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PROTOZOOLOGY - ORIGINAL PAPER

Artemisinin-resistant *Leishmania* parasite modulates host cell defense mechanism and exhibits altered expression of unfolded protein response genes

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Abstract

Artemisinin, extracted from a medicinal herb *Artemisia annua*, is widely used to treat malaria and has shown potent anticancer activity. Artemisinin has been found to be effective against experimental visceral and cutaneous leishmaniasis. Despite extensive research to understand the complex mechanism of resistance to artemisinin, several questions remain unanswered. The artesunate (ART)-resistant line of *Leishmania donovani* was selected and cellular mechanisms associated with resistance to artemisinin were investigated. ART-resistant (AS-R) parasites showed reduced susceptibility towards ART both at promastigote and amastigote stage compared with ART sensitive (WT) parasites. WT and AS-R parasites were both more susceptible to ART at the early log phase of growth compared with late log phase. AS-R parasites were more infective to the host macrophages ($p < 0.05$). Evaluation of parasites' tolerance towards host microbicidal mechanisms revealed that AS-R parasites were more tolerant to complement-mediated lysis and nitrosative stress. ROS levels were modulated in presence of ART in AS-R parasites infected macrophages. Interestingly, infection of macrophages by AS-R parasites led to modulated levels of host interleukins, IL-2 and IL-10, in addition to nitric oxide. Additionally, AS-R parasites showed upregulated expression of genes of unfolded protein response pathway including methyltransferase domain-containing protein (HSP40) and flagellar attachment zone protein (prefoldin), that are reported to be associated with ART resistance in *Plasmodium falciparum* malaria. This study presents in vitro model of artemisinin-resistant *Leishmania* parasite and cellular mechanisms associated with ART resistance in *Leishmania*.

Keywords *Leishmania donovani* · Artemisinin resistance · Parasite fitness · Immune modulation · HSP 40 · Prefoldin

Introduction

Artemisinin, a sesquiterpene lactone, is isolated from an annual herb *Artemisia annua* that has been used in traditional Chinese medicine for over 2000 years (van Agtmael et al. 1999). Artemisinin and derivatives (ARTs) have been found to be effective against parasitic infections (Yang and Liew 1993; Keiser et al. 2006; Danso-Appiah et al. 2009). Recent studies have documented the potential anticancer effect of ARTs in colorectal carcinoma, breast cancer, cervical cancer, and others (Jansen et al. 2011; Krishna et al. 2015; Hagens et al. 2017). Artemisinin-based combination therapies have been recommended by WHO for the treatment of malaria to prevent development of resistance (WHO, world malaria report 2016).

There are studies demonstrating anti-leishmanial activity of ARTs with high safety index (Want et al. 2015). In vitro studies with *Leishmania* have shown that ARTs cause externalization of phosphatidylserine, loss of mitochondrial membrane potential, and cell-cycle arrest at the sub-G0/G1 phase, resulting in programmed cell death of *Leishmania donovani* (Sen et al. 2007). In vivo studies have reported that anti-leishmanial activity of ART is associated with increased IL-4 and IFN-γ levels in the host (Ghaffarifar et al. 2015). However, there are limited studies investigating anti-leishmanial properties and possible mechanism of resistance to ARTs in *Leishmania* parasite.

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Plasmodium falciparum resists ARTs by evoking increased cell stress response. The enhanced adaptive response against oxidative stress and protein damage are hallmark of ARTs resistance observed in clinical as well as lab adapted ARTs resistant *P. falciparum* (Mok et al. 2015; Tilley et al. 2016; Rocamora et al. 2018). Several gene sets and pathways related to protein metabolism such as protein export, post-translational translocation, endoplasmic reticulum retention sequences, protein folding, unfolded protein binding, signal recognition particle, proteasome, and phagosome were upregulated in ARTs resistant *P. falciparum.* Most of these pathways are reported to contribute to unfolded protein response (UPR) in other eukaryotes (Mok et al. 2011, 2015). Further, it has been demonstrated that coordinated transcription of multiple chaperon partners plays important role in ARTs resistance in *Plasmodium*. The mRNA levels for the two putative chaperonin complexes, *Plasmodium* reactive oxidative stress complex (PROSC) and TCP-1 ring complex (TRiC), that participate in UPR of other species, were correlated with ARTs resistance (Mok et al. 2015).

