disrupt SL-dependent signaling pathways in macrophages. It has been reported that Leishmania donovani infection led to elevated ceramide levels in macrophages, which were responsible for the downregulation of classical PKC activity and the induction of PKCζ (an atypical Ca-independent stress kinase), as well as the ceramide-activated protein phosphatas.

These changes were associated with the inhibition of NF-kappaB transactivation and the suppression of nitric oxide generation [52–54]. In the future, molecular interactions between iscl mutants and host phagocytes will be evaluated to determine whether sphingomyelin degradation is required for the inhibition of proinflammatory cytokine release. In addition, the ability of iscl mutants to persist without pathology provides an excellent platform to study the long-term, asymptomatic infection where the interaction between Leishmania and host is poorly understood.

Summary

Our study revealed an essential yet previously unrecognized role of SL degradation in L. major virulence. Despite the lack of sphingomyelin biosynthesis, Leishmania parasites possess an ISCL-dependent neutral SMase activity. Deletion of ISCL completely abolished acute disease pathology in mice. ISCL is also required for the turnover of IPC, but is not required for the production of EiN. Future studies will determine the molecular mechanism by which host-SL degradation contributes to acute virulence.
Materials and Methods

Materials

BALB/c (female, 7–8 weeks old) mice were purchased from Charles River Laboratories International. All procedures involving mice were approved by the Animal Care and Use Committee at Texas Tech University (PHS Approved Animal Welfare Assurance No. A3629-01). N-[6-(7-nitro-2,1,3-benzoxadiazol-4-yl)aminoo]hexanoyl]-sphingosine-1-phosphocholine (NB6 C6-sphingomyelin) was purchased from Invitrogen Corporation. N-[12-(7-nitro-2,1,3-benzoxadiazol-4-yl)aminool]odecanoyl]-sphingosine-1-phosphinositol (NB6 C12-IPC) was custom-synthesized by Avanti Polar lipids. All other chemicals were purchased from VWR International unless specified otherwise.

Molecular constructs

The open reading frame (ORF) of ISCL (LmjF08.0200) was amplified by PCR from L. major genomic DNA using primer pairs 5’ ISCL ORF BamHI (attactGGATCCACCATGTCGCACGCATGGATCTTCTTCAGCT, P58) and 3’ ISCL ORF BamHI (attactGGATCCACCATGTCGCACGCTATCCCTACAACTTCTTCAGCT, P59). The resulting DNA fragment was digested with BamHI and cloned in the pXG vector [55] as pXG-ISCL (B83) or the pIR1SAT vector [56] as pIR1SAT-ISCL (B106). After confirming its sequence, ISCL ORF was cloned into the pXG-GFP-2’ vector to generate pXG-GFP-ISCL (B103), which was used in localization studies. To generate the knock-out constructs for ISCL, the predicted 5’- and 3’-untranslated regions (∼1 Kb each) were PCR amplified and cloned in tandem in the pUC18 vector; genes conferring resistance to puromycin (PAC) and blasticidin (BSD) were inserted between the 5’- and 3’-untranslated regions to generate pUC-KO-ISCL:PAC (B85) and pUC-KO-ISCL:BSD (B84). ORFs of human neutral SMase 1 (accession #NM_003071) and mouse neutral SMase 2 (accession #NM_021491.3), derived from constructs generated in Dr. Yusuf Hannun’s lab (Medical University of South Carolina), were cloned in the pIR1SAT vector as pIR1SAT-SSU::hNSM1 (B142) and pIR1SAT-ssu::mNSM2 (B141), respectively. ISCL genes from S. cerevisiae (ScISC1, accession #P40015) and C. neoformans (CnISC1, accession #DQ407762) were PCR amplified and cloned in the pIR1SAT vector as pIR1SAT-ScISC1 (B108) and pIR1SAT-CNISC1 (B107), respectively.

