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The iron-dependent mitochondrial superoxide dismutase SODA promotes *Leishmania* virulence

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Running Title: *SODA mediated redox signaling promotes Leishmania virulence*

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ABSTRACT

Leishmaniasis is one of the leading global neglected diseases, affecting millions of people worldwide. *Leishmania* infection depends on the ability of insect-transmitted metacyclic promastigotes to invade mammalian hosts, differentiate into amastigotes and replicate inside macrophages. To counter the hostile oxidative environment inside macrophages, these protozoans contain anti-oxidant systems that include iron-dependent superoxide dismutases (SODs) in mitochondria and glycosomes. Increasing evidence suggests that in addition to this protective role, *Leishmania* mitochondrial SOD may also initiate H_2O_2-mediated redox signaling that regulates gene expression and metabolic changes associated with differentiation into virulent forms. To investigate this hypothesis, we examined the specific role of SODA, the mitochondrial SOD isoform in *Leishmania amazonensis*. Our inability to generate *L. amazonensis SODA* null mutants and the lethal phenotype observed following RNAi-mediated silencing of the *T. brucei* SODA ortholog suggests that SODA is essential for trypanosomatid survival. *L. amazonensis* metacyclic promastigotes lacking one SODA allele failed to replicate in macrophages and were severely attenuated in their ability to generate cutaneous lesions in mice. Reduced expression of SODA also resulted in mitochondrial oxidative damage and failure of *SODA/*Δ*sodA* promastigotes to differentiate into axenic amastigotes. SODA expression above a critical threshold was also required for the development of metacyclic promastigotes, as *SODA/*Δ*sodA* cultures were strongly depleted in this infective form and more susceptible to reactive oxygen species (ROS)-induced stress. Collectively, our data suggests that SODA promotes *Leishmania* virulence by protecting the parasites against mitochondria-generated oxidative stress and by initiating ROS mediated signaling mechanisms required for the differentiation of infective forms.

Leishmaniasis affects an estimated 12 million people worldwide, with an estimated 350 million at risk of infection (1). Depending on the *Leishmania* species, symptoms vary from self-healing skin lesions to a visceralizing form that can be lethal. The absence of efficacious and cost-effective drugs, combined with the emergence of drug-resistance, accentuates the critical need for new therapeutic targets.

The life-cycle of *Leishmania spp.* alternates between non-virulent promastigotes inside the insect vectors and virulent amastigotes inside mammalian hosts. Transmission of the parasites to mammals occurs through sand fly bites (2). Amastigotes are adapted to survive and

replicate inside acidic parasitophorous vacuoles (PV) of macrophages. After ingestion by sand flies during a blood meal amastigotes transform into promastigotes, which replicate in the fly digestive tract. As nutrients become depleted promastigotes cease to replicate and move up the sand fly gut towards the proboscis, where they mature into infective metacyclic forms. Metacyclics are reintroduced into new hosts during the next feeding cycle and enter host macrophages where they transform into amastigotes. To adapt to the rapidly changing environmental conditions during its life cycle, *Leishmania* undergoes extensive morphological and metabolic changes orchestrated at the post-transcriptional and post-translational levels. (3-5). Despite significant progress in characterizing these life cycle associated developmental changes, the molecular pathways that initiate differentiation in *Leishmania* are still poorly understood.

Over the last two decades, a role for reactive oxygen species (ROS) as regulators of physiological and biological responses (redox biology) has emerged (6-8). Two of the most abundant ROS generated as by-products of mitochondrial respiration or as end-products of metabolic reactions, $O_2^{\bullet-}$ and H_2O_2, have been linked to cell fate determination. Subtle increases in intracellular $O_2^{\bullet-}$ levels promote cell proliferation, whereas low level accumulation of H_2O_2 can inhibit cell growth and initiate differentiation (9-11). Recent studies specifically implicate mitochondria generated ROS in intracellular signaling (12-14). $O_2^{\bullet-}$, the highly toxic ROS resulting from reduction of O_2 by complexes I, II and III of the mitochondrial electron transport chain (ETC)(15), is rapidly converted by cytosolic or mitochondrial dismutases (SOD) into H_2O_2. Due to its higher stability, membrane diffusibility and ability to promote target-specific thiol-modifications, H_2O_2 is considered a primary intracellular signaling ROS molecule (7,10).

Recent evidence indicates that H_2O_2 generated within mitochondria through the action of SOD plays a central role in regulating differentiation of *Leishmania amazonensis* promastigotes into infective amastigotes (16,17). Given that SOD enzymes in trypanosomatid protozoa exclusively utilize iron as an essential co-factor(18), these enzymes have emerged as important links between iron and ROS-regulated differentiation pathways in *L. amazonensis*. Notably, the role of H_2O_2 in triggering promastigote to amastigote differentiation was directly demonstrated during studies of iron uptake in *L. amazonensis* promastigotes (16). Subsequent studies with *L. amazonensis* lines defective in mitochondrial iron import (*LMIT1/*Δ*lmit1*) implicated mitochondrial iron-dependent SOD (SODA) and mitochondria-generated ROS in the generation of virulent forms (17).

Further supporting a role for SODA and H_2O_2 in the development of virulence in *L. amazonensis*, two cues known to trigger *in vitro* amastigote differentiation, low pH and /high temperature (19), are also effective triggers of ROS generation (20-22). Exposure to elevated temperature is thought to lead to hyper-polarization of mitochondria, increased respiratory rate and a ROS surge that is not only tolerated by *Leishmania*, but is actually used as a differentiation signal. Stationary phase promastigotes show increased resistance to oxidative stress and enhanced SOD activity as they differentiate into amastigotes. Low SOD activity, on the other hand, induces logarithmic phase promastigotes to accumulate higher levels of ROS and to undergo apoptosis, a process that can be reversed by overexpression of mitochondrial SODA (21).

Here we confirm the prediction that iron import into mitochondria is required for activity of *L. amazonensis* SODA. Furthermore, by targeting the SODA gene for allelic knockout and examining the phenotype of mutant lines, we conclude that SODA expression is required for maintaining mitochondrial redox balance and also for the development of parasite virulent forms. .

RESULTS

Amastigote differentiation is Associated with Mitochondrial Iron Import and Activation of Mitochondrial SODA - In earlier work we identified LMIT1, a *L. amazonensis* mitochondrial iron transporter, and suggested that the reduced ability of *LMIT1/* Δ*lmit1* promastigotes to import iron into mitochondria and activate the iron-dependent SODA might be

responsible for their failure to differentiate into amastigotes (17). This hypothesis was largely based on the reduced SOD activity observed in total parasite extracts and on morphological evidence for mitochondrial oxidative damage in *LMIT1/ Δlmit1* parasites subjected to the low pH/ high temperature protocol for axenic amastigote differentiation. To directly investigate the specific role of SODA in the signaling process leading to amastigote differentiation, we developed antibodies capable of distinguishing the mitochondrial SODA from glycosomal SODB, and compared SOD activity in mitochondrial fractions of wild type and *LMIT1/ Δlmit1* parasites undergoing low pH/high temperature-induced axenic differentiation.

Specific polyclonal antibodies against mitochondrial SODA and SODB were generated using purified recombinant proteins (Fig. S1). The immunofluorescence localization of anti-SODA antibodies was identical to the staining pattern of MitoTracker Red, a mitochondria-specific dye (Fig. 1A). This result demonstrates the mitochondrial localization of endogenous SODA, as proposed earlier based on overexpression of GFP-tagged SODA (23).

To obtain mitochondria-enriched fractions free of glycosomal contaminants, sub-cellular fractionation of promastigotes expressing a 3xFLAG-tagged form of the LMIT1 mitochondrial iron transporter was carried out with increasing concentrations of digitonin, as previously described (17). Western blot detection of organelle specific markers (adenosuccinate lyase for cytosol (24), LMIT1-FLAG for mitochondria (17) and arginase for glycosomes (25)) showed that pellets obtained after 1 mg/ml digitonin treatment contained ~98% of the total LMIT1-FLAG and less than 5% of the total cellular arginase, reflecting mitochondrial enrichment in fractions mostly devoid of glycosomal contaminants (Fig. 1B). Immunoblot with the specific antibodies against SODA or SODB confirmed that >90% of the endogenous SODA co-fractionated with LMIT1 in mitochondria-enriched fractions, while >95% of endogenous SODB was found in the soluble fraction (Fig. 1B).

SOD activity was then quantified in mitochondria-enriched fractions prepared from wild type (WT) and *LMIT1/ Δlmit1* promastigotes. These two lines were subjected to the low pH/high temperature conditions previously shown to trigger amastigote differentiation in WT but not in *LMIT1/ Δlmit1* (17). WT parasites showed a gradual increase in mitochondria-associated SOD activity and SODA protein levels during differentiation (Fig. 2A-C), consistent with the SOD activity increase we reported earlier for whole cell extracts (17). In contrast, although SOD activity in *LMIT1/ Δlmit1* cells was slightly higher during early differentiation (6 h), it was ~2.5 fold lower than WT at the 48 h time point when most WT parasites had already assumed the rounded morphology typical of amastigotes (Fig. 2A). Western blot analysis showed comparable amounts of SODA protein and only trace levels of SODB in both WT and *LMIT1/ Δlmit1* mitochondrial fractions (Fig. 2B,C), indicating that the reduced SOD activity in *LMIT1/ Δlmit1* cells is likely to reflect SODA inactivation as a result of impaired LMIT1-mediated iron import into mitochondria. Importantly, we also observed down-regulation (of about two fold) in the expression of ascorbate dependent peroxidase (APX) in *LMIT1/ Δlmit1* mitochondrial lysates, when compared to WT (Fig. 2B,C). Expression of APX, a mitochondrial protein required for the enzymatic breakdown of H_2O_2 in *Leishmania*, is known to be up-regulated in response to H_2O_2 accumulation (26). Collectively, these results demonstrate that differentiation of *L. amazonensis* promastigotes into infective amastigotes is associated with activation of the mitochondrial iron-dependent SODA and accumulation of H_2O_2, a ROS previously shown to directly trigger differentiation (16).

