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Review Article

The Complex World of Cellular Defense in the *Leishmania* Parasite

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Reactive oxygen species (ROS) and their products are the main agents used by cells to kill invading pathogens. Therefore, to establish a successful infection, pathogens require a robust defensive system to counteract host-generated oxidative stress and their products. *Leishmania* parasites with a digenetic life cycle, cause Kala-azar and are exposed to conditions of moderate to severe oxidative stress in both the insect and the mammalian hosts. Natural selection has endowed these parasites with multiple defense systems including a peroxiredoxin system of defense, a robust system of thiols, arginases, ascorbate peroxidases, kinases and phophatases, all of which can be used for thwarting potential threats. In addition to these, the parasites are able to defend themselves by altering the host defense mechanisms through the manipulation of host cytokines and other signaling pathways. The peroxiredoxin system is distinct from mammalian peroxiredoxin system and absence of the parasite components in the mammalian host makes them potential drug targets. Trypanothione, a thiol unique to the *Leishmania* parasite, serves as a central molecule for a cascade of enzymes through which the electrons are shuffled and finally used by the terminal enzyme, the tryparedoxin peroxidase (TXNPx) to eliminate peroxides. Tryapanothione can directly interact with the products of the oxidative stress and can be formed from cellular glutahione. This review discusses the relevance of the parasite defense systems in the context of cell death in the *Leishmania* parasites.

Key Words: *Leishmania*; Tryparedoxin Peroxidase; Thiols; Reactive Oxygen Species; Ascorbate

Introduction

Cellular defense responses are essential for protection against a variety of stresses. During evolution, processes of cellular defense mechanisms were selected according to the survival needs of a particular cell. In contrast to protective mechanisms of normal body cells required to maintain regular homeostasis, the pathogens invading host organisms require very specialized forms of defense. This is because, they face host cell generated defensive arsenal in the form of oxidative stress that is produced to eliminate pathogens and invaders with the best defense ability to modulate host cell protective response become the deadliest of pathogens. Oxidative stress can generate a variety of reactive oxygen species (ROS) capable of damaging cellular macromolecules through direct interaction or through toxic products generated by them, resulting in pathogen death. Concomitantly, natural selection also worked upon the cellular defense mechanisms of the host for it to survive during a pathogen attack. Thus, this effort to eliminate each other during the course of evolution has resulted in the development of robust defense systems in both organisms. In this particular review, we discuss the defensive mechanisms and death processes of a Kinetoplastid parasite, the *Leishmania* species that deviated as a separate branch from the base of the main line that generated the eukaryotic crown during evolution. Evolution has endowed these parasites with sophisticated defense mechanisms to survive within the mammalian macrophages, the very cells that are supposed to eliminate them.

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The order Kinetoplastida of Trypanosomatidae family, have parasite species of the genus *Trypanosoma* and the *Leishmania* which are among, one of the first to acquire the mitochondria through engulfment of the alpha-proteobacteria (Arnoult *et al.,* 2002). These first mitochondrial eukaryotes express eukaryotic features but share some prokaryotic characters as well and hence make good model systems for the study of cellular processes that evolved early. While the introduction of mitochondria within the cells helped them to survive in an increasingly aerobic atmosphere, this also required the evolution of processes to deal with toxic products generated intracellularly by the ROS produced within the cells. The *Leishmania* parasite causes Leishmaniasis, which kills about half a million people per year (Flohe, 2012). The disease is transmitted by the female phlebotomine flies of the genera *Phlebotomus* and *Lutzomyia*. In humans, the disease occurs in four different forms, the life threatening visceral leishmaniasis, commonly known as kala-azar, mucosal leishmaniasis, the self-healing cutaneous leishmaniasis and post-kala-azar dermal leishmaniasis (Chang and Fong, 1983). Human infections are caused by about 21 of 30 species of *Leishmania*. These include the *L. donovani* complex with 3 species (*L. donovani*, *L. infantum* and *L. chagasi*); the *L. mexicana* complex with 3 main species (*L. mexicana*, *L. amazonensis* and *L. venezuelensis*); *L. tropica*; *L. major*; *L. aethiopica* and the subgenus *Viannia* with 4 main species (*L.* (*V.) braziliensis, L.* (*V.) guyanensis, L.*(*V.) panamensis* and *L.*(*V.) peruviana*). Having originated about 140 million years ago in Africa and infected the reptiles, the spread and evolution of these parasites have been affected by the geographical changes in the continents and appearance of new species of mammals (Momen and Cupolillo, 2000).

