# Using LEXSY plasmids for sodb1 gene silencing in Leishmania tropica parasites

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# Abstract

**Background**: Genetic modifications and manipulations have topped the list of recent research, as they target the causes, not the results, and are currently considered one of the most powerful methodologies used to study the biology of the *Leishmania* parasites. These methods have expanded since the publication of the first study in which the genetic replacement of one of the *Leishmania* genes was done, which provided an opportunity to analyze and study the biology of the parasite genomically.

In this study, the SODB1 gene was targeted by antisense RNA. This gene encodes one of the important enzymes in the infectivity of *Leishmania tropica* parasites within macrophages, which is the superoxide dismutase enzyme (SODB1).

**Methods**: An inverted sequence of part of SODB1 ORF and 3'UTR were cloned in LEXSY plasmid, and after obtaining the silencing constructions, the *Leishmania tropica* parasites were transfected by electroporation. Western Blot analysis of SODB1 expression and the infectivity of these mutant parasites in human macrophages was studied and evaluated in comparison with a wild-type negative control and another control containing the GFP gene that codes for Green Fluorescent Protein (GFP).

**Results**: The results showed that using LEXSY plasmids for SODB1 gene silencing was efficient and the knocking-down was clear forasmuch the decrease in both infectivity and parasite load in human macrophages in vitro. On the other hand, western blot analysis revealed a lower expression level of SODB1 in the mutant parasites than wild-type. The results were subjected to statistical analysis and the decreases in the infectivity and parasites load of the mutant parasites were very significant in comparison with the wild-type.

**Conclusion**: Our study confirms the efficiency of the produced silencing system, and the importance of the SODB1 enzyme in the amastigote parasites' ability to grow and survive within the host's macrophages despite the presence of many other SODs enzymes. This is the first study that affirms the success of using the LEXSY gene expression system for gene silencing according to the antisense RNA approach. Thus, this work will allow using of the produced silencing system to target other important genes in *Leishmania*, and continue studying the knocked-down strain in vivo.

# Background

Genetic manipulation in protozoa such as *Leishmania* parasites enables us to analyze deeply the consequences of genetic changes and their impact on many aspects of the parasite's life. Especially, in exploring the immune responses formed during infection. The use of genetically modified *Leishmania* parasites can solve the mystery of the parasite's contribution in pathogenesis, cellular mechanisms, host-parasite interaction, and the responses of the innate and adaptive immune systems against this pathogen [1]. The possibility of modeling attenuated parasites has been readily available forasmuch the efficiency of the modification processes, the availability of various vectors for genetic manipulations, and due to the abundance of sequences on bioinformatics websites that provide different databases [2].

These mutant parasites which genetically manipulated could serve to investigate the efficacy of new drug targets, and can also be candidates for use in vaccines, depending on the targeted genes.

Through these modifications, self-genes or external genes can be inserted. This could be either by episomal vectors or by genome integrated vectors. In the episomal vectors, the ectopic gene expression is temporary, because this type of expression is under the control of the vector's promoter and it can be inducible in certain conditions. As for the case of integrated vectors, the inserted genes are directed to merge within a specific region that is located downstream of rRNA promoter site in the genome, to study the effects of the persistent expression during all stages of parasite life and in subsequent cells resulting from its division [3]. This genetic manipulation can be made to target a gene family or multiple different genes at the same time, through the concept of replacement that was generally according to the principle of Homologous Recombination [4]. This methodology has shown great success in elucidating the functions of several *Leishmania* genes.

The *Leishmania* expression system (LEXSY) is a protein expression platform developed for *Leishmania tarentolae* and was designed to combine eukaryotic protein synthesis, folding and modification with higher growth rates comparison with mammalian cells. In Vivo, LEXSY is available in two principal types of expression: constitutive (persistent) or inducible. The constitutive system leads to efficient and consistent production of proteins. It is based on the integration of an expression cassette into the highly repeated chromosomal 18S rRNA (ssu-locus). This site of integration will permit persistent transcription by the endogenous RNA Polymerase I [5, 6].

