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Involvement of the *Leishmania donovani* virulence factor A2 in protection against heat and oxidative stress

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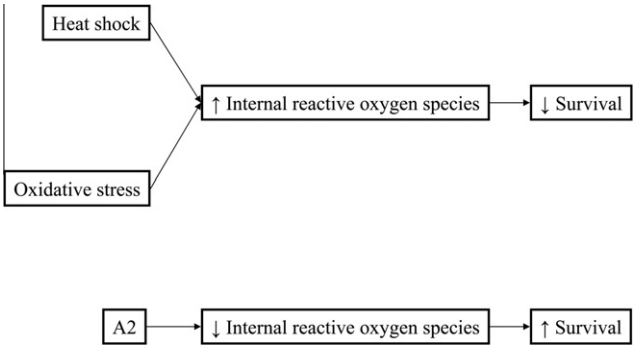
HIGHLIGHTS

- A2 proteins protect against host defences, including heat stress (fever) and oxidative stress.
- Protection is associated with decreased intracellular levels of reactive oxygen species.
- A2 does not protect against unfolded protein stress caused by dithiothreitol.
- These results help to explain the increased virulence of A2-expressing cells in visceral organs.

GRAPHICAL ABSTRACT



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ABSTRACT

Leishmania is an obligate intracellular protozoan parasite that infects cells of the reticulo-endothelial system. Host defences against *Leishmania* include fever and oxidant production, and the parasite has developed a number of defence mechanisms to neutralize the host response. The *Leishmania donovani* A2 family of proteins has been shown to be essential for survival in mammalian visceral organs. Here we provide evidence that A2 proteins protect the parasite against host defences, namely heat stress (fever) and oxidative stress. A2 is however unable to protect the cells from endoplasmic reticulum stress induced by dithiothreitol. To downregulate A2 protein expression, *L. donovani* was transfected with an A2 antisense RNA expressing-vector, resulting in significant reduction of A2 levels. The resulting A2-deficient cells were more sensitive to heat shock and this was associated with increased production of internal oxidants during heat shock. Moreover, axenic amastigotes with downregulated A2 expression had increased internal oxidants and decreased viability following treatment with hydrogen peroxide or a nitric oxide donor when compared to control cells. Overall, these results suggest that A2 protects *L. donovani* from a variety of stresses, thereby allowing it to survive in the internal organs of the mammalian host and to cause visceral disease.

1. Introduction

Leishmania are protozoan parasites that have a dimorphic lifecycle where the promastigote stage proliferates in the sandfly vector and is transmitted to its mammalian host during a blood meal. Within the mammalian host, the promastigotes differentiate into amastigotes and multiply predominantly inside macrophages. Amastigotes can then infect other macrophages or be taken up by feeding sandflies. Human infection is associated with a variety of disease manifestations depending mainly on the infecting *Leishmania* species. In particular, *Leishmania donovani* causes visceral infections and *Leishmania major* cutaneous disease (Murray et al., 2005).

Since *Leishmania* specifically multiply inside macrophages, they are exposed to a battery of host defences, including reactive oxy-

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gen and nitrogen species (Wilson et al., 2005). Nitric oxide in particular is especially important for parasite clearance (Wei et al., 1995). Infection with visceralizing species such as *L. donovani* is also associated with high fever (Pearson and Sousa, 1996). The parasite must therefore be able to withstand oxidative stress and heat stress. *Leishmania* has evolved a broad spectrum of mechanisms to protect itself against these host defences, including enzymes such as superoxide dismutase (Mehlotra, 1996), 2-cys peroxiredoxins, ascorbate peroxidases and glutathione peroxidase-like enzymes (Castro and Tomas, 2008). These enzymes detoxify reactive oxygen and nitrogen species using electrons from NADPH transferred via trypanothione and tryparedoxin, ascorbate and glutathione (Castro and Tomas, 2008). Non-enzymatic scavengers such as thiols also play a key role in antioxidant defenses (Mehlotra, 1996). Finally, *Leishmania* can also inhibit host intracellular pathways, thereby leading to downregulation of oxidant production by the macrophage (Olivier et al., 2005).

A2 genes are arranged in tandem arrays on chromosome 22 (LinJ.22.0670) (Charest et al., 1996), with up to four such arrays per diploid genome (Zhang and Matlashewski, 2001). A2 proteins in *L. donovani* have 40 to 90 copies of a repetitive ten amino acid sequence, leading to a family of proteins with sizes ranging from 42 to 100 kDa (Zhang et al., 1996). The number of proteins varies between strains, with up to nine different-sized A2 proteins detected in the *L. donovani* 1S/Cl2D strain. Although A2 was originally considered amastigote-specific and expressed at low levels in promastigotes (Charest and Matlashewski, 1994), A2 has since been shown to be induced in promastigotes by a variety of stresses, including heat shock, protein misfolding and induction of the unfolded protein response (Barak et al., 2005; Gosline et al., 2011; McCall and Matlashewski, 2010). A2 is localized to the endoplasmic reticulum (ER) during mammalian infection and in heat-shocked promastigotes (McCall and Matlashewski, 2010).