Several anti-leishmanial drugs used to treat leishmaniasis generate ROS to kill these parasites, however, inspite of substantial number of deaths due to leishmaniasis in humans and a fair understanding of mechanism of drug action, the therapeutic efforts remain frustrating. Most of the anti-leishmanial drugs show heavy cytotoxicity; have limited potency and are prone to develop resistance (Flohe, 2012). Among them pentavalent antimonials (meglumine antimonate and sodium stibogluconate) being the first line drug has been used for the last several decades but because of the increasing incidence of resistance cases combinatorial therapy is currently prescribed. In the endemic region of Bihar, antimony resistance is most prevalent (Croft *et al.,* 2006b; Croft *et al.,* 2006a). For biological activity, pentavalent antimonials need reduction to the trivalent form that has been shown to interfere with thiol metabolism (Wyllie *et al*., 2004). Our studies have shown that trivalent antimony mediated deaths occur through generation of ROS in the *Leishmania* cells as a consequence of which glutathione levels are lowered resulting in cell death (Mehta and Shaha, 2006). Amphotericin B - a polyene antibiotic used as an anti-leishmanial drug also generates ROS but is not preferred due to high toxicity. Miltefosine, an alkylphosphocholine is the first oral anti-leishmanial drug, also acts through ROS; and is approved for use in this country (Vincent *et al*., 2014). Paromomycin, an aminoglycoside antibiotic that does not generate ROS or result in apoptotic death (Moreira *et al*., 2011) is an anti-leishmanial drug registered for the treatment of VL in India. Pentamidine and Sitamaquin are the other two drugs effective against Leishmaniasis (Seifert *et al*., 2011) and act through the production of ROS (Mehta and Shaha, 2003). In the context of the parasite eliminating potential of ROS and their products during host parasite interaction; and drug treatment makes the study of defense mechanisms of the parasites very important for improvement of current therapy and future drug development.

Life-Cycle of the *Leishmania* Parasite

The *Leishmania* parasite exists in two forms, as the extracellular free swimming promastigote form found in the invertebrate host and blood stream of the mammalian host; and as the intracellular round immotile amastigote form found in the mammalian host. The amastigote forms are accountable for manifestation of the disease features. The sandfly vector ingests the macrophages housing the amastigotes when feeding on the blood of an infected organism. In the sandfly gut, the released amastigotes mature to motile infective promastigote forms and

are transmitted to the vertebrate host through sandfly bites (Garcia *et al*., 2012). Neutrophils are the first cells to arrive at the site of bite under the skin and are believed to serve as the primary host cells and possibly are used by the parasites as Trojan horses to infect macrophages (Mocsai, 2013). It is at this point of entry that the parasites are exposed to ROS and various ROS derived toxic products and if they are successful in combating these elements, the parasite transforms rapidly into the amastigote form within the macrophages and proceeds to replicate intracellularly. Although most of the pathogens evade the microbicidal responses of the host macrophages by subverting or escaping from the phagocytic pathway, *Leishmania* parasites manages both to survive and to proliferate within the mature phagolysosomal compartment of the macrophages (McConville *et al*., 2007).

Oxidative Stress in the Macrophages

The phagocytic properties of macrophages along with the capability to generate oxidative stress are critical for innate immune recognition and subsequent clearance of pathogens. Molecular oxygen is the parent molecule for all of the ROS inside a cell and reduction of O_2 leads to formation of superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^\bullet$). The *Leishmania* parasite has evolved elegant mechanisms to subvert these host responses and survive within the extreme conditions of the macrophage phagolysosomes. The ROS producing system of the macrophage consists of plasma membrane-bound enzyme complexes, the NADPH oxidase (NOX) system. As the phagolysosomes form during pathogen engulfment, NOX expressed on the phagolysosomal membrane starts generating ROS to kill the invaders. The primary radical generated by the NOX is the superoxide that further generates other ROS. The reaction of NO with superoxide is extremely rapid resulting in peroxynitrite ($OONO^-$) production which is a potent oxidant (Koppenol *et al.*, 1992). *Leishmania* are susceptible to killing by both ROS and reactive nitrogen species (RNS) (Roach *et al*., 1991; Wilson *et al*., 1994). While activated mouse peritoneal macrophages kill ingested *Leishmania donovani* amastigotes by secreting either ROS or NOS (Murray, 1982; Murray, 1990) or RNS (Roach *et al*., 1991), inhibition of these reactive species *in vitro* prevents this killing (Iyer *et al.*, 2008; Murray, 1982). Therefore, defense systems competent to neutralize these radicals are essential for the parasites to survive.

