Identification of *Leptospira interrogans* Phospholipase C as a Novel Virulence Factor Responsible for Intracellular Free Calcium Ion Elevation during Macrophage Death

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Abstract

**Background:** *Leptospira*-induced macrophage death has been confirmed to play a crucial role in pathogenesis of leptospirosis, a worldwide zoonotic infectious disease. Intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\text{\textit{i}}) elevation induced by infection can cause cell death, but [Ca\(^{2+}\)]\text{\textit{i}} changes and high [Ca\(^{2+}\)]\text{\textit{i}}-induced death of macrophages due to infection of *Leptospira* have not been previously reported.

**Methodology/Principal Findings:** We first used a Ca\(^{2+}\)-specific fluorescence probe to confirm that the infection of *L. interrogans* strain Lai triggered a significant increase of [Ca\(^{2+}\)]\text{\textit{i}} in mouse J774A.1 or human THP-1 macrophages. Laser confocal microscopic examination showed that the [Ca\(^{2+}\)]\text{\textit{i}} elevation was caused by both extracellular Ca\(^{2+}\) influx through the purinergic receptor, P\(_{2\alpha X_{\gamma}}\), and Ca\(^{2+}\) release from the endoplasmic reticulum, as seen by suppression of [Ca\(^{2+}\)]\text{\textit{i}} elevation when receptor-gated calcium channels were blocked or P\(_{2\alpha X_{\gamma}}\) was depleted. The LB361 gene product of the spirochete exhibited phosphatidylinositol phospholipase C (L-PI-PLC) activity to hydrolyze phosphatidylinositol-4,5-bisphosphate (PIP\(_{2}\)) into inositol-1,4,5-trisphosphate (IP\(_{3}\)), which in turn induces intracellular Ca\(^{2+}\) release from endoplasmic reticulum, with the Km of 199 \(\mu\)M and Kcat of 8.566E-5 S\(^{-1}\). Secretion of L-PI-PLC from the spirochete into supernatants of leptospire-macrophage co-cultures and cytosol of infected macrophages was also observed by Western Blot assay. Lower [Ca\(^{2+}\)]\text{\textit{i}} elevation was induced by infection with a LB361-deficient leptospiral mutant, whereas transfection of the LB361 gene caused a mild increase in [Ca\(^{2+}\)]\text{\textit{i}}. Moreover, PI-PLCs (PI-PLC-\(\beta3\) and PI-PLC-\(\gamma1\)) of the two macrophages were activated by phosphorylation during infection. Flow cytometric detection demonstrated that high [Ca\(^{2+}\)]\text{\textit{i}} increases induced apoptosis and necrosis of macrophages, while mild [Ca\(^{2+}\)]\text{\textit{i}} elevation only caused apoptosis.

**Conclusions/Significance:** This study demonstrated that *L. interrogans* infection induced [Ca\(^{2+}\)]\text{\textit{i}} elevation through extracellular Ca\(^{2+}\) influx and intracellular Ca\(^{2+}\) release cause macrophage apoptosis and necrosis, and the LB361 gene product was shown to be a novel PI-PLC of *L. interrogans* responsible for the [Ca\(^{2+}\)]\text{\textit{i}} elevation.

Citation: Zhao J-F, Chen H-H, Ojcius DM, Zhao X, Sun D, et al. (2013) Identification of *Leptospira interrogans* Phospholipase C as a Novel Virulence Factor Responsible for Intracellular Free Calcium Ion Elevation during Macrophage Death. PLoS ONE 8(10): e75652. doi:10.1371/journal.pone.0075652

Editor: Yung-Fu Chang, Cornell University, United States of America

Received March 4, 2013; Accepted August 17, 2013; Published October 4, 2013

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Funding: This work was supported by grants (81171534 and 81261160321) from the National Natural Science Foundation of China and a grant (2010ZZ09) from the National Key Lab for Diagnosis and Treatment of Infectious Diseases of China. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Leptospirosis caused by pathogenic *Leptospira* species is a world-wide spread zoonotic infectious disease [1]. The disease has been prevalent in most countries in Southeast Asia and South America [2,3]. However, in recent years, human leptospirosis cases have also been frequently reported in North America and Europe [4–6], and the disease was identified as an emerging global public health problem [1,7].

Many animals serve as the natural hosts of pathogenic *Leptospira* species [8]. When individuals come in contact with soil or water contaminated with leptospire-containing urine from infected animals, the leptospires invade into human body through the skin or mucosa to cause leptospirosis [9,10]. The mild cases of infection exhibit influenza-like manifestations such as fever and myalgia, while the severe cases frequently result in death due to respiratory failure caused by pulmonary diffuse hemorrhaging and meningitis or renal failure due to renal injury and jaundice [3,4,9,10].
However, until now, the pathogenic mechanisms of Leptospira infection remain poorly understood.

Macrophages and neutrophils play an important role in innate immunity against infection through phagocytosis of microbial pathogens including Leptospira. However, unlike many other bacterial pathogens, only macrophages can kill the phagocytosed leptospires in individuals who do not have specific antibodies against Leptospira [9,11,12]. Therefore, macrophages act as a crucial phagocyte in the host defense mechanisms against leptospiral infection in unvaccinated individuals, and ability to evade phagocytosis by macrophages contributes to virulence of pathogenic Leptospira species [13–15].

Infection results from interaction between microbial pathogens and hosts [16,17]. In order for the hosts to respond to pathogen, or for the pathogen to resist the innate immune response of the host, both the pathogens and hosts must modify significantly their metabolism and gene expression profiles [18,19]. For instance, intracellular free calcium ion (Ca$^{2+}$), an important intracellular messenger with multiple physiological functions, is increased when cells are infected with some bacterial pathogens [20]. Thus, Helicobacter pylori or Campylobacter jejuni cause an elevation of intracellular free Ca$^{2+}$ concentration ([Ca$^{2+}$]i) through Ca$^{2+}$ release and/or influx mechanisms in gastric mucous epithelial cells or in intestinal epithelial cells during infection [21,22]. The high [Ca$^{2+}$]i in macrophages caused by infection with Listeria monocytogenes or Brucella abortus are involved in bacterial invasion and escape from phagocytic vesicles for intracellular replication [23,24]. In particular, high [Ca$^{2+}$]i can induce cell death, in which a mild [Ca$^{2+}$]i increases typically stimulates cell apoptosis while a high [Ca$^{2+}$]i change results in cell necrosis [25]. Our previous studies confirmed that L. interrogans, a predominant pathogenic Leptospira species, could be phagocytized by human or mouse macrophages, but infection induces macrophage apoptosis and necrosis [14–16]. However, the change of [Ca$^{2+}$]i in L. interrogans-infected macrophages and the role of high [Ca$^{2+}$]i in Leptospira-induced macrophage death had not been investigated yet.