Genetically, after years of knock-out attempts, only about two hundred genes out of the 9,000 genes in *Leishmania* have been targeted [7]. But Recently, with the discovery and application of the CRISPR-Cas9 system, the number of targeted genes has increased dramatically. This system is a very efficient and time saver for studying different genes and their functions by knocking out them in previously transfected parasites for expressing Cas9 protein [8]. Genes also can be targeted by knocking down using the antisense RNA (asRNA) approach, which leads to repressing the translation process to some extent. This mechanism was first reported in 1984 in *E.coli* bacteria [9]. It has been applied as a technology that can control the gene expression processes, and affect the nature of the studied organism such as the *Leishmania* parasite without making any genomic modifications at the genome level [10]. asRNA sequence is engineered to retard the ribosome binding site (RBS), and to bind the initiation codon of the target mRNA [11]. This specific region is important for initiating translation and thus will prevent the ribosome from binding to the RBS site of the target mRNA. Furthermore, the target mRNA blocked by asRNA may tend to be rapidly degraded in the cell by nuclease enzymes [12]. The efficiency of asRNA varies greatly depending on the target gene, and in general, it is low [13]. Regression and loss of silencing are possible to occur, when culturing and growing for a long time and that's, in the case when using an episomal vector [14]. To get a successful silencing, the asRNA transcribed by the plasmid vector must be hybridized with the target mRNA, this process will impede the translation of the target protein [15], as well as by increasing the number of transcribed copies of asRNAs in the cell, the higher number of copies achieve the higher silencing efficiency [16].

A set of the crucial proteins in *Leishmania* are Iron Superoxide dismutase enzymes, these proteins are metalloenzymes that act as antioxidants and protect the cell by converting the superoxide radical into $H_2O_2$ [17, 18]. Then peroxidoxine enzyme continues the reaction to get rid of $H_2O_2$ by converting it to water and molecular oxygen (O2) [19, 20]. SOD enzymes form a large family of proteins in most organisms, that use metals such as manganese, iron, nickel, copper and zinc as cofactors [21]. In *Leishmania*, FeSODs were classified into SOD-A which is expressed in the mitochondria as dimeric form, glycosome-specific SODB1 andSODB2 enzymes, and SOD-C recently detected in the mitochondria[18]. The genes encoding SODB1, SODB2, are organized tandemly on the same chromosome, their sequences show a significant similarity of more than 92% in several species of *Leishmania* parasites (*L.major, L.chagasi, L.donovani*). In addition, the amino acid sequences of SODB1 and SODB2 are approximately 90% similar, with the main difference between them due to the presence of about 13 codons at the 3' end of the SODB2 gene [22]. When *Leishmania* parasites infect the host's macrophages, these macrophages go through consequent respiratory oxidation process producing ROS intermediates such as: $H_2O_2$, OH. radicals, O2.$^-$ and peroxynitrite, as a part of the oxygen-dependent mechanisms. these mechanisms destroy the invading pathogens of phagocytes [23]. It can also activate NO, which is a small organic radical with a strong antimicrobial effect [24, 25]. In humans, the reactive oxygen species (ROS) dominates the elimination weapons of *Leishmania* parasites. In the early stages of infection after phagocytosis, phagocytes release lethal particles such as O2.$^-$ and $H_2O_2$ [26−28], so that, FeSODs could play a crucial role in the parasite's survival.

Although macrophages are the primary host facing the flagellated parasites, neutrophils and dendritic cells can also recognize and phagocytose these parasites [29].

In this study, *Leishmania tropica* parasites were transfected by two linear cassettes of LEXSY plasmid, the first contained a reporter gene that encodes the green fluorescent protein (GFP), and the second contained a cloned specific designed adverse sequence of SODB1 gene so that it was integrated into the genome in the opposite arrangement, and the antisense RNA molecules could lead to repression of gene expression either by inhibiting the initiation of translation or by stimulating RNase enzymes to degrade the target mRNA [30], then we evaluated the efficiency of the SODB1 down regulating in the transfected strains by western blot analysis followed by infectivity experiment of human peripheral blood macrophage cells (PBMC).

# Methods

# Parasite sampling and genotyping:

# Cultivation and genotyping of Leishmania:

Samples were collected from a positive patient for Cutaneous Leishmaniasis (CL) from the Dermatology Hospital in Damascus with verbal consent. Aspirated samples were taken from lesions for culturing at

26°C in a culture medium (RPMI/10% FBS/ Pen-Strep1x) [31]. Genomic DNA was extracted from 2ml of *Leishmania* parasites suspension (~ 30×10$^6$ cell/ml) using Wizard Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instructions. Then Polymerase Chain Reaction (PCR) was carried out according to a commonly used protocol in *Leishmania* species genotyping [32]. Another genomic DNA was isolated from reference strains *L.major* (MHOM/SY/91/LEM2397) and *L.tropica* (MHOM/SY/90/LEM2066) was used as a positive control (Reference strains are a gift from French National Reference Centre for *Leishmania*, Montpellier, France).