Due to their intrinsic properties, each ROS reacts with their own preferred biological targets within the cell. H_2O_2 is actually a poor oxidant and is relatively stable, however, its toxicity is essentially the consequence of its reduction to $HO^\bullet$ by metal catalyzed Fenton chemistry (Kozlowski *et al.*, 2014). The high reactivity of the $HO^\bullet$ radical limits its area of action but can cause severe cellular damage.

Cellular Defense Systems Against Oxidative Stress in the *Leishmania* Parasite

The cellular defense system of the *Leishmania* parasite consists of multiple elements, however, some of them are more active at a given situation than others. It is believed that many aspects of the defense mechanisms in these parasites are yet to be discovered fully. However, in the post genome era, it has become easier to identify potential new targets that may be playing a defensive role for the parasites against stress. In the following paragraphs we describe the components of the defensive arsenal of the *Leishmania* parasite.

(a) Thiols and Ovothiol A

Trypanothione (N1, N8-bis(glutathionyl)-spermidine adduct) is the redox mediator in trypanosomatids synthesized *de novo* from spermidine, glutathione and ATP (Henderson *et al.*, 1990; Krauth-Siegel & Comini, 2008). Trypanothione is also regenerated by trypanothione reductase from its oxidized cyclic disulfide form produced during its activity in the peroxide reducing enzyme cascade that also contains dithiol proteins like thioredoxin and tryparedoxin (Fig. 1). It is the primary thiol in these parasites and adopts the metabolic role of glutathione. Although the order Kinetoplastida to which the *Leishmania* parasites belong, lack glutathione reductase, they still maintain significant levels of glutathione from which trypanothione can be generated (Krauth-Siegel and

Comini, 2008). An increase in the levels of trypanothione was also correlated with metal resistance in different *Leishmania* species though the mechanism seems to differ (Legare *et al.*, 1997).

Ovothiol A acts as a non-enzymatic scavenger of H_2O_2 though it is much less efficient than trypanothione (Ariyanayagam and Fairlamb, 2001). In *Leishmania* promastigotes, ovothiol A can exceed trypanothione content particularly in late logarithmic and stationary phases of growth. In other trypanosomatids, it represents less than 10% of the total thiol pool. Although amastigotes of *L. major* and *L. donovani* contain equivalent amounts of glutathione and trypanothione, ovothiol A is present in the former but absent in the latter. Given the presence of an active trypanothione peroxidase system in the trypanosomatid parasites, it is thought that under physiological conditions, ovothiol is unlikely to play a major role in the metabolism of hydrogen peroxide in intact cells (Ariyanayagam and Fairlamb, 2001).

(b) Trypanothione Reductase and Tryparedoxin

Trypanothione reductase (TR), is a member of the disulfide reductase family and is the enzyme responsible for maintaining trypanothione in its reduced form (Fig. 1) (Dumas *et al.*, 1997). This enzyme is a part of a cascade of enzymes that form the primary defense system of the parasite. This enzyme shows a high sequence homology to glutathione reductase. Mutants of *L. donovani* and *L. major* possessing only one wild-type TR allele express less TR mRNA and have lower TR activity compared to wild-type cells carrying two copies of the TR gene. Significantly, these mutants show attenuated infectivity with a markedly decreased capacity to survive intracellularly within macrophages producing reactive oxygen intermediates (Dumas *et al.*, 1997). Tryparedoxin is the proximal reaction partner for trypanothione and serves as a reductant for different types of thiol peroxidases. They are proteins characterised by having the CxxC motif. Functionally, TXNs are protein disulphide reductants.

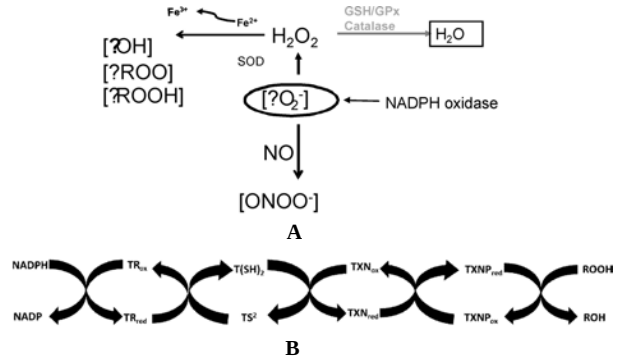

Fig. 1. ROS generation and the trypanothione mediated elimination of peroxides. (A) A schematic representation of the ROS generated by the NADPH oxidase present on the phagolysosomal membrane of the mammalian macrophages. The GPx and catalase are not present in the *Leishmania* parasite; (B) Figure shows the enzyme cascade for elimination of peroxides like hydrogen peroxide, Peroxinitrite and organic hydroperoxides. TR, trypanothione reductase; TS, trypanothione; TXN, tryparedoxin; TXNP, tryparedoxin peroxidase