Phospholipase (PL) is a group of enzymes in eukaryotes and prokaryotes that can be classified into the PLA, PLC, PLD and sphingomyelinase (SMase) subfamilies according to the specificity of their substrates and diversity of products [26]. PLC is also divided into PC-PLC, which hydrolyzes phosphatidyl choline (PC) of their substrates and diversity of products [26]. PLC is also divided into PC-PLC, which hydrolyzes phosphatidylcholine (PC) into phosphorylcholine and diacylglycerol (DAG), and PI-DAG, which hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP2) to produce inositol-1,4,5-trisphosphate (IP3) and DAG. The PLC activity of the PL-domain-containing genes (LA0543, LA2250 and LB361) in the endoplasmic reticulum to trigger release of Ca$^{2+}$ from the endoplasmic reticulum into cytosol, resulting in[Ca$^{2+}$]i elevation [27].

Until now, except for a PI-PLC produced by L. monocytogenes, few bacterial PLC have been well characterized [28]. Our bioinformatic analysis indicated that there are three PLC-domain-containing genes (LA0543, LA2250 and LB361) in chromosomal DNA of L. interrogans serogroup Icterohaemorrhagiae serovar Lai strain Lai (GenBank accession No.: NC_004342) were predicted by using SWISS-MODEL and InterProScan softwares [32,33]. Signal peptides and transmembrane regions in sequences of the three genes were analyzed using SignalP 3.0 and TMHMM 2.0 softwares.

Detection of Target Genes in Different Leptospiral Strains

The distribution of LA0543, LA2250 and LB361 genes in seven pathogenic L. interrogans strains and two non-pathogenic L. biflexa strains belonging to different serogroups and serovars were detected by PCR and sequencing. The experimental details are given in Materials S1.

Expression and Extraction of Target Recombinant Proteins

The signal peptide sequence-lacking LA0543 and LA2250 genes and the entire LB361 gene of L. interrogans strain Lai were amplified by PCR. After sequencing, the LA0543, LA2250 and LB361 gene segments were linked with the linearized pET42a plasmid (Novagen, USA) to form recombinant expression vectors, respectively, and then transformed into E. coli BL21DE3 (Novagen) for expression. The expressed soluble recombinant proteins were examined by SDS-PAGE and then extracted by Ni-NTA affinity chromatography (Figure S2B). The details for the expression and extraction of recombinant proteins expressed by the three leptosporial genes are given in Materials S1.

Preparation of Antisera and IgGs

Details about the preparation of antiserum and IgGs against the recombinant proteins expressed by the LA0543, LA2250 and LB361 genes of L. interrogans strain Lai are given in Materials S1.
Detection of PLC Activity of Leptospiral Recombinant Proteins

The PC-PLC or PI-PLC activity of recombinant proteins expressed by the LA0543, LA2250, and LB361 genes of L. interrogans strain Lai was detected by the r-nitrophenylphosphorylcholine (NPPC) assay or IP3 fluorescence polarization assay as previously reported [34,35]. Briefly, in the NPPC assay, 90 μl borax-HCl buffer (100 mM, pH 7.5) containing 0.1, 1, or 10 μg each of the recombinant proteins was mixed with 10 μl borax-HCl buffer containing 100 mM NPPC (Sigma) for a 30-min incubation at 37°C. The r-nitrophenol released due to NPPC hydrolysis was quantified using a spectrophotometer (Bio-Rad, USA) at OD410, and the PC-PLC activity was calculated based on the standard curve created with different concentrations of r-nitrophenol (Sigma). In the IP3 fluorescence polarization assay, 50 μl reaction buffer (100 mM KCl, 1.9 mM CaCl2, 2 mM EGTA, 0.5 mg ml−1 bovine serum albumin and 0.1% sodium deoxycholate in 50 mM HEPES buffer, pH 7.0) containing 0.1, 1, or 10 μg each of the recombinant proteins was added with 400 μM PIP2 substrate (Echelon, USA). After incubation at 37°C for 10 min, the mixture was added with 250 μl chloroform-methanol-HCl (500:500:3, V:V:V) for a short vortex, and then added with 100 μl 5 mM EGTA-1 M HCl solution to terminate the reaction. The mixture was centrifuged at 500×g for 10 min and the aqueous phase was harvested to detect the IP3 concentration using a HitHunter™ IP3 Assay Kit (DiscoveRx Corp, USA) and a SpectraMax M5 Reader (Molecular Devices, USA) with fluorescence polarization spectrograph at FP-model according to the manufacturer’s protocol. The PI-PLC activity was calculated based on the standard curves created with different concentrations of IP3 (DiscoveRx Corp). In these assays, bovine serum albumin (BSA, Sigma) was used as the control, while 10 μM U73122, a mammalian cell PI-PLC blocker [36], was used to inhibit the PI-PLC activity of recombinant proteins.

Determination of Km and Kcat Values of rL-PI-PLC

To determine the enzyme kinetic parameters (Km and Kcat) of recombinant protein expressed by the LB361 gene of L. interrogans strain Lai (rL-PI-PLC), 60, 80, 100, 200, 400, 600 or 800 μM of PIP2 substrate (Echelon) in 50 μl of the reaction buffer, as described above, was mixed with 1 μg rL-PI-PLC for a 10-min...
incubation at 37°C. IP$_3$ concentrations in the mixtures due to PI-PLC-based hydrolysis of PIP$_2$ substrate were detected by the IP$_3$ fluorescence polarization assay, as described above. The Km and Kcat values of rL-PI-PLC were calculated using the double reciprocal Lineweaver-Burk plot according to the standard curves created with different concentrations of IP$_3$ (DiscoverRx Corp) [37].

Identification of Membrane Calcium Channels in Macrophages

J774A.1 or THP-1 cells were collected by a 500×g centrifugation for 15 min at 4°C. After washing with PBS and centrifugation, total membrane proteins of the cells were extracted using a Membrane Protein Extraction Kit (AbCam, USA). The protein concentration in the membrane protein samples was quantified using a BCA Protein Quantitative Kit (Beyotime Biotech, China). Using 1:500 diluted rabbit anti-voltage-gated Cav3.1, Cav3.2, Cav3.3 or Cav2.3 calcium channel protein-IgG (AbCam), or anti-receptor-gated P$_2$X$_1$, P$_2$X$_4$, P$_2$X$_7$, P$_2$X$_9$, or P$_2$X$_7$r calcium channel protein-IgG (Santa Cruz, USA) as the primary antibody, and 1:3000 diluted HRP-conjugated goat anti-rabbit-IgG (Jackson ImmunoResearch, USA) as the secondary antibody, Western Blot assays were performed to detect the membrane calcium channels of J774A.1 or THP-1 cells.

Detection of Leptospiroses in Macrophages during Infection

J774A.1 or THP-1 cells (10$^6$ cells per well) were seeded in 6-well culture plates (Corning, USA) for a 12-h incubation at 37°C to form monolayers. Freshly cultured L. interrogans strain Lai in EMJH medium was precipitated by a 17,200×g centrifugation at 15°C for 15 min. After washing twice with PBS, the leptosomal pellet was suspended in antibiotic-free 2.5% FCS RPMI-1640 medium for counting leiptospires with a Petroff-Hausser counting chamber under a dark-field microscope (Fisher Scientific, USA) [38]. The cell monolayers were infected with the spirochete at a multiplicity of infection (MOI) of 100 (100 leiptospires per cell) for 0.5 or 1 h [15]. The leiptospires in macrophages were detected by transmis-sion electron microscopy and laser confocal microscopy as previously described [19].

