Leishmania major Methionine Sulfoxide Reductase A Is Required for Resistance to Oxidative Stress and Efficient Replication in Macrophages

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Abstract

Leishmania are protozoan parasites that proliferate within the phagolysosome of mammalian macrophages. While a number of anti-oxidant systems in these parasites have been shown to protect against endogenous as well as host-generated reactive oxygen species, the potential role of enzymes involved in the repair of oxidatively damaged proteins remains uncharacterized. The Leishmania spp genomes encode a single putative methionine sulfoxide reductase (MsrA) that could have a role in reducing oxidized free and proteinogenic methionine residues. A GFP-fusion of L. major MsrA was shown to have a cytoplasmic localization by immunofluorescence microscopy and subcellular fractionation. An L. major msrA null mutant, generated by targeted replacement of both chromosomal alleles, was viable in rich medium but was unable to reduce exogenous methionine sulfoxide when cultivated in the presence of this amino acid, indicating that msrA encodes a functional MsrA. The ΔmsrA mutant exhibited increased sensitivity to H2O2 compared to wild type parasites and was unable to proliferate normally in macrophages. Wild type sensitivity to H2O2 and infectivity in macrophages was restored by complementation of the mutant with a plasmid encoding MsrA. Unexpectedly, the ΔmsrA mutant was able to induce normal lesions in susceptible BALB/c indicating that this protein is not essential for pathogenesis in vivo. Our results suggest that Leishmania MsrA contributes to the anti-oxidative defences of these parasites, but that complementary oxidative defence mechanisms are up-regulated in lesion amastigotes.

Introduction

Leishmania are sand fly-transmitted protozoa that cause a spectrum of diseases in humans, ranging from localized cutaneous lesions to disseminating mucocutaneous and visceral infections [1]. More than 350 million people are at risk of infection worldwide, with an estimated 1.5 to 2 million new cases per year. Visceral leishmaniasis is associated with high mortality and an estimated 50,000 deaths each year [2]. While there have been recent advances in treatment options, current front line anti-leishmanial therapies are limited by their high cost, limited availability and/or toxicity and the widespread resistance in endemic areas [3].

Leishmania develop as extracellular promastigotes in the mid-gut of the Phlebotomine sand fly vector. Non-dividing metacyclic promastigotes that accumulate in the sandfly mouthparts are injected into the mammalian host when infected, female sandflies take a blood meal and are phagocytosed by host macrophages, either directly or after passage through neutrophils [4]. Phagocytosed promastigotes are delivered to the mature phagolysosome compartment of macrophages where they differentiate to the non-motile, obligate intracellular amastigote stage [5]. Both developmental stages are likely to be exposed to oxidative stresses. In the sandfly mid-gut, reactive oxygen species (ROS) are likely to be generated during the digestion of blood hemoglobin, while in the mammalian host, ROS are generated during parasite invasion of naive macrophages and in immune-activated host cells. Leishmania combat these stresses via a number of different and well-studied mechanisms. For example, invading promastigotes and amastigotes have been shown to actively inhibit recruitment of the NADPH oxidase complex to the phagolysosome membrane and to express a number of surface glycoconjugates and intracellular proteins that either scavenge extracellular and/or neutralize intracellular ROS species [6–8]. The latter include multiple isoforms of superoxide dismutase [9], ascorbate peroxidase [10–12], periderine reductase [13,14] as well as enzymes involved in the synthesis and regulation of the major cellular thiols, trypanothione and ovathiol [6,15–17]. Genetic disruption of most of these enzymes leads to partial or complete attenuation of parasite growth and survival in the mammalian host [18–21].

In contrast to the mechanisms listed above, nothing is known about oxidative defense mechanisms that involve the repair of damaged proteins. Methionine, a sulfur-containing amino acid, is...
particularly vulnerable to oxidative damage and, in organisms such as yeast and *E. coli*, mechanisms exist to reverse oxidized methionine. The product of methionine oxidation, methionine sulfoxide (MetO), exists as two epimers, methionine-(S)-sulfoxide (Met-S-O) and methionine-(R)-sulfoxide (Met-R-O) [22], and three separate repair enzymes, known as methionine sulfoxide reductases (Msrs) have been identified. Methionine sulfoxide reductase A (MsRA) acts on both free and protein-bound Met-S-O [23], methionine sulfoxide reductase B (MsRB) primarily repairs protein-bound Met-R-O [24] and methionine sulfoxide reductase C (MsRC) is able to reduce free Met-R-O [25,26]. Of these enzymes MsRA is perhaps the best characterized, and is involved in resistance to oxidative stress and virulence for a number of bacteria including *Salmonella* [27], *Enteroctococcus fassalis* [28] and *Mycoplasma genitalium* [29,30].