(c) Tryparedoxin Peroxidases

Tryparedoxin peroxidases or peroxiredoxins are a family of peroxidases that reduce H_2O_2 and alkyl hydroperoxides to water and alcohol respectively, with the use of reducing equivalents provided by thiol containing proteins (Rhee *et al*., 2001)**.** Tryparedoxin peroxidases are the terminal peroxidase of the cascade. These enzymes are ubiquitous, present in yeast, plant and animal cells, including both protozoan and helminth parasites, and most, if not all, eubacteria and archaea. They exert their protective antioxidant role in cells through their peroxidase activity, whereby H_2O_2, peroxynitrite and a wide range of organic hydroperoxides are reduced and detoxified (Castro *et al*., 2002). They appear to be fairly promiscuous with respect to the hydroperoxide substrate; the specificities for the donor substrate vary considerably between the subfamilies. Because of their ability to eliminate a variety of toxic species generated by the host cell machinery to destroy pathogens, they have been implicated in the virulence of mycobacteria and trypanosomatids (Levick *et al*., 1998). They have also been designated as potential drug targets as these enzymes are not present in the mammalian host. Peroxiredoxins use redox-active cysteines to reduce peroxides and were originally

divided into two categories, the 1-Cys and the 2-Cys peroxiredoxins, based on the number of cysteinyl residues directly involved in catalysis. The TXNPxs of Trypanosomatidae belong to the 2-Cys peroxiredoxin based on the sequence homology to higher eukaryotic peroxiredoxins and presence of two catalytic cysteine residues and associated motifs (Flohe *et al*., 1999).

Pronounced differences exist between the antioxidant machinery of trypanosomatids and other eukaryotes. Trypanosomatids do not express essential anti-oxidant enzymes like catalase and selenium-containing glutathione peroxidases (Castro *et al*., 2002). As much as 70% of their glutathione is converted to trypanothione. The component accepting the reduction equivalents from trypanothione is tryparedoxin (TXN), which is related to the thioredoxin family (Fig. 1). Tryparedoxin itself is a substrate for the tryparedoxin peroxidase (TXNPx) which reduces H_2O_2 and organic hydroperoxides. All trypanosomatid organisms studied so far possess 2-Cys peroxiredoxins and 1-Cys peroxiredoxins but are not present in the genomes of *T. brucei*, *T. cruzi* and *L. major* (Harder *et al.,* 2006).

There are two types of 2-Cys peroxiredoxins, one located in the cytosol (cytosolic tryparedoxin peroxidase) (cTXNPx) and the other in the mitochondria (mitochondrial tryparedoxin peroxidase) (mTXNPx). The trypanosomatid genomes encode multiple and almost nearly identical copies for cytosolic proteins and on another chromosome a single copy gene for a mitochondrial 2-Cys peroxiredoxin. Homologous enzymes of cytosolic as well as mitochondrial peroxidase have been identified in *L. major* (Levick *et al.,* 1998), *T. brucei* (Tetaud *et al.,* 2001), *L. infantum* (Castro *et al.,* 2002) and *L. donovani* (Iyer *et al.,* 2008). The cytosolic enzymes contain the two classical VCP motifs. The mitochondrial enzyme has an N-terminal mitochondrial pre-sequence and an IPC motif as second redox center. This motif is similar to the LPC sequence in yeast TSA I and II and is not a general feature of mitochondrial 2-Cys-peroxiredoxins. The mitochondrial peroxidase is coded by a 226 amino acid coding gene present on the chromosome 23 of both *L. major* and *L. infantum*. Many studies on developmentally induced changes using microarray analysis (Holzer *et al.,* 2006) as well as proteomic analysis (Bente *et al.,* 2003) on lesion derived promastigotes and axenic amastigotes in various *Leishmania* spp. have shown different profiles of expression for the protein.