Measurement of Target Leptospiral Gene-mRNAs during Infection

J774A.1 or THP-1 cell monolayers (10$^6$ cells per well) were infected with L. interrogans strain Lai (10$^8$) at a MOI of 100 for 0.5, 1, 2, 4 or 6 h. The cultures were treated with 0.05% NaTDC-PBS to lyse cells [19], followed by a 17,200×g centrifugation at 4°C for 15 min to precipitate leiptospires. Total RNAs in the leiptospires were extracted using Trizol reagent (Invitrogen, China) plus digestion with RNase-free DNase (TaKaRa, China). cDNAs from the RNAs were synthesized by reverse transcription (RT) using a PrimeScript® RT reagent Kit (TaKaRa). Using each of the cDNAs as the template, mRNA levels of the LA0543, LA2250 or LB361 gene in the leiptospires were assessed by real-time quantitative PCR (qPCR) using a SYBR® Premix Ex-Taq™ II Kit (TaKaRa) in an ABI 7500 Real-Time PCR System (ABI, USA). The primers used in the RT-qPCR are shown in Table S1. In the RT-qPCRs, the leptosomal 16S rRNA gene was used as the internal reference [39]. The RT-qPCR data were analyzed using the ΔΔCt model and randomization test in REST 2005 software [40].

Determination of Leptospiral Protein Secretion during Infection

J774A.1 or THP-1 cell monolayers (10$^6$ cells per well) were infected with L. interrogans strain Lai (10$^8$) at a MOI of 100 for 0.5, 1, 2 or 4 h as above. The supernatants of cultures were collected to lyse cells [19], followed by a 17,200×g centrifugation at 4°C for 15 min to precipitate leiptospires. Total proteins in all the supernatant and cytosol samples were extracted by trichloroacetic acid precipitation as previously described [41].
used as a control.

Lane C2: immunoblotting result of the recombinant macrophages after infection with the spirochete (0.5–4 h). Lane C1: before infection (0 h) or from leptospire-free lysates of the two cytosol samples were collected from lysate of the J774A.1 or THP-1 cells cultured of the spirochete with J774A.1 or THP-1 cells (0.5–4 h). The supernatant samples are leptospire-free or cell-free liquids collected from the culture of the spirochete in EMJH medium (0 h) or from co-supernatant samples are leptospire-free or cell-free liquids collected from the culture of the spirochete before infection (0 h). The statistically significant differences were determined by ANOVA variance analysis plus Dunnett’s multiple comparison test. (B) Secretion of the proteins encoded by the LA0543, LA2250 and LB361 genes of L. interrogans strain Lai during infection of J774A.1 and THP-1 cells for the indicated times. The supernatant samples are leptospire-free or cell-free liquids collected from the culture of the spirochete in EMJH medium (0 h) or from co-cultures of the spirochete with J774A.1 or THP-1 cells (0.5–4 h). The cytosol samples were collected from lysate of the J774A.1 or THP-1 cells before infection (0 h) or from leptospire-free lysates of the two macrophages after infection with the spirochete (0.5–4 h). Lane C1: blank control. Lane C2: immunoblotting result of the recombinant protein expressed by the LB361, LA0543 or LA2250 gene used as the positive control. HlyX is a cytoplasmic protein of the spirochete also used as a control.

doi:10.1371/journal.pone.0075652.g003

Using 1:200 diluted rabbit-IgG against the recombinant protein of the LA0543, LA2250 or LB361 gene as the primary antibody, and 1:3000 diluted HRP-conjugated goat anti-rabbit IgG [Jackson ImmunoResearch] as the secondary antibody, Western Blot assays were performed to detect the proteins expressed by the LA0543, LA2250 and LB361 genes. In these assays, a leptospiral nonsecreted cytoplasmic protein, HlyX, was used as the control, and the recombinant HlyX protein (rHlyX) and rHlyX-IgG were provided by our laboratory [41].

Generation and Identification of the LB361 Gene-deleted or Complemented Mutant

Plasmid pUC19 was used for LB361 gene deletion (ΔLB361) and complementation (CALB361) since only the LB361 gene product was confirmed to have PI-PLC activity. Briefly, a suicide plasmid pUC19arm-kanArm was constructed and then electroporated into wild-type L. interrogans strain Lai to replace the LB361 gene with the kanamycin-resistant cassette sequence (kan) through allelic exchange by the 5’ and 3’ homologous arm sequences from the upstream and downstream regions of the gene to generate a ΔLB361 mutant. Conversely, a recombinant plasmid pUC19arm-kanArmΔLB361-spc-5’arm was constructed and then electro-transformed into the ΔLB361 mutant to replace the kan gene with the LB361-spc segment through allelic exchange by the 5’ and 3’ homologous arm sequences to generate a CALB361 mutant. The deletion in the ΔLB361 mutant and the complementation in the CALB361 mutant were confirmed by PCR, sequencing, and Western Blot assay (Figure S3 and S4A). The details for the generation and identification of the ΔLB361 and CALB361 mutants are given in Materials S1.

Generation and Identification of LB361 or chpI Gene-transfected Macrophages

pCMV-Tag2C, a prokaryote-eukaryote shuttle plasmid, is often used to transfect prokaryotic genes into different mammalian cells. Briefly, a recombinant pCMV-Tag2C containing the LB361 gene of L. interrogans strain Lai (pCMV-Tag2CLB361) was constructed and then transfected into J774A.1 or THP-1 cells using a Lipofectamine 2000 Transfection Kit (Invitrogen) or a Human Monocyte Nucleofector Kit (Lonza, Germany). The expression of the LB361 gene product in the LB361 gene-transfected macrophages was confirmed by Western Blot and immunofluorescence assays (Figure S4B and E). In addition, the chpI gene of L. interrogans strain Lai was transfected into J774A.1 or THP-1 cells, and ChpI protein expression in the transfected macrophages was determined by Western Blot assay (Figure S4C). Our recent study confirmed that the chpI gene product has no cytotoxicity to macrophages (the manuscript has been submitted to the journal of BMC Microbiology), and the chpI gene-transfected macrophages were used as the negative controls to determine the function of the LB361 gene product in host macrophages. The details about the generation and identification of LB361 or chpI gene-transfected J774A.1 and THP-1 cells are given in Materials S1.

Generation and Identification of P2X7-depleted Macrophages

To determine the role of the P2X7 calcium channel in extracellular Ca\(^{2+}\) influx, the P2X7 gene in J774A.1 or THP-1 cells was depleted with siRNA interference. The absence of P2X7 protein in the P2X7-depleted J774A.1 or THP-1 cells was confirmed by Western Blot assay (Figure S4D). The details about the generation of P2X7-depleted J774A.1 and THP-1 cells with siRNA interference and their characterization are given in Materials S1.