SODA is an Essential Gene in L. amazonensis and in T. brucei Procyclics - To further understand how mitochondrial SODA regulates the ROS signaling pathway leading to amastigote differentiation, we proceeded to generate SODA null mutants. Knockout constructs carrying drug-resistance gene cassettes flanked by 5' and 3' UTR regions of the *SODA* gene were generated and used for gene replacement through homologous recombination (Fig. S2A). Replacement of a single *SODA* allele with *HYG* or *PHLEO* drug resistance gene cassettes was possible, and allelic integration into

the desired locus was confirmed in each case by PCR and Southern blot analysis (Fig. S2B). *SODA/*Δ*sodA* promastigotes showed approximately 53% reduction in SODA protein levels when the parasites reached early stationary growth, when compared to WT (Fig. 3). During the log phase of growth both SODA and SODB were expressed at lower levels, and no significant difference was observed between WT and *SODA/*Δ*sodA* (Fig. 3A,B). Repeated attempts to generate a *SODA* null line by replacing the second allele were unsuccessful. Similar to our previous experience with *LMIT1*, an *SODA* ORF was consistently detected in lines resistant to both phleomycin and hygromycin, even when the intended *in situ* integration of the targeting drug-resistance markers was confirmed. Thus, SODA appears to be essential for the viability of *L. amazonensis* promastigotes.

Providing further evidence that *SODA* is an essential gene in trypanosomatid parasites, tetracycline-inducible RNAi-mediated ablation of SODA in *Trypanosoma brucei* procyclic forms (Fig. S3A) markedly impaired the parasites' ability to sustain replication after 72 h in culture (Fig.S3B). *T. brucei* procyclics were reported to be partially dependent on mitochondrial metabolism (27,28), suggesting that the phenotype we observed may be related to gradual mitochondrial damage as accumulated ROS reach toxic levels in the absence of SODA.

SODA Deficiency Severely Impairs the Ability of Leishmania Promastigotes to Differentiate into Virulent Forms - Given our inability to generate *SODA* null mutants, we compared the properties of single knockout *SODA/*Δ*sodA* (*hyg*) and WT promastigotes. When promastigote cultures were shifted to axenic amastigote growth conditions (pH 4.5 /32^{o}C), after an initial lag period of 48 h WT parasites replicated steadily throughout the monitored period (144 h) (Fig. 4A). In contrast, parasites lacking one *SODA* allele showed very poor growth and a gradual loss in viability, as indicated by propidium iodide staining (data not shown). Marked differences were also observed in parasite morphology 48 h after the low pH/high temperature shift. About 95% of the WT population showed the expected change into the rounded/aflagellated amastigote form, while the majority (~55%) of the viable *SODA/*Δ*sodA* parasites retained long flagella and showed marked morphological abnormalities (Fig. 4B,C). Transmission electron microscopy (TEM) analysis revealed extensive mitochondrial alterations in *SODA/*Δ*sodA* parasites (Fig. 4D) that were reminiscent of what we previously observed in *LMIT1/*Δ*lmit1* promastigotes (17). Mitochondria were enlarged and deformed, with an overall reduction in electron density and accumulation of dense aggregates in the matrix. Normal mitochondrial morphology and a normal ability to differentiate and replicate as amastigotes were restored when *SODA/*Δ*sodA* parasites were complemented with episomally expressed SODA (*SODA/*Δ*sodA+SODA*) (Fig. 4A-C).

In addition to the low pH/high temperature stimuli, in previous work we showed that upregulation of the iron uptake machinery in response to iron deprivation also triggers differentiation of *L. amazonensis* promastigotes into amastigotes in a process dependent on mitochondrial H_2O_2, the product of SODA (16,17). Thus, we also examined the effects of iron deprivation on the growth and differentiation of *SODA/*Δ*sodA* promastigotes. When cultured in iron-deficient medium both WT and SODA-deficient parasites were initially able to replicate, but reached a maximum cell density (~$3x10^7$ /ml) that was lower than what is normally observed in complete growth medium (Fig. 5A, compare with WT in Fig. 6A). However, in contrast to WT, the *SODA/*Δ*sodA* promastigote count steadily declined after reaching the peak density (Fig. 5A), resembling the "population crash" we previously reported for Δ*lit1/*Δ*lit1* (lacking the ferrous iron transporter LIT1) and *LMIT1/*Δ*lmit1* (partially deficient in the mitochondrial iron importer LMIT1) parasites grown in iron-depleted medium (16,17). Microscopic analysis of the cultures showed that on day 5 of iron deprivation >55% of WT parasites lacked a visible flagellum and had assumed the rounded amastigote-like morphology, while only 20% of viable *SODA/*Δ*sodA* cells were able to undergo this transformation (Fig. 5B). Complementation with episomally expressed SODA (*SODA/*Δ*sodA+SODA*) partially restored both the growth pattern and the parasite's ability to transform into amastigote-like forms (Fig. 5A,B).

Taken together, the results described in this section show that mitochondrial SODA is important for the differentiation of *L. amazonensis* promastigotes into amastigotes after both types of stimulation – low pH/high temperature, and iron deprivation.

*Stationary Phase SODA/Δ*sodA *Promastigotes are More Susceptible to ROS Stress and are Impaired in Metacyclogenesis* - We next examined if a single copy of *SODA* was sufficient to sustain growth and development during the promastigote stage. When grown in complete promastigote culture medium (containing iron and 10% FBS) *SODA/Δ*sodA promastigotes grew at a similar rate as WT during the early logarithmic phase of growth, but entered stationary phase earlier at a density of 5-6x10^7 cells/ml (day 5). In contrast, WT parasites kept growing until reaching stationary phase at 7-8x10^7 cells/ml (day 7) (Fig. 6A). Moreover, after day 5 the *SODA/Δ*sodA parasite population showed a steady decline, a process that was partially reverted in the complemented *SODA/Δ*sodA+SODA line. These results suggest that SODA activity is also important for promastigote viability during the stationary phase of growth.

To investigate if an impaired ability to detoxify mitochondrial ROS products due to SODA deficiency might cause promastigote death, we exposed WT, *SODA/Δ*sodA and *SODA/Δ*sodA+SODA mid-log promastigotes to increasing concentrations of menadione, a drug that induces $O_2^{\bullet-}$ generation in mitochondria. *SODA/Δ*sodA parasites showed markedly higher sensitivity to the drug, with a drop in viability from >60% in WT to <30% in *SODA/Δ*sodA after exposure to 4 μM menadione. As expected, complemented *SODA/Δ*sodA+SODA promastigotes were more resistant to menadione toxicity when compared to *SODA/Δ*sodA parasites (Fig. 6B). Collectively, our data suggests that normal levels of SODA expression are required for protection against the accumulation of mitochondria-generated ROS during the late-log and stationary phases of promastigote growth.

Environmental stress (e.g. nutrient and oxygen deprivation) experienced by *Leishmania* promastigotes attached to the sand fly midgut is thought to be important for their transformation into infective metacyclic forms (29). However, very little is known about the actual signaling pathway involved in *Leishmania* metacyclogenesis *in vivo*. Considering our evidence for a role of SODA-mediated redox signaling in the promastigote-amastigote transition, and the increased SODA protein levels observed in stationary phase promastigotes in culture, we investigated whether SODA was also required for metacyclic promastigote development. The number of metacyclic forms was quantified on day 6 WT, *SODA/Δ*sodA and *SODA/Δ*sodA+SODA stationary promastigote cultures after selective agglutination with a *L. amazonensis* promastigote-specific antibody. *SODA/Δ*sodA cultures showed ~3 fold reduction in the yield of metacyclics compared to WT, a phenotype partially restored by SODA complementation (*SODA/Δ*sodA+SODA) (Fig. 7A). Scanning electron microscopy (SEM) analysis revealed a normal promastigote morphology for all three lines during the log phase of growth (day 3) but during stationary phase (day 7) the elongated and slender forms characteristic of metacyclic forms were only observed in WT and *SODA/Δ*sodA+SODA (Fig. 7B). Thus, the *SODA/Δ*sodA stationary phase promastigote population was largely devoid of slender metacyclic forms and contained a large number of cells with abnormal morphology.

To assess mitochondrial function we treated promastigotes with JC-1, a lipophilic cationic dye whose rate of accumulation inside mitochondria is directly dependent on the maintenance of an active mitochondrial membrane potential ($\Delta\psi_m$) (17,30). The shift of JC-1 from a monomeric form under low concentrations to an aggregated form inside mitochondria is detected as a shift from green (emission 530 nm) to red (emission 590 nm) fluorescence. Thus, the 590nm/530nm ratio in this assay provides an accurate quantification of the $\Delta\Psi_m$ dependent amount of dye imported into mitochondria. Increase in the 590nm/530nm fluorescence ratio was observed for all three lines on days 2 and 3 of culture, indicating healthy mitochondrial activity during the logarithmic phase of growth. However, *SODA/Δ*sodA parasites showed a significant reduction in the 590nm/530nm fluorescence ratio on day 7, an

effect reversed by *SODA* complementation (Fig. 7C). This result suggests that SODA expression from a single allele is not sufficient to maintain normal mitochondrial function as the parasites enter the stationary phase of growth, when differentiation into virulent metacyclic forms is initiated.

Deletion of One SODA Allele Reduces Mitochondrial SOD Activity in Stationary Phase Promastigotes - Whole cell extracts from wild type, *SODA/*Δ*sodA* and *SODA/*Δ*sodA+SODA* were prepared at different phases of promastigote growth and assayed for SOD activity. A reduction in the total SOD activity was observed in *SODA/*Δ*sodA* parasites in late-log (day 5) and stationary phase (day 7) (Fig. 8A). Since this biochemical activity assay does not distinguish between the mitochondrial SODA and the glycosomal SODB, we analyzed the same parasite extracts by immunoblot with antibodies specific for each of the enzymes. As observed for the total SOD activity, SODA protein expression in the WT and *SODA/*Δ*sodA+SODA* parasites was similar and increased gradually as the cells entered stationary phase, while significantly less SODA was detected in *SODA/*Δ*sodA* extracts at the same time points (days 5 and 7) (Fig. 8B). In contrast, the levels of SODB protein were more stable and comparable between the three lines, showing only slightly elevated levels on days 5 and 7 (Fig. 8B). These results suggest that the abnormal mitochondrial function observed in *SODA/*Δ*sodA* promastigotes is likely to be a consequence of reduced SODA expression during the stationary phase of growth. This finding is in agreement with the mitochondrial localization of SODA (Fig. 1A) and with a lack of involvement of the glycosomal SODB isoform.