Both the cytosolic and the mitochondrial forms of the tryparedoxin peroxidases exist as decamers (Alphey *et al*., 2000). The three dimensional structure of the two enzymes are very similar although they share about 52% primary sequence identity. Studies from this laboratory have shown that overexpression of the cytosolic enzyme rescue parasites from oxidative and drug induced stress (Iyer *et al*., 2008). The parasites are more sensitive to the combined stress of H_2O_2 and NO that can be overcome through overexpression of the cTXNPx. The elimination of peroxides by the overexpressed enzyme prevents entry of extracellular Ca^{2+} and release of intracellular Ca^{2+} induced by the oxidative stress, thus, reducing the forces capable of precipitating cell death and consequently increasing the cell survival (Iyer *et al.,* 2008). Parasites overexpressing the cTXNPx could infect macrophages *in vitro* in higher numbers as compared to only vector transfected parasites (Iyer *et al*., 2008) showing that the presence of excess cTXNPx rendered the parasites more capable of combating host defense. Data from this laboratory show that overexpression of the mitochondrial enzyme also renders the parasites more capable of infecting macrophages. Our studies have shown that the mitochondrial targeting sequence (MTS) of the TXNPx is essential for transport to the mitochondria and is cleaved upon entry into the organelle (Aich and Shaha, 2013). Interestingly, the MTS contains a calmodulin binding sequence and *in silico* studies show that calmodulin binds to the MTS (Fig. 2). Our investigations using site directed mutagenesis studies clearly demonstrate that substitution of specific residues in place of calmodulin binding amino acids, impedes the translocation of the protein to the mitochondria. The calmodulin essentially helps HSP70 to bind to the protein for translocation to the mitochondria (Aich and Shaha, 2013). Data from the laboratory show that if translocation of the protein is

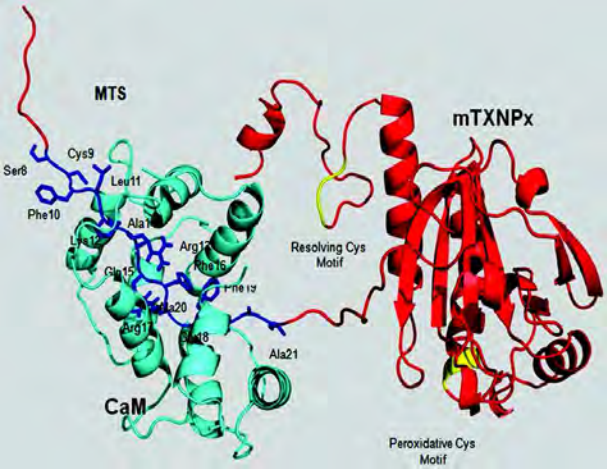

Fig. 2. Docking of calmodulin to mTXNPx. The docked model of closed conformation state calmodulin (in cyan) as a ligand protein and mTXNPx (in red) as a receptor protein using the online protein-protein docking server ClusPro 2.0 visualized using PyMOL. The residues marked in blue are the predicted CaM binding residues of mTXNPx. CaM, calmodulin; MTS, mitochondrial targeting signal

impeded and bulk of the enzyme resides within the cytosol, the parasites become sensitive to mitochondria generated oxidative stress produced by inhibition of respiratory chain complexes. Interestingly, it has been reported that the mitochondrial enzyme by itself can act as a chaperone and mTXNPx can be a determinant for pathogenicity that is independent of the peroxidative activity of the enzyme (Castro *et al.*, 2011).

Therefore, in a nutshell, these two most essential enzymes of the *Leishmania* defense system, the tryparedoxin peroxidases, cytosolic as well as the mitochondrial one are responsible for eliminating peroxides thus protecting the cells from ROS. However, they also have other functions not related to their anti-oxidant activity. Importantly, overexpression of both enzymes rendering the parasites resistant to drug induced death and increase in the enzyme levels upon relevant oxidative stress in wild-type cells suggests that a close look is necessary at clinical isolates of parasites to determine if they are linked to drug resistance (Wyllie *et al.*, 2010).