Detection of PI-PLC Phosphorylation of J774A.1 and THP-1 Cells during Infection

J774A.1 or THP-1 cell monolayers (10\(^6\) cells per well) were infected with L. interrogans strain Lai (10\(^8\)) at a MOI of 100 for 0.5, 1, 2 or 4 h. After trypsinization, washing with PBS and centrifugation, the precipitated cells were lysed with 0.05% NaTDC-PBS [19], followed by a 17,200 g centrifugation at 4°C for 15 min to harvest leptospire-free supernatants. Using 1:500 diluted rabbit-IgG against total PLC-β3 or PLC-γ1, pSer537 in PLC-β3, or pTyr759, pTyr783 or pTyr1217 in PLC-γ1 (Santa Cruz) as the primary antibody, and 1:3000 diluted HRP-conjugated goat anti-rabbit-IgG [Jackson ImmunoResearch] as the secondary antibody, Western Blot assays were performed to detect the phosphorylation of PLC-β3 and PLC-γ1 in the supernatant samples. In this assay, β-actin was used as the control.
Measurement of [Ca²⁺]i in Macrophages during Infection

J774A.1 or THP-1 cells (10⁵ cells per well) were seeded in 12-well culture plates (Corning) for a 12-h incubation at 37°C. The cell monolayers were washed thoroughly with D-Hank's buffer and then incubated in 100 µl of 0.2% BSA RPMI-1640 medium containing 10 µM fluorescent calcium indicator fluo-4/AM (Molecular Probes, USA) at 37°C for 1 h, followed by a 30-min incubation with 2.5% FCS RPMI-1640 medium for AM de-esterification to release the indicator. After washing with D-Hank's buffer again, the cell monolayers were infected with L. interrogans strain Lai (10⁷) for a 120-min contiguous detection at 37°C in a laser confocal microscope (type LSM510, Zeiss, Germany) to measure the fluorescence signal intensity reflecting intracellular free Ca²⁺ concentration ([Ca²⁺]i) (494 nm excitation wavelength).
and 516 nm emission wavelength) according to the manufacturer’s protocol. In the detection procedure, the images of leptospire-cell co-cultures incubated for 15, 30, 45, 60, 90 and 120 min were analyzed. The change of \([\text{Ca}^{2+}]_{\text{i}}\) in the leptospire-infected J774A.1 or THP-1 cells was calculated as the following formula: the fluorescence intensity in 500 cells infected with the spirochete for

**Figure 5. Reduced elevation of \([\text{Ca}^{2+}]_{\text{i}}\) in \(\Delta\text{LB361}^{*}\) mutant-infected macrophages.** (A). Change of \([\text{Ca}^{2+}]_{\text{i}}\) in J774A.1 or THP-1 cells during infection with different leptospires for the indicated times determined by laser confocal microscopy. The intensity of green fluorescence reflects the \([\text{Ca}^{2+}]_{\text{i}}\) in macrophages. The images at “0 h” indicate the \([\text{Ca}^{2+}]_{\text{i}}\) in the J774A.1 and THP-1 cells before infection. EGTA is an extracellular \(\text{Ca}^{2+}\) chelator to block extracellular \(\text{Ca}^{2+}\) influx. U73122 is a mammalian cell PI-PLC inhibitor. Neomycin is a blocker of IP3 production. (B). Statistical summary of \([\text{Ca}^{2+}]_{\text{i}}\) changes in the macrophages during infection with different leptospires. Data from experiments such as shown in A. Bars show the means ± SD of three independent experiments. The values at “0 h” indicate the \([\text{Ca}^{2+}]_{\text{i}}\) in the J774A.1 and THP-1 cells before infection. Five hundred cells were analyzed for each of the samples. *: \(p<0.05\) vs the fluorescence intensity reflecting \([\text{Ca}^{2+}]_{\text{i}}\) in the J774A.1 or THP-1 cells before infection (0 h). #: \(p<0.05\) vs the fluorescence intensity reflecting \([\text{Ca}^{2+}]_{\text{i}}\) in the EGTA-untreated J774A.1 or THP-1 cells infected with wild-type \(L.\) interrogans strain Lai. #: \(p<0.05\) vs the fluorescence intensity reflecting \([\text{Ca}^{2+}]_{\text{i}}\) in the EGTA-treated J774A.1 or THP-1 cells infected with wild-type \(L.\) interrogans strain Lai. The statistically significant differences were determined by ANOVA variance analysis plus Dunnett’s multiple comparison test.

doi:10.1371/journal.pone.0075652.g005
Figure 6. Macrophage death caused by Leptospira-induced [Ca^{2+}]_{i} elevation. (A). High [Ca^{2+}]_{i}-related apoptosis and necrosis in the J774A.1 or THP-1 cells during infection with different leptospires for the indicated times, determined by flow cytometry. The Annexin V^+/PI^- cells represent early-apoptotic death while the Annexin V^-/PI^- cells represent late-apoptotic or necrotic death. The images at "0 h" indicate the early or late apoptosis of the normal and P2X7-depleted J774A.1 or THP-1 cells before infection. EGTA is an extracellular Ca^{2+} chelator, BAPTA/AM is an intracellular Ca^{2+} chelator, and P2X7-depleted cells do not express P2X7.
free Ca\(^{2+}\) chelator. Neomycin is a blocker to inhibit IP\(_3\) production. U73122 is a mammalian cell PI-PLC inhibitor. (B). Statistical summary of early or late apoptotic/necrotic ratios in macrophages during infection with different leptospires. Data from experiments such as those shown in A. Bars show the means ± SD of three independent experiments. The values at "0 h" indicate the early or late apoptosis of the normal or P2X\(_7\)-depleted J774A.1 or THP-1 cells before infection. Five thousand cells were analyzed for each of the samples. *: p < 0.05 vs the early apoptotic or late apoptotic/necrotic ratios in the J774A.1 or THP-1 cells before infection (0 h). #: p < 0.05 vs the early apoptotic or late apoptotic/necrotic ratios in the J774A.1 or THP-1 cells infected with wild-type \(L.\) interrgans strain Lai but untreated with the chelators, blockers or inhibitors. The statistically significant differences were determined by ANOVA variance analysis plus Dunnett’s multiple comparison test. (C). Diversity of viability of different leptospires in macrophages, determined by CFU enumeration. Bars show the means ± SD of three independent experiments. *: p < 0.05 vs the CFUs of the \(C\). \(L.\) isolate but untreated with the chelators, blockers or inhibitors. The statistically significant differences were determined by ANOVA variance analysis plus Dunnett’s multiple comparison test.

doi:10.1371/journal.pone.0075652.g006

Determination of Intracellular free Ca\(^{2+}\) Source in Macrophages during Infection

EGTA is an extracellular Ca\(^{2+}\) chelator, while BAPTA/AM is an intracellular free Ca\(^{2+}\) chelator [43,44]. Neomycin is a blocker of IP\(_3\) production [45]. SKF96365 is a receptor-gated Ca\(^{2+}\) channel blocker [46], while verapamil or mibefradil acts as blocker of L-type or T-type voltage-gated Ca\(^{2+}\) channels [47,48]. To determine the source of intracellular free Ca\(^{2+}\) in J774A.1 or THP-1 cells (10\(^5\) per well) during infection with \(L.\) interrogans strain Lai (10\(^7\)), the two macrophage monolayers were pre-treated with 2 mM EGTA (Sigma), 100 \(\mu\)M BAPTA/AM (Sigma), 1.2 mM neomycin (Sigma), 20 \(\mu\)M SKF96365 (Sigma), 100 \(\mu\)M verapamil (Sigma) or 10 \(\mu\)M mibefradil (Sigma) for 30 min at 37°C [43–48]. The subsequent experimental steps for [Ca\(^{2+}\)]\(_i\) detection were the same as described above. In this assay, the normal J774A.1 or THP-1 cells before infection, the chelator or blocker-untreated, and P2X\(_7\)-depleted J774A.1 or THP-1 cells infected with the spirochete were used as the controls.