Consistent with this view, progression into the stationary phase of growth was associated with a reduction in both the total SOD activity and the amount of SODA protein in mitochondrial fractions isolated from *SODA/*Δ*sodA* parasites, when compared to WT (Fig. 9A,B). As expected, only trace amounts of SODB were detected in the same mitochondrial fractions. Slightly elevated levels of SOD activity were observed in mitochondrial extracts from *SODA/*Δ*sodA* on day 3, possibly as a response to elevated ROS stress in SODA deficient parasites during the logarithmic phase of growth.

Mitochondrial ROS Generation is Required for Development of Virulent Forms - The data discussed above suggested that the inability of stationary phase *SODA/*Δ*sodA* promastigotes to efficiently differentiate into metacyclic forms might be due to inadequate generation of H_2O_2, which is known to function as an amastigote differentiation signaling molecule in *L. amazonensis* (16). However, an alternative explanation is that the differentiation defect of *SODA/*Δ*sodA* promastigotes was merely a result of mitochondrial dysfunction, caused by $O_2^{\bullet-}$ induced damage to Fe-S cluster ETC proteins. To distinguish between these possibilities, wild type and *SODA/*Δ*sodA* promastigotes were treated with MitoTempo, a mitochondria targeted anti-oxidant that acts as a SOD mimetic, converting available $O_2^{\bullet-}$ to H_2O_2 (31-33). Importantly, MitoTempo also reduces the overall leakage of electrons from the mitochondrial respiratory chain, thereby inhibiting production of all ROS, including $O_2^{\bullet-}$, H_2O_2 and peroxynitrite (31). Accordingly, we found that treatment of both WT and *SODA/*Δ*sodA* promastigotes with MitoTempo improved parasite survival during the stationary phase of growth (Fig.10A). MitoTempo also restored the $\Delta\psi_m$ of *SODA/*Δ*sodA* stationary phase promastigotes to wild type levels (Fig. 10B). These effects on promastigote survival and mitochondrial membrane potential were observed with both low (15 μM) and high (50 μM) concentrations of MitoTempo. Interestingly, when we examined the yield of metacyclic forms, an improvement was observed after treatment of *SODA/*Δ*sodA* parasites with 15 μM, but not with 50 μM MitoTempo (Fig. 10C). We observed a similar concentration-dependent effect in the expression of the H_2O_2 reporter protein APX: 15 μM MitoTempo restored APX expression to WT levels, but such effect was not seen after treatment with 50 μM. In WT parasites, both concentrations of MitoTempo caused >two-fold reduction in both the yield of purified metacyclics (Fig. 10C) and in APX protein levels (Fig.10D). Thus, although mitochondrial function was restored in *SODA/*Δ*sodA* promastigotes with 50

μM MitoTempo, this treatment did not increase the yield of metacyclic stage differentiation – suggesting that mitochondrial dysfunction is not the primary cause for the inability to differentiate of SODA deficient parasites. In WT parasites, where a normal complement of SODA may reduce the availability of $O_2^{\bullet-}$, MitoTempo may not stimulate H_2O_2 production, as indicated by the lower levels of APX expression under these conditions (Fig. 10D). Collectively, the results of these experiments reinforce the view that SODA-mediated H_2O_2 production plays a central role in the development of *Leishmania* virulent forms.

Deletion of One SODA Allele Results in Strong Loss of Virulence - Metacyclic promastigotes from WT, *SODA/*Δ*sodA* and *SODA/*Δ*sodA+SODA* promastigotes were purified from day 5 stationary cultures, their viability assessed with the dye FDA, and compared for their ability to establish infections in mouse bone marrow macrophages (BMMs). Because of the reduced ability of *SODA/*Δ*sodA* to undergo metacyclogenesis, larger culture volumes were used to obtain comparable numbers of viable metacyclic forms. BMM infection with WT parasites progressed normally, and after the typical 24 h lag period the parasites replicated intracellularly as amastigotes, progressively increasing in number (Fig. 11A). In contrast, a steep decline in the number of intracellular parasites was observed with the *SODA/*Δ*sodA* line between 3 and 24 h after BMM infection, with only very few intact parasites being detectable after 72 h. Episomal expression of SODA enhanced the ability of the parasites to survive intracellularly, as indicated by a markedly increased number of parasites detected after infection of BMM with the *SODA/*Δ*sodA+SODA* line. The *SODA* complemented parasites were also able to replicate intracellularly, albeit at a slower rate when compared to the WT line (Fig. 11A). These results indicate that normal levels of SODA expression are necessary for *L. amazonensis* to establish successful infections in host macrophages.

We also examined the role of SODA in the parasite's ability to induce cutaneous lesions in mice. Purified viable metacyclic forms were injected into the footpads of C57BL/6 mice and the progression of cutaneous lesions was quantified over a period of 9 weeks (Fig. 11B). A steady growth in lesion size was recorded for WT *L. amazonensis* up to week 9. In contrast, mice infected with *SODA/*Δ*sodA* metacyclics showed no evidence of lesion formation. Quantification of the parasite tissue load after 9 weeks showed a $>10^4$ fold higher number of parasites in the footpad tissues of mice injected with WT parasites, when compared to the group infected with the *SODA/*Δ*sodA* line. This phenotype was also partially restored in the complemented *SODA/*Δ*sodA+SODA* line (Fig. 11C). Inability to fully restore virulence was not unexpected, considering that lack of robust complementation is commonly observed in transgenic *Leishmania* (17,34-38). These findings demonstrate that *L. amazonensis* must maintain normal levels of expression of the mitochondrial enzyme SODA in order to replicate inside host macrophages and establish cutaneous infections *in vivo*.

DISCUSSION

Leishmania spp. are among the few organisms that can survive and replicate in the hostile environment of macrophage phagolysosomes, where degradative enzymes and reactive oxygen species (ROS) function as effective mechanisms of protection against pathogens. $O_2^{\bullet-}$ and $^{\bullet}NO$ are two key ROS molecules generated by macrophages to neutralize invading microorganisms (39). In order to successfully evade this onslaught, *Leishmania* species have developed multiple adaptive features that include an antioxidant defense repertoire that includes trypanthione /trypanthione reductase, peroxidases and three iron dependent SODs (39-41). The *Leishmania* glycosomal iron dependent SOD isoforms SODB1 and SODB2 are developmentally regulated, as indicated by reports of elevated SODB1 transcripts in amastigotes and of SODB2 in promastigotes, respectively (42,43). *L. chagasi* SODB1 null mutants are not viable and parasite lines lacking one SODB1 allele have markedly reduced viability inside macrophages (42). The mitochondrial SOD isoform SODA, on the other hand, was previously proposed to protect *Leishmania* mitochondria from oxidative stress (21,44), but prior to our present study its

physiological function had not been investigated. Here we investigated the physiological role of *L. amazonensis* SODA in light of recent evidence implicating this iron-dependent enzyme in ROS generation inside mitochondria, a process proposed to play a key role in the developmental of *Leishmania* virulent life-cycle stages (17).

We previously identified and functionally characterized the *L. amazonensis* mitochondrial iron importer LMIT1 (17). Similar to what we report here for SODA, *LMIT1* null mutants are not viable, consistent with the importance of iron for the assembly of Fe-S cluster proteins and ETC function. We also found that *LMIT1/*Δ*lmit1* promastigotes partially impaired in mitochondrial iron import are markedly defective in the ability to differentiate into infective amastigote forms. *LMIT1/*Δ*lmit1* parasites also showed a significant drop in mitochondrial iron content, a decrease in aconitase (a Fe-S cluster protein) and SOD activity, and extensive damage to mitochondria following oxidative stress. Based on these findings, we hypothesized that mitochondria is the major site where $O_2^{\bullet-}$ is generated and then converted by the iron-dependent SODA to H_2O_2, previously shown to act as a signal for differentiation (17). However, our initial studies of the role of SOD in *L. amazonensis* differentiation did not distinguish between the mitochondrial SODA and the glycosomal SODB1 and SODB2 isoforms. In this study, by measuring SOD activity in SODA-enriched mitochondrial fractions with little or no contaminating SODB, we clearly demonstrate that the >50% reduction in SOD activity observed in *LMIT1/*Δ*lmit1 L. amazonensis* promastigotes in response to a differentiation stimuli can be attributed to SODA.

Our inability to generate *SODA* null mutants suggests that SODA is also essential for the long-term survival of *L. amazonensis*. This conclusion is consistent with the presence of functioning mitochondria in both promastigote and amastigote forms, and the well established role of SOD in detoxifying ROS generated through electron leakage from the respiratory chain (6,8). The critical role played by SODA in protecting mitochondria from endogenous ROS accumulation is further evident from the lethal phenotype we observed following RNAi mediated knockdown of the SODA ortholog *Tb927.5.3350* in procyclic forms of *T. brucei*. No phenotype was observed following RNAi mediated silencing of the *T. brucei* SODA ortholog *Tb927.5.3350* in bloodstream forms (18), in agreement with the dependence on active mitochondrial metabolism of *T. brucei* procyclics but not bloodstream forms, which possess only rudimentary mitochondria (27,28).

SODA expression in *L. amazonensis* promastigotes increased progressively during culture reaching maximal levels on the stationary phase, presumably as a direct consequence of build-up in oxidative stress. Such priming of the antioxidant defense system was proposed to facilitate virulence development, by preparing *Leishmania* parasites for the invasion of host macrophages (17,26,42). The ~50% reduction in both SODA activity and SODA protein levels following entry into stationary phase is probably responsible for the sudden decline in cell viability we observed in *SODA/*Δ*sodA* promastigote cultures. On the other hand, the slight but reproducible increase in SOD activity observed in early log phase cultures of *SODA/*Δ*sodA* parasites is consistent with a putative surge in $O_2^{\bullet-}$ levels during the initial phase of promastigote growth. In this scenario, impaired conversion of $O_2^{\bullet-}$ into H_2O_2 by SODA might explain the faster growth rate observed for *SODA/*Δ*sodA* promastigotes during the log-phase, given the known role of H_2O_2 in arresting cell growth and promoting cell differentiation in *Leishmania* and other organisms (9,16,26,45,46).