In this study, we adapted a field isolate of *L. donovani* for artesunate (ART) resistance and exploited this ART resistant line to explore possible cellular mechanisms and altered parasite behavior linked to resistance. Additionally, expression of genes representing PROSC and TRiC was analyzed in ART sensitive and resistant *Leishmania* parasites since ARTs resistance in *P. falciparum* is highly correlated with the expression of these genes.

Material and methods

Parasite culture and selection of ART-resistant parasites

A cryopreserved field isolate of *Leishmania donovani* (WT), obtained from a visceral leishmaniasis patient, described earlier (Sreenivas et al. 2004), was propagated in Medium M199 with 25-mM HEPES (pH 7.4) supplemented with 10% heat-inactivated FBS, 100-IU penicillin G, and 100-μg/ml streptomycin (complete medium) at 26 °C (Bhandari et al. 2014). Parasites were selected for ART resistance by gradually exposing to increasing ART (Sigma Aldrich) concentration (10–50 μM) in culture medium at promastigote stage (Bhandari et al. 2014; Verma et al. 2017). The ART-resistant parasite was designated as AS-R.

ART susceptibility of AS-R and the corresponding wild type (WT) parasite

(i) ART susceptibility at promastigote stage Susceptibility of the WT and AS-R parasites towards ART were determined using resazurin-based fluorometric assay, as described earlier (Kulshrestha et al. 2013). The log phase promastigotes (5×10^5 parasites/well) were incubated in 96 well plates with 200-μl complete medium or serial dilutions of ART at concentration ranging from 1 to 650 μM. Cell viability was measured fluorimetrically (Tecan, Switzerland). The percentage reduction in the parasite viability compared with that in untreated control wells was determined. Fifty percent inhibitory concentration (IC_{50}) and 90% inhibitory concentration (IC_{90}) were calculated using sigmoidal regression analysis.

(ii) ART susceptibility at intracellular amastigote stage In vitro ART susceptibility was evaluated at intracellular amastigote stage using primary peritoneal macrophages (Bhandari et al. 2014; Verma et al. 2017). Briefly, female BALB/c mice were injected with 1 ml of 2% starch suspension via i.p. injection. After 48 h, mice were sacrificed, disinfected with 70% ethanol, and 5 ml of PBS was injected into peritoneal cavity of the mice. The primary peritoneal exudate cells (PECs) were harvested with PBS solution, centrifuged at 2000 rpm for 10 min at 4 °C and suspended in RPMI 1640 medium (Sigma, USA), supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). PECs (2×10^5 cells) were incubated at 37 °C in presence of 5% CO_2 and were allowed to adhere in 16-well chamber slides. After 24 h, the medium was gently removed, and the macrophages were infected with late log phase promastigotes at a ratio of 10 parasites per macrophage in a volume of 200 μl complete RPMI 1640 medium. Twenty-four hours later, infected macrophages were exposed to ART (13, 26, 52, 104, 208, and 260 μM) for 48 h. After staining with Diff-Quik solutions, 100 macrophages were examined at × 1000 magnification to count the number of intracellular amastigotes. The survival rate of parasites relative to untreated macrophages was calculated, and IC_{50} and IC_{90} values were determined.

Assessment of infectivity of WT and AS-R parasites

Infectivity of the parasites was assessed using primary peritoneal macrophages as described earlier (Deep et al. 2017). Briefly, macrophages were infected with the parasites at 1:10 ratio and washed after 6 h of infection to remove non-internalized parasites. The slides were further incubated for 42 h (group 1) and 66 h (group 2) and stained with Diff-Quik solutions. A total of 500 macrophages were counted in randomly selected fields for each group at × 1000 magnification for calculating the percentage of infected macrophages.

Metacyclogenesis in WT and AS-R parasites

Stationary phase promastigotes (2×10^8 cells/ml) were suspended in 10 ml of complete medium and allowed to agglutinate at room temperature for 30 min in presence of 50-μg/ml peanut agglutinin (PNA) (Sigma Aldrich). The sediment and

the supernatant were recovered. The sediment was diluted to 10 ml in fresh complete medium-containing 50-μg/ml PNA, and supernatant was recovered after centrifugation at 200*g* for 10 min. The supernatants collected were centrifuged at 2000*g* to obtain PNA^- (metacyclic) promastigotes. All steps were monitored under a light microscope (Nikon ECLIPSE TS100). PNA^- population was counted and percent metacyclic population was calculated (Deep et al. 2017).