Determination of the Role of Cellular or Leptospiral PI-PLC in [Ca\(^{2+}\)]\(_i\) Elevation of Macrophages

J774A.1 or THP-1 cell monolayers (10\(^5\) cells per well) were infected with the \(\Delta\)LB361 or \(\Delta\)LB361 mutant or wild-type \(L.\) interrogans strain Lai (10\(^7\)) for a 120-min contiguous incubation at 37°C. To further determine the roles of cellular PI-PLC or L-Pi-PLC in [Ca\(^{2+}\)]\(_i\) elevation of macrophages during infection with the spirochete, J774A.1 or THP-1 cell monolayers were pre-treated with 2 mM extracellular Ca\(^{2+}\) chelator EGTA (Sigma) [43], 10 \(\mu\)M mammalian cell PI-PLC blocker U73122 (Sigma) [36] and/or 1.2 mM IP\(_3\) production blocker neomycin (Sigma) [45], and then infected with the \(\Delta\)LB361 or \(\Delta\)LB361 mutant or wild-type strain (10\(^7\)). The subsequent experimental steps for [Ca\(^{2+}\)]\(_i\) detection were the same as described above. In this detection, the normal J774A.1 or THP-1 cells before infection were used as the controls.

Detection of Macrophage Death by Flow Cytometry

J774A.1 or THP-1 cell monolayers (10\(^5\) cells per well) were infected with the \(\Delta\)LB361 or \(\Delta\)LB361 mutant or wild-type \(L.\) interrogans strain Lai (10\(^7\)) for 0.5, 1 or 2 h. After trypsinization, washing with PBS and centrifugation, the harvested cell pellets were lysed with 0.05% Na\(\)TDC-PBS and then centrifuged at 17,200 \(\times\) g for 15 min at 4°C. The cytosol specimens were harvested to measure the intracellular IP\(_3\) levels by IP\(_3\) fluorescence polarization spectrography as described above. In the detection, the normal J774A.1 and THP-1 cells without transfection, and wild-type pCMV-Tag2C or \(\Delta\)hhf gene-transfected J774A.1 or THP-1 cells were used as the controls.

Detection of IP\(_3\) Levels in LB361 Gene-transfected Macrophages

The LB361 gene-transfected J774A.1 or THP-1 cells were incubated in 10% FCS RPMI-1640 at 37°C for 0.5, 1 or 2 h. After trypsinization, washing with PBS and centrifugation, the cell pellets were lysed with 0.05% Na\(\)TDC-PBS and then centrifuged at 17,200 \(\times\) g for 15 min (4°C) to precipitate intracellular leptospires. Serial dilutions of the leptospires were inoculated onto EMJH-agar plates and then incubated at 28°C for three weeks [16]. The leptospiral colony-forming units (CFUs) on plates were enumerated after incubation.

Detection of [Ca\(^{2+}\)]\(_i\) and Apoptosis of in LB361 Gene-transfected Macrophages

To avoid the influence of extracellular Ca\(^{2+}\) influx on [Ca\(^{2+}\)]\(_i\), J774A.1 and THP-1 cells were pretreated with 2 mM extracellular Ca\(^{2+}\) chelator EGTA (Sigma) at 37°C for 30 min [43]. The [Ca\(^{2+}\)]\(_i\) or apoptosis of the macrophages at different times was detected by laser confocal microscopy or flow cytometry as described above. In the detection, the normal J774A.1 or THP-1 cells without transfection, and wild-type pCMV-Tag2C-transfected or \(\Delta\)hhf-gene-transfected J774A.1 or THP-1 cells were used as the negative controls. In addition, the pCMV-Tag2C LB361L.
transfected J774A.1 or THP-1 cells pretreated with 1.2 mM IP3 production blocker neomycin were also used as the controls to further determine the function of the LB361 gene product.

Data Analysis
Data from a minimum of three experiments were averaged and presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparisons test were used to determine significant differences. Statistical significance was defined as p<0.05.

Results

The LA0543, LA2250 and LB361 genes in different leptospiral strains

The distribution of the LA0543, LA2250 and LB361 genes in different pathogenic or non-pathogenic Leptospira strains was examined as a way to predict the potential role of the genes in leptospiral pathogenicity. The PCR and sequencing data confirmed that all the seven tested pathogenic L. interrogans strains but not the two non-pathogenic L. biflexa strains belonging to different serogroups and serovars possessed the LA0543 and LB361 genes with high sequence identities, while the LA2250 gene was only detectable in genomic DNA from L. interrogans serogroup Icterohaemorrhagiae serovar Lai strain Lai (Figure S2A), suggesting a possible correlation of expression of the three genes with the pathogenicity of L. interrogans. On the other hand, our bioinformatic analysis revealed that there is a PLC domain in the amino acid sequences of the LA0543, LA2250 and LB361 genes of L. interrogans strain Lai (Figure 1A). However, only the LB361 gene was predicted as a PI-PLC due to its sequence containing X and Y box domains (Figure 1A). The formation of X and Y dimers has been shown to be necessary for enzymatic activity of PI-PLC in order to hydrolyze the PIP2 substrate [49].

PI-PLC Activity of the Recombinant Protein Encoded by the LB361 Gene

The NPPC assay or IP3 fluorescence polarization assay was used to determine the PC-PLC or PI-PLC activity of recombinant proteins expressed by the LA0543, LA2250 and LB361 genes of L. interrogans strain Lai. The results of the NPPC assay demonstrated that none of the recombinant proteins expressed by the LA0543, LA2250 and LB361 genes of L. interrogans strain Lai expressed PC-PLC activity (data not shown). The IP_{3} fluorescence polarization assay confirmed that only the recombinant protein of the LB361 gene had PI-PLC activity to hydrolyze PIP_{2} into IP_{3} (Figure 1B), with the Km of 199 μM and Kcat of 8.56E-5 S^{-1} (Figure 1C). The product of the LB361 gene was designated as L-PI-PLC. However, U73122, a mammalian PI-PLC inhibitor [42], did not inhibit the PIP_{2} hydrolytic activity of rL-PI-PLC (Figure 1B).
P2X7 Expression in J774A.1 and THP-1 Cells

Extracellular Ca$^{2+}$ influx through membrane calcium channels can cause the increase of [Ca$^{2+}$]i. Until now, at least fifteen calcium channels in mammalian cells have been identified [50]. However, among the four voltage-gated and seven receptor-gated calcium channels tested, only P2X7, a receptor-gated calcium channel, was detectable in both J774A.1 and THP-1 cells by Western Blot assay (Figure 2A). This result indicates that P2X7 is the unique calcium channel expressed by J774A.1 and THP-1 cells, and receptor-gated calcium channel blockers, such as SKF96365 [46], could be used to determine extracellular Ca$^{2+}$ influx through the P2X7 calcium channel in J774A.1 or THP-1 cells during infection.