Metacyclic promastigotes derived from single allele deletion mutants of *SODA* showed a drastically reduced ability to establish infections in macrophages or to induce cutaneous lesions in mice. Since the parasite's ability to invade host cells was apparently not compromised, this loss of virulence may have resulted from enhanced susceptibility to macrophage-generated toxic oxygen radicals, or from an inability to generate sufficient amounts of the amastigote differentiation signal, H_2O_2 (17). The latter possibility is strengthened by the failure of *SODA/*Δ*sodA* promastigotes to differentiate axenically into amastigotes when exposed to two different differentiation stimuli: low pH/high temperature, or iron deprivation. Promastigote to amastigote differentiation involves a major shift

in metabolism characterized by increased dependence on the TCA cycle and mitochondrial respiration for energy production, and less dependence on glycolysis (47). In addition, to maintain a neutral intracellular pH while replicating in the acidic PV environment, intracellular amastigotes have to establish a strong intracellular proton gradient and actively pump H^+ out of the cells (4,19). Both processes require upregulation of electron transport, which is a major source of $O_2^{\bullet-}$ generation. Thus, the high mortality rate we observed as *SODA/*Δ*sodA* promastigotes failed to differentiate into amastigotes is consistent with a requirement for a threshold level of active SODA to counter the $O_2^{\bullet-}$ surge and initiate H_2O_2 mediated signaling. This view is in agreement with our earlier characterization of the *LMIT1/*Δ*lmit1* line that is deficient in mitochondrial iron import (17).

An additional finding that emerged from this present study is the importance of SODA and its product H_2O_2 in the generation of infective metacyclic promastigotes (a process that occurs in nature within the digestive tract of sand fly vectors (48)). Although it was possible to obtain sufficient viable metacyclic promastigotes to perform virulence assays, we observed a sharp decline in the yield of these infective stages - a phenotype that was reversed by episomal expression of SODA and by treatment with SOD mimetic MitoTempo. This finding significantly expands the evidence supporting the important role of mitochondria-generated ROS as a regulator of virulence in *Leishmania* parasites.

EXPERIMENTAL PROCEDURES

Leishmania culture - L. amazonensis (IFLA/BR/67/PH8) was a kind gift from Dr. David Sacks (Laboratory of Parasitic Diseases, NIAID, NIH). Promastigote forms were cultured *in vitro* at 26°C in M199 media (pH 7.4) supplemented with 10% heat inactivated FBS, 0.1% hemin (Frontier scientific; 25 mg/ ml in 50% triethanolamine), 10 mM adenine (pH 7.5), 5 mM L-glutamine and 5% penicillin-streptomycin (36). To induce axenic differentiation into amastigote form, promastigote cultures (~2-4x10^7 /ml) were mixed with equal volumes of acidic amastigote media (M199 containing 0.25% glucose, 0.5% trypticase and 40 mM sodium succinate pH 4.5) and incubated at 32°C. Differentiated amastigotes were maintained in amastigote media at 32°C. Parasite viability was determined by fluorescence microscopy following fluorescein diacetate (FDA; Sigma-Aldrich) and propidium iodide (PI; Sigma-Aldrich) staining as described previously (16). Ability to differentiate was quantitated as the percentage of promastigotes with long flagella (undifferentiated) versus rounded forms with short flagella (differentiated) parasites via phase contrast microscopy. At least 200 viable cells per sample were scored.

Estimation of growth and differentiation of *L. amazonensis* promastigotes in iron depleted growth media were done as described earlier (16,17). Briefly, mid-log phase *L. amazonensis* promastigotes (~2x10^7 / ml) were harvested and resuspended in iron-depleted media at a concentration of 2x10^6 /ml. Cell growth was quantitated by microscopic counting of FDA stained cells at different times, as indicated.

Menadione sensitivity was determined by seeding promastigotes in log-phase culture (~2x10^7 / ml) at 4x10^5/ml with or without increasing concentrations of menadione. Following 48 h of incubation at 26°C, parasite viability was determined by fluorescence microscopy following FDA staining .

To assess the effect of the mitochondrial SOD mimetic MitoTempo (Sigma Aldrich, USA) on *L. amazonensis* growth and differentiation, the antioxidant was added daily to promastigote cultures at the indicated concentrations, for the whole duration of the experiment.

T. brucei culture and RNAi - T. brucei procyclic the 29–13 strain (49) that stably express T7 polymerase and Tet repressor were cultured at 27°C in SM9 media containing 15 μg/ml G418 (Gibco) and 50 μg/ml hygromycin (Invitrogen) and supplemented with 10% tetracycline free FBS (Atlanta Biological) (50). Growth of *T. brucei* cultures were monitored by counting using a Beckman Coulter counter.

Generation of RNAi cell lines- A 427 bp gene sequence targeting the *T. brucei SODA* gene (*Tb927.5.3350)* for RNAi-mediated knockdown was identified using RNAit software (51) and amplified from *T. brucei* genomic DNA using the following oligonucleotides *FD: TbSODA-*

HindIII (G*AAAGCTT*GTTGGAGCTGCACTACACGA) ; *RV: TbSodA-XbaI* (GA*TCTAGA*CCCACACATCCACTGTGAAG) (introduced *HindIII* and *XbaI* restriction sites are indicated as italicized and underlined nucleotides). The amplified gene fragment was then cloned into p2T7-LMIT1 (17) by replacing the *LMIT1* gene using corresponding restriction sites to create the RNAi construct pTbSODA-KD. The resulting plasmid linearized with *NotI* was electroporated into *T. brucei* and stable transfectants (*2T7/SODA*) were obtained after limiting dilution in 96-well plates with Phleomycin (2.5 μg/ml) and selection as described (50).

RNAi mediated knockdown- dsRNA synthesis was induced by the addition of 1 μg/ml tetracycline to cultures of clonal cell lines at $1x10^6$/ml starting concentration. Cells growing in presence or absence of tetracycline were counted daily using a hemocytometer and diluted to the initial starting concentrations. SODA knockdown was confirmed by performing Western blot analyses of whole cell lysates at different time points following tetracycline addition, using anti-SODA rabbit polyclonal antibodies raised against *Leishmania* SODA.

Generation of L. amazonensis SODA single knockout cell lines - The *L. amazonensis SODA* open reading frame (ORF) was genetically targeted for replacement with gene deletion constructs containing the hygromycin resistance gene (HYG) or Neomycin phosphotransferase (NEO) through homologous recombination, as described earlier (17,52). Sequences upstream and downstream of the *SODA* ORF were cloned using the following primers containing *SfiI* restriction enzyme sites (underlined): *LamSODA 5'SfiI-A:FD-* GAGGCCACCTAGGCCCGAAGAGGGAGTT GTG and *LamSODA 5'SfiI B:RV-* GAGGCCACGCAGGCCGAGTAGTGAGGTG CTTT to amplify 5' sequence; *LamSODA 3'SfiI-C:FD* – GAGGCCTCTGTGGCCTGCTTGGTGCCAA CGCG and *LamSODA 3'SfiI-D:RV* – GAGGCCTGACTGGCCGCTGACAACTGCA CG for 3' UTR. Four-part ligation using the PCR amplified 5' and 3' flanking sequences, drug resistance cassettes and the plasmid backbone were carried out. Positive clones were identified by analyzing *SfiI* restriction digests of plasmid DNA samples and confirmed by sequencing with specific primers as described (52). The targeting fragment used to transfect *L. amazonesis* promastigotes was released by *PacI* digestion, gel purified and used for electroporation. *Leishmania* clones with *a* single SODA allele deletion (*SODA/*Δ*sodA)* were isolated based on the ability of transformants to grow on agar plates containing hygromycin (100 μg/ml) or neomycin (50 μg/ml) and analyzed by Southern blot and PCR to verify integration of the drug cassette in the desired location.

For generation of a rescue plasmid expressing Leishmania *SODA* with C-terminal hemaglutinin (HA) tag, a two-step PCR amplification strategy was employed. In the first round, a 693 bp fragment of the *SODA* ORF was amplified with primers *FD-SODA HA*: AACCCGGGACAT*ATG*TTCCGCCGTGTCTC GATG (*SmaI* site underlined) and *RV-SODA HA*: CTGGGACGTCGTATGGGTAAAGCTTCTTC GTGGC that allowed for removal of the endogenous stop codon and introduction of an in-frame HA tag. The PCR product was used as template in a second round of amplification using *FD-SODA-HA* as sense and *RV:HA TAG2:* TTGGATCCTTAAGCGTAGTCTGGGACGTC GTATGG (*BamHI* site underlined) as antisense primers. The final PCR products were digested with *BamHI* and *SmaI* and cloned into pXG-SAT (courtesy of Prof. S. Beverley, Washington University). Transfected *Leishmania* clones were selected in plates containing 50 μg/ml nourseothricin (Jena Biosciences) and expression of HA -tagged SODA was confirmed by Western blot.

Expression of recombinant SODA,SODB and APX proteins and generation of antibodies - To produce recombinant histidine tagged SOD proteins, the genes encoding SODA, SODB and APX were PCR amplified from *L. amazonensis* genomic DNA using primers *FD:SODAexp* (GG*CATATG*TTCCGCCGTGTCTCG) and *RV:SODAexp* (GG*AAGCTT*CTTCGTGGCCTTTTC) for SODA, *FD:SODBexp* (AA*CATATG*CCGTTCGCTGTTCAGCCGCT) and *RV:SODBexp* (TT*AAGCTT*CAGATCACTGTTGACGTAGTG

) for SODB and *FD:APXexp*-(CCA*CACATG*TTCGGCACCTCGCGG) and *RV:APXexp* (TTCA*AAGCTT*GCTCCCCGACGCGG) for APX. Forward primers for SODA and SODB contained an *NdeI* restriction site and the APX gene contained a *PciI* site, contiguous with the start codon. Reverse primers were engineered to include a *HindIII* restriction site that removed the endogenous stop codon and allowed for synthesis of a six histidine tag. The resulting PCR products were cloned in the pET28b(+) expression plasmid (Novagen) using *NdeI* and *HindIII* restriction sites and the resulting plasmids were used to transform *E.coli* BL21(DE3)pLysS (Novagen). Expression of 6x-His tagged SODA, SODB and APX protein in soluble form was achieved by inducing transformed *E.coli* strains with 0.1mM IPTG (overnight at room temperature in media supplemented with 2% ethanol). 6x-His tagged SODA,SODB or APX proteins were purified from bacterial cell extracts by nickel column chromatography with His60 Ni superflow resin (Clontech) followed by elution with 0.3M imidazole buffer, according to the manufacturer's protocol. Homogeneity of the purified proteins was ascertained by SDS-PAGE.

