Leishmania donovani induced Unfolded Protein Response delays host cell apoptosis in PERK dependent manner

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Abstract

Background

Endoplasmic reticulum (ER) stress generated unfolded stress response (UPR) is a basic survival mechanism which protects cell under unfavourable conditions. Leishmania parasite modulates host macrophages in various ways to ensure its survival. Modulation of PI3K-Akt pathway in delayed apoptotic induction of host; enables parasite to stabilize the infection for further propagation.

Methodology

Infected RAW macrophages were exposed to camptothecin or thagsigargin and phosphorylation status of PERK, Akt, BAD and Cyt-C was determined through western blotting using phospho specific antibody. Expression at transcriptional level for cIAP1 &2, ATF4, CHOP, ATF3, HO-1 and sXBP1 was determined using real time PCR. For inhibition studies, RAW macrophages were pre-treated with PERK inhibitor GSK2606414 before infection.

Findings

Our studies in RAW macrophages showed that induction of host UPR against L. donovani infection activates Akt mediated pathway which delays apoptotic induction of the host. Moreover, Leishmania infection results in phosphorylation and activation of host PERK enzyme and increased transcription of genes of inhibitor of apoptosis gene family (cIAP) mRNA. In our inhibition studies, we found that inhibition of infection induced PERK phosphorylation under apoptotic inducers reduces the Akt phosphorylation and fails to activate further downstream molecules involved in protection against apoptosis. Also, inhibition of PERK phosphorylation under oxidative exposure leads to increased Nitric Oxide production. Simultaneously, decreased transcription of cIAP mRNA upon PERK phosphorylation fates the host cell towards apoptosis hence decreased infection rate.
Conclusion

Overall the findings from the study suggests that Leishmania modulated host UPR and PERK phosphorylation delays apoptotic induction in host macrophage, hence supports parasite invasion at early stages of infection.

Author summary

Visceral Leishmaniasis or Kala-azar is one of the severe tropical neglected parasitic diseases caused by Leishmania donovani in Indian subcontinent. Modulation of host in terms of delayed apoptotic induction is one of the aspects which favours disease establishment; however the mechanism is not clearly understood yet. In the present study, we tried to explore the connection between L. donovani infection induced UPR in host with delayed onset of apoptosis. We found that L. donovani infection phosphorylates the PERK and Akt molecule in host along with delayed apoptosis. Simultaneously, the levels of cellular IAP (cIAP1 & 2) genes were also up-regulated in infected macrophages. To assess the involvement of PERK in delayed apoptosis of host, we inhibited the phosphorylation of PERK under the exposure to apoptotic inducers. We found that PERK inhibition decreased the Akt phosphorylation and fails to activate other associated downstream molecules involved in delayed apoptosis of host. Also, a significant reduction in cIAP levels was observed. Under oxidative exposure, inhibition of PERK phosphorylation debilitates infected RAW cell's ability to maintain redox homeostasis leading to higher nitric oxide production. Altogether, L. donovani infection modulates host apoptosis in a PERK dependent manner and favours infection.

Introduction

Leishmania ability to defy the host immune response is a major cause for persistence of Leishmaniasis. Parasite modulates host in various aspects and hampers the activation of adaptive immune responses against infection [1]. Expression of LPG on parasite surface and TLR 2 modulates the host immune response [2, 3] by providing resistance to complement, attachment and entry into macrophages, protection against proteolytic damage within acidic vacuoles [4] or inhibition of phagosomal maturation [5]. Leishmania parasite has to counter the oxidative and nitrosative pressure generated in host macrophage against invasion. Parasite modulates host NO and IL-12 production [6, 7, 8, 9] and induces host HO-1 which suppress the production of superoxide and increases parasitic burden [10].

Unfolded protein response (UPR) is an evolutionary conserved mechanism that restores cellular homeostasis and ensures cell survival during Endoplasmic Reticulum stress. UPR consists of 3 signalling pathways: activation of transcription factor (ATF)-6, inositol-requiring enzyme (IRE)-1, and PKR-like endoplasmic reticulum kinase (PERK) [11]. As a downstream consequence, activated IRE1 increases cytosolic concentration of spliced XBP1 (sXBP1); an active transcription factor which in turn activates an array of genes to counter ER stress for cell survival [12, 13, 14]. L. amazonensis induces the IRE1α/XBP1 pathway to escape cellular defence; particularly oxidative stress by sXBP1 induced expression of antioxidant molecules such as SOD-1 and catalase, and increases expression of IFN1-β, an important cytokine that favour’s L. amazonensis infection [15].
In another aspect of modulation, *Leishmania* parasite induces a mild ER stress in host macrophages and activates the PI3K-Akt pathway [16]. However, a very few studies have been conducted in correlation with induced UPR and leishmaniasis, but the PI3K-Akt phosphorylation is of great importance in parasite biology. As a part of host modulation, *Leishmania* promastigote activates host PI3K-Akt pathway, phosphorylates BAD, checks cytochrome-C leakage from mitochondria, inhibits caspase activation; hence delays apoptotic induction in host macrophage [17].

PERK being an important component of integrated endoplasmic reticulum stress response (IERSR) [18]; its activation influences cellular adaptation through multiple mechanisms [19, 20]. Induction of mammalian inhibitor of apoptosis proteins (IAP proteins) is one of the PERK-dependent survival mechanisms which protects cell from ER stress- induced apoptosis [21, 22]. Also, activation of the PI3K–Akt pathway by ER stress is dependent on PERK [23]. Also, PERK phosphorylation under oxidative stress is crucial for protection against oxidative stress and maintaining redox balance for cell survival [24, 25].

So, in this study we first confirms the previous findings that *Leishmania* infection delays host cell apoptosis under exposure to apoptotic inducers by phosphorylating Akt, BAD protein which inhibits the caspase-3 activity, resulting delayed apoptosis. Further we investigated the effect of *L. donovani* infection in imparting endoplasmic reticulum (ER) stress in host and activation of unfolded protein response (UPR). Here, we first time reported that *Leishmania* infection induced host UPR relays on PERK activation. Our findings suggest that inhibition of PERK phosphorylation render RAW cells more prone to apoptosis ultimately leading to decreased infection rate. Moreover, activation of IAP (inhibitor of apoptosis proteins) class of proteins which protects cell from various apoptotic stimulus; depends upon PERK phosphorylation. Overall we conclude that UPR activated against *L. donovani* infection is a PERK dependent process and have an important role in delayed onset of apoptosis in host macrophages; hence contributes to *Leishmania* infectivity.

**Methods**

**Leishmania cell culture**

*Leishmania donovani* promastigotes used in all experiments were of clones AG83 (MHOM/IN/1983/AG83). The promastigotes were grown in fresh M199 media (Gibco) in 25 cm² flasks (Nunc) at 25°C supplemented with 10% FBS (Gibco). Cultures were allowed to reach stationary phase (5–6 days post inoculation), as determined by growth curve analysis (S1 Fig), prior to inoculation into fresh medium. Syrian golden hamsters were used to maintain the infectivity of the clone regularly [26].

**Cell line maintenance**

The RAW 267.4 cell line (obtained from NCCS, Pune) was maintained in a 25-cm2 flask in RPMI-1640 medium (Gibco) supplemented with 10% FBS and antibiotics (streptomycin and penicillin) in a humidified 5% CO₂ incubator (Sanyo, Japan) at 37°C. The growth and condition of cultures were routinely checked under an inverted microscope (Nikon, USA). The cells were sub cultured in every 72 hrs.