The related trypansomatid parasite, *Trypanosoma cruzi*, has recently been shown to express a functional MsRA that confers increased resistance to oxidative stress when overexpressed in pathogenic stages [31]. However, the potential role of MsRA enzymes in the pathogenesis of *T. cruzi* or other protozoan parasites remains unknown. Here, we provide evidence that *L. major* encodes a functional MsRA and have investigated its role in resistance to oxidative stress, intracellular replication and parasite virulence within a mammalian host.

**Results**

**Identification of the *Leishmania* Gene Encoding a Putative MsRA Enzyme with a Non- Classical Catalytic Site Sequence**

Analysis of the *L. major* strain Friedlin genome with the NCBI BLAST suite of programs [32] revealed the presence of a single open reading frame, *LmjF07.1140*, encoding a protein with 40% identity to human MsRA and 38% identity to MsRA from *E. coli*. Based on the analyses described below, we refer to this gene as *msrA* and the encoded protein as LmMsRA. Alignment of the predicted amino acid sequence with putative and known MsRA enzymes from other trypansomatids revealed that LmMsRA is highly conserved across different species of *Leishmania* with greater than 90% identity between *L. major*, *L. infantum*, *L. donovani* and *L. mexicana* MsRA homologues (Figure 1). The sequence similarity with the more evolutionary divergent *Leishmania* species, *L. braziliensis*, was 76%, and lower identity (59–64%) was observed to MsRA enzymes from the related *Trypanosoma* parasite species. Intriguingly, all of the *Leishmania* MsRA sequences contained a single amino acid substitution within the catalytic site, in which a tyrosine residue that has been shown to be critical for enzymatic activity in other eukaryotic and bacterial MsRA [33] was replaced with a phenylalanine residue (GC to WG) (Figure 1).

This sequence change was not observed in the *T. cruzi* or *T. brucei msrA* homologues. Genes encoding a putative MsRB enzyme (*LmjF28_2660*) and a putative MsRC (*LmjF23_1460*) enzyme, with 37% and 39% identity to *E. coli* MsRB and MsRC, respectively, were also identified in these analyses. As studies in other microbial pathogens have highlighted the role of MsR in pathogenesis [27,34,35], we focused our studies on defining the potential role of this enzyme in *L. major*.

**MsRA of *L. major* is Localized to the Cytoplasm of the Parasite and is not Essential for Parasite Viability**

MsRA has been localized to both the cytosol and organellar compartments of eukaryotic cells [36,37]. The LmMsRA amino acid sequence lacks conspicuous organellar targeting signals, indicating a possible cytoplasmic localization. To confirm the localization of this protein, an LmMsRA-GFP fusion protein was expressed in *L. major* promastigotes. As expected, episcopal expression resulted in production of a 47 kDa protein, the expected size, as detected by Western blotting using an anti-GFP antibody (Figure 2A). No GFP breakdown by-product was observed in the cells expressing pXG-msrA-GFP. Consistent with the lack of targeting signals, epifluorescent microscopy of live parasites revealed a cytosolic location for LmMsRA, with bright green fluorescence distributed evenly through the cytoplasm (Figure 2C). To confirm the absence of low level organelar targeting that might be masked by the strong cytoplasmic localization, *L. major* promastigotes expressing the fusion protein were lyzed and organellar fractions separated from cytosol by differentiation centrifugation. As shown in Figure 2B, full length LmMsRA-GFP was recovered in the cytosolic fraction. In contrast, the organellar marker, FBP, was only detected in pellet fractions (Figure 2B). These results suggest that *L. major* MsRA is exclusively localized in the cytosol, as has been proposed for an isoform of *T. cruzi* MsRA [31].

To further investigate the role of LmMsRA, an *L. major* null mutant was generated using standard homologous recombination techniques in which the two chromosomal alleles encoding LmMsRA were replaced with drug resistance cassettes. A number of isolates were recovered after two rounds of selection. Deletion of the msrA gene and correct integration of bleomycin and puromycin resistance cassettes was confirmed using PCR (data not shown). Promastigote stages of these *L. major* ΔmsrA null mutants displayed similar growth kinetics in standard culture medium as wild type *L. major* promastigotes (data not shown). These results suggest that *Leishmania msrA* is not essential during cultivation in rich medium.