Leptospire Levels in Macrophages and Activation of Cellular PI-PLC during Infection

Macrophages can phagocytose bacterial pathogens, and the phagocytosis results in activation of cellular PI-PLC [51]. In the present study, when J774A.1 and THP-1 cells were infected with L. interrogans strain Lai for 30 min, leptospires in the two macrophage types could be observed by electron microscopy (Figure 2B) and confocal microscopy (Figure 2C). Compared to the J774A.1 and THP-1 cells before infection, the phosphorylation levels at the serine 537 (pSer537) on PI-PLC-b3 and tyrosine 783 (pTyr783) on PI-PLC-c1 of the two leptospire-infected macrophages were significantly increased (Figure 2D). However, phosphorylation at the Tyr759 and Tyr1217 of PI-PLC-c1 was undetectable. The data suggest that PI-PLC-b3 and PI-PLC-c1 of macrophages play a role in [Ca$^{2+}$] elevation in macrophages during infection.

Elevation of LB361-mRNA Levels during Infection

The expression levels of a bacterial gene will increase if the gene is required by the pathogen during infection of hosts [19]. Our RT-qPCR analysis showed that the LB361-mRNA levels of L. interrogans strain Lai increased significantly during infection of J774A.1 or THP-1 cells with the maximal mRNA levels (4.04 or 5.83 fold increase) observed at the 2 h of post-infection (Figure 3A). Our previous microarray detection results showed the increase in LA0543-mRNA levels, the decrease in LA2250-mRNA levels, but...
no significant change in LB361-mRNA levels in the spirochetes during infection of macrophages [18]. However, RT-qPCR is more sensitive and accurate than microarray analysis. Therefore, the RT-qPCR data suggest that the LB361 gene could play a role in infection by the spirochete.

External Secretion of L-PI-PLC in Macrophages during Infection

External secretion is necessary for L-PI-PLC to play a functional role in host cells. Our Western Blot assay demonstrated that none of the proteins expressed by the LA0543, LA2250 and LB361 genes were detectable in the supernatants of cultures and cytosol of macrophages (Figure 3B). However, HlyX, a leptospiral nonsecreted cytoplasmic protein that was used as the control, was undetectable in all the supernatant and cytosol samples (Figure 3B). Although the protein expressed by the LA0543 gene was also detectable in the supernatants of cocultures and cytosol of leptospire-infected J774A.1 or THP-1 cells (Figure 3B), this protein did not exhibit PI-PLC or PC-PLC activity (Figure 1B). However, the protein encoded by the LA2250 gene was undetectable in both the supernatant and cytosol samples. The data suggest that the L-PI-PLC of L. interrogans could play a direct role in macrophages during infection.

[Ca^{2+}]_i Elevation Due to Extracellular Ca^{2+} Influx and Intracellular Ca^{2+} Release

Extracellular Ca^{2+} influx and intracellular Ca^{2+} release are the most common ways to increase [Ca^{2+}]_i [20]. Compared to the [Ca^{2+}]_i in uninfected J774A.1 cells and THP-1 cells, the [Ca^{2+}]_i in the two macrophages increased rapidly after infection with L. interrogans strain Lai (Figure 4). The maximal [Ca^{2+}]_i in the leptospire-infected J774A.1 or THP-1 cells was observed at the 1 h of post-infection (Figure 4). Pretreatment with the extracellular Ca^{2+} chelator EGTA or IP3 production blocker neomycin caused a significant decrease of the [Ca^{2+}]_i elevation during infection (Figure 4). The receptor-gated calcium channel blocker SKF96365, but not the L-type or T-type voltage-gated calcium channel blockers (verapamil or mibefradil), could inhibit the increase in [Ca^{2+}]_i, and the P2X7-depleted J774A.1 or THP-1 cells also displayed a decrease in the [Ca^{2+}]_i elevation similar to the EGTA-pre-treated macrophages (Figure 4). However, the heat-killed L. interrogans strain Lai did not induce an increase in [Ca^{2+}]_i in the macrophages. The data suggest that infection of L. interrogans induces an increase in [Ca^{2+}]_i in macrophages, and both extracellular Ca^{2+} influx through P2X7 receptor-gated calcium channel and intracellular Ca^{2+} release from endoplasmic reticulum contribute to the increase in [Ca^{2+}]_i.

Reduced [Ca^{2+}]_i Elevation in Macrophages Infected with the ΔLB361 Mutant

Phospholipase C can regulate the [Ca^{2+}]_i by hydrolysis of PIP2 into IP3, an inducer of intracellular Ca^{2+} release from the endoplasmic reticulum [27]. Our fluorescent calcium indicator-based laser confocal microscopic examination revealed that the J774A.1 or THP-1 cells infected with the ΔLB361 mutant displayed a smaller [Ca^{2+}]_i elevation than the J774A.1 or THP-1 cells infected with wild-type L. interrogans strain Lai and the CALB361 mutant, but the [Ca^{2+}]_i in the two macrophages infected with the CALB361 mutant was similar to that in the two wild-type strain-infected macrophages (Figure 5). Pretreatment with the extracellular Ca^{2+} chelator EGTA plus the mammalian cell PI-PLC blocker U73122 caused a significant decrease of [Ca^{2+}]_i elevation in the ALB361 mutan-infected J774A.1 or THP-1 cells and a smaller decrease of [Ca^{2+}]_i elevation in the wild-type strain or CALB361 mutant-infected macrophages (Figure 5). The data suggest that both the product of LB361 gene and host cell PI-PLC contribute to the [Ca^{2+}]_i elevation in macrophages through intracellular Ca^{2+} release during infection with L. interrogans.

Macrophage Death Due to [Ca^{2+}]_i Changes on Macrophage Death

High [Ca^{2+}]_i had been shown to induce cell apoptosis or necrosis [52]. The flow cytometric analysis showed that the early-apoptotic and late-apoptotic/necrotic ratios of the ALB361 mutant-infected J774A.1 or THP-1 cells were significantly lower than that of the two macrophages infected with the CALB361 mutant or wild-type L. interrogans strain Lai (Figure 6A and B). When J774A.1 or THP-1 cells were pretreated with the chelator of extracellular Ca^{2+}, BAPTA/AM, the extracellular Ca^{2+} chelator, EGTA, EGTA plus the IP3 production blocker neomycin, or the mammalian cell PLC inhibitor, U73122, there was a significant decrease of apoptosis or necrosis of the two macrophages infected with the different leptospires. The lowest apoptotic and necrotic ratios were obtained with BAPTA/AM-pre-treated J774A.1 or THP-1 cells (Figure 6A and B). In particular, the apoptotic and necrotic ratios of P2X7-depleted J774A.1 or THP-1 cells were similar to those of the two EGTA-treated macrophages during infection. These results suggest that the [Ca^{2+}]_i elevation due to extracellular Ca^{2+} influx and intracellular Ca^{2+} release during infection with L. interrogans contribute to apoptosis or necrosis of macrophages.