Polyclonal antibodies were raised against purified SODA, SODB and APX proteins by periodic injection of rabbits with purified protein samples (the Pocono Rabbit Farm and Laboratory). Specificity of the antisera was assessed by Western blot.

Isolation of mitochondrial fractions - Mitochondrial enrichment was performed as described previously (17) using $5x10^8$ promastigotes from stationary phase cultures. The cells were washed three times with MES buffer (20 mM MOPS pH 7.0, 250 mM sucrose and 3mM EDTA) and resuspended in 500 µl of MES supplemented with 1 mg/ml digitonin and protease inhibitor cocktail (Roche). Following 5 min incubation at room temperature, the cell suspensions were centrifuged for 5 min (10,000g at 4°C) and the supernatant collected as the cytoplasmic fraction. The pellet was washed once with MES buffer and used for further analysis as the mitochondrial fraction or stored at -80°C until further use.

Determination of SOD activity in whole cell and mitochondrial extracts - SOD activity was determined in whole cell extracts as described earlier (16,17). To estimate mitochondrial SOD activity, mitochondrial fractions obtained following subcellular fractionation as described above were resuspended in buffer A and lysed by sonication. SOD activity in the lysates was measured using the SOD Assay Kit-WST (Dojindo Molecular Technologies, Inc.) according to the manufacturer's protocol. Standard curves were generated using known concentrations of horseradish SOD (Sigma-Aldrich). Protein content was determined using BCA™ protein assay kit (Thermo Scientific).

Localization of SODA by immunofluorescence microscopy - Immunolocalization of SODA was performed as described previously (17). To confirm the mitochondrial localization of SODA, promastigotes were incubated with MitoTracker Red CMXRos (Invitrogen) followed by fixation with 4% paraformaldehyde and attachment to poly L-lysine coated slides (multitest 8-well; MP Biomedicals). Following treatment with 50 mM NH_4Cl, the cells were permeabilized with 0.1% triton in PBS, blocked with PBS 5% horse serum and 1% bovine serum albumin (BSA) for 1 h at room temperature and incubated with anti-SODA rabbit polyclonal antibodies (1:10,000 dilution in PBS-1% BSA) for 1 h followed by anti-rabbit IgG AlexaFluor 488 (Invitrogen) 1:5,000 dilution in PBS-1% BSA for 1 h and staining with 2 µg/mL DAPI for 1 h. Slides were mounted with ProLong Gold antifade reagent (Invitrogen), images were acquired through a Deltavision Elite Deconvolution microscope (GE Healthcare) and processed using Volocity Suite (PerkinElmer).

Assays for mitochondrial activity - Mitochondrial membrane potential ($\Delta\psi_m$) was estimated using the MitoProbe JC-1 assay kit (Invitrogen). $1x10^7$ promastigote cells were incubated with 10 µM JC-1 for 15 min at 27°C, washed and resuspended in PBS. Fluorescence measured at 530 and 590 nm using a SpectraMaxM5^e microtiter plate reader (Molecular Devices) was used determine the $\Delta\psi_m$ (530/590 ratio).

To visualize the mitochondrial staining pattern promastigotes were placed in glass-bottom dishes (MatTek corporation) for live imaging on a Nikon Eclipse Ti inverted microscope with a

100x NA 1.4 objective (Nikon) equipped with a Hamamatsu C9100-50 camera and mCherry and FITC filters. Acquired images were analyzed with the Volocity Software Suite (PerkinElmer).

Electron microscopy - For scanning EM, parasites fixed in 2.5% (v/v) glutaraldehyde in 0.1M sodium cacodylate buffer pH 7.4 for 60 min and attached to poly-L-lysine coated coverslips were rinsed briefly with PBS, fixed with 0.1 M cacodylate buffer pH 7.4, treated with osmium tetroxide for 1 h, acetone dehydrated and critical point dried from CO_2. After sputter coating with Au/Pd the preparations were imaged in an Amray 1820D scanning electron microscope. For transmission EM, fixed parasite cells were post-fixed with osmium tetroxide and cell sections were prepared as described before (38). Final images were obtained using a Zeiss EM10CA electron microscope.

Quantification of Leishmania Intracellular Growth in Macrophages - Macrophage infection assays were carried out as described previously (16,17,37,38). A total of 1×10^5 BMMs from C57/BL6 mice (Charles River Laboratories), plated on glass coverslips in 3 cm dishes 24 h prior to the experiment, were infected with a at 1:5 multiplicity of infection (MOI) with metacyclic forms purified from stationary phase promastigote cultures (5-day old) using the m3A1 monoclonal antibody (53). After allowing 3 h for invasion BMM were washed three times in PBS and incubated for the indicated times at 34°C. Coverslips were retrieved after 3 (initial infection) 24, 48, and 72 h of incubation, fixed in 4% PFA, permeabilized with 0.1% Triton X-100 for 10 min and stained with 10 µg/ml DAPI for 1 h. The total number of macrophages and the total number of intracellular parasites per microscopic field (100× N.A. 1.3 oil immersion objective, Nikon E200 epifluorescence microscope) were determined and the results expressed as intracellular parasites per 100 macrophages. At least 300 host cells, in triplicate, were analyzed for each time point. The data were analyzed for statistical significance using an unpaired Student's t test ($p < 0.05$ was considered significant).

In vivo virulence and parasite load estimation - A total of 1 $X10^6$ infective metacyclics purified from *WT*, *SODA/*Δ*sodA* or *SODA/*Δ*sodA+SODA* stationary phase promastigote cultures and resuspendend in a volume of 50 µl PBS, were used to inoculate six-week-old female C57BL/6 mice (n=5 per group) in the left hind footpad. Progression of footpad lesion development was monitored through weekly measurements with a caliper (Mitutoyo Corp., Japan), quantitating the difference between the left and right hind footpads. The parasite load was estimated in infected tissue collected from footpads of mice sacrificed 11 weeks post infection using a limiting dilution assay (54).

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Conflict of interest. The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions. BM and NWA conceived and designed the research. BM, MFLS, DCM and JPBM performed the experiments; BM, MFLS, DCM and JPBM analyzed the data; BM and NWA prepared the manuscript.

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FOOTNOTES

This work was supported by National Institutes of Health grant R01 AI067979 to NWA.

The abbreviations used are: SOD, superoxide dismutase; PV, parasitophorous vacuole; ASL, adenosuccinate lyase; LMIT1, Leishmania mitochondrial iron transporter 1; LIT1, Leishmania iron transporter 1; TEM, transmission electron microscopy; SEM, scanning electron microscopy; TCA cycle, tricarboxylic acid cycle; BMM, bone marrow-derived macrophages; FDA, fluorescein diacetate.

FIGURE LEGENDS

FIGURE 1. SODA localizes to mitochondria. (A) Immunolocalization of SODA in *L. amazonensis* promastigotes was performed using polyclonal antibodies against SODA (green) and mitochondria were stained with MitoTracker Red (red). Merging the two images (merge) confirmed the mitochondrial localization of SODA. Bar = 4μm. (B) SODA is enriched in mitochondrial fractions. Subcellular

fractionation of *L. amazonensis* promastigotes expressing FLAG-tagged LMIT1 was performed using increasing concentrations of digitonin as indicated. Proteins in the supernatant (S) and pellet (P) fractions were detected by Western blot using antibodies against cytoplasmic adenosuccinate lyase (ASL), mitochondrial FLAG-tagged LMIT1 and glycosomal arginase. SODA and SODB were detected with specific polyclonal antisera and LMIT1-3xFLAG with an anti-FLAG monoclonal antibody.

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FIGURE 3. Deletion of a single *SODA* allele reduces the amount of SODA protein in promastigotes entering stationary phase. (A) Protein samples (10 µg protein /lane) prepared from whole cell lysates of wild type (*WT*) or *SODA/*Δ*sodA* promastigotes at log phase (day 2) or early stationary phase (day 4) of growth were analyzed by Western blot using antibodies specific for SODA or SODB. Tubulin expression levels in the lysates were used as loading controls. (B) SODA and SODB protein levels relative to tubulin estimated by quantification of the band intensities on Western blots. The data represents the mean ±SD of triplicate determinations and are representative of three independent experiments (Student's t test ** p=0.006).

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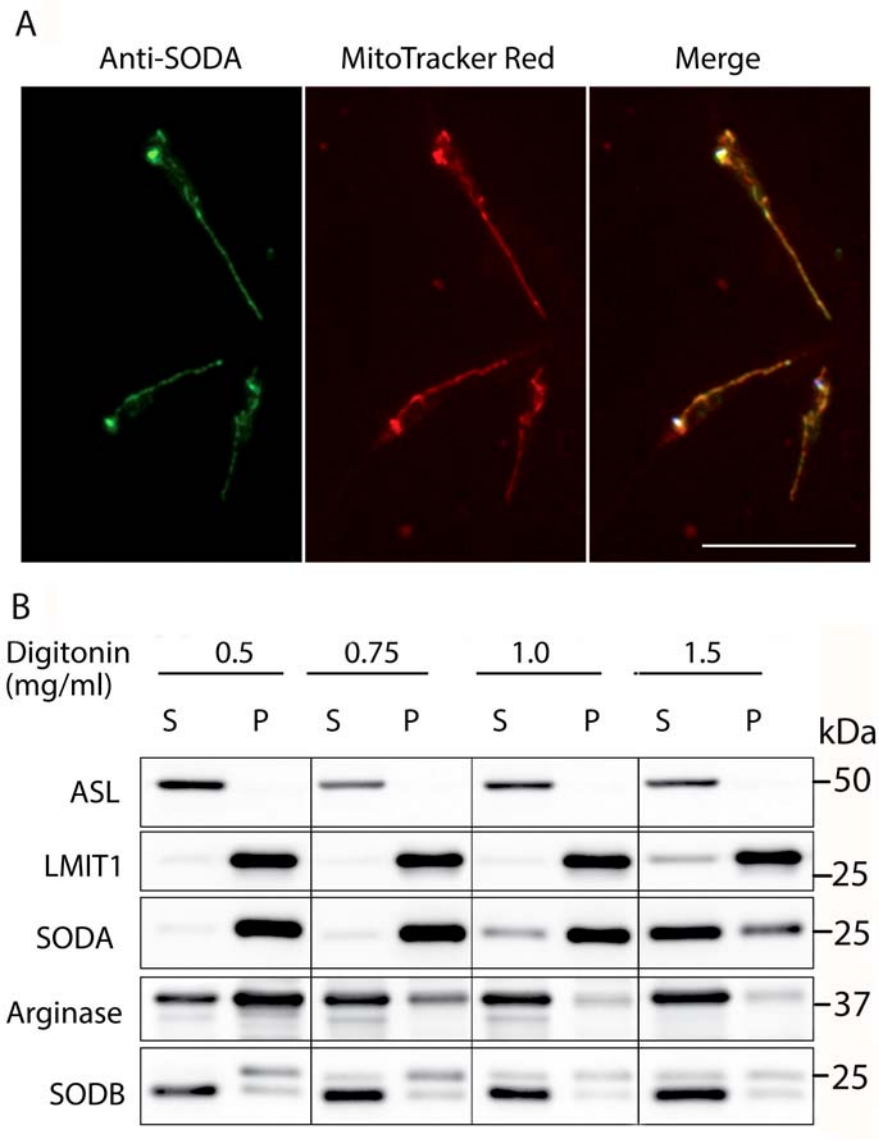
Figure 1