**LmMsRA is a Functional Methionine Sulfoxide Reductase, as Loss of msrA Leads to Higher Levels of Methionine Sulfoxide within the Parasite**

The *E. coli* and *Salmonella* MsRA enzymes can reduce both free- and protein-bound Met-S-O back to methionine [25,27]. In order to establish if LmMsRA has similar enzymatic activity, despite the alteration in the catalytic site, *L. major* wild type and ΔmsrA strains were cultured in the presence of varying concentrations of Met and MetO (final concentration 100 μM). *Leishmania* are methio-nine auxotrophs and are therefore dependent on uptake of methionine for growth. We reasoned that wild type, but not ΔmsrA mutant promastigotes would be able to reduce any MetO taken up by the methionine transporters. Parasites were grown in minimal media containing Met and/or MetO for five days and polar metabolites extracted and analyzed by liquid chromatography-mass spectrometry. Both strains contained very low levels of MetO (0.1–0.2 percent of Met levels) when grown in the absence of exogenously added MetO (Figure 3C). Wild type *L. major* promastigotes maintained low intracellular levels of MetO even exogenously added MetO (Figure 3C). Wild type *L. major* promastigotes maintained low intracellular levels of MetO even when cultured in high concentrations of MetO (>50 μM) (Figure 3B). In contrast, intracellular levels of MetO in *L. major* ΔmsrA promastigotes increased as the MetO concentration in the media was increased. Specifically, ΔmsrA promastigotes cultured in the presence of 100 μM MetO had 10-fold higher levels of MetO than wild type parasites cultured under the same conditions (Figure 3B). Significantly, only one of the two MetO isomers identified in the LC-MS analysis was elevated in the MetO-treated parasites (Figure 3A, lower panel). This finding is consistent with the observation that MsR enzymes typically display a strong preference for the Met-S-O isomer and have minimal activity against the Met-R-O isomer. The low levels of the second isomer in both the wild type and ΔmsrA parasites likely reflects the continued expression of MsRB and/or MsRC enzymes in both
The latter has been shown to reduce free Met-R-O [25,26]. Collectively, these results indicate that LmMsrA is a functional methionine reductase A.

Deletion of \( \text{msrA} \) Leads to a Greater Sensitivity to Oxidative Stress in vitro

Loss of MsrA results in greater susceptibility to oxidative stresses in a number of organisms [27,38]. Conversely, overexpression of \( \text{msrA} \) in \( T. \text{cruzi} \) increased resistance to exogenous oxidative stress, such as hydrogen peroxide treatment [31]. We therefore examined whether the \( \Delta \text{msrA} \) mutant exhibited increased sensitivity to exogenous oxidative stress. Parasites were treated with 0.2 mM H\(_2\)O\(_2\) for 24 hours and parasite viability measured by following parasite outgrowth in fresh media. Wild type \( L. \text{major} \) exhibited minimal growth retardation after H\(_2\)O\(_2\) treatment compared to untreated, control parasites (Figure 4A). In contrast, growth of \( L. \text{major} \) \( \Delta \text{msrA} \) was significantly reduced (≈50% of untreated parasites) following H\(_2\)O\(_2\) treatment, demonstrating a heightened sensitivity of parasites to exogenous oxidative stress in the absence of LmMsrA.

To confirm that the observed difference was due to the absence of the LmMsrA, the \( L. \text{major} \) \( \Delta \text{msrA} \) mutant was transfected with a pXG1A plasmid encoding the \( \text{msrA} \) gene. Transfection with pXG1A-msrA largely restored resistance to exogenous H\(_2\)O\(_2\), as seen in Figure 4A.

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**Figure 1.** Sequence alignment of \( L. \text{major} \) MsrA with known and putative MsrA enzymes from other \( Leishmania \) species and \( Trypanosoma \) species, as well as human and \( E. \text{coli} \) MsrA. The catalytic site is boxed, and the non-conserved residue within the site marked by an ". The degree of amino acid conservation is indicated by shading intensity. Sequence alignment was performed using ClustalW [55,56] and the resulting alignment viewed and edited using JalView software [57]. The following sequence accession numbers were used: \( L. \text{major} \) XP_001681026.1, \( L. \text{donovani} \) CBZ31842.1, \( L. \text{infantum} \) XP_001463331.1, \( L. \text{mexicana} \) CBZ23737.1, \( L. \text{braziliensis} \) XP_001562615.1, \( T. \text{brucei} \) XP_846892.1, \( T. \text{cruzi} \) XP_814806.1, \( \text{Homo sapiens} \) AAG09689.1, \( E. \text{coli} \) XP_001746621.1.

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Leishmania Protein Repair Mechanisms
the viability of the complemented strain after three days of exposure to H₂O₂ was not significantly different from the wild type parasites (Figure 4A). In contrast, transfection of the ΔmsrA mutant with the empty pXG1A plasmid did not increase the resistance of the mutant to oxidative stress (Figure 4B). These results provide direct evidence for msrA having a role in parasite resistance to oxidative stress.