**Real-time PCR**

RAW267.4 macrophages were allowed to infect with *L. donovani* promastigotes for 4 h, washed to remove unattached promastigotes and further infection was carried out for mentioned time periods (4 h/12 h/ 36 h) before camptothecin (2mM/ 6 h) or thapsigargin (1μM/ 1hr)
treatment. For inhibition studies, RAW267.4 macrophages were pre-treated with GSK2606414 for 2 hours before allowing infection. Reverse transcription was performed using 0.2 mg total RNA using an anchored oligo (dT) (H-dT11M, where M represents A, C, or G; Gen-Hunter). Real-time PCR, was performed in the Light Cycler 480 (Roche) using SYBR green (Roche) chemistry. The cycling conditions were; 1 cycle at 95˚C for 3 min and 40 cycles of 95˚C for 15s (denaturation), 58˚C for 30s (annealing), and 72˚C for 30s (extension). The fluorescence signal was captured at the end of each cycle using the SYBR channel (excitation wavelength 490-nm and emission wavelength 525-nm). Results expressed as target/reference ratios of each sample, normalized by the target/reference ratio of the calibrator. Here, the target/reference value of untreated/normal RAW macrophages was used as the calibrator and the GAPDH gene was used internal control/reference to normalize the qRT-PCR in the experiments. The primers used for RT-PCR are mentioned in tabular form. (Table 1).

Western blotting

RAW 264.7 cells were plated in 6-well polystyrene plates 1 d before infection and were either pre-treated with GSK2606414 or untreated. Total protein extracts were prepared from infected cells 4 h, 12 h or 36 h after infection. For preparation of total cellular extracts, macrophages were washed 3 times with PBS and then lysed in 100 ml lyses buffer [50mMTris- HCl (pH 7.5), 5 mM EDTA, 10 mM EGTA, 50 mM NaF, 20 mM b-glycerophosphate, 250mM NaCl, 0.1% Triton X-100, and 1mg/ml bovine serum albumin) supplemented with 1:100 dilution of protease inhibitor cocktail (Sigma-Aldrich). Total protein (50μg) was separated by SDS-PAGE. The proteins were transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA) and blotted with antibodies against phosphos Akt (Santa Cruz Biotechnology, Dallas, TX, USA), phosphos PERK (Cell Signaling), phosphos BAD (Santa Cruz Biotechnology), GAPDH (Santa Cruz Biotechnology), anti-PERK (Cell Signaling) overnight. Membranes were incubated with horseradish peroxidase–conjugated IgG (1:5000) for 1 h in room temperature and washed extensively with Tris-buffered saline- Tween (TBST). Antibody antigen complexes were detected by enhanced chemiluminescence kit (Thermo Fischer Scientific, USA) was used for detection. The intensity of bands was analyzed using Quantity One software (Bio-Rad).

Caspase-3 activity assay

RAW 267.4 macrophages either untreated or pre-treated with GSK2606414 were infected with *L. donovani* promastigotes. After desired time periods, the cells were induced with camptothecin (2μM/ 6 h) in six well plates (1x10^6 cells). The cells were then scraped and harvested, lysed in cell lysis buffer supplied with the caspase-3 Fluorometric Assay Kit (BioVision) and the concentration of protein in lysates was determined. Equal amount of protein from each set was used to determine caspase-3 activity. The detection was based on the cleavage of AFC.

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**Table 1. Primer sequences for real-time PCR.**

| Gene  | Forward primer (5’-3’)                        | Reverse primer (5’-3’)                        |
|-------|-----------------------------------------------|-----------------------------------------------|
| ATF3  | GCCATCCAGAAGACTGCTGTCACTCCCATG              | GGCTACCTGAGCTTTTGTGAT                        |
| ATF4  | CCCTTCCTCCTTAACCTCT                       | TGAAGGATAGGACCGACAC                         |
| sXBP1 | CTGAGTCGCGAGCAAGGT                      | TGTCCAGAATGCCCAAGG                         |
| CHOP  | GAGACATCATGTCCTCCACCTT                       | TGGGATGAGGTCACACT                          |
| dAP1  | GAAGAAATGCTGACCCACCATAGA                      | GCTCATGACGCACTTC                           |
| dAP2  | CGATGCAGAGACGAGATGA                          | TTTGGTCCTCCGAGATGC                         |
| HO-1  | GCAGAGAATGCTGATTCATG                      | CCTGTCACCAGGCCACATAGTG                      |

https://doi.org/10.1371/journal.pntd.0006646.t001
(7-amino-4-trifluoromethyl coumarin) from DEVD-AFC by caspase-3 and the relative fluorescence was measured in the PerkinElmer LS-55 spectrometer (Perkin Elmer, USA) at excitation of 390 to 400 nm and emissions of 510 to 550 nm.

**Detection of DNA fragmentation**

Raw cells (1×10^6) were allowed to adhere into 6-well (Corning, USA) and incubated at 37°C in 5% CO_2 incubator (Sanyo, Japan). Once macrophages were adhered, pre-treatment with GSK2606414 was given and washed. Further infection was carried out by adding L.donovani promastigotes to each well and maintained at 37°C in 5% CO_2 overnight. Non-internalized promastigotes were removed by gentle washing twice with PBS. Infected macrophages were treated with camptothecin (6 h/2μM) or thapsigargin (1.0μM / 1hr) in 5% CO_2 at 37°C. Untreated infected macrophages were taken as a control. Total genomic DNA was isolated using an apoptotic DNA laddering kit (Roche, USA) as described earlier [27].

**Inhibition assay**

GSK2606414 (Calbiochem), a PERK inhibitor, was added at a 30nM final concentration to RAW 267.4 macrophages, 2 h prior to the infection, washed and the macrophages were allowed to infect with L.donovani promastigotes (MOI 10:1). Further treatment of camptothecin (2μM/6 h) [17] or thapsigargin (1.0μM / 1hr) [15] was given.

**Infection study**

For infection studies, RAW 264.7 cells were plated in 6-well polystyrene plates 1 day before infection. The infection index was calculated by multiplying the percentage of infected macrophages by the average number of parasites per macrophage on Giemsa-stained slides [15].

**Apoptotic detection**

Untreated or GSK2606414 pre-treated RAW 267.4 macrophages were plated in six well tissue culture plates overnight and then infected with late stationary phase Leishmania promastigotes. After 4 h, free parasites were washed off with PBS and the treatment of either 2mM camptothecin for 6 h or thapsigargin (1μM/1hr) was given. Cells were trypsinized and resuspended in phosphate-buffered saline. Propidium iodide was added to a concentration of 2.5 mg/ml [20]. Propidium iodide fluorescence was measured with a Becton Dickinson FACS ARIA.

**Determination of nitric oxide production**

Nitric oxide (NO) concentration was determined by analysing nitrite content in cell supernatant with the Griess reaction. L.donovani infected RAW cells either GSK 2606414 pre-treated or untreated were exposed to H_2O_2 (200μM/4hr) and Culture supernatant from each set (50μL) was mixed with 50μL of a solution containing sulphanilamide (10 mg/mL), N-[naphthyl] ethylenediamine dihydrochloride (NEED; 1 mg/mL) and 5% phosphoric acid. The absorbance was measured by spectrophotometry at 540 nm [28].