Leishmania culture and genetic manipulations

L. major LV39 clone 5 (Rho/SU/59/P) promastigotes were grown in M199 medium with 10% fetal bovine serum and other supplements as described [57]. L. amazonensis (MHOM/BR/77/LTB0016) promastigotes and axenic amastigotes were cultured as previously described [43, 58]. Growth rate was determined by monitoring the density of culture over time using a hemacytometer. Viability was measured by flow cytometry using an Accuri C6 Flow Cytometer after staining with 5 µg/ml of propidium iodide. Metacyclics were isolated from stationary phase culture using the density gradient method [34] and/or the peanut agglutination method [33]. The ISCL alleles were sequentially replaced by puromycin (PAC) and blasticidin (BSD) resistance genes to generate the isl” mutant (AISCL::BSD/AISC1::PAC). Transfection and selection of promastigotes was performed as previously described [57]. To confirm the deletion of ISCL, genomic DNA was digested with NotI plus BamHI, resolved on a 0.8% agarose gel, transferred to a nitrocellulose membrane, and hybridized with a [32P]-labeled DNA probe. To reconstitute ISCL expression, pXG-ISCL was introduced into isl” and referred to as isl” /+ISCL (AISCL::BSD/AISC1::PAC+/pXG-ISCL). To test whether isl” can be complemented by mammalian neutral SMases, linearized DNA (using Sall) from pIR1SAT-kNSM1 or pIR1SAT-ssu::mNSM2 were integrated into the small ribosomal subunit site of isl” mutants to generate isl” SSU::hNSM1 or isl” SSU::mNSM2. To test whether isl” can be complemented by fungal ISCL genes, mutants were transfected with pIR1SAT-ISCL1 or pIR1SAT-CNISC1 to generate isl” /+ScISC1 or isl” /+CnISC1. To study the localization of GFP-tagged ISCL protein, WT parasites were transfected with pXG-GFP-ISCL and WT (pXG-GFP-ISCL) cells were selected based on resistance to G418.

Microscopy and macropage infection

To analyze the morphology of isl” mutants, log and stationary phase promastigotes were fixed in 3.7% formaldehyde and percentages of round cells (defined as those with the length of the long axis less than twice the length of the short axis) were determined using a hemacytometer. About 200 randomly selected cells were counted in each experiment. For fluorescence microscopy, parasites were stained with 350 nM of Mitotracker Red 580 (Invitrogen) for 30 min in darkness. Cells were then attached to poly-lysine coated cover slips, washed twice with phosphate buffered saline (PBS), once with 50% ethanol, and stained with 2.5 µg/ml of Hoechst 33342 for 10 min. Images were acquired using an Olympus BX50 Upright Fluorescence Microscope equipped with a digital camera. Localizations of LPG (primary antibody: WIC79.3 [42]; secondary antibody: goat anti-mouse IgG-Texas Red) and GP63 (primary antibody: monoclonal anti-L. major GP63 antibody from Cedarlane Inc.; secondary antibody: goat anti-mouse IgG-FITC) were determined by indirect immunofluorescence microscopy as we previously described [12]. Peritoneal macrophages from BALB/c mice were isolated and infected with purified stationary phase promastigotes (opsonized with C57BL/6 mouse serum) at a ratio of 10 parasites per one macrophage as previously described [38]. Percentages of infected macrophages and the number of parasites per 100 macrophages were determined microscopically after staining with 2.5 µg/ml of Hoechst 33342.

Mouse infections and analyses

Virulence of promastigotes was evaluated in BALB/c mice using footpad infection [59] and ear lobe infection [60, 61]. For footpad assay, late stationary phase promastigotes (3 days after the onset of stationary phase) or purified metacyclics (prepared from 3–4 days old stationary phase culture) were resuspended in DMEM and injected into the footpads of 8-week old female BALB/c mice (6–10 mice per group) at 1 x 10⁶ cells/mouse (stationary phase promastigotes) or 2 x 10⁵ cells/mouse (metacyclics). For ear lobe infection, stationary phase parasites were inoculated intradermally into the ear lobes of 8-week old female BALB/c mice (5 mice per group) at 1 x 10⁶ cells/mouse. Lesion sizes were measured weekly using a vernier caliper and parasite numbers in the infected tissue were determined by limiting dilution assay [59]. Mice infected with WT or isl” /+ISCL parasites were sacrificed when their lesions became overly large (over 2.5 mm for footpad infection and over 5.0 mm for ear infection).

Neutral SMase assay and IPCase assay

Log phase promastigotes were suspended in a lysis buffer (25mM Tris pH7.5, 0.1% Triton X100, 1 x protease inhibitor) at 2.0 x 10⁶ cells/ml and incubated for 5 min on ice. Protein concentration was determined by the micro-BCA assay. Triton
Degradation of sphingomyelin by intact L. major promastigotes

Promastigotes were inoculated in M199 medium without fetal bovine serum at 7.0 × 10^6 cells/ml and labeled with 5 μM of NBD C6-sphingomyelin. After 48 h, cells were washed with PBS and total lipids were extracted and analyzed by TLC as described above for neutral SMase assay.