Loss of msrA Results in Decreased Replication within Macrophages in vitro, but is Not Required for Pathogenesis in the Mouse Model of Cutaneous Leishmaniasis

*L. major* parasites proliferate within the mature phagolysosome of mammalian macrophages, where they are likely to be exposed to high levels of reactive oxygen species. Although *L. major* has a number of mechanisms to avoid the oxidative defense mechanisms of the host, these parasites are unable to completely inhibit the macrophage oxidative burst during invasion and subsequent proliferative phases [7] raising the possibility that LmMsrA may be important for parasite proliferation within this compartment. Murine RAW 264.7 macrophages were infected with *L. major* wild type and ΔmsrA stationary phase promastigotes and parasite proliferation assessed over 4 days. Both wild type and ΔmsrA parasites exhibited similar rates of macrophage invasion and the number of macrophages infected with both strains remained similar over 4 days (data not shown). However, proliferation of *L. major* ΔmsrA strain was markedly attenuated compared to *L. major* wild type parasites after four days (Figure 3A). A similar result was observed when macrophages were infected with *L. major* wild type+pXG1A and *L. major* ΔmsrA+pXG1A, with the number of *L. major* wild type+pXG1A parasites again approaching twice that of *L. major* ΔmsrA+pXG1A (Figure 3B).

In contrast, growth of the complemented mutant *L. major* ΔmsrA+pXG1A more closely resembled that of *L. major* wild type+pXG1A (Figure 5B), indicating that the observed defect in intracellular growth was due to loss of msrA.

To investigate whether this replicative defect was important in the pathogenesis of the parasites in vivo, susceptible BALB/c mice were subcutaneously infected with either stationary-phase promastigotes or lesion-derived amastigotes. Following infection, the size of the inflammatory lesion was assessed weekly. Unexpectedly, no difference in average lesion size was observed for *L. major* ΔmsrA compared to *L. major* wild type, when either promastigote or amastigote stages were used to initiate infections (Figures 6A and B). These studies suggest that additional oxidative stress protective mechanisms are activated in *in vivo* parasite stages, compared to amastigotes in *ex vivo*-infected macrophages, which can compensate for loss of MsrA.

Discussion

*Leishmania* parasites have developed a number of strategies for dealing with reactive oxygen and nitrogen species generated within the host macrophage despite lacking a number of key antioxidant enzymes, such as catalase and glutathione peroxidase. These strategies can be broadly grouped into avoidance of the host antioxidant response, neutralization of host generated reactive oxygen and nitrogen species and repair of oxidatively damaged proteins. While the first two lines of oxidative defense have been extensively studied [6], little is known about the protein repair mechanisms of *Leishmania*. Proteinogenic methionine residues are highly susceptible to oxidation and in this study we have investigated the potential role of a putative methionine sulfoxide reductase A in *Leishmania* pathogenesis. *Leishmania* MsrA is encoded
by a single gene that is well conserved, both in terms of sequence homology (76–90%) and synteny across the *Leishmania* genus. MsrA gene homologues are also present in the genomes of other trypanosomatid parasites and exhibit ∼40% sequence homology to human and *E. coli* MsrA. The amino acid sequences of *L. major* MsrA is notable in containing a tyrosine residue instead of phenylalanine in an otherwise highly conserved sequence in the catalytic site. This change was conserved in all species of *Leishmania* and has not been reported in any other organism. Mutation of this Phe residue to Ala or His in the bacterial MsrA proteins is associated with a complete loss of enzymatic activity [33]. The Phe to Tyr change observed in the *Leishmania* sequences is more conservative, but nonetheless results in addition of an extra hydroxyl group and increased polarity in the catalytic motif. Thus

![Figure 3](image.png)

**Figure 3.** LC-MS quantification of intracellular MetO and Met levels in *L. major* grown in varying concentrations of Met and MetO. A. Abundance of free MetO (comprising a doublet of Met-S-O/Met-R-O isomers) and Met in total extracts of wild type (upper panel) and ΔmsrA (lower panel) promastigotes cultivated in media containing 80 μM MetO and 20 μM Met. B. Ratio of MetO to Met levels in *L. major* wild type (black columns) and ΔmsrA (white columns) grown in varying concentrations of MetO.

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it was important to establish if LmMsrA was functional. An L. major strain lacking both alleles of msrA was readily generated indicating that LmMsrA is not essential for growth under standard culture conditions. When wild type and ΔmsrA mutant parasites were cultivated in the presence of exogenous methionine sulfoxide (MetO), the mutant parasites, but not the wild type parasites, accumulated significant levels of MetO, indicating that MsrA is functional. The ΔmsrA parasites accumulated a single isomer of MetO providing further evidence that the msrA gene encodes a classic MsrA with selectivity for the Met-S-O isomer, as well as indicating the presence of other methionine reductases with selectivity for the Met-R-O isomer. Interestingly, neither wild type nor ΔmsrA parasites were able to grow in methionine-free medium supplemented with MetO (data not shown). The rate of reduction of MetO may therefore be insufficient to supply the nutritional needs of these parasites and/or the rapid incorporation of MetO into other metabolic intermediates or proteins may result in growth inhibition.