(d) Arginases

The enzyme arginase is the part of host as well as parasite defense. Phagocytosis of promastigotes leads towards the two opposing forms of classical and alternative activation of host macrophages which results in differential L-arginine metabolism by two key enzymes i.e. inducible nitric oxide synthase (iNOS) and arginase (Kropf *et al.*, 2003; Iniesta *et al.*, 2001). Arginase hydrolyzes L-arginine to urea and ornithine through alternative macrophage activation (Gordon, 2003). Ornithine as a result, actively participates in synthesis of polyamines, that are essential nutrients for growth and proliferation of *Leishmania* parasites (Iniesta *et al.*, 2001; Iniesta *et al.*, 2002; Kropf *et al.*, 2005). Classical activation of macrophages occurs through iNOS oxidizing L-arginine to NO in a two step process (Gordon, 2003). NO has a potent role in parasite clearance. Since the two pathways compete for arginine, therefore, activation of one pathway down regulates the other. For example, hydroxyl arginine being an intermediate of classical activation pathway is a powerful arginase inhibitor and treatment of *L. major*-infected mice with its synthetic analog Nω-hydroxy-L-arginine (NOHA) causes a significant reduction in lesion size and as well as parasite burden (Kropf *et al.*, 2005). Similar to the host, the *Leishmania* parasites express their own arginase which modulates infectivity. It appears that *Leishmania*-encoded arginase increases progression of the disease by enhancing the host arginase activity. *L. major* null mutant for arginase is relatively less efficient to infect macrophages both *in* vitro and *in vivo* (Muleme *et al.*, 2009).

(e) Kinases and Phosphatases

Host macrophages, neutrophils and dendritic cells engulf parasites and start immune responses against them through multiple signalling pathways. Phosphorylation and dephosphorylation processes mediated by kinases and phosphatases play an important role in this process. *Leishmania* parasites being effective in modulating macrophage signalling and antimicrobial function, possesses surface protein kinases which phosphorylate members of complement system thus inactivating cellular

cascades. This helps the parasite to evade the innate immune responses and ensure a safe environment for its proliferation. Forget and coworkers in 2001 demonstrated that *in vivo* inhibition of host PTP (Protein Tyrosine Phosphatases) can control the disease progression by NO production (Forget *et al.*, 2001). Apart from this, host's serine/threonine phosphatase PP2 as well as MAPK phosphatases MKP1 and MKP3 are observed to be modulated by *Leishmania* during murine Leishmaniasis (Kar *et al.*, 2010). Further, Kar and coworkers in 2010 demonstrated that *Leishmania* infection increases expression, activity, and membrane translocation of two PKC isoforms, PKCε and PKCζ, that are implicated in the up-regulation of DSP (Dual Specific Phosphatases) and STP (Serine Threonine Phosphatases) expression and activity which further inhibit macrophage leishmanicidal activity along with higher IL-10 production. The best anti-leishmanial drugs developed to date are arsenic-based compounds that have been shown to target PTPs. Thus kinases and phosphatases also form an important component of *Leishmania* defense against the innate immune system.

(f) Modulation of Host Cytokines

The T helper cell type 1 (Th1) response is indispensable for leishmaniasis resistance, while the Th2 response favors development of the disease (Campos-Neto, 2005). Parasites can inhibit the activation of several inflammatory cytokines like IL-12 (involved in T-cell activation), IFNγ, IL-1 and TNFα that strengthens parasite survival. *L. donovani* infection inhibits IL-1β secretion, and the parasite LPG can also repress IL-1β through promoter repression sequence (Hatzigeorgiou *et al.*, 1996; Reiner and Malemud, 1985; Reiner *et al.*, 1990). *L. donovani* are inferior activator of proinflammatory reactions as compared to *L. major* (Matte *et al.*, 2001). Both the species induce heterologous population of host inflammatory cells like neutrophils and monocytes/macrophages which are effective in controlling/clearing infections. IL-12 being a lead player in regulation of cellular immune responses (T-cell activation and IFN-γ secretion) is inhibited by *L. donovani*, *L. major* and *L. mexicana* amastigotes for securing a safe environment for parasites (Carrera *et al.*, 1996; Weinheber *et al.*, 1998). Cytokine inhibition is further augmented by production of immuno suppressive signalling molecules, like arachidonic acid metabolites (like PGE2) and Th2 stimulating cytokines like TGFβ and IL-10 (via interaction with FCγ receptor) (Matte *et al.*, 2001; Reiner and Malemud, 1984; Reiner and Malemud, 1985). As a result, decreased expression of iNOS and reduced activity of NK cells has been observed. Th2 being involved in disease progression pathway also down regulates the Th1 associated pathways (microbicidal effects) by suppressing several key players of Th1 like IL-1, IL-12 and TNFα. Whereas prostaglandin (PGE2) favors parasite survival by inhibiting TNFα, IL-1 and ROI.