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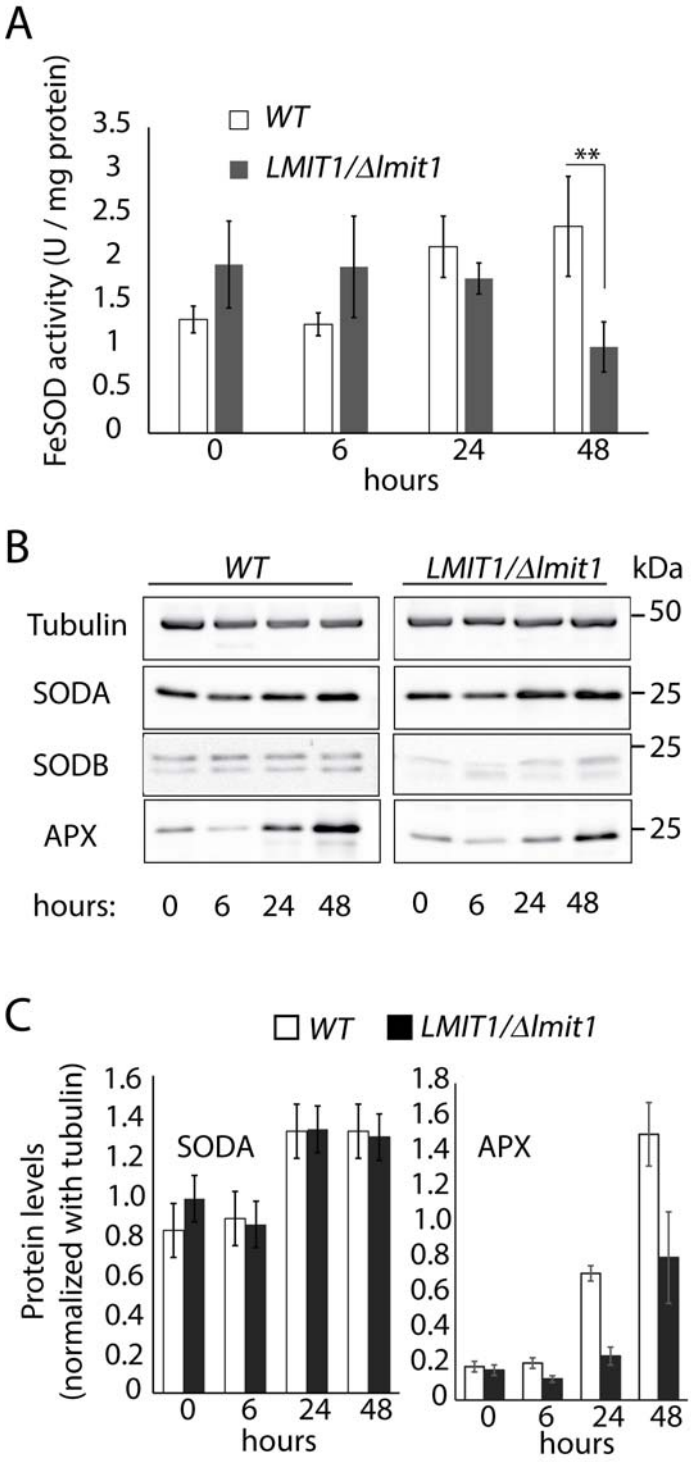

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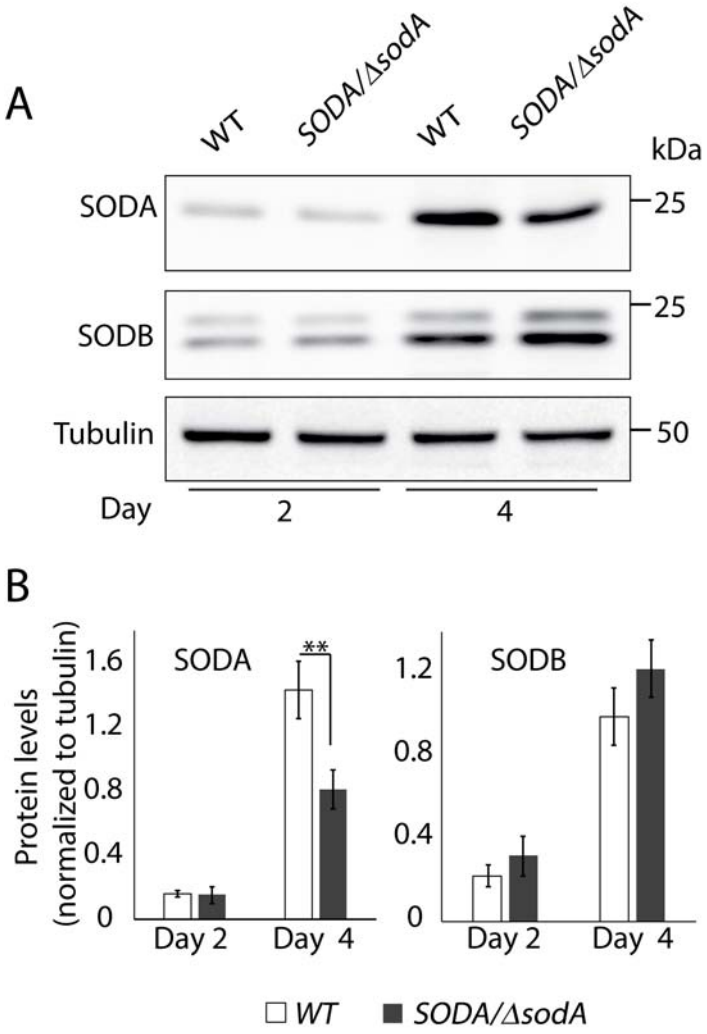

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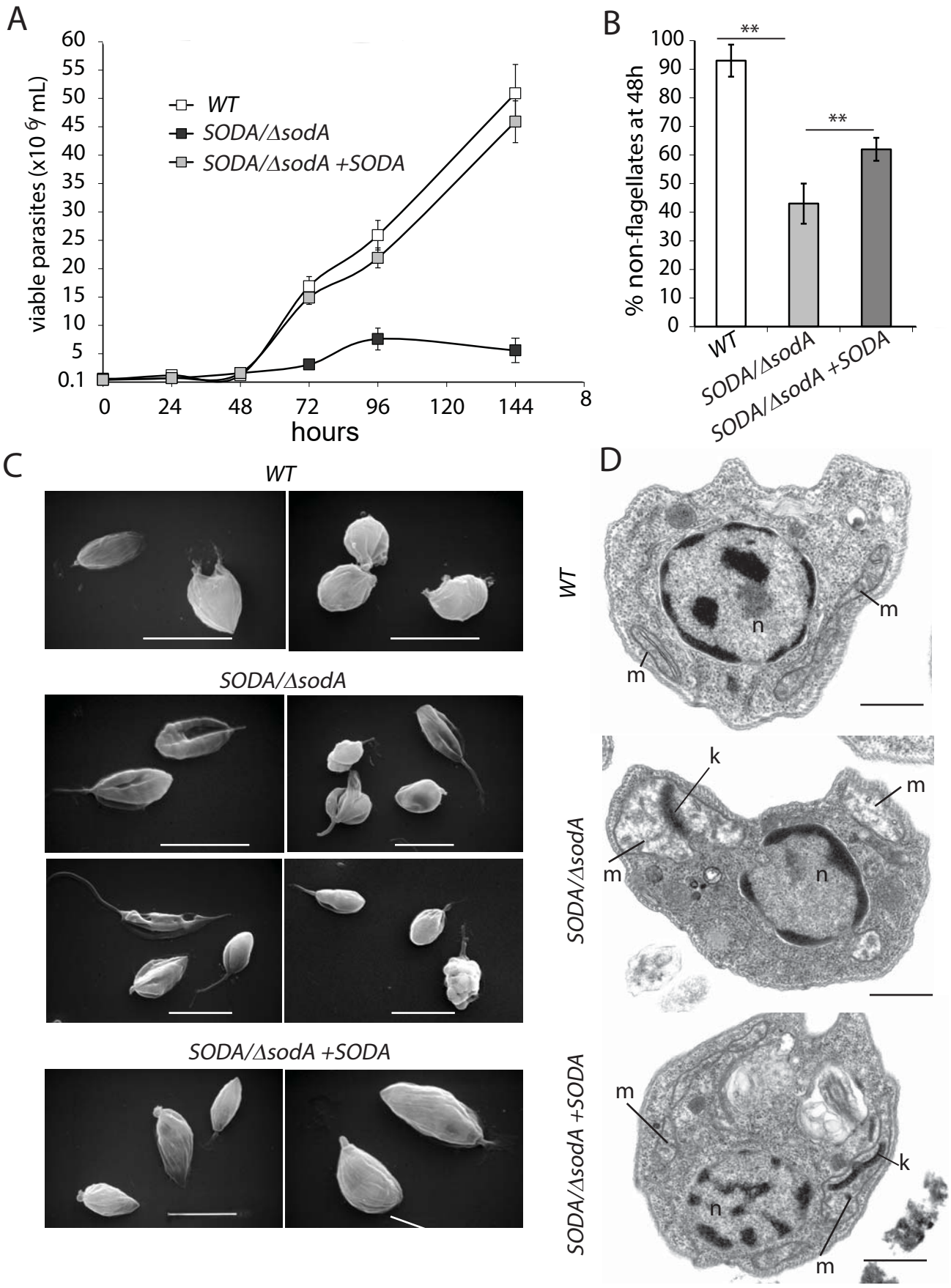