The L. major MsrA protein lacked any recognizable organelle-targeting signals indicating that it is primarily or exclusively localized in the cytosol. This localization was confirmed by localization of an MsrA-GFP-fusion protein in live promastigotes by fluorescence microscopy and by subcellular fractionation. The MsrA proteins of T. cruzi and S. cerevisiae have also been shown to have a cytosolic localization [31,39]. In contrast, two isoforms of MsrA are expressed in mammalian cells, which are targeted to the cytosol and the mitochondrion, respectively [37,40]. Whether other methionine reductases in Leishmania and other trypanosomatids are targeted to the mitochondrion remains to be defined.

Deletion of the L. major msrA gene resulted in increased promastigote sensitivity to external oxidative stress (H2O2). These findings are consistent with a recent report showing that over-

Figure 4. Recovery of L. major parasites following exposure to hydrogen peroxide treatment. Growth is expressed as a percentage of the growth of untreated control parasites (for each cell line) 3 days following H2O2 removal, as a measure of cell viability following H2O2 treatment. Error bars represent S.E.M. of three experiments. A. L. major ΔmsrA is more susceptible to H2O2 treatment, with a significant decrease in cell viability compared to L. major wild type (P = 0.02, unpaired t-test). B. L. major ΔmsrA+pXG1A is also more susceptible to H2O2 treatment, with a significant decrease in cell viability compared to L. major+pXG1A (P = 0.01, unpaired t-test). In contrast, no significant decrease in cell viability is observed for the complemented mutant strain ΔmsrA+pXG1A-msrA when compared to L. major+pXG1A (P = 0.22, unpaired t-test).

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Figure 5. Infection of RAW 264.7 murine macrophages with L. major promastigotes. Data is presented as the number of parasites per infected macrophage, as observed by microscopy. Error bars represent S.E.M. from three independent experiments performed in triplicate. A. Macrophages infected with L. major wild type (squares) or L. major ΔmsrA (triangles). A significant decrease in parasite numbers is observed at day 4 for L. major ΔmsrA (P = 0.01, unpaired t-test). B. Macrophages infected with L. major+pXG1A (squares), L. major ΔmsrA+pXG1A (triangles) or L. major ΔmsrA+pXG1A-msrA (inverted triangles).

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expression of MsrA in T. cruzi epimastigotes results in increased resistance to oxidative stress [31]. The mutant line also exhibited a reduced capacity to replicate as amastigotes in murine macrophages, consistent with a number of expression profiling studies indicating that MsrA is constitutively expressed in both major developmental stages [41,42]. Collectively, these findings suggest that MsrA has a significant role in repairing oxidatively damaged parasite proteins and/or reversing the oxidation of potentially limiting levels of methionine in intracellular stages [43]. Unexpectedly, deletion of L. major msrA had no discernable impact on lesion development in the highly susceptible BALB/c mouse model of cutaneous leishmaniasis. L. major infection in BALB/c induces a strong anti-inflammatory Th2 response, with concomitant suppression of classic activation and induction of the oxidative burst in infected macrophages (reviewed in [44]). Under these conditions, MsrA mediated repair of proteins may not be essential for parasite proliferation. Alternatively, the reduced virulence of the L. major ΔmsrA mutant in ex vivo infected macrophages but not in the BALB/c murine models, could reflect the up-regulation of additional parasite stress responses in the mammalian host. These could include increased expression of heat shock proteins, proteasome activity, lysosomal enzymes or autophagy [45–47], all of which may have a role in either preventing protein mis-folding or removing damaged proteins and have been shown to be essential for Leishmania amastigote survival in vivo.

The absence of a strong loss of virulence phenotype in the ΔmsrA mutant may also reflect redundancy in methionine protein repair mechanisms. Specifically, the L. major genome contains genes for putative MsrB (LmjF28_2660) and MsrC (LmjF23_1460) enzymes [48]. While MsrA is responsible for reversing the oxidation of the free and proteinogenic Met-S-O epimer, MsrB and MsrC are primarily responsible for repairing the Met-R-O epimer in damaged proteins and free Met-R-O, respectively [27,49,50]. MsrA has been shown to be important for the virulence of several bacterial pathogens, including Helicobacter pylori [51], Salmonella typhimurium [27], Mycobacteria genitalium [29] and M. smegmatis [34,35]. In S. typhimurium and M. smegmatis, the msrB enzyme appeared to play a limited role in resistance to oxidative stress as deletion of the corresponding genes had little effect on the intracellular growth of these pathogens [27,34,35]. However, deletion of both msrB and msrC genes in S. typhimurium resulted in a severe attenuation of virulence indicating that these enzymes may have redundant roles in repairing the Met-R-O epimer [27]. In considering the role(s) of MsrA/B/C in microbial pathogenesis, it is important to note that these proteins may become essential under certain growth conditions or during specific stages of infection. For example, expression of MsrA is required for the proper functioning of extracellular adhesins [29,52,53], motility [54] and biofilm production [55] in several bacterial pathogens, reflecting the oxidative sensitivity of specific proteins involved in pathogenesis. The reduced virulence of the L. major ΔmsrC mutant in ex vivo-infected macrophages could therefore reflect a dependency on one or more oxidatively sensitive proteins that are not required for infection in vivo.