(g) Selenoproteins

Selenocysteine (Sec-U), the 21st amino acid is present in a number of proteins of organisms in the three domains of life, bacteria, archaea and eukarya (Bock *et al.*, 1991; Forchhammer and Bock, 1991). Sec is analogous to a cysteine residue but having sulfur substituted by selenium. In Eukarya the Sec insertion element (SECIS) element is located in the mRNA 3´ untranslated region (3´-UTR). In recent years, several selenoprotein families, some with antioxidant properties, like glutathione peroxidase and thioredoxin glutathione reductase (TGR) appear to be essential in flatworms that have been described and characterized (Maiorino *et al.*, 1996; Williams

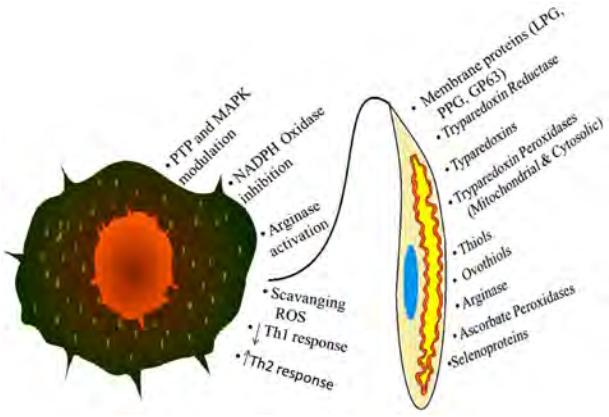

Fig. 3. Defense systems of the parasite. A summary of the defense systems in the parasite and the systems in the host modulated by the parasite

et al., 1992). One such protein selenophosphate synthetase in *P. falciparum* and other Plasmodia has been characterized and is important. In *Leishmania* parasites, several genes were reported to contain a Sec codon coding for a homolog of selenophosphate synthetase (*Leishmania major* (accession no. AAG35734), *Trypanosoma cruzi* (XM_805940) and *Trypanosoma brucei* (XP_823164)), an enzyme that generates selenophosphate, a selenium donor compound used for biosynthesis of Sec (Jayakumar *et al.,* 2004; Lobanov *et al.,* 2006). Selenoproteins from protozoan parasites needs to be characterized in greater detail and their exact role in the defense processes needs to be explored in more detail.

(h) Ascorbate Peroxidases

Although catalase and selenium-containing glutathione peroxidases are not present in the parasite, ascorbate peroxidase (LmAPX) from *L. major* presents itself to be a potential candidate for scavenging of ROS and has been shown to be central to the redox defense system of *Leishmania* (Dolai *et al*., 2009). Ascorbate peroxidase is a heme peroxidase identified in the *Leishmania* parasite. This enzyme is localized to the inner mitochondrial membrane. Overexpression of this enzyme in *L. major* confers tolerance to oxidative stress-mediated cardiolipin oxidation and thus protects the parasites from extensive protein damage. Double knockout of this enzyme shows higher intracellular H_2O_2 as compared to wild type parasites. Protection against host cell induced apoptosis is also accorded by ascorbate peroxidase in *Leishmania* (Dolai *et al.,* 2009; Pal *et al.,* 2010).

(i) NADPH Oxidase and iNOS Expression

Amastigotes resistant to hydrolytic environment prevail well in the phagolysosomal compartment of host macrophages. *Leishmania* promastigotes inhibit phagolysosome biogenesis via LPG, which causes periphagosomal accumulation of F_actin and impaired assembly of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex and exclusion of vesicular proton ATPase from phagosomes. Amastigote harboring vacuoles are composed of endoplasmic reticulum like calnexin and Sec 22b as well as endocytic pathway components like Rab7, LAMP-1 and LAMP-2 (Antoine *et al.,* 1990; Antoine *et al.,* 1998; Vinet *et al.,* 2009). Proteolysis within the phagosome is key to a competent antigen processing and presentation which is again challenged by the parasites. Disruption in antigen presentation (Mantegazza *et al.,* 2008; Rybicka *et al.,* 2012; Savina *et al.,* 2009) by *L. donovani* promastigotes occurs through NADPH oxidase complex which significantly contributes to the fudging of the immune system. NADPH oxidase complex assembly requires the cytosolic phosphorylated p47phox and p40/p67 phox heterodimers which associate to form p47/p67/p40phox hetero-trimers prior to their membrane translocation, where they interact with membrane-associated flavor cytochromeb558 (DeLeo *et al*., 1999; El-Benna *et al*., 2009). To avoid ROS exposure amastigotes avoids the phosphorylation of cytosolic p47 phox, which is necessary for NADPH oxidase activation during phagocytosis (Lodge and Descoteaux, 2006).