Determination of parasite tolerance towards oxidative and nitrosative stress

WT and AS-R promastigotes (1×10^5 cells/well in 96 well plates) were subjected to oxidative stress using hydrogen peroxide (H_2O_2) (9.76 to 10,000 μM) and nitrosative stress using S-nitroso-N-acetyl-DL-penicillamine (SNAP) (0.98 to 1000 μM). The plates were incubated at 25 °C for 72 h, followed by addition of 50-μl resazurin (0.0125%) into each well and further incubation for 18 to 24 h. Cell viability was measured fluorometrically. The results were expressed as percent reduction in parasite viability, compared with untreated controls, and the 50% inhibitory concentration (IC_{50}) was calculated (Carter et al. 2005; Bhandari et al. 2014).

Complement-mediated cell lysis

Both WT and AS-R promastigotes (1×10^6 cells/ml in M199 medium with 20% FBS) were incubated with serial dilutions of freshly isolated human serum (0.78 to 50%) in 96-well plates at 37 °C for 60 min. After 1 h, cold EDTA and resazurin were added, plates were incubated for another 24 h at 25 °C, and the fluorescence was measured (Ouakad et al. 2011).

Assessment of intracellular ROS production in WT/AS-R parasites–infected macrophages

Mice peritoneal macrophages infected with WT/AS-R parasites were incubated for 48 h without or with ART (20 μM). Intracellular ROS levels (expressed as mean fluorescence intensity unit) were measured fluorometrically using H_2DCFDA dye (Deep et al. 2017).

Assessment of NO production in WT/AS-R parasites–infected macrophages

Lipopolysaccharide (1 μg/ml)-stimulated mice peritoneal macrophages were infected with WT or AS-R parasites and incubated for 48 h. The NO levels were estimated in cell culture supernatant by Griess reaction, measuring the absorbance at 540 nm (Kulshrestha et al. 2011). The amount of nitrite was calculated from a standard curve constructed with different concentrations of sodium nitrite (linear range between 10 and 80 μM).

Multiplex ELISA for cytokine estimation

Levels of Th1/Th2 cytokines were evaluated in mice peritoneal macrophages as described earlier (Bhandari et al. 2014; Avishek et al. 2016). Briefly, macrophages stimulated with LPS were infected with WT or AS-R parasites. After 48 h of infection, the culture supernatant was collected and IFN-γ, IL-12, TNF-α, IL-2, IL-10, and IL-4 cytokine levels were evaluated by Bio-PlexPro™ (BIO-RAD) kit and multiplex array reader, Luminex™ Instrumentation System (Bio-Plex workstation, BIO-RAD Laboratories).

Quantitative real time PCR

The expression profile of genes involved in UPR was analyzed in WT and AS-R *Leishmania* parasites by quantitative real time PCR (Q-RT-PCR). These included genes homologous to *Plasmodium* TRiC, viz. T-complex protein 1 (TCP1), and prefoldin (also known as flagellar attachment zone protein, FAZP) and PROSC, viz., peptidyl-prolylcis-trans isomerise (cyclophilin 2), heat shock protein 40 (HSP 40; also known as methyltransferase, METase domain-containing protein) and heat shock protein 70 (HSP 70, also known as luminal-binding protein 1, BiP). Total RNA isolated from promastigotes was reverse transcribed to cDNA, and reactions were carried out in 25-μl volume containing 1 X Fast SYBR Green master mix (Applied Biosystems, USA), 100-ng/ml forward and reverse primers and cDNA. Constitutively expressed genes encoding cystathionine-β-synthase (CBS) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as internal control for normalization. The relative changes in target gene expression were calculated using $2^{-\Delta\Delta Ct}$ method (Verma et al. 2017; Kumar et al., 2012). The list of primers, designed using Primer express software version 3.0, is given in Table S1 (Supplementary Information).

Ethics approval

The procedures for the care, use, and euthanasia of experimental animals was carried out under ethical approval by the Institute Animal Ethics Committee of the ICMR-National Institute of Pathology (Project No. NIP/IAEC-1502) following the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Results

Generation of artemisinin (ART)-resistant parasite

L. donovani parasites (WT) were exposed stepwise to increasing ART pressure up to 50 μM and stable resistance was achieved in

48 weeks. The resistant parasite (designated AS-R) exhibited morphology similar to wild type *L. donovani*. The resistance in parasites was stable without drug pressure up to 8 passages. The AS-R parasites showed adequate growth in the absence and presence of ART (25 μM). However, WT parasites showed reduced growth in the presence of ART (25 μM) (Fig. 1a).