**Statistical analysis**

Graph Pad Prism (Version 6.0, GraphPad Software, USA) was used to analyse the data statistically. Each experiment was repeated three times in separate sets and the results were expressed as Mean ± SD. Statistical differences were determined using Mann–Whitney U test for comparing two groups or Kruskal–Wallis with Dunn’s multiple comparison test for comparing three or more groups. A P-value of <0.05 was considered significant.
Results

*L. donovani* infection induces UPR in RAW macrophages

*Leishmania* infection is known to elicit ER stress and activates UPR response in host. [16]. To ensure the same, expression level of ER stress marker genes viz; CHOP, ATF3, ATF4 and sXBP1 was monitored by qPCR at 4hr, 12hr and 36hr post infection. A significant increase in the expression of ER stress marker gene was observed after infection. At 4hr and 12hr post infection, the increase in level of marker genes was more prominent i.e. 1.5–2.0 folds higher (Fig 1A). At later stage of infection (36hr), a general decrease in all ER stress marker genes was observed however the value was higher compared to uninfected RAW cells. As a positive control, RAW cells treated with an ER stress inducer (thapsigargin 1.0 μM/ 1hr) were considered. The selected ER stress marker genes were found to be significantly upregulated with higher magnitude of induction (Fig 1B). GAPDH was chosen as reference gene since its expression did not change significantly following infection.

ER stress inducers enhance *L. donovani* infection

As *Leishmania* infection induces ER stress in host macrophages, we further investigated the effect of generated ER stress on infection rate. RAW macrophages treated with thapsigargin (Thg) or tunicamycin (Tu) for 1 hour were infected with *L. donovani*. After 36 hours, the infection index showed significantly higher parasite burden in thapsigargin (Thg) or tunicamycin (Tu) treated cells compared with that in non-treated cells (UNS) (Fig 1C). The result suggests that ER stress induced RAW macrophages have enhanced parasite infection.

*L. donovani* induces host PERK phosphorylation

Phosphorylation and simultaneous activation of PERK under stress is a major UPR which influences cellular adaptation and survival under stress through multiple mechanisms [18]. As the *L. donovani* infection induces ER stress in host macrophages, we further investigated the status and involvement of the host PERK in mounting UPR. Western blotting using phospho anti-PERK antibody revealed that in infected RAW cells, host PERK was phosphorylated (Fig 2A). At early stages of infection (4hr & 12hr) the degree of phosphorylation was higher compared to 36 hours post infection as shown in densitometry (Fig 2B). In case of thapsigargin treatment the phosphorylated status of PERK was confirmed and was taken as positive control for the experiment. No any phosphorylation was detected in untreated RAW macrophages.

*L. donovani* infection induces Akt phosphorylation with delayed apoptosis of RAW cells

*Leishmania* parasites modulate the host cell by activating PI3K/Akt signalling and inhibiting caspase-3 activity which delays host cell apoptotic process. To confer this delayed apoptosis, Akt phosphorylation status, caspase3 activity and percentage of cells undergoing apoptosis was determine in RAW cells infected with *L. donovani*. A significant rise in Akt phosphorylation was observed in infected RAW cells compared to uninfected controls. The figure (Fig 2C) shows phosphorylation of Akt at early stages of infection (4hr & 12hr) and was well maintained at 36 hours post infection. The infection had no effect on native Akt levels as represented in densitometric analysis of western blot bands (Fig 2D). Further, caspase-3 activity was accessed in uninfected or infected RAW cells or infected RAW macrophages treated with camptothecin (2mM) or thapsigargin as inducers of apoptosis. The level of caspase-3 activity was found significantly high in uninfected macrophages subjected to camptothecin (RAW+Cmpt) or thapsigargin (RAW+Thg) exposure than untreated control. In contrary, there was lower/limited
activity of caspase-3 in macrophages that were first infected for 4hr, 12hr or 36hr with *L. dono*vanii promastigotes before exposure to camptothecin or thapsigargin (Fig 3A). However, at 36hr post infection, the level of caspase-3 activity was higher than early stages of infection. Simultaneously, percentage of macrophages undergoing apoptosis was also determined in case of infected RAW macrophages compared to uninfected ones under exposure to apoptotic inducers. In uninfected RAW macrophages, the percentage of cells undergoing apoptosis was recorded significantly high compared to infected RAW cells (Fig 3B). Hence, the results were in agreement with previous findings that *Leishmania* infected macrophages are more tolerant to apoptotic induction [17].

ER stress induces host IAP expression

The mammalian inhibitor of apoptosis (IAP) gene family, particularly cellular IAPs (cIAP1 and cIAP2), provides cell protection during infection and against a variety of apoptotic stimuli [29, 30, 31, 32, 33]. It has been demonstrated that ER stress also induces the expression of cIAP1 and cIAP2 [34, 35] in a PERK-dependent manner [23]. As *L. donovani* infection delays host apoptosis induction, we accessed the level of cIAP1 and cIAP2 in infected RAW macrophages. Real time data revealed that the expression of cIAP1 and cIAP2 was induced in case of
infected RAW cell, whereas no such induction was detected in uninfected macrophages. Induction of both cIAP1 and cIAP2 was recorded transiently highest at 4 hours post infection which gradually declined at latter stages of infection. Similar up regulation of cIAP1 and cIAP2 was also observed at mRNA level in RAW macrophages subjected to thapsigargin (1h/1μM) or campothecin (6h/2mM) treated RAW cell and were taken as control (Fig 4A and 4B).

**Host PERK inhibition promotes apoptosis**

The above results reveal that *L. donovani* infection induces ER stress, phosphorylates host Akt molecule and delays host macrophage apoptosis which facilitates infection. Also, induced ER stress phosphorylates host PERK and leads to accumulation of cIAP mRNA. So to further explore the connection between *L. donovani* infections induced ER stress and host PERK phosphorylation mediated UPR in modulating host cell apoptosis, inhibition studies was carried out. For inhibiting PERK mediated UPR response, RAW 267.4 macrophages were pre-treated with GSK2606414, a specific inhibitor of PERK phosphorylation [36, 37, 38, 39, 40]; followed by washing before any infection or exposure as it is also effective against *Leishmania* PERK [26]. However, GSK2606414 pre-treatment did not affect the parasite entrance as initially at 4 hours, no any significant difference in terms of parasite entrance was observed in the GSK2606414 pre-treated RAW macrophages than untreated/normal one (S2 Fig).