A2 proteins are expressed by the visceral disease-causing *L. donovani*, but not by *L. major* (Zhang et al., 2003). Downregulation of A2 is associated with decreased macrophage infection levels *in vitro* and decreased parasite levels in the spleen and liver during experimental mouse infection (Zhang and Matlashewski, 1997, 2001). In contrast, experimental introduction of an ectopic A2 gene into *L. major* leads to increased survival in visceral organs (Zhang et al., 2003). More recently, it has been demonstrated that experimental introduction of A2 into *L. major* resulted in increased protection of *L. major* from heat shock (McCall and Matlashewski, 2010). Based on these observations involving ectopic A2 expression in *L. major*, it was necessary to confirm the role of endogenous A2 in *L. donovani* as described within. Since there are too many A2 gene families to perform gene deletions in *L. donovani* (Zhang and Matlashewski, 2001), we have used antisense A2 RNA to downregulate A2 protein expression as previously described (Zhang and Matlashewski, 1997). This approach leads to stable downregulation of all sizes of A2 in *L. donovani* (Zhang and Matlashewski, 1997).

Downregulation of A2 expression in *L. donovani* was associated with decreased resistance to heat shock. Interestingly, it was further observed that downregulation of A2 resulted in increased internal oxidant levels following heat shock and decreased resistance against reactive oxygen species (ROS) and nitric oxide. A2 could not however protect from unfolded protein stress in response to dithiothreitol (DTT). Given that heat shock is associated with the generation of internal ROS (Kampinga, 2006) and changes in membrane fluidity (Park et al., 2005), this may be one way in which A2 promotes survival at higher temperatures. Overall, these results provide a possible process to explain the role of A2 in supporting *L. donovani* survival within visceral organs and indicate that A2 may be a key determinant of the *L. donovani*-*L. major* dichotomy.

2. Material and methods

2.1. Parasite culture and transfections

L. donovani 1S/Cl2D strain promastigotes were maintained at 26 °C pH 7 as previously described (McCall and Matlashewski, 2010). Briefly, cells were grown in M199 medium (Sigma) supplemented with 10% fetal bovine serum (FBS), 25 mM HEPES, streptomycin, penicillin, adenosine, glutamine and folic acid, at pH 7.2.

Promastigotes were transfected as described in (Robinson and Beverley, 2003) with the KSneo-control plasmid and KSneo-A2 (R) antisense plasmid generated by Zhang and Matlashewski (Zhang and Matlashewski, 1997). Briefly, the KSneo-A2 (R) plasmid contains a 1.6-kb A2 coding sequence and part of the A2 3′ untranslated region (UTR), inserted in the antisense orientation in between the A2 5′ UTR and 3′ UTR. The KSneo-control plasmid contains only the A2 5′ and 3′ UTR in the sense orientation. Drug selection is provided by a neomycin resistance gene. Transfected parasites were maintained in media supplemented with 200 μg/mL G418 (Wisent), leading to stable maintenance of the plasmid in transfected parasites.

To induce promastigote to amastigote differentiation, promastigotes were resuspended in amastigote media (M199 medium supplemented with 25% FBS, streptomycin, penicillin, succinic acid, adenine, glycerol, L-proline and folic acid, at pH 5.5) at a cell density of 5×10^6 cells/mL and transferred to 37 °C, 5% CO_2.

2.2. Heat shock and promastigote viability assay

Promastigotes (1×10^7 cells) were resuspended in fresh promastigote media at 5×10^6 cells/mL and incubated at 26 °C or 40 °C for 12 h. Following heat shock, promastigotes were diluted to a cell density of 1×10^6 cells/ml, incubated at 26 °C in the presence of Alamar blue reagent (Invitrogen) for 72 h and absorbance was determined at 570 and 600 nm as previously described (McCall and Matlashewski, 2010; Mikus and Steverding, 2000).

2.3. Amastigote viability assays

For heat shock, amastigotes were incubated at 37 °C or 40 °C for 12 h and then diluted into fresh amastigote media. For oxidative stress assays, amastigotes were washed twice in Hepes-NaCl buffer (Mukherjee et al., 2009), resuspended in Hepes-NaCl buffer and treated with H_2O_2 (BioShop) or *S*-nitroso-*N*-acetylpenicillamine (SNAP, Sigma) for 4 h at 37 °C or 40 °C. The cells were then resuspended in amastigote media. OD600 was measured 48 h after stress treatments, except for DTT treatment where amastigotes were treated for 72 h with dithiothreitol (DTT, Fisher Scientific) dissolved in water. To account for any possible discrepancies in growth rates between A2-expressing and A2-deficient cells, the % survival was calculated for each strain as: (OD600 value of stressed sample/OD600 value of unstressed sample) × 100 as described in (Moreira et al., 2009). For DTT treatments, the OD600 of the treated samples were divided by the OD600 of water-treated cells. We used OD600 measurements for amastigote viability assays rather than Alamar blue because the optimal pH for Alamar blue assays is between pH 6.8 and 7.4 as per manufacturer's instructions.

2.4. Dichlorofluorescein diacetate (DCFDA) assay

DCFDA assays were performed as described in (Moreira et al., 2009). Briefly, cells were collected, washed twice with Hepes-NaCl buffer, and incubated in Hepes-NaCl buffer containing 5 μM CM-H_2DCFDA (Invitrogen) for 45 min. Promastigotes were incubated in DCFDA-containing buffer at 26 °C and amastigotes at 37 °C.