ART susceptibility of AS-R parasites at promastigote stage The AS-R parasites, at promastigote level, showed 3.73 fold increase in IC_{50} (mean $IC_{50} = 78.63 \pm 9.17$ μM) as compared to WT (mean $IC_{50} = 21.08 \pm 3.15$ μM) and 1.80 fold increase in IC_{90} value (mean $IC_{90} = 254.94 \pm 14.8$ μM) in comparison with the WT isolates (mean $IC_{90} = 141.44 \pm 1.82$ μM) for ART (Fig. 1b).

ART susceptibility of AS-R parasites at amastigote stage

Susceptibility towards ART was also determined at amastigote stage. An increase of > 3 fold in IC_{50} (mean $IC_{50} = 73.09 \pm 1.14$ μM) and approx. 2 fold increase in IC_{90} (mean $IC_{90} = 119.39 \pm 7.21$ μM) value was observed at intracellular amastigote level as compared with WT parasites (mean $IC_{50} = 21.62 \pm 3.24$ μM and mean $IC_{90} = 58.91 \pm 9.02$ μM) (Fig. 1c).

Comparison of ART susceptibility of parasites from day 3 to day 6 of growth The comparative sensitivity of -WT and AS-R *L. donovani* towards artesunate was determined daily from day 3 to day 6. The IC_{50} values for WT isolate were 4.60 ± 0.33 μM (day 3), 14.27 ± 0.13 μM (day 4), 23.38 ± 0.23 μM (day 5), and 24.41 ± 1.55 μM (day 6). Similarly, the IC_{50} values AS-R isolates were 34.86 ± 3.09 μM (day 3), 51.59 ± 1.36 μM (day 4), 86.65 ± 0.48 μM (day 5), and 83.63 ± 3.06 μM (day 6) (Fig. 1d). Thus, an increase in IC_{50} of parasites from day 3 (early log phase) to day 5 (end of log phase) was observed indicating that the early log phase parasite is highly susceptible to ART. On day 6 (stationary phase), both WT and AS-R parasites showed similar IC_{50} as on day 5 Fig. 1d.