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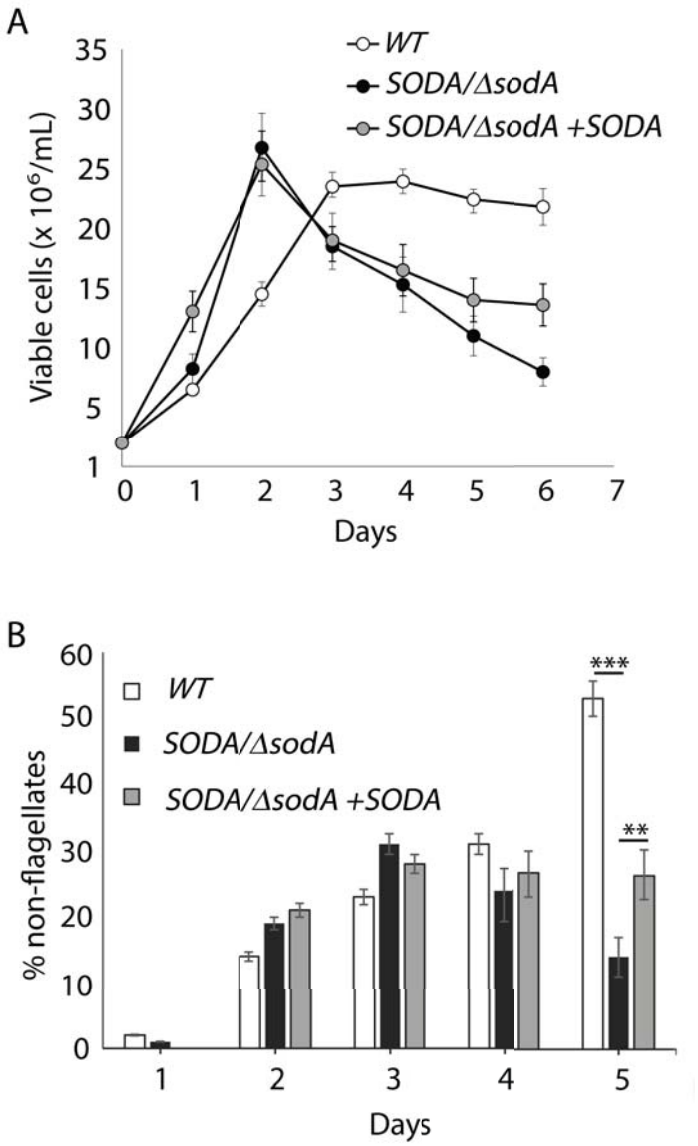

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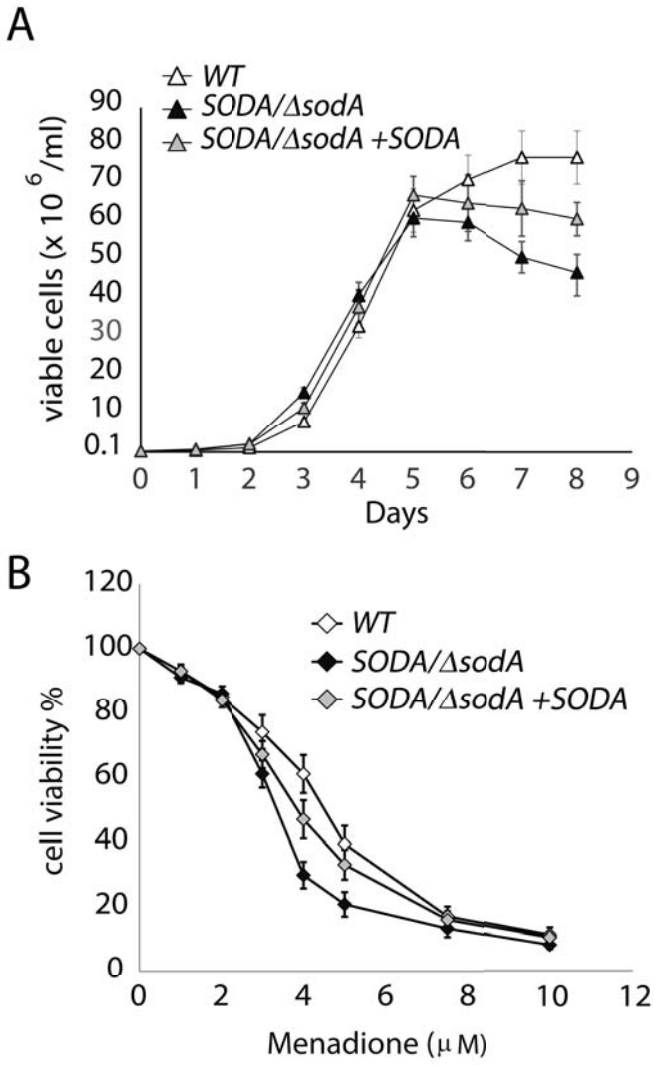

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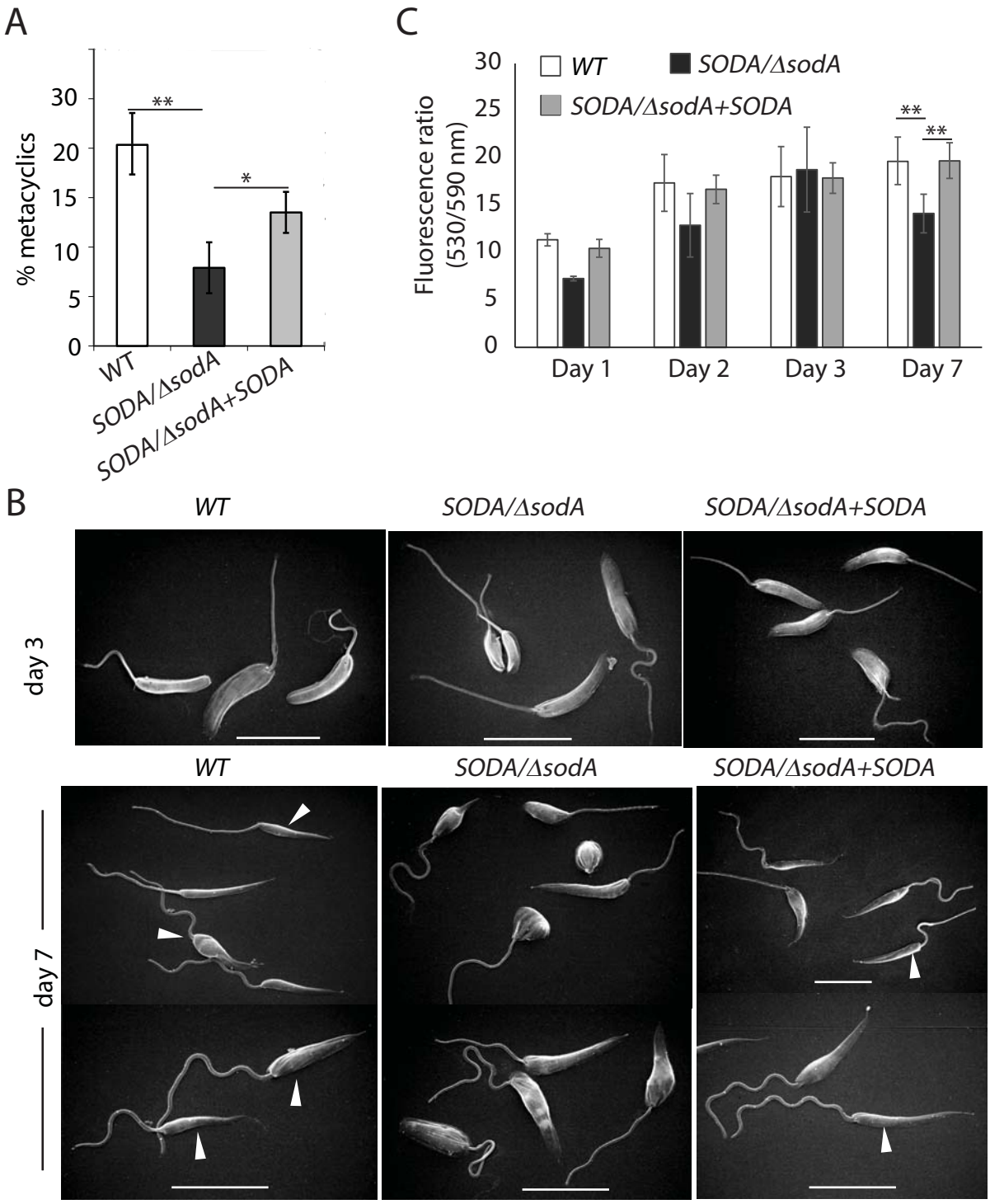

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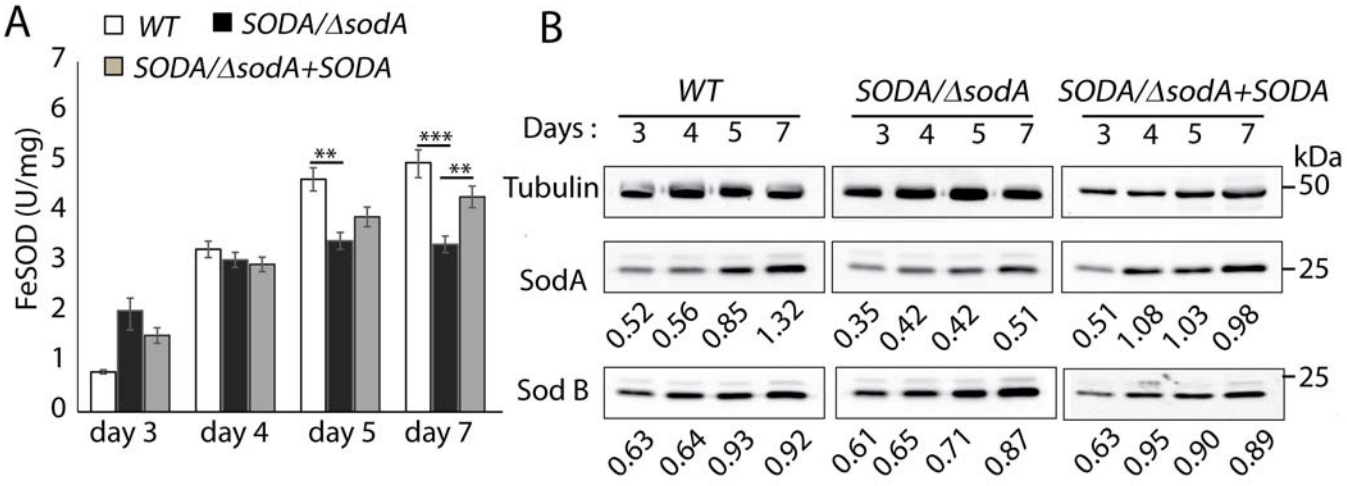