Attenuated Viability of ΔLB361 Mutant in Macrophages

Compared to the CALB361 mutant and wild-type L. interrogans strain Lai, the CFUs of the ΔLB361 mutant from the infected J774A.1 or THP-1 cells did not change significantly at the 0.5 or 1 h of post-infection, but displayed a significant decrease of CFU at the 2 h of post-infection (Figure 6C). The data suggest that the LB361 gene product contributes to survival of L. interrogans in macrophages.

Elevation of IP3 Levels in LB361 Gene-transfected Macrophages

PI-PLC hydrolyzes PIP2 to produce IP3 to cause the increase of intracellular IP3 [27]. The IP3 fluorescence polarization assay demonstrated that the IP3 levels in the LB361 gene-transfected J774A.1 or THP-1 cells were significantly higher than in the wild-type pCMV-Tag2C-transfected macrophages (Figure 7A). The data suggest that the product of the L. interrogans LB361 gene (L-PI-PLC) hydrolyzes PIP2 into IP3 in macrophages.

L-PI-PLC-induced [Ca^{2+}]_i Elevation and Apoptosis of Macrophages

To obtain direct evidence that the LB361 gene product of L. interrogans strain Lai can cause [Ca^{2+}]_i-dependent death of macrophages, we measured the [Ca^{2+}]_i and apoptosis/necrosis of the LB361 gene-transfected J774A.1 or THP-1 cells. The results showed that the LB361 gene-transfected J774A.1 or THP-1 cells displayed a detectable increase in [Ca^{2+}]_i compared to the two normal or wild-type pCMV-Tag2C-transfected macrophages, but the IP3 production blocker neomycin inhibited the [Ca^{2+}]_i.
such as *L. monocytogenes* modulates cellular stress responses to environmental stimuli [54]. Although an earlier study only express P2X7 protein. In the present study, we demonstrated that pretreatment with EGTA, an extracellular Ca\(^{2+}\) chelator, or neomycin, a blocker of IP\(_3\) production [45], repressed the [Ca\(^{2+}\)]\(_i\) elevation in the J774A.1 and THP-1 cells during infection with *L. interrogans* strain Lai. Moreover, the receptor-gated calcium channel blocker (SKF96365), but not the voltage-gated Ca\(^{2+}\) channel blockers, could inhibit the *Leptospira*-induced [Ca\(^{2+}\)]\(_i\) elevation in the two macrophages, while the depletion of P\(_2\)X\(_7\) also prevented the [Ca\(^{2+}\)]\(_i\) elevation during infection. The data imply that *L. interrogans* causes the increase in [Ca\(^{2+}\)]\(_i\) in the infected macrophages due to extracellular Ca\(^{2+}\) influx through membrane calcium channels or intracellular Ca\(^{2+}\) release from endoplasmic reticulum [53–55]. The intracellular Ca\(^{2+}\) release occurs when the IP\(_3\)-receptor (IP\(_3\)R) on endoplasmic reticulum is ligated with cytoplasmic IP\(_3\) [27,56]. So far, many different calcium channels that belong to voltage-gated or receptor-gated family have been characterized [47,48,50]. A previous study reported that mouse J774 macrophages express both P\(_2\)X\(_7\) and P\(_2\)X\(_4\) receptor-gated calcium channels [57]. However, J774A.1 and THP-1 cells used in this study only express P\(_2\)X\(_7\) protein. In the present study, we demonstrated that pretreatment with EGTA, an extracellular Ca\(^{2+}\) chelator, or neomycin, a blocker of IP\(_3\) production [45], repressed the [Ca\(^{2+}\)]\(_i\) elevation in the J774A.1 and THP-1 cells during infection with *L. interrogans* strain Lai. Moreover, the receptor-gated calcium channel blocker (SKF96365), but not the voltage-gated Ca\(^{2+}\) channel blockers, could inhibit the *Leptospira*-induced [Ca\(^{2+}\)]\(_i\) elevation in the two macrophages, while the depletion of P\(_2\)X\(_7\) also prevented the [Ca\(^{2+}\)]\(_i\) elevation during infection. The data imply that *L. interrogans* causes the increase in [Ca\(^{2+}\)]\(_i\) in the infected macrophages due to extracellular Ca\(^{2+}\) influx through membrane calcium channels or intracellular Ca\(^{2+}\) release from endoplasmic reticulum.

**Discussion**

Intracellular free Ca\(^{2+}\) is an important mediator of cell signaling pathways that regulate many physiological functions such as metabolism, protein secretion, cell division, phagocytosis, cell death, and muscle contraction [53]. In particular, Ca\(^{2+}\) also modulates cellular stress responses to environmental stimuli [54]. Infection can also be viewed as a source of stress for both microbial pathogens and their infected hosts. Several pathogenic bacteria, such as *L. monocytogenes*, *H. pylori*, *C. jejuni* and *B. abortus*, can stimulate an increase in [Ca\(^{2+}\)]\(_i\) in different host cells including macrophages [21–24]. The results of this study also found that infection with *L. interrogans* strain Lai induces a significant increase in [Ca\(^{2+}\)]\(_i\) in macrophages from murine or human origin.

Intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) can increase due to extracellular Ca\(^{2+}\) influx through membrane calcium channels or intracellular Ca\(^{2+}\) release from endoplasmic reticulum [53–55]. The intracellular Ca\(^{2+}\) release occurs when the IP\(_3\)-receptor (IP\(_3\)R) on endoplasmic reticulum is ligated with cytoplasmic IP\(_3\) [27,56]. So far, many different calcium channels that belong to voltage-gated or receptor-gated family have been characterized [47,48,50]. A previous study reported that mouse J774 macrophages express both P\(_2\)X\(_7\) and P\(_2\)X\(_4\) receptor-gated calcium channels [57]. However, J774A.1 and THP-1 cells used in this study only express P\(_2\)X\(_7\) protein. In the present study, we demonstrated that pretreatment with EGTA, an extracellular Ca\(^{2+}\) chelator, or neomycin, a blocker of IP\(_3\) production [45], repressed the [Ca\(^{2+}\)]\(_i\) elevation in the J774A.1 and THP-1 cells during infection with *L. interrogans* strain Lai. Moreover, the receptor-gated calcium channel blocker (SKF96365), but not the voltage-gated Ca\(^{2+}\) channel blockers, could inhibit the *Leptospira*-induced [Ca\(^{2+}\)]\(_i\) elevation in the two macrophages, while the depletion of P\(_2\)X\(_7\) also prevented the [Ca\(^{2+}\)]\(_i\) elevation during infection. The data imply that *L. interrogans* causes the increase in [Ca\(^{2+}\)]\(_i\) in the infected macrophages due to extracellular Ca\(^{2+}\) influx through membrane calcium channels or intracellular Ca\(^{2+}\) release from endoplasmic reticulum.