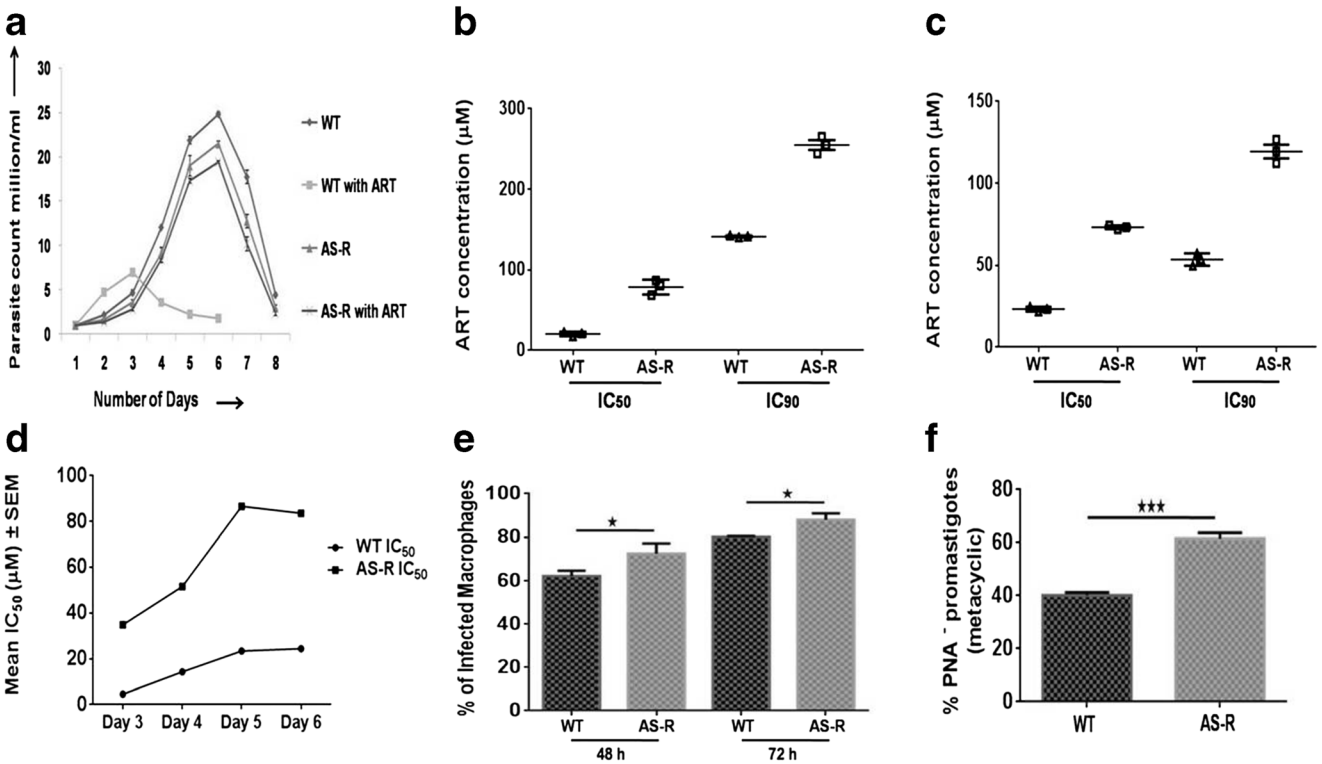

Fig. 1 Selection and characterization of ART-resistant *Leishmania* parasites. **a** Growth profile of cultured AS-R and WT promastigotes in presence or absence of ART. Error bars indicate standard error of mean (SEM) based on 3 independent experiments. **b** Sensitivity profile of AS-R and WT parasites towards ART at promastigote and **c** amastigote stage. IC_{50} and IC_{90} values are the mean of three independent experiments performed in triplicate. **d** Comparative susceptibility of WT and AS-R parasites at early and late log phase of growth. IC_{50} values represented are the mean ± SEM of three independent experiments performed in quadruplicate. **e** Infectivity of WT and AS-R parasite. Mice peritoneal-derived macrophages infected with WT or AS-R parasites at a 1:10 (cell/parasite) ratio. The percent infectivity was determined at 48 h and 72 h post-infection by counting number of infected cells out of 500 macrophages at × 1000 magnification after staining with Diff-Quik. Data represents mean ± SEM of three independent experiments each in duplicate. **f** Percent metacyclogenesis of promastigote population estimated based on negative selection of peanut agglutinin (%PNA^- promastigote). Values represent mean ± SEM of three independent experiments

Infectivity of WT and AS-R isolates

The infectivity of AS-R parasites was higher than that of WT parasites as evidenced by the percentage of infected macrophages at both 48 h (AS-R = 72.4 ± 2.74, WT = 62.03 ± 1.47, $P < 0.05$) and 72-h post-infection (AS-R = 88 ± 1.73, WT = 80.2 ± 0.23, $P < 0.05$) (Fig. 1e).

Metacyclogenesis in WT and AS-R isolates

The percent of metacyclic promastigotes was determined based on negative selection with PNA in culture. A significantly higher (1.53 fold; $P < 0.001$) proportion of metacyclic promastigotes was observed in AS-R parasites (61.50 ± 1.29%) compared with WT (40.12 ± 0.64%) (Fig. 1f).

Fitness of WT and AS-R towards nitrosative and oxidative stress

Responses of the WT and AS-R strains towards oxidative and nitrosative stress were compared. Both WT and AS-R parasites were similarly susceptible ($p = 0.33$) towards H_2O_2 (mimicking oxidative stress) with mean IC_{50} values of 167.39 ± 0.76 μM and 178.16 ± 2.3 μM, respectively (Fig. 2a). However, AS-R promastigotes were significantly ($P < 0.0001$) more tolerant to SNAP (NO donor) than WT promastigotes. The resistant parasites were > 8 fold more tolerant to NO stress, with a mean IC_{50} of 442.67 ± 40.01 μM, in comparison with the WT strain (IC_{50}, 50.25 ± 4.24 μM) (Fig. 2b).

Complement-mediated lyses

The AS-R parasites showed higher tolerance (1.68 fold) to complement-mediated lysis than WT parasites. The mean IC_{50} for AS-R was 3.05 ± 0.06% while the mean IC_{50} for WT was 1.81 ± 0.03% (Fig. 2c).

Modulation in production of ROS and NO upon infection

There was no significant difference in ROS levels produced by WT or AS-R infected mice PECs. However, ROS levels were significantly increased in both WT and AS-R infected macrophages upon addition of ART drug. Moreover, ROS levels in AS-R-infected macrophages were significantly lower ($p < 0.0001$) as compared with WT-infected macrophages in the presence of ART (Fig. 3a). NO production in AS-R-infected mice macrophages was significantly lower (1.58 fold; $p < 0.0001$) in comparison with WT parasites–infected macrophages (Fig. 3b).