In conclusion, this is the first study to examine the role of MsrA, or indeed any oxidative damage repair mechanisms, in human pathogenic protozoan parasites. We show that LmMsrA confers protection against exogenous oxidative stress, and is required for normal growth in macrophages. Surprisingly, we find that MsrA is not essential for lesion development in mice. In future studies, it will be of interest to delineate the potentially redundant roles of MsrB and MsrC in Leishmania infectivity.

Materials and Methods

Ethics Statement

Use of mice was approved by the Institutional Animal Care and Use Committee of the University of Melbourne (ethics number 0811011.1). All animal experiments were performed in accordance with the Australian National Health Medical Research council (Australian code of practice for the care and use of animals for scientific purposes, 7th Edition, 2004, ISBN: 1864962638).

Bioinformatic Analyses

BLAST [32] searching of the Leishmania major strain Friedlin genome [48] was used to identify putative Msr enzymes. Protein sequence alignments were performed using ClustalW [55,56] and edited using JalView software [57].

Parasite Strains and Culture Conditions

L. major substrain MHOM/SU/73/5-ASKH was used to create all mutant and transfected lines. Parasites were routinely cultured as axenic promastigotes in Medium-199 (M199, Gibco, Invitrogen, Australia) supplemented with 10% heat-inactivated foetal bovine serum (FBS, Invitrogen) at 27°C or, prior to infection experiments, in SDM-79 medium supplemented with 10% FBS.
G418 (Invitrogen, 100 μg/mL) was used to maintain selection pressure on parasites transfected with pXG1A plasmids, while puromycin (Invitrogen, 20 μg/mL) and/or bleocin (Calbiochem, 10 μg/mL) were used to select transformants during mutagenesis. Lesion amastigotes used in infection studies were prepared by disrupting murine lesions (diameter 5–10 mm) by passage through a stainless steel sieve and repeated passage through a 27 G needle to lyse macrophages and release parasites. Cell debris was removed by centrifugation (500×g, 10 min, 4°C) and amastigotes in the supernatant collected by a second centrifugation step (2000×g, 10 min, 4°C). Amastigotes were washed in sterile PBS and counted using a haemocytometer prior to use in infection studies.

Genetic Manipulation of L. major

L. major ΔmsrA was created via sequential homologous gene replacement in a manner similar to that previously described [58,59]. Briefly, a 935 bp 5’ untranslated region (UTR) containing a 5’ HindIII site and a 3’ BamHI/EcoRI linker region was amplified using primers 5’-CCCAAGCTT CTAGCTGCG-3’ and 5’-CCGGTTGAGATCCGAATTCTCGAGCGGTGTTGGTG-3’, and a 980 bp 3’ UTR region containing a 5’ BamHI/EcoRI linker region and a 3’ HindIII site was amplified using primers 5’-GAATTCGGATCC-3’ and 5’-CCCGGATCCAC-3’. An overlap PCR was then performed using these PCR products as template, and the resultant product cloned into the HindIII/NotI sites of the pBluescript II SK vector (Stratagene, CA, USA). Puromycin or bleocin resistance cassettes were excised from either pXG-PAC or pXG-PHLEO [60] respectively using BamHI and EcoRI, and bleocin resistance cassettes were excised from either pXG-PAC or pXG-PHLEO [60] respectively using BamHI and EcoRI, and cloned into the engineered BamHI/EcoRI site. Deletion mutant constructs were verified by restriction digest profiles and DNA sequencing. The PAC- and BLE-containing msrA targeting constructs were excised by HindIII/NotI digest, gel purified and 5 μg of each sequentially electroporated into L. major as described previously [61]. Clonal transformants resistant to both puromycin and bleocin were selected and deletion of msrA confirmed via triplicate PCR (data not shown). To generate the pXG-msrA vector for complementation studies, full-length msrA gene was amplified by primers 5’-CTGCCGGATGCCCAGCCTATCTC-GAGCC-3’ and 5’-CGGGATCCTAAAGGTA- CAGCGGTTGAGCC-3’ and cloned into the Smal/BamHI sites of the pXG1A vector. To create the MsrA-GFP fusion protein, full length msrA was amplified using primers 5’-CCGGATCCACCATGCCCAGCCTATCGGAGG-3’ and 5’-CGATAT-CAAAGTACAAAGGGTGTCAGGACG-3’ and cloned into the BamHI and EcoRV sites of pXG-GFP [62]. The resulting pXG-derived constructs were confirmed via DNA sequencing and electroporated into L. major as previously described [61].