![Fig 2. L. donovani infection induces host PERK and Akt phosphorylation.](https://doi.org/10.1371/journal.pntd.0006646.g002)
a. The Akt mediated signalling pathway requires PERK phosphorylation.
An ER stress-inducing agent activates the PI3K–Akt signalling pathway [34, 41 and 42]. To determine whether PERK-dependent signals trigger Akt activation, RAW cells treated with camptothecin or thapsigargin (Thg) in presence or absence of GSK were harvested at the indicated intervals and Akt phosphorylation was determined by western analysis using antibodies specific for Akt phosphorylated at serine 473. Akt phosphorylation was observed in RAW cells exposed to camptothecin or thapsigargin to alone, whereas significantly a low level of phosphorylated Akt was detected in GSK treated cells (RAW+GSK) (Fig 5A & 5B).

b. PERK inhibition regulate the expression of IAP mRNA.
Earlier work has revealed that PERK and Akt regulate the expression of IAP at transcriptional level [23]. Also in the above results we found that L. donovani infection induces cIAP1 and cIAP2 mRNA translation. Since IAP is directly related to anti-apoptotic mechanism, we determine the involvement of host PERK in regulation of cIAP expression under apoptotic stimulus. RAW macrophages untreated or pre-treated with GSK2606414 were allowed to infect by L. donovani promastigotes. After 4 hours post infection, infected cells were exposed to camptothecin or thapsigargin for apoptotic induction. RNA was prepared
and cDNA was reversed transcribed for further real time analysis of cIAP1 and cIAP2 mRNA levels. It was found that in GSK2606414 untreated cells, the level of cIAP1 and cIAP2 was significantly up-regulated by ~3.0 fold high compared to GSK pre-treated RAW macrophages (Fig 5C) under. So the result suggests that the mRNA expression of cIAP1 and cIAP2 is regulated in a PERK-dependent manner.

c. *L. donovani* infection phosphorylates BAD protein in a PERK dependent manner.

Phosphorylation of Akt may activate cascade of downstream molecules of the apoptotic pathway. To investigate the same, BAD protein phosphorylation status was monitored in *L. donovani* infected RAW macrophages. BAD protein is a member of proapoptotic Bcl-2 family which serves as an important regulator of apoptotic cell death [43]. Phosphorylation of BAD protein under various stimuli leads to its inactivation and divert cell fate against apoptosis. First we investigated the phosphorylation status of BAD in RAW macrophages under infected conditions. It was observed that the BAD protein was phosphorylated in RAW macrophages (Fig 6A) under exposure to campothecin. Further, the involvement of PERK in BAD phosphorylation was investigated in presence of GSK2606414. RAW macrophages either untreated or pre-treated with GSK2606414 were infected with *L. donovani* promastigotes. After 4 hours post infection, the macrophages were exposed to campothecin...
or thapsigargin and the phosphorylation of BAD protein in lysates was determined through western blot. In GSK2606414 treated RAW macrophages, the level of phosphorylated BAD was found to be significantly decreased than untreated infected RAW macrophages (Fig 6C & 6D). Hence the result summarises that PERK phosphorylation is required for BAD phosphorylation.

d. Inhibition of host PERK in infected RAW cells induces release of Mitochondrial Cytochrome C with increased caspase-3 activity.

Fig 5. Inhibition of host PERK decreases Akt phosphorylation and inhibits transcription of host cIAP mRNA expression under exposure to apoptotic inducers. (A) RAW 267.4 macrophages pre-treated with GSK2606414 (2 hours) or untreated were infected with L. donovani promastigotes. After 4 h of infection, GSK pre-treated infected cells were exposed to camptothecin (Cmpt+GSK) or thapsigargin (Thg+GSK). Similarly, untreated infected RAW macrophages were also exposed to camptothecin (Cmpt) or thapsigargin (Thg). After treatment, cells were harvested and western analysis probed with phospho-anti Akt, anti-Akt and GAPDH was performed from harvested cell lysates. (B) Band intensities of western blot bands after densitometry. (C) Relative expression of cIAP1 and cIAP2 mRNAs levels as analysed through real time in infected RAW 267.4 macrophages (4 h) either pre-treated with GSK2606414 or untreated, followed by camptothecin (Cmpt+Gsk) / (Cmpt) or thapsigargin (Thg+GSK) / (Thg) exposure. Infected RAW cell without any exposure were also taken in account. RAW 267.4 macrophages without any treatment or exposure were taken as control. Data are represented as the mean ± SEM of at least three experiments. (* p < 0.05).

https://doi.org/10.1371/journal.pntd.0006646.g005
Release of cytochrome C from mitochondria after disruption of outer mitochondrial membrane is an important marker indicating triggering of apoptosis in metazoans. RAW cell pre-treated with GSK or without it were infected with *L. donovani* promastigotes and in infected RAW macrophages (4 h post infection) released cytochrome C in cytoplasmic protein fraction was determined through western blotting using anti-Cyt C antibody after camptothecin (2mM/6h) exposure. It was observed that the level of cytochrome C in GSK2606414 pre-treated infected RAW cells (Cmpt+GSK) was ~ 2.1 folds higher than in GSK2606414 untreated infected RAW cells exposed to camptothecin (Cmpt) as revealed by densitometric analysis (Fig 7A & 7B). Similarly results were obtained in case of thapsigargin exposure. Normal RAW cells without any exposure or treatment were taken as control and did not show Cyt-C release. To further access the progression of apoptosis, caspase-3 activity was determined in cell lysates of infected RAW macrophages either pre-treated or untreated with GSK2606414 under camptothecin (Cmpt+LD+RAW+GSK) or thapsigargin (Thg+GSK) exposure respectively (Fig 7C). Hence the results showed that the inhibition of host PERK phosphorylation leads to significant increased caspase-3 activity.
e. Inhibition of PERK in infected RAW cells results in increased DNA fragmentation.

One of the important identification features of apoptosis in mammalian cells is the fragmentation of cell DNA in multiples of 180–200 bp. Similarly, we also measure the DNA fragmentation in L. donovani infected RAW267.4 in presence of GSK2606414 after exposure to apoptotic inducers. Agarose gel runs in Fig 7D shows that induction of apoptosis with camptothecin or thapsigargin in L. donovani infected cells pre-treated with GSK2606414 (Cmpt+GSK) results in increased DNA fragmentation as compared to untreated or uninfected RAW macrophages (UNS) or RAW cells treated with GSK2606414 (RAW+GSK) taken as control. Data are represented as the mean ± SEM of at least three experiments (*p < 0.05).

https://doi.org/10.1371/journal.pntd.0006646.g007
+GSK or Thg+GSK) resulted in an increase in DNA fragmentation as compared to GSK2606414 untreated infected RAW cells (Cmpt or Thg). Control untreated macrophages (UNS) or L. donovani infected RAW cells (LD+RAW) did not show any DNA fragmentation. The DNA fragmentation assay revealed that inhibition of infection induced PERK phosphorylation resulted in an increased DNA fragmentation upon apoptotic induction.

f. Oxidative stress induces PERK Phosphorylation in RAW macrophages.