Modulation in generation of Th1/Th2 cytokines upon infection

Th1/Th2 cytokine levels in culture supernatant of WT or AS-R-infected and LPS-stimulated mice PECs were evaluated. There was a significant reduction (2.95 fold; $p = 0.0005$) in IL-2 levels in AS-R infected macrophages (5.49 ± 0.11 pg/ml) compared with WT-infected macrophages (16.19 ± 1.04 pg/ml). IL-10 level was significantly higher ($p = 0.0046$) in AS-R-infected macrophages (42.08 ± 0.29 pg/ml) as compared with WT-infected macrophages (35.54 ± 1.21 pg/ml). The level of TNF-α in WT-infected macrophages was 39.21 ± 0.98 pg/ml; however, it was undetectable in AS-R-infected macrophages (Fig. 3c). IFN-γ, IL-12 and IL-4 levels were not detectable in either WT- or AS-R-infected macrophages.

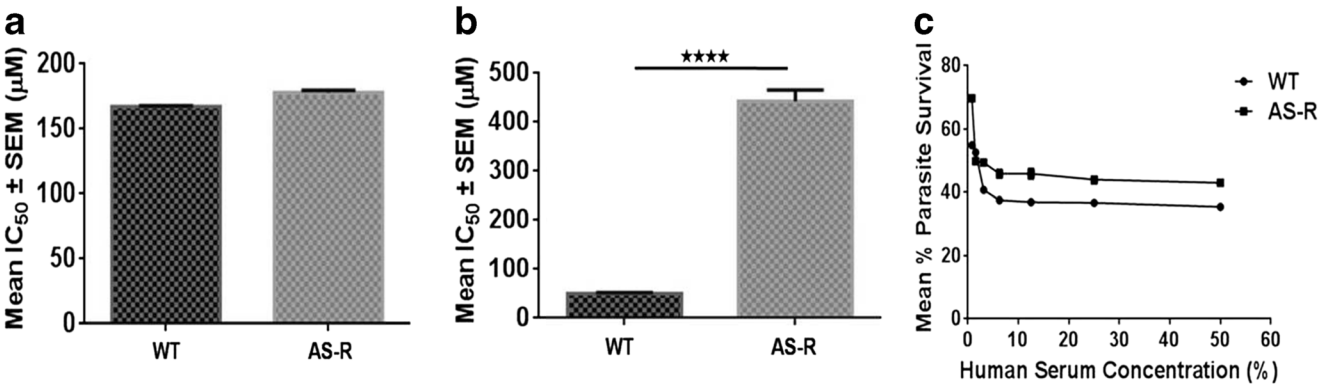

Fig. 2 In vitro susceptibility of ART resistant and sensitive parasites towards oxidative stress, nitrosative stress and complement-mediated lysis. **a** In vitro susceptibility of WT and AS-R isolates to H_2O_2 (oxidative stress) and **b** SNAP (nitrosative stress) at the promastigote stage was determined using the resazurin assay. Each bar represents the mean IC_{50} ± SEM of two independent experiments performed in quadruplicate. **c** Complement-mediated lysis both WT and AS-R parasites were incubated with fresh human serum, and parasite survival rates were measured at different serum concentrations. The assay was performed twice in quadruplicate. Values given are mean percent survival ± SEM