Detection of LmMsrA-GFP Using Immunoblotting and Microscopy

Subcellular fractionation of parasites was performed as described previously [63]. Briefly, log phase parasites were harvested via centrifugation, washed in PBS and resuspended in hypotonic lysis buffer (1 mM NaHEPES-NaOH, pH 7.4, 1 mM DTT and 2 mM EGTA, protease inhibitor cocktail (Complete-EDTA free, Roche, Germany), 10 min 0°C) and cell lysis achieved by passage through a 27 G syringe needle. The lysis buffer was adjusted to 50 mM NaHEPES-NaOH, pH 7.4, and cell debris was removed by low speed centrifugation (500×g, 15 min, 0°C), and the supernatant subjected to sequential centrifugation at 10,000×g, and 100,000×g (30 min, 4°C). The pellet and supernatant fractions were analyzed by SDS-PAGE and MsrA-GFP fusion protein detected using the anti-GFP antibody (clones 7.1 and 13.1, Roche, Germany) at 1:1000 dilution with the signal detected using standard chemiluminescent techniques. The membrane was then probed with an antibody to the organellar marker, fructose-1,6-bisphosphatase (FBP) [59] (diluted 1:100). For microscopy studies, live cells were immobilized on poly-L-lysine coated coverslips and visualized using a Zeiss Axioplan 2 epifluorescence microscope. Images were obtained using an AxiCam MRm camera and AXIOVISION 4.3 software.

LC-MS Measurement of Methionine/Methionine Sulfoxide

L. major promastigotes were cultured in completely defined media [64] containing 0–100 μM methionine (Met, Sigma, USA) and 0–100 μM methionine sulfoxide (MetO, MP Biomedicals, Australia) at a final concentration of 100 μM. Cells were incubated for 5 days and metabolites extracted as previously described with rapid metabolic quenching [65]. Briefly, parasite metabolism was quenched by rapid cooling to 0°C in an ethanol/dry-ice slurry and parasites rapidly harvested by centrifugation (3,200×g, 10 min, 0°C). The pellet was washed in cold PBS and parasites lysed in chloroform:methanol:water (1:3:1, v/v, 250 μL) with sonication and incubation on ice for 15 minutes. Insoluble material was removed by centrifugation (10,000×g, 5 min, 0°C) and water (100 μL) added to generate a biphasic mixture after centrifugation (10,000 g, 5 min, 0°C). The upper, aqueous phase was dried under nitrogen in 250 μL glass vials and amino derivatives derivatized with aminoquinolyl-N-hydroxy succinimidyl carbamate (AQC) reagent [66] prior to liquid chromatography-mass spectrometry (LC-MS) analysis [66]. Multiple reaction monitoring was used to detect methionine and methionine-S/R-sulfoxide peaks using m/z 320/171 and m/z 336/171 transitions, respectively.

Induction of Oxidative Stress in vitro

L. major promastigotes (2×10⁵) were exposed to fresh H₂O₂ (20 μM) for 24 h, then harvested by centrifugation and resuspended into fresh M199 media. Untreated control parasites were included for each strain tested. Parasites were incubated at 27°C for 5 days and viable numbers assessed every 24 h by counting using a haemocytometer, as an indirect measure of the viable number of parasites surviving H₂O₂ treatment.

Macrophage and Mouse Infections

Infection of RAW 264.7 murine macrophage cells was performed as described previously [67]. Briefly, macrophages growing on coverslips were infected at a multiplicity of infection (MOI) of 10 with stationary phase promastigotes and incubated at 33°C in RPMI medium supplemented with 10% FBS and 5% CO₂. Macrophages were washed after 4 h to remove extracellular parasites and coverslips fixed with 4% paraformaldehyde every 24 hours for four days. Intracellular parasites were stained with Hoechst and triplicate coverslips were counted at each time point, with a minimum of 100 macrophages per coverslip, to determine parasite infection levels. Three independent infections were performed in triplicate. To assess virulence in mice, 6–8 week old (age-matched) female susceptible BALB/c mice were injected subcutaneously at the tail base with either 5×10⁶ amastigotes or 1×10⁶ stationary phase promastigotes. Lesion size was assessed weekly and scored 0–4, as described previously [68]. All parasite cell lines had been passaged previously in mice to ensure loss of...
virulence unrelated to the known genetic mutations, and parasites re-isolated from mice as described previously [61].