PERK mediated integrated endoplasmic reticulum stress response (IERSR) has a crucial role in redox homeostasis of the cell [44, 45]. To investigate the effect of oxidative stress on PERK of RAW macrophages in correlation with L. donovani infection, phosphorylation status of PERK was determined. Western blot analysis using phospho-PERK antibody against protein lysates of RAW macrophages exposed to H_2O_2 either untreated (H_2O_2) or pre-treated with GSK2606414 (H_2O_2+GSK) or incubated with NAC (H_2O_2+NAC) revealed that the PERK enzyme was phosphorylated in H_2O_2 exposed RAW cells (Fig 8A); however the

![Western blot analysis to determine host PERK phosphorylation status in L. donovani infected RAW 267.4 macrophages under oxidative exposure. GSK2606414 pre-treated or untreated RAW 267.4 macrophages, infected with L. donovani were exposed to H_2O_2. The cells were then harvested; lysed and equal amount of protein (50μg) was resolved on SDS-PAGE followed by probing with anti-phospho PERK antibody. Normal RAW macrophages (RAW) or L. donovani infected (LD+RAW) were considered as control. B) Densitometric analysis of western blot bands. C) Nitrite concentration was measured by Griess reaction in cell supernatant of infected RAW macrophages (LD+RAW) or in infected RAW cells exposed to H_2O_2 (LD+RAW+H_2O_2) or pre-treated with GSK2606414 (LD+RAW+H_2O_2+GSK) or in uninfected (H_2O_2+RAW) cells without any treatment. Untreated (UNS) or RAW macrophages exposed to H_2O_2 in presence of NAC (H_2O_2+NAC) were taken as control. D) Real time plot to determine relative expression of HO-1 at mRNA level under above mentioned conditions.](https://doi.org/10.1371/journal.pntd.0006646.g008)
level of phosphorylation found to be significantly decreased in GSK pre-treatment or NAC incubation as represented in densitometric analysis of western blot bands (Fig 8B). The result suggests that oxidative stress equally serves as potent inducer of PERK phosphorylation compared to \textit{L. donovani} infection which was considered as positive control in the same study. Normal RAW macrophages without any treatment or incubated along with GSK2606414 were taken as negative control in which no any phosphorylation was detected.

g. PERK phosphorylation regulates HO-1 expression and protects \textit{L. donovani}-infected macrophages from oxidative stress.

PERK phosphorylation plays a critical role in protection against oxidative stress by promoting expression of HO-1, a fundamental anti-oxidative enzyme of eukaryotic system. During \textit{Leishmania} infection, host PERK-ATF4-NRF2 mediated pathway regulates HO-1 expression to maintain redox balance [28]. To determine the effect PERK inhibition on \textit{L. donovani} infected host cells during oxidative exposure, GSK2606414 pre-treated or untreated infected RAW 267.4 macrophages (4hr) were exposed to H$_2$O$_2$ and nitrite production as an indirect measure of nitric oxide (NO) levels was determined in cell supernatants. It was observed that NO level in H$_2$O$_2$ exposed uninfected RAW cell (H$_2$O$_2$+RAW) was ~ 5 folds higher compared to infected RAW macrophages (UNS) but was ~2 folds higher in case of \textit{L. donovani} infected RAW (H$_2$O$_2$+LD+RAW) macrophages (Fig 8C). However, the level of NO was maximum ~ 7.0 times higher in infected RAW cells pre-treated with GSK2606414 (H$_2$O$_2$+LD+RAW+GSK). Uninfected RAW macrophages without any treatment were taken as control (UNS). \textit{Leishmania} infection promotes expression of heme oxygenase (HO-1), a key antioxidant enzyme of host which facilitates parasite survival during oxidative burst to maintain redox homeostasis of host cell [28]. We further determined the expression of HO-1 in infected RAW267.4 under oxidative environment. GSK2606414 pre-treated or untreated RAW macrophages; infected with \textit{L. donovani} promastigotes (4hr) were exposed to H$_2$O$_2$. Following exposure, total mRNA was isolated, cDNA was prepared and HO-1 expression was determined. It was observed that the level of HO-1 was ~ 3.2 folds higher compared in H$_2$O$_2$ exposed RAW (H$_2$O$_2$+RAW) macrophages than normal untreated RAW cells (UNS). However in infected RAW macrophages (H$_2$O$_2$+LD+RAW) the level of was ~ 5.4 folds higher which results in lower NO production (Fig 8D). In GSK2606414 pre-treated infected RAW macrophages, a significant decreased level of HO-1 was observed; also reflected in higher NO production (Fig 8C). These data demonstrate that PERK phosphorylation mediated signaling pathway has an important role in the anti-oxidative cell response during \textit{L. donovani} infection.

\textbf{Inhibition of host PERK induces apoptosis in infected RAW macrophages and decreases infection rate}

Finally, the percentage of infected RAW macrophages undergoing apoptosis after camptothecin or thapsigargin or H$_2$O$_2$ exposure was determined in presence or absence of GSK2606414. The results revealed that GSK2606414 inhibition significantly increases the percentage of cells undergoing apoptosis (Fig 9A). Under H$_2$O$_2$ exposure, inhibition of PERK phosphorylation leads to significant increase in apoptotic cell percentage. \textit{L. donovani} infected RAW macrophages in presence of Wortamanian also showed increased apoptotic rate; however the change was less significant compared to inhibitory effect of GSK2606414, suggesting that the effect of PERK inhibition is more prominent.

As the ER stress favours \textit{L. donovani} infection in RAW macrophages, infection rate after inhibition of host PERK was investigated. RAW macrophages pre-treated with GSK2606414...
were infected with *L. donovani* promastigotes. After 36 hours, infection status was determined in terms of infection index. In case of PERK inhibition, a drastic decrease in parasite burden was observed compared to untreated normal RAW macrophages subjected to infection as represented in infection index (Fig 9B). The similar pattern was also reported in thapsigargin induced RAW cells. This finding suggests that PERK mediated UPR is required for successful infection.

### Discussion

Various intracellular parasites deploy different strategies for their survival inside host; however one thing common among them is delayed/resistance against induced apoptosis via wide range of agents [46, 47]. Leishmaniasis disease is mainly contributed to the parasite ability to modulate host machinery to thrive the harsh phagolysosomal environment. *Leishmania* parasite deploy different strategies like; increases expression of host redox homeostasis enzymes like HO-1, SOD-1 or catalase to sustain oxidative burst [10, 15], delays host cell apoptosis via PI3K-Akt pathway [17], induces ER stress to escape cellular defence, increases expression of IFN1-b etc.

In accordance with previous findings, we observed that *L. donovani* infection induces ER stress in RAW macrophages and consecutively activates UPR of the host as the level of marker genes like ATF3, ATF4, sXBP1 and CHOP were found upregulated (Fig 1A). A similar trend of gene expression was observed in thapsigargin treated RAW macrophages (Fig 1B), which further validated the observation. The magnitude of UPR induction during infection was comparatively lower than thapsigargin treatment which gradually decreases at later time points; might be a requirement for spread and progression of infection after successful establishment. Moreover, a general down regulation of gene expression at later stages of infection has been reported in literature [48, 49].
Akt mediated signalling pathway has its unique importance in apoptosis induction. *Leishmania* parasite, as a survival strategy, delays the apoptotic induction in host macrophages via phosphorylation and activation of Akt molecule [17]. ER stress also is capable of inducing Akt phosphorylation [23] which establishes a connection between UPR and PI3K-Akt pathway. In our studies, we observed Akt phosphorylation under apoptotic inducers exposure and *L. donovani* infection (Fig 2C). Simultaneously, with Akt phosphorylation, PERK was also found phosphorylated (Fig 2A) in infected macrophages. So the result bridges a connection between *Leishmania* infections, PERK mediated UPR activation and Akt pathway activation.