**Supporting Information**

**Figure S1** Strategy for generation of LB361 and CALB361 mutants. See Materials S1 for details. (TIF)

**Figure S2** Amplification and expression of LA0543, LA2250 and LB361 genes. (A): Amplification of LA0543, LA2250 and LB361 genes in different leptospiral strains. Lane M: DNA marker. Lane 1: blank control. Lanes 2 to 8: amplicoms of the LA0543 gene (1320 bp) and LB361 gene (384 bp) from pathogenic *L. interrogans* serovar Lai strain Lai, serovar Grippotyphosa strain Lin-6, serovar Autumnalis strain Lin-4, serovar Pomona strain Luo, serovar Hebdomadis strain 56069, serovar Australis strain 65-9 and serovar Canicola strain Lin, respectively, but only *L. interrogans* strain Lai provided an amplicom (918 bp) of LA2250 gene. Lanes 9 and 10: no amplification products of the LA0543, LA2250 and LB361 genes from non-pathogenic *L. biflexa* serovar Patoc strain Patoc-1 and serovar Adamana strain CH-11. (B): Expression of LA0543, LA2250 and LB361 genes of *L. interrogans* strain Lai and purification of recombinant proteins. Lane M: protein marker. Lane 1: blank control of wild-type pET42a-transformed *E. coli* BL21DE3. Lanes 2 to 4: the recombinant proteins expressed by LA0543, LA2250 and LB361 genes, respectively. Lanes 5 to 7: the purified recombinant proteins of LA0543, LA2250 and LB361 genes by Ni-NTA affinity chromatography, respectively. (TIF)

**Figure S3** Confirmation of LB361 and CALB361 mutants by PCR and sequencing. (A): PCR results for identification of the LB361 mutant. Lane M: DNA marker. Lane 1: blank control. Lane 2: amplicon (2668 bp) of the 5’armkan-3’arm (2428 bp) plus two extending regions (120 bp each) can be activated by phosphorylation [60,61]. In the present study, the phosphorylation elevation of PI-PLC-β3 and PI-PLC-γ1 in the J774A.1 or THP-1 cells was also observed during infection with *L. interrogans* strain Lai, and pretreatment with a cellular PI-PLC blocker (UT73122) also dampened the elevation of [Ca\(^{2+}\)]\(_i\) and IP\(_3\) levels in the two macrophages during infection with the spirochete. These data imply that the cellular PI-PLCs also play a role in [Ca\(^{2+}\)]\(_i\) elevation in macrophages during infection with *L. interrogans*.

Infection-induced macrophage death is viewed as a common strategy used by different pathogens to evade the host immune response [62]. Since macrophages are the only phagocytes that can kill phagocytosed intracellular leptospires in the absence of Leptospira-specific antibodies [9–12], *Leptospira*-induced macrophage death is important for the ability of the spirochetes to survive in infected hosts [15,16]. Previous studies reported that a mild increase in [Ca\(^{2+}\)]\(_i\) induces cell apoptosis through a mitochondrion-dependent apoptotic pathway, while large [Ca\(^{2+}\)]\(_i\) increases cause cell necrosis through activation of Ca\(^{2+}\)-dependent phospholipases and proteinases [25,52,63]. Our results showed that infection of murine and human macrophages with *L. interrogans* strain Lai caused both apoptosis and necrosis. However, blockage of extracellular Ca\(^{2+}\) influx and/or intracellular Ca\(^{2+}\) release favored apoptosis over necrosis. In addition, the LB361 gene-transfected macrophages only exhibited cell apoptosis, which may be due to L-PI-PLC causing a mild increase in [Ca\(^{2+}\)]\(_i\). Taken together, our data show that infection with *L. interrogans* induced an increase in [Ca\(^{2+}\)]\(_i\) and [Ca\(^{2+}\)]\(_i\)-dependent apoptosis and necrosis of macrophages, and the LB361 gene product is a novel leptospiral PI-PLC that contributes to [Ca\(^{2+}\)]\(_i\)-dependent macrophage death.
from the ΔLB361 mutant. Lane 3: amplicon (1981 bp) of the 5′arm-LB361-3′arm (1741 bp) plus two extending regions (120 bp each) form wild-type *L. interrogans* strain Lai. (B). PCR results for identification of the CALB361 mutant. Lane M: DNA marker. Lane 1: blank control. Lane 2: amplicon (3220 bp) of the 5′arm-LB361-3′arm segment (2986 bp) plus two extending regions (120 bp each) from the CALB361 mutant. Lane 3: amplicon (2668 bp) of the 5′arm-kan-3′arm (2428 bp) plus two extending regions (120 bp each) from the CALB361 mutant. Lane 4: amplicon (1981 bp) of the 5′arm-LB361-3′arm (1741 bp) plus two extending regions (120 bp each) form wild-type *L. interrogans* strain Lai. (C). Schematic diagram of sequencing result of the ΔLB361 mutant. The positions of PCR primers used are marked below. (D). Schematic diagram of sequencing result of the CALB361 mutant. The positions of PCR primers used are marked below.

**Figure S4** Confirmation of ΔLB361 and CALB361 lepto-spiral mutants and LB361 or chpI gene-transfected and P2X7-depleted macrophages. (A). Expression of LB361 gene in the ΔLB361 and CALB361 mutants determined by Western Blot assay. Lane 1: the protein expressed by LB361 gene in wild-type *L. interrogans* strain Lai. Lane 2: no LB361 gene-encoding protein detectable in the ΔLB361 mutant. Lane 3: the protein expressed by LB361 gene in the CALB361 mutant. Lane 4: blank control. (B). Expression of the LB361 gene in the LB361 gene-transfected macrophages determined by Western Blot assay. Lane 1 or 3: the protein expressed by LB361 gene in the LB361 gene-transfected J774A.1 or THP-1 cells. Lane 2 or 4: no LB361 gene-expressed protein detectable in the normal J774A.1 or THP-1 cells without transfection. Lane 5: blank control. (C). Expression of ChpI protein in the chpI gene-transfected macrophages determined by Western Blot assay. Lane 1 or 3: the expressed ChpI protein in the chpI gene-transfected J774A.1 or THP-1 cells. Lane 2 or 4: no ChpI protein detectable in the normal J774A.1 or THP-1 cells without transfection. Lane 5: blank control. (D). Expression of the LB361 gene product in the LB361 gene-transfected J774A.1 or THP-1 cells, determined by laser confocal microscopy. The small green spots correspond to the protein expressed by the LB361 gene in the transfected J774A.1 or THP-1 cells. The large blue plaques correspond to the cell nucleus. The images at “0 h” indicate the results of laser confocal microscopic examination of normal J774A.1 or THP-1 cells before LB361 gene transfection.

**Table S1** Sequences of the primers used in this study.

**Materials S1** Detection and expression of LA0543, LA2250 and LB361 genes, and generation and identification of LB361 gene deletion and transfection.

**Author Contributions**
Congeved and designed the experiments: JY LJL XAL. Performed the experiments: JFZ HHC NZ YMG LLZ. Analyzed the data: JFZ DS YMG JY. Contributed reagents/materials/analysis tools: JFZ XAL DMO. Wrote the paper: JFZ DMO JY.

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