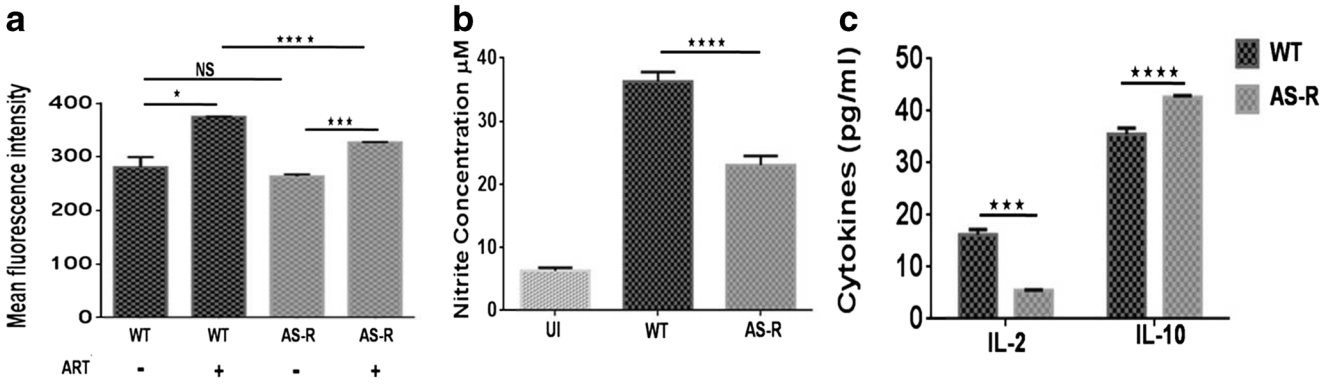

Fig. 3 Assessment of ROS, NO, and Th1/Th2 cytokines levels in WT- and AS-R-infected macrophages. **a** Accumulation of ROS in mice peritoneal macrophages infected with WT or AS-R parasites with or without ART exposure (20 μM). **b** Levels of NO produced by WT and AS-R-infected macrophages. **c** Th1 and Th2 cytokines levels in WT- and AS-R-infected macrophages. IL-2 and IL-10 levels were in detectable amount and represented here. Data represents mean ± SEM of three independent experiments, each in triplicate (one asterisk (*) indicates $p \leq 0.5$, three asterisks (***) indicate $p \leq 0.001$, four asterisks (****) indicate $p \leq 0.0001$)

Gene expression analysis

The expression of FAZP (prefoldin homolog of *Plasmodium*) and METase (HSP 40 homolog of *Plasmodium*) was upregulated by 3.55 and 1.67 fold, respectively, in ART resistant parasites. Bip (HSP 70 homolog of *Plasmodium*) showed 3.36 fold downregulated expression in AS-R parasites as compared with WT parasites. However, expression of TCP 1 and cyclophilin 2 (peptidyl-prolylcis-trans isomerase homolog of *Plasmodium*) was similar in WT and AS-R parasites **(**Fig. 4**)**.

Discussion

ARTs have potent antileishmanial activity and can be an effective treatment option in the future. Studies aimed towards understanding the mechanism of action/resistance to ARTs have been mostly confined to *P. falciparum*. The present study aimed to unravel the possible mechanisms of resistance towards this drug in *L. donovani* parasites. The experimental ART-resistant parasites showed decreased susceptibility to ART at both promastigote and amastigote stages. *Leishmania* parasites at early log phase were more susceptible to ART as compared with parasites at late log phase of growth, similar to the observation in *P. falciparum*, where an early ring stage of the parasite was hypersensitive to ARTs, although the reason was unidentified (Klonis et al. 2013).

Drug resistance in *Leishmania* holds a strong association with parasitic infectivity. Previous studies have reported that drug-resistant *L. donovani* strains have higher virulence as compared with drug-sensitive parasites (Rezai et al. 1969; Deep et al. 2017). In the present study, we observed significant increase in population of metacyclic promastigotes and higher infectivity of ART-resistant parasites in comparison with ART-sensitive parasites, suggesting association of virulence with ART resistance.

The role of the complement system in the eradication of promastigotes in the bloodstream of an infected host is well established (Vanaerschot et al. 2011). ART-resistant *Leishmania* parasites had greater survival capacity against complement-mediated lysis, similar to that observed in antimony and paromomycin resistant parasites (Ouakad et al. 2011; Bhandari et al. 2014). The drug-resistant *Leishmania* parasites have been reported to be more tolerant to macrophage-killing mechanisms (Carter et al. 2005; Bhandari et al. 2014). AS-R parasites significantly modulated NO levels in infected macrophages and showed higher tolerance to nitrosative stress in comparison with WT parasites. In presence of ART, significant decrease in ROS level was observed in AS-R-infected macrophage as compared with WT-infected macrophages.

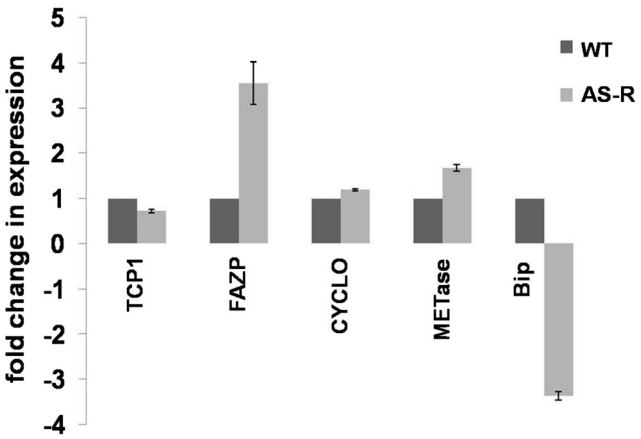

Fig. 4 Expression analysis of TCP1, FAZP, Cyclo 2, METase, and Bip in WT and AS-R isolates. Real-time quantitative PCR expression analysis of TCP1, FAZP (TCP-1 ring complex) and Cyclo 2, METase, and Bip (reactive oxidative stress complex) in *L. donovani* was performed using GAPDH and CBS as internal controls. The graph shows the expression index, defined as gene expression relative to WT parasites. Data represent the mean ± SEM of the results of three independent experiments performed in triplicate