*Leishmania* infection confers host cell resistance to apoptosis by modulating PI3K/Akt signalling pathway [17] which we also confirmed in our studies using camptothecin as apoptotic inducer. As compared to uninfected RAW macrophages, *Leishmania* infection brings about phosphorylation of host Akt with low caspase-3 activity in infected RAW cells. The level of caspase-3 activity was found higher in uninfected RAW cells which indicates early onset of apoptosis (Fig 3A). The mRNA level of cIAP1 and cIAP2 during *Leishmania* infection was comparatively higher than under apoptotic inducers (Fig 4) however; the induction was transient which decreases at later stages of infection. It has also been reported that at translational level both cIAP1 and cIAP2 protein continues to accumulate even at later stages and have key role in delayed apoptosis [23].

To further investigate the importance of PERK mediated UPR in *L. donovani* infected macrophages in connection with delayed apoptosis of host macrophages; inhibition studies using GSK2606414 was performed. RAW macrophages pre-treated with GSK2606414 were infected with *L. donovani* promastigotes. After 4 hours post infection, infected RAW macrophages were exposed to camptothecin or thapsigargin for apoptotic induction and the molecular mechanism involved in apoptosis was investigated.

The level of Akt phosphorylation was found to be decreased in case of GSK treated macrophages compared to untreated one under thapsigargin exposure signifies that UPR-dependent Akt activation requires PERK phosphorylation (Fig 5A). Similar results were obtained in case of camptothecin exposure; hence it can be concluded that induction of Akt phosphorylation under apoptotic stimulus is mediated via PERK phosphorylation.

Akt activation is implicated in the regulation of IAP gene transcription [34] and PERK regulates IAP expression in an Akt-dependent manner [23]. Similarly in GSK treated RAW macrophages, the level of cIAP1 and cIAP2 mRNAs was relatively in lower amount signifies that inhibition of host PERK hampers cIAP1 and cIAP2 accumulation (Fig 5C). Following the consequence, Akt phosphorylation brings about deactivation of downstream BAD protein. BAD being a proapoptotic molecule forwards the apoptosis process. Akt phosphorylation further phosphorylates BAD protein which checks its activity by facilitating its binding with 14.3.3 protein. In our findings, we observed decreased phosphorylation of BAD in GSK2606414 pre-treated infected macrophages than infected RAW macrophages without GSK2606414 (Fig 6C). Hence, PERK inhibition decreases infection induced BAD phosphorylation.

Phosphorylation of BAD render it inactive whereas, unphosphorylated BAD alters mitochondrial membrane permeability leading to cytochrome C leakage from inner mitochondrial space to cytosol which in turn activates proteases i.e. caspase like protein. Camptothecin being an apoptotic activator enhances the release of cytochrome C in RAW macrophages. In *Leishmania* infected RAW macrophages, a low level of released cytochrome C suggests that infected macrophages are more tolerant to apoptotic inducers however, PERK inhibition leads to increased cytochrome C release even in infected RAW cell (Fig 7A).

For further determination of apoptotic progression, caspase-3 activity in lysates of infected RAW macrophages was accessed after camptothecin or thapsigargin treatment. In our studies, we confirm the delayed onset of apoptosis in *L. donovani* infected RAW macrophages by

interfering caspase-3 activity. However GSK2606414 treatment abolishes the effect of infection as higher caspase-3 activity was observed. In GSK pre-treated RAW macrophages, PERK inhibition alters the Akt mediated anti-apoptotic mechanism resulting higher caspase-3 activity (Fig 7C). Increased caspase-3 activity finally damage cell DNA to ensure apoptotic death of cell. DNA fragmentation analysis following camptothecin treatment suggests that PERK inhibition leads to increased DNA damage even in infected macrophages (Fig 7D).

https://doi.org/10.1371/journal.pntd.0006646.g010
Apart from activation of caspase cascade, UPR induced Akt phosphorylation also regulates the level of cIAP mRNA in PERK dependent manner. In infected RAW cells, inhibition of PERK phosphorylation interferes with mRNA accumulation. A decreased cIAP1 and cIAP2 mRNA level due to initial inhibition of PERK; reflects the same controlling mechanism. Contrary to this, in *L. donovani* infected RAW cells, an upregulated level of cIAP1 and cIAP2 mRNA was observed (Fig 5C). Hence it can be extracted that parasite induces expression of IAP to delay apoptotic induction of host macrophages.

Increased level of released Cyt-C from mitochondria as a result of altered mitochondrial membrane potential; propagates the apoptosis [50]. ROS is one of the important reasons which alter mitochondrial membrane potential and leads to increased leakage of Cyt-C [51] responsible for apoptotic induction [52, 53]. Since PERK mediated signaling pathway has a distinct protective role against ROS; inhibition of the same resulted in increased NO level (Fig 8C). Also, inhibition of oxidative stress induced PERK phosphorylation leads to higher apoptotic cell percentage (Fig 9A) with less chances of survival of infected cells; hence decreased infection rate (Fig 9B).

So overall it can be concluded that inhibition of host PERK during *L. donovani* infection, hampers the parasite ability to induce ER stress mediated host Akt phosphorylation and consecutively the phosphorylation of host BAD protein; which in turn alters mitochondrial membrane, increases cytochrome C release and finally DNA fragmentation. Simultaneously, decrease in IAP production in RAW macrophages due to PERK inhibition leads to an early onset of apoptotic process which ultimately results in decreased the parasitic burden (Fig 10).

Hence, findings from this study will help in better understanding of host-parasite interaction in relation with UPR activated PERK mediated mechanism in providing host cell resistance against apoptosis. It will open a new therapeutic option for drug target.

**Supporting information**

**S1 Fig. Growth Curve of *L. donovani* promastigotes.** Graph showing growth curve of *L. donovani* promastigotes in M199 media supplemented with 10% FBS. Initially, 10^5 promastigotes per ml of culture was taken and grown for 8 days in BOD incubator. Cell viability was determined by trypan blue dye exclusion method at an interval of 24 hours. (TIF)

**S2 Fig. Infection index at 4 hours.** RAW macrophages either thapsigargin exposed (LD +RAW+Thg) or GSK2606414 pre-treated or normal/ untreated RAW macrophages (LD +RAW) were infected with *L. donovani* in 10:1 MOI. After 4 h of infection, the cells were fixed with methanol and stained with Giemsa, and the infection index was calculated (percent *L. donovani* infected cells X number of amastigotes per cell). Uninfected RAW macrophages (RAW) were taken as control. (Significant difference ^\* (P < 0.05).

(TIF)

**Acknowledgments**

The authors are extremely grateful to Mr. Amar Kant Singh for providing technical assistance throughout this study.