Lipid analysis by electrospray ionization mass spectrometry

Lipid extraction and analysis by ESI/MS (negative ion mode) was performed as previously described [15] with minor modifications. A N-octanoyl-D-erythro-sphingosine-1-phosphate (d18:1/8:0 ceramide phosphate, FW = 505.5, 1.0 × 10^6 molecules/cell) was used as a standard for IPC; a 1-2-O-β-D-octadecenyl-2-arachidonyl-sn-glycero-3-phosphoethanolamine (p18:0/20:4-PE, FW = 751.6, 2.0 × 10^6 molecules/cell) was used as a standard for PLE; and a N-octanoyl-D-erythro-sphingosine (d18:1/8:0 ceramide, FW = 425.7, 0.0 × 10^6 molecules/cell) was used as a standard for Ceramide. All three internal standards were added prior to lipid extraction.

Western-blot of LPG and GP63

Promastigotes were collected and resuspended in PBS at 1 × 10^6 cells/ml. Cell extracts were prepared and western-blotting were performed as previously described [12]. Primary antibodies include the rabbit anti-GP63 polyclonal antiserum (a generous gift from Dr. KP Chang at Rosalind Franklin University of Medicine and Science) (1:10000), monoclonal anti-L. major LPG antibody WIC79.3 [34] (1:5000), and monoclonal anti-β-tubulin antibody (Sigma) (1:8000); secondary antibodies include the goat anti-rabbit or anti-mouse IgG-Ab–HRP conjugated (1:2000).

Supporting Information

Figure S1 Metabolism of SLs in L. major. SPT: serine palmitoyltransferase; SPL: sphingosine-1-phosphate lyase; ISCLp: inositol phosphophingolipid phospholipase C 1 protein or IPCase; IPCS: IPC synthase. Note that phosphoethanolamine can be produced via an IPC-independent pathway or an indirect pathway that requires the synthesis and degradation of IPC.

Found at: doi:10.1371/journal.ppat.1000692.s001 (0.07 MB PDF)

Figure S2 Degradation of sphingomyelin (A) and IPC (B). In mammals, the degradation of sphingomyelin by SMase is a major route to produce ceramide, an important signaling molecule. Leishmania parasites do not synthesize sphingomyelin but contain high abundance of IPC. In fungi (which also synthesize IPC), hydrolysis of IPC is mediated by inositol phosphophingolipid phospholipase C (ISCLp, B), a homolog of mammalian neutral SMase.

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Figure S3 Alignment of the amino acid sequences of LmISCLp (L. major geneDB system ID LmjF08.0200), human neutral SMase 1 (Genbank accession #NP_003071), and Saccharomyces cerevisiae ISCLp (Genbank accession #P40015). Highly conserved regions are shaded. The P-loop domain is underlined. The two boxed regions near the C-terminus of LmISCLp (at aa 447–466 and 612–634) represent predicted transmembrane helices. Asterisks represent amino acids essential for catalysis based on studies of B. cereus SMase [26], SISCLp [27], and HNSMase 1 [28].

Found at: doi:10.1371/journal.ppat.1000692.s003 (0.11 MB PDF)

Figure S4 Targeted deletion of ISCL. Southern blot analysis of L. major wild type (WT), heterozygote clones (ISCL+/− #1 and 2), homozygote clones (ISCL−/− #1-1 and #2-1), and the reconstituted strain (ISCL+/+ISCL) was performed as described in Materials and Methods using a probe corresponding to the ISCL ORF. Bands corresponding to the endogenous (>10 Kb, 24 hours exposure) and episomal alleles of ISCL (~2.0 Kb, 3 hours exposure) are indicated.

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Figure S5 L. major promastigotes do not secrete ISCL protein. Promastigotes of iscl−/−ISCL were grown in the absence or presence of C16-sphingomyelin (provided at 1 μM final concentration daily) and neutral SMase assay was performed using whole cell lysate or culture supernatant. Positive control (+): 0.1 unit of B. cereus SMase; negative control (−): boiled WT lysate.

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Table S1 Parasite number in BALB/c mice infected with iscl−/−ISCL. L. major, iscl−/−ISCL, and iscl−/−SSU::pIR (as described in Fig. 6B) Limiting dilution assays were performed at 4–5 weeks post infection (two mice per group).

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Table S2 Parasite numbers in BALB/c mice infected with L. major promastigote (as described in Fig. 6D). Limiting dilution assays were performed at 6–7 weeks post infection (two mice per group).

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Author Contributions

Conceived and designed the experiments: KZ. Performed the experiments: OZ MCW WX FFH YW KZ. Analyzed the data: OZ MCW WX FFH JT LS SMB KZ. Contributed reagents/materials/analysis tools: FMK PK. Wrote the paper: OZ SMB KZ.
References