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References
1. Murray HW, Berman JD, Davies CR, Saravia NG (2005) Advances in leishmaniasis. Lancet 366: 1561–1577.
2. den Boer M, Argaw D, Jannin J, Alvar J (2011) Leishmaniasis impact and transmission. Clinical microbiology and infection: the official publication of the European Society of Clinical Microbiology and Infectious Diseases 17: 1471–1477.
3. Croft SL, Olliaro P (2011) Leishmaniasis chemotherapy-challenges and opportunities. Clinical microbiology and infection: the official publication of the European Society of Clinical Microbiology and Infectious Diseases 17: 1478–1483.
4. Peters NG, Egen JG, Secundino N, Debrabant A, Kimblin N, et al. (2008) In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. Science 321: 970–974.
5. McGonville MJ, Naderer T (2011) Metabolic pathways required for the intracellular survival of Leishmania. Annu Rev Microbiol 65: 343–361.
6. Van Assche T, Deschacht M, da Luz RA, Maes L, Cos P (2011) Leishmaniasis-macrophage interactions: insights into the redox biology. Free radical biology & medicine 51: 337–351.
7. Spath GF, Garraway LA, Turco SJ, Beverley SM (2003) The role(s) of lipophosphoglycan (LPG) in the establishment of Leishmania major infections in mammalian hosts. Proc Natl Acad Sci U S A 100: 9536–9541.
8. Chan J, Fujiwara T, Brennan P, McNeil M, Turco SJ, et al. (1989) Microbial glycolipid: possible virulence factors that scavenge oxygen radicals. Proc Natl Acad Sci U S A 86: 2453–2457.
9. Plewes KA, Barr SD, Gedamu L (2003) Iron superoxide dimutase targets to the glycoosomes of Leishmania chagasi are important for survival. Infection and immunity 71: 5910–5920.
10. Pal S, Dola S, Yadav RR, Adak S (2010) Ascorbate peroxidase from Leishmania major controls the virulence of oxidative stress by regulating oxidative stress. PloS one 5: e12177.
11. Dola S, Yadav RK, Pal S, Adak S (2009) Overexpression of mitochondrial Leishmania major ascorbate peroxidase enhances tolerance to oxidative stress-induced programmed cell death and protein damage. Eukaryote cell 8: 1721–1731.
12. Dola S, Yadav RK, Pal S, Adak S (2008) Leishmania major ascorbate peroxidase overexpression protects cells against reactive oxygen species-mediated cardiolipin oxidation. Free radical biology & medicine 45: 1520–1529.
13. Nare B, Garraway LA, Vickers TJ, Beverley SM (2009) PTP1B-dependent synthesis of tetrahydrobiopterin contributes to oxidant susceptibility in the protozoan parasite. Leishmania major. Current genetics 55: 287–299.
14. Moreira W, Leblanc E, Ouellette M (2009) The role of reduced pterins in immunity 71: 5910–5920.
15. McConville MJ, Naderer T (2011) Metabolic pathways required for the intracellular survival of Leishmania. Annu Rev Microbiol 65: 543–561.

Author Contributions
Conceived and designed the experiments: FMS LT MJM. Performed the experiments: FMS LT JER. Analyzed the data: FMS LT JER MJM. Contributed reagents/materials/analysis tools: ECS TN. Wrote the paper: FMS LT MJM.
45. Besteiro S, Williams RA, Coombs GH, Mottram JC (2007) Protein turnover and differentiation in *Leishmania*. International journal for parasitology 37: 1063–1075.

46. Morales MA, Watanabe R, Dacher M, Chafey P, Osorio y Fortea J, et al. (2010) Phosphoproteome dynamics reveal heat-shock protein complexes specific to the *Leishmania donovani* infectious stage. Proc Natl Acad Sci U S A 107: 8381–8386.

47. Williams RA, Smith TK, Cull B, Mottram JC, Coombs GH (2012) ATG5 is essential for ATG8-dependent autophagy and mitochondrial homeostasis in *Leishmania major*. PLoS pathogens 8: e1002695.

48. Ivens AC, Peacock CS, Woofhey EA, Murphy L, Aggarwal G, et al. (2005) The genome of the kinetoplastid parasite, *Leishmania* major. Science 309: 436–442.

52. Wizemann TM, Moskovitz J, Pearce BJ, Martin DM, Clamp M, Barton GJ (2009) Jalview Version 2—a multiple sequence alignment editor and analysis workbench. Bioinformatics 25: 1189–1191.

57. Waterhouse AM, Procter JR, Martin DM, Clamp M, Barton GJ (2009) Jalview Version 2—a multiple sequence alignment editor and analysis workbench. Bioinformatics 25: 1189–1191.