When the Defense Mechanisms Fail

Our studies as well as the work done by others have shown that when the promastigotes or the free swimming parasite forms of *Leishmania* parasites are exposed to oxidative stress and their defense mechanism fails, they undergo an apoptosis-like death. When death is induced by H_2O_2, they show DNA fragmentation, mitochondrial potential fall, Ca^{2+} increase and nuclear condensation similar to mammalian apoptosis (Das *et al.,* 2001; Lee *et al.,* 2007; Smirlis and Soteriadou, 2011). This H_2O_2 induced parasite death is an interesting model to study the mechanism of stress response as it mimics the ROS generated by the host. In response to H_2O_2, cTXNPx levels increase in cells in a time dependent manner showing an attempt by the cell to mount a defensive response. One of the early events associated with excess H_2O_2 exposure is the dysfunction of the single long mitochondrion resulting in a dose dependent fall in mitochondrial potential. Interestingly, this fall of potential occurs in a heterogeneous pattern with areas of high and low potential overlapping each other in the mitochondria

ensuring a minimal supply of ATP required for apoptotic death (Mukherjee *et al.,* 2002; Sen *et al.,* 2004). This shows that requirement of mitochondrial generation of energy during apoptosis-like death was possibly selected early during evolution. One of the prominent agents that causes changes inside the cells after a stress response is Ca^{2+}. Ca^{2+} elevation occurs after oxidative stress through non-selective cationic channels and importantly, blocking of Ca^{2+} entry prevents cell death clearly indicating a functional role of Ca^{2+} in precipitating cell death (Sudhandiran and Shaha, 2003; Mukherjee *et al.,* 2002). Other studies have also shown involvement of Ca^{2+} in *Leishmania* cell death (Dolai *et al.,* 2011). Overexpression of cTXNPx precipitates a blockade of ROS induced Ca^{2+} entry from various sources, thus protecting the cells from oxidative damage. In the free-swimming forms, oxidative stress induced death does not occur in iron depleted conditions and worsens with addition of iron (Mehta and Shaha, 2006). Ca^{2+} increase occurs in intracellular amastigotes as well (Sudhandiran and Shaha, 2003). Mitochondrial generation of ROS occurs at the respiratory chain complexes and inhibiting these complexes in the *Leishmania* parasite by using specific inhibitors of the complexes can induce ROS (Mehta and Shaha, 2004). Protection from the effects of mitochondrial defensive enzymes strategically located in the mitochondria presumably accord protection from locally generated ROS and our studies with mTXNPx confirm this. Therefore, the ability of the *Leishmania* parasites to undergo cell death with apoptosis like features ensures that unnecessary inflammatory reactions are not initiated when cells die within the host. Interestingly, it has been shown that during infective bites by the sandfly, the infective inoculum contains apoptotic cells that help establish an infection without initiating an inflammatory response (El-Hani *et al.,* 2012). Therefore, deficiency of defensive enzymes or other protective molecules like thiols during stress drives the death processes within these parasites.

Future Trends

The current repertoire of anti-leishmanial drugs is not sufficient as they are inefficient and toxic. Interference with defensive mechanisms form an ideal target area as many of the proteins may not be similar to host proteins. It is obvious from the above discussion that a sizable amount of investment has to be made in further studies on defense mechanisms of these parasites as many unknown areas are apparent. TXNPx has been used as a vaccine candidate in murine models of cutaneous leishmaniasis caused by *L. major* with DNA/MVA delivery where long term memory was elicited (Stober *et al.,* 2007). Therefore, further studies with the TXNPxs in larger animal models will provide clues as to whether these molecules can be pursued further either as targets for blockage or as vaccine candidates. Vaccines against leishmaniasis have been slow in development because of the complexity of parasite survival within the host. Immune cells from the site of bite pick up the parasites and then they disperse to various organs. To target these parasites through vaccines, the antibody has to reach interior of the cells and then cross the cell as well as the phagolysosomal membrane. This makes success of vaccines very difficult. On the other hand, the components of the trypanothione dependent redox metabolism system being distinct from their host, form potential targets for development of therapeutics. However, complete knowledge about these enzymes is required to launch plans to develop drugs against the trypanosomatid parasites. Some of these enzymes show structural similarity to mammalian enzymes and therefore, specific inhibitors targeting exclusive parasite specific sites on these enzymes may be required. Intense studies on these molecules using small molecule libraries are one of the ways for successful drug development.

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