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References
1. Gantt KR., Goldman TL, McCormick ML, Miller MA, Jeronimo SM, Nascimento ET, et al. Oxidative responses of human and murine macrophages during phagocytosis of Leishmania chagasi. J. Immunol. 2001; 167: 893–901 PMID: 11441096
2. Becker I, Salaiza N, Aguirre M, Delgado J, Carrillo-Carrasco N, Kobeh LG, et al. Leishmania lipophosphoglycan (LPG) activates NK cells through toll-like receptor-2. Mol. Biochem. Parasitol. 2003; 130: 65–74. PMID: 12946842
3. de Veer MJ, Curtis JM, Baldwin TM, DiDonato JA, Sexton A, McConville MJ, et al. MyD88 is essential for clearance of Leishmania major: possible role for lipophosphoglycan and Toll like receptor 2 signaling. Eur. J. Immunol. 2003; 33: 2822–2831. https://doi.org/10.1002/eji.200324128 PMID: 14515266
4. Bogdan C, and Rollinghoff M. How do protozoan parasites survive inside macrophages? Parasitol. Today. 1999; 15: 22–28. PMID: 10234174
5. Dermine JF, Scianimancio S, Prive C, Descoteaux A, and Desjardins M. Leishmania promastigotes require lipophosphoglycan to actively modulate the fusion properties of phagosomes at an early step of phagocytosis. Cell. Microbiol. 2000; 2: 115–126. PMID: 11207568
6. Brittingham A, and Mosser DM. Exploitation of the complement system by Leishmania promastigotes. Parasitol. Today. 1996; 12: 444–447. PMID: 15275279
7. Proudfoot L, Nikolaev AV, Feng GJ, Wei WQ, Ferguson MA, Brimacombe JS, et al. Regulation of the expression of nitric oxide synthase and leishmanicidal activity by glycoconjugates of Leishmania lipophosphoglycan in murine macrophages. Proc. Natl. Acad. Sci. 1996; USA 93:10984–10989. PMID: 8855295
8. Piedrafita D, Proudfoot L, Nikolaev AV, Xu D, Sands W, Feng GJ, et al. Regulation of macrophage IL-12 synthesis by Leishmania phosphoglycans. Eur. J. Immunol. 1999; 29: 235–244. https://doi.org/10.1002/(SICI)1521-4141(199901)29:01<235::AID-IMMU235>3.0.CO;2-S PMID: 9933105
9. Coelho-Finamore JM, Freitas VC, Assis RR, Melo MN, Novozhilova N, Secundino NF, et al. Leishmania infantum: Lipophosphoglycan intraspecific variation and interaction with vertebrate and invertebrate hosts. Int. J. Parasitol. 2011; 41: 333–342. https://doi.org/10.1016/j.ijpara.2010.10.004 PMID: 21118695
10. Luz NF, Andrade BB, Feijó DF, Araújo-Santos T, Carvalho GQ, Andrade D, et al. Heme oxygenase-1 promotes the persistence of Leishmania chagasi infection. J Immunol. 2012; 188: 4460–4467. https://doi.org/10.4049/jimmunol.1103072 PMID: 22461696
11. Walter P and Ron D. The unfolded protein response: from stress pathway to homeostatic regulation. Science. 2011; 334, 1081–1086. https://doi.org/10.1126/science.1209038 PMID: 22116877
12. Lee AH, Iwakoshi NN and Glimcher LH. XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response. Mol. Cell. Biol. 2003; 23, 7448–7459 https://doi.org/10.1128/MB.23.21.7448-7459.2003 PMID: 14559994
13. Acosta-Alvear D, Zhou Y, Blais A, Tsikitis M, Lents NH, Arias C, et al. XBPs control diverse cell type- and condition-specific transcriptional regulatory networks. Mol. Cell. 2007; 27, 53–66 https://doi.org/10.1016/j.molcel.2007.06.011 PMID: 17612490
14. Hertz C, Martinon F, Rodríguez D, and Glimcher LH. The unfolded protein response: integrating stress signals through the stress sensor IRE1a. Physiol. Rev. 2011; 91, 1219–1243. https://doi.org/10.1152/physrev.00001.2011 PMID: 22013210
15. Dias-Teixeira KL, Calegari-Silva TC, dos Santos GR, Vitorino Dos Santos J, Lima C, Medina JM, et al. The integrated endoplasmic reticulum stress response in Leishmania amazonensis macrophage infection: the role of X-box binding protein 1 transcription factor. The FASEB Journal 2016; 30(4):1557–1565. https://doi.org/10.1096/fj.15-281550 PMID: 26678450
16. Galluzzi L, Diotallevi A, De Santi M, Ceccarelli M, Vitale F, Brandi G, et al. *Leishmania infantum* induces Mild Unfolded Protein Response in Infected Macrophages. PLoS ONE 2016; 11(12): e0168339. https://doi.org/10.1371/journal.pone.0168339

17. Ruhland A, Leal N, Kima PE. *Leishmania* promastigotes activate PI3K/Akt signalling to confer host cell resistance to apoptosis. Cell Microbiol 2007; 9:84–96. https://doi.org/10.1111/j.1462-5822.2006.00769.x

18. Donnelly N, Gorman AM, Gupta S, Samali A. The eIF2α kinases: their structures and functions. *Cell. Mol. Life Sci.* 2013; 70:3493–3511 https://doi.org/10.1007/s00018-012-1252-6

19. Harding HP, Zhang Y, Bertolotti A, Zeng H, Ron D. Perk is essential for translational regulation and cell survival during the unfolded protein response. Mol. Cell. 2000b; 5: 897–904.

20. Cullinan SB, Zhang D, Hannink M, Arvisais E, Kaufman RJ, Diehl JA. Nrf2 is a direct PERK substrate and effector of PERK dependent cell survival. Mol Cell Biol. 2003; 23: 7198–7209. https://doi.org/10.1128/MCB.23.20.7198-7209.2003 PMID: 14517290

21. Crook NE, Clem RJ, Miller LK. An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. J Virol. 1993; 67: 2168–2174. PMID: 8445726

22. Simons M, Beinroth S, Gleichmann M, Liston P, Korneluk RG, MacKenzie AE et al. Adenovirus-mediated gene transfer of inhibitors of apoptosis protein delays apoptosis in cerebellar granule neurons. J Neurochem. 1999; 72: 292–301 PMID: 9886081

23. Hamanaka RB, Marjon EB, Ji X, Liebhaber SA, Diehl JA. PERK-dependent regulation of IAP translation during ER stress. Oncogene. 2009; 28: 910–920. https://doi.org/10.1038/onc.2008.428 PMID: 19029953

24. Niture SK, Kaspar JW, Shen J, Jaiswal AK Nrf2 signaling and cell survival. Toxicol Appl Pharmacol. 2010; 244:37–42 https://doi.org/10.1016/j.taap.2009.06.009 PMID: 19538984

25. Rouschop KM, Dubois LJ, Keulers TG, van den Beucken T, Lambin P, Bussink J, et al. PERK/eIF 2α phosphorylation protects therapy resistant hypoxic cells through induction of glutathione synthesis and protection against ROS. Proc Natl Acad Sci USA. 2013; Mar 19; 110(12):4622–7. https://doi.org/10.1073/pnas.1206331110 PMID: 23471998

26. Abhishek K, Sardar AH, Das S, Kumar A, Ghosh AK, Singh R, et al. Phosphorylation of translation initiation factor 2-alpha in *Leishmania donovani* under stress is necessary for parasite survival. Mol Cell Biol. 2017; 37:e00344–16

27. Kumar A, Das S, Purkait B, Sardar AH, Ghosh AK, Dikhit MR, et al. Ascorbate Peroxidase, a Key Molecule Regulating Amphoter cin B Resistance in Clinical Isolates of *Leishman ia donovani*. Antimicrob. Agents Chemother. 2014; 58(10):6172. https://doi.org/10.1128/AAC.02834-14 PMID: 25111428

28. Dias-Teixeira KL, Calegari-Silva TC, Medina JM, Vivarini ÁC, Cavalcanti Á, Teteo N, et al. Emerging Role for the PERK/eIF2α/ATF4 in Human Cutaneous Leishmaniasis. Sci Rep. 2017; Dec 6; 7(1);17074 https://doi.org/10.1038/s41598-017-17252-x