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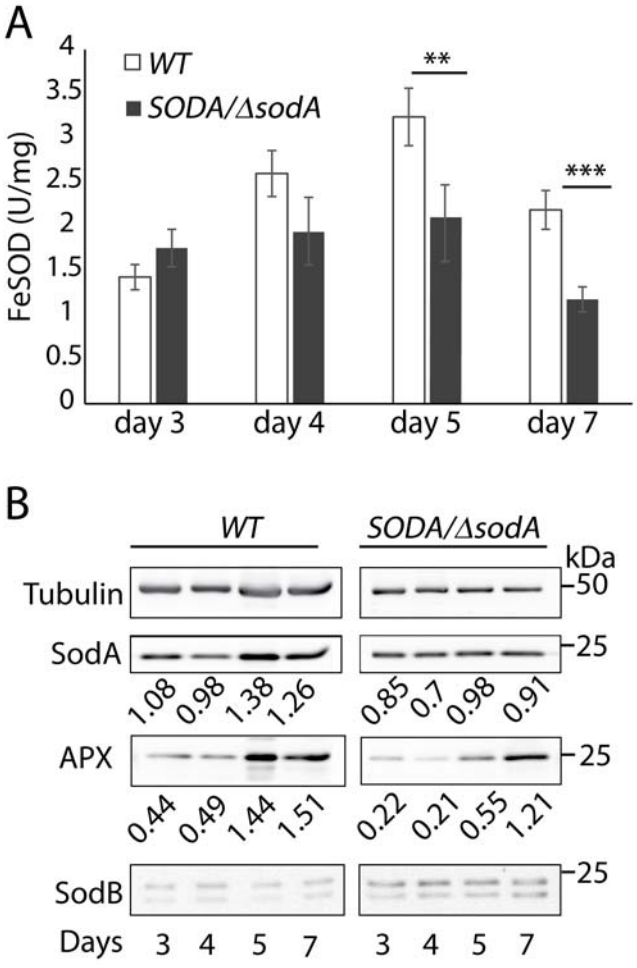

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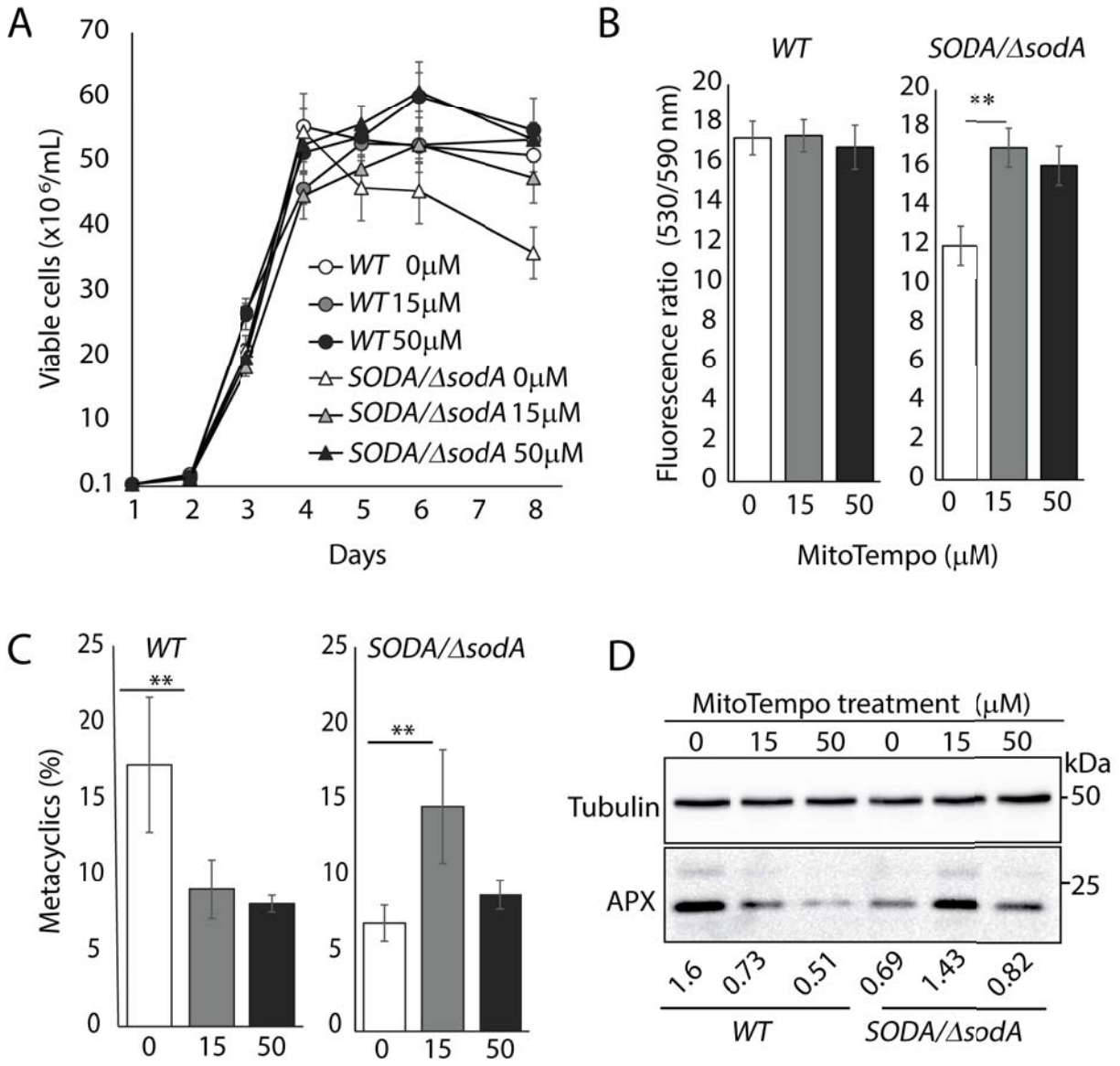

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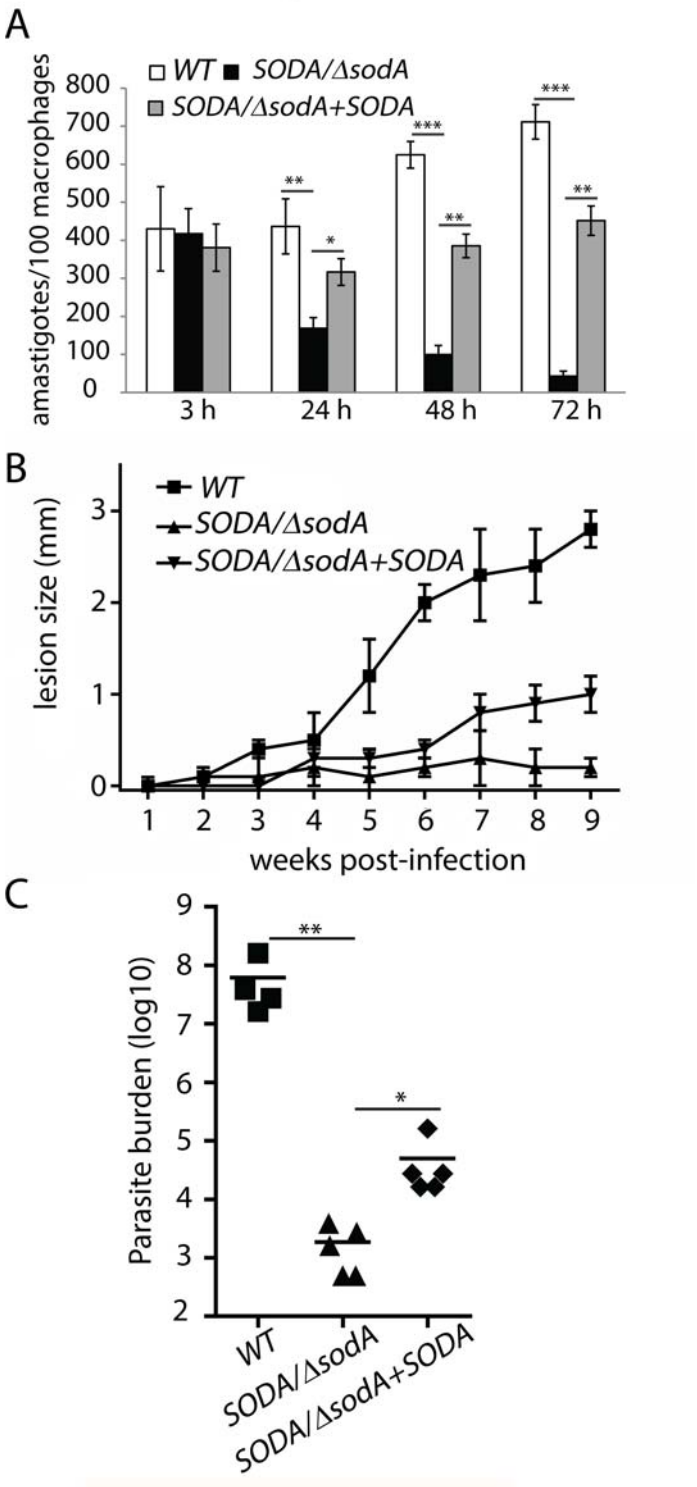
Figure 11



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The iron-dependent mitochondrial superoxide dismutase SODA promotes Leishmania virulence

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