AS-R *Leishmania* infection in macrophages resulted in enhanced proinflammatory response as evidenced by significant decrease in IL-2 and increase in IL-10 in comparison with WT parasite infection. Collectively, these findings suggest that ART-resistant *Leishmania* parasites successfully capitalized host defense mechanism to favor their survival within host macrophages.

The unfolded protein response (UPR) is an important set of signaling events that protect cells from pharmacological or environmental stress. In *Plasmodium*, ARTs resistance is associated with increased UPR (Paloque et al. 2016) and upregulated mRNA expression of genes of PROSC and TRiC was observed in ART-resistant malaria parasites (Mok et al. 2011, 2015). In the present study, ART-resistant *Leishmania* exhibited upregulated expression of FAZP (prefoldin, TRiC) and METase (HSP 40 PROSC) while expression of Bip (HSP 70, PROSC) was downregulated. Prefoldin is a key regulator of cell morphogenesis in *Trypanosoma brucei* which is linked to pathogenicity and proliferation in both the mammalian host and insect vector (Sunter et al. 2015). The increased infectivity and metacyclogenesis of AS-R parasites observed in this study may be associated with upregulated expression of FAZP in AS-R *Leishmania*. HSPs have been shown to play important role in pathogenesis of protozoan parasites such as *Leishmania* (Wiesgigl and Clos 2001; Reiling et al. 2006), *Trypanosoma* (Graefe et al. 2002), and *Plasmodium* (Banumathy et al. 2003). HSP 40 is believed to stimulate the ATPase activity of HSP 70 and act as the primary substrate recruiter for HSP70. Further, it prevents unfolding and misfolding of proteins in protozoan parasites (Seraphim et al., 2014). Upregulated expression of HSP 40 may be an adaptation of AS-R parasites to prevent unfolding and misfolding of proteins under drug-induced stress. HSP 70 is involved in housekeeping role of protein folding and refolding in non-stressed cells; however, under stressed conditions, it is essential for protection and survival of the cells (Requena et al. 2015). In *T. gondii*, HSP 70 plays an important role in evading host proinflammatory responses and thereby contributes to its virulence (Dobbin et al. 2002). In *Leishmania* parasites, HSP 70 is involved in resistance to macrophage-induced oxidative stress (Miller et al. 2000). AS-R parasites were not superior to WT parasites in terms of fitness towards oxidative stress; this may be attributed to the downregulated expression of Bip in drug-resistant parasites.

Researchers have investigated the utility of artemisinin for treating *Leishmania* infection in in vivo mouse model and found that its nanoliposomal formulation is highly effective in treating the infected animals (Want et al. 2015, 2017). The present study has characterized the infectivity and fitness of artemisinin-resistant *Leishmania* parasite in vitro using mouse peritoneal macrophages. Further studies on characterizing the infectivity and resistant nature of ART-resistant *Leishmania* parasite may be explored using in vivo model. Based on the observation in the present studies regarding infectivity and host cell immunomodulation using mouse peritoneal macrophages model, it is expected that the AS-R parasite, with increased metacyclogenesis potential, will be highly infective and may cause the exacerbated infection in the animal model. The emergence of artemisinin-resistant *Leishmania* parasites in endemic area following the use of artemisinin for treatment of leishmaniasis seems plausible and may pose a serious threat.

To conclude, this is the first study to explore resistance towards artemisinin in *L. donovani* and demonstrate that ARTs resistance in *Leishmania* is a multifactorial phenomenon. ART-resistant *L. donovani* is more virulent, modulates host cell defense mechanism, and exhibits modulated expression of genes involved in unfolded protein response, compared with sensitive parasite. The present study provides artemisinin-resistant *Leishmania* parasite as an experimental model for investigating various aspects of resistance due to artemisinin.

Author's contribution Conceived and designed the experiments: AV, RS, PS. Performed the experiments: AV, SG. Analyzed the data: AV, SG, RS. Contributed reagents/materials/analysis tools: RS, PS. Wrote the paper: AV, RS, PS. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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