29. Salvesen GS, Duckett CS. IAP proteins: blocking the road to death’s door. Nat Rev Mol Cell Biol. 2002; 3: 401–410. https://doi.org/10.1038/nrm830 PMID: 12042762

30. Orth K, Dixit VM. Bik and Bak induce apoptosis downstream of CrmA but upstream of inhibitor of apoptosis. J Biol Chem. 1997; 272:8841–8844 PMID: 9082997

31. Roy N, Deveraux QL, Takahashi R, Salvesen GS, Reed JC. The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. EMBO J. 1997; 16: 6914–6925 https://doi.org/10.1093/emboj/16.23.6914 PMID: 9384571

32. Clem RJ, Sheu TT, Richter BW, He WW, Thomberry NA, Duckett CS et al. c-IAP1 is cleaved by caspases to produce a procaspotic C-terminal fragment. J Biol Chem 2001; 276: 7602–7608. https://doi.org/10.1074/jbc.M010259200 PMID: 11106668

33. Vucic D, Fairbrother WJ. The inhibitor of apoptosis proteins as therapeutic targets in cancer. Clin Cancer Res. 2007; 13: 5995–6000. https://doi.org/10.1158/1078-0432.CCR-07-0729 PMID: 17947460

34. Hu P, Han Z, Couvillon AD, Exton JH. Critical role of endogenous Akt/IAPs and MEK1/ERK pathways in counteracting endoplasmic reticulum stress-induced cell death. J Biol Chem. 2004; 279:49420–49429 https://doi.org/10.1074/jbc.M407700200 PMID: 15339911

35. Wamakulasuriyachchidi D, Cerquozzi S, Cheung HH, Holcik M. Translational induction of the inhibitor of apoptosis protein HIAP2 during endoplasmic reticulum stress attenuates cell death and is mediated via an inducible internal ribosome entry site element. J Biol Chem. 2004; 279: 17148–17157 https://doi.org/10.1074/jbc.M308737200 PMID: 14960583

36. Moreno JA, Halliday M, Molloy C, Radford H, Verity N, Axten JM, et al. Oral treatment targeting the unfolded protein response prevents neurodegeneration and clinical disease in prion-infected mice. Sci Transl Med. 2013; 5(206): 206–138
37. Axten JM, Medina JR, Feng Y, Shu A, Romaneri SP, Grant SW et al. Discovery of 7-methyl-5-(1-[[3-(tri-fluoromethyl)phenyl]acetetyl]-2,3-dihydro-1H-indol-5-yl]-7H-pyrrolo[2,3-d]pyrimidin-4-amine (GSK2606414), a potent and selective first-in-class inhibitor of protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK). J Med Chem 2012; 55: 7193–7207. https://doi.org/10.1021/jm300713s PMID: 22827572

38. Ji Yun, Zhaolai Dai, Guoyao Wu, and Zhenlong. 4-Hydroxy-2-nonenal induces apoptosis by activating ERK1/2 signaling and depleting intracellular glutathione in intestinal epithelial cells. Sci Rep. 2016; 6: 32929 https://doi.org/10.1038/srep32929 PMID: 27620528

39. Yan F, Cao S, Li J, Dixon B, Yu X, Chen J, Gu C, Lin W, Chen G. Pharmacological Inhibition of PERK Attenuates Early Brain Injury After Subarachnoid Hemorrhage in Rats Through the Activation of Akt. Mol Neurobiol. 2017 Apr; 54(3):1808–1817. https://doi.org/10.1007/s12035-016-9790-9 PMID: 28879783

40. Hsieh YY, Lo HL and Yang P.M. EZH2 inhibitors transcriptionally upregulate cytotoxic autophagy and cytoprotective unfolded protein response in human colorectal cancer cells. Am J Cancer Res. 2016 Aug 1; 6(8):1661–80. PMID: 27648357

41. Hosoi T, Hyoda K, Okuma Y, Nomura Y, Ozawa K. Akt up and down-regulation in response to endoplasmic reticulum stress. Brain Res. 2007; 1152: 27–31 https://doi.org/10.1016/j.brainres.2007.03.052 PMID: 17434462

42. Kazerni S, Mounir Z, Baltzis D, Raven JF, Wang S, Krishnamoorthy JL et al. A novel function of eIF2alpha kinases as inducers of the phosphoinositide-3 kinase signaling pathway. Mol Biol Cell. 2007; 18: 3635–3644. https://doi.org/10.1091/mbc.E07-01-0053 PMID: 17596516

43. Forde JE, Dale TC. Glycogen synthase kinase 3: a key regulator of cellular fate. Cell Mol Life Sci. 2007; Aug 64(15):1930–44, https://doi.org/10.1007/s00018-007-7045-7 PMID: 17530463

44. Cullinan S. B. & Diehl J. A. PERK-dependent activation of Nrf2 contributes to redox homeostasis and cell survival following endoplasmic reticulum stress. J. Biol. Chem. 2004; 279, 20108–20117 https://doi.org/10.1074/jbc.M314219200 PMID: 14978030

45. Avivar-Valderas A, Salas E, Bobrovnikova-Marjon E, Diehl JA, Nagi C, Debnath J, et al. PERK integrates autophagy and oxidative stress responses to promote survival during extracellular matrix detachment. Mol Cell Biol. 2011; 31(17):3616–29 https://doi.org/10.1128/MCB.05164-11 PMID: 21709020

46. Gao L and Abu Kwaik Y. Hijacking of apoptotic pathways by bacterial pathogens. Microbes Infect. 2000; 2: 1705–1719 PMID: 11137044

47. Heussler VT, Kuenzi P and Rottenberg S. Inhibition of apoptosis by intracellular protozoan parasites. Int J Parasitol. 2001a; 31: 1166–1176

48. Buates S, Matlashewski G. General suppression of macrophage gene expression during Leishmania donovani infection. J Immunol. 2001; 166: 3416±3422. PMID: 11207299

49. Ovalle-Bracho C, Franco-Munoz C, Londono-Barbosa D, Restrepo-Montoya D, Clavijo-Ramirez C. Changes in Macrophage Gene Expression Associated with Leishmania (Viannia) braziliensis Infection. PLoSOne 2015; 10: e0128934. https://doi.org/10.1371/journal.pone.0128934 PMID: 26052705

50. Ott M, Robertson J D, Gogvadze V, Zhivotovsky B, and Orrenius S. Cytochrome c release from mitochondria proceeds by a two-step process. PNAS. 2002; (5) 99; 1259–1263

51. Petrosillo G, Ruggiero FM, Paradies G. Role of reactive oxygen species and cardiolipin in the release of cytochrome c from mitochondria. FASEB J. 2003; 17(15):2202–8 https://doi.org/10.1096/fj.03-0012com PMID: 14656982

52. Magdalena LC and Tak Yee Aw. Reactive Oxygen Species, Cellular Redox Systems and Apoptosis. Free Radic Biol Med. 2010; 15; 48(6): 749–762. https://doi.org/10.1016/j.freeradbiomed.2009.12.022 PMID: 20045723

53. Simon HU, Haj-Yehia A, Levi-Schaffer F. Role of reactive oxygen species (ROS) in apoptosis induction. Apoptosis. 2000; Nov; 5(5):415–8 PMID: 11256882