# Preparing the antisense construct:

# amplifying the target sequence:

A couple of primers 5'-ATATCTCGAGGAGGGAGATTTCACTTCTGGC-3' and 5'-TATAGGTACCGAGCCATCGGGCAATTTGG-3' was designed using (uber Geneious 4.8.4) according to *L.major* genomic DNA (Accession Number: FR796428.1). these primers are amplifying a sequence of around 671bp which contains 331bp from SODB1 ORF and 340bp from 3'UTR. 3' and 5' adapters for the restriction enzymes (*XhoI/KpnI*) were added to the primers to ensure the insertion of this sequence at its inverted orientation into the vector. To amplify the target sequence a Polymerase Chain Reaction (PCR) was performed on the genomic DNA of *L.tropica* strain, using other primers that targeted a sequence of the rRNA-18S gene as positive control. The total volume of PCR reaction is 50µl including 25µl (1×) master mix (Vivantes, Malaysia), 1µl (10 µM) of each primer, and 0.3µg of DNA template. DNA amplified for 35 cycles after initial heating at 95 °C for 5min. Each cycle was divided into three stages: denaturation (95 °C – 30sec), annealing (50 °C – 30sec), and elongation (72 °C–1min). Then one cycle of the final extension was performed at 72°C for 7 min. PCR products were visualized by electrophoresis on 1% agarose gel. The expected length of the amplified PCR products was about 671bp.

# Insertion of target sequence into pLEXSY-neo-2.1 expression vector:

The pLEXSY-neo-2.1 and pLEXSY-GFP-sat2.1 expression vectors (Jena bioscience, Germany) was replicated in *E.coli Top10* and a mini prep process was performed (Genedirex, Taiwan). The target sequence and the cloning plasmid pLEXSY-neo-2.1 was digested by (*XhoI/KpnI*) restriction enzymes. then the trimmed target sequence was purified by PCR Clean up kit (Vivantes, Malaysia), and digested plasmids were extracted by Gel extraction kit (Invitrogen, USA) after agarose gel electrophoresis had been carried out. thereafter, a ligation reaction was performed with T4 DNA Ligase (Thermo, UK).The reaction product was inserted into *E.coli* TOP10 strain by heat shock transformation [33]. The transformation and cloning of plasmid construction were confirmed by colony PCR, restriction sites analysis, and sequencing (Macrogen, Korea). The sequencing was carried out by using LEXSY primers (P-1442/A264) according to the manufacturer's instructions (Jena bioscience, Germany). The results were compared with three *Leishmania tropica* strains sequences retrieved from the GeneBank database (KY366316, CM024318, OM718775).

Transfection of Leishmania:
# Preparing the vectors for the recombination:

The two vectors pLEXSY-GFP-sat2.1 and the cloned vector pLEXSY-neo-2.1 was digested with *SwaI* enzyme (Thermo, Lithuania) to get a linear cassette for GFP expression and for SODB1 silencing tools, respectively. The amount of each vector was 17 µg digested by 50 U of the enzyme and the final volume was 60 µl. these reactions were incubated at 37°C overnight. both reactions then have heated at 95°C for 15min before transfection.

# Transfection of parasites by electroporation:

*Leishmania* parasites were grown to mid-log phase at a density of (80-100$10^6$×cell/ml) and 460 µl from this suspension was transfected with 17µg/60µl of digested plasmid pLEXSY-neo-sodb1, and the plasmid pLEXSY-GFP-sat2.1 by electroporator ECM-399 (BTX, USA), these parasites pulsed twice at 2200V with 10sec between the two pulses (pulse time 1 m.sec), then the parasites were chilled on ice for 10min and then incubated at 26°C in 5ml RPMI medium supplied with FBS 10% and Pen/Strep 1x.

# Selection of transfected strains:

After 24h of transfection, the cultures get turbid, and the selective antibiotics were added at the recommended final concentration, LEXSY neomycin at 50µg/ml to *L.tropica* transfected with pLEXSY-neo-sodb1 (Lt-asSOD), and Nourseothricin at 100µg/ml to *L.tropica* transfected with pLEXSY-GFP-sat2 (Lt-GFP), side by side with two wild-type cultures to each transfected parasite culture. The GFP fluorescence was detected using a fluorescent microscope (Olympus, Japan) for the promastigote parasites and then for the amastigotes after incubation of the promastigotes at 37°C for 48h [34]. The parasites were incubated at 26°C and examined daily, and every 5 days centrifuged at 2000g/3min then renewed the medium by adding the appropriate antibiotic. 10 days later, the wild-type parasites dead so the medium was clear, and the live transfected parasite cultures still get turbid. The antibiotics were added twice after this step.

# Confirmation of genomic integration by PCR:

To confirm the integration of the silencing cassette into the chromosomal 18S rRNA (*ssu*) locus, a diagnostic PCR using LEXSY special primers (F3001 + A1715) was done. One of these primers hybridizes within the silencing cassette, and the other primer hybridizes to a *Leishmania* chromosomal ssu-flanking sequence not present on the cassette. For more assurance, another PCR reaction was done with LEXSY primers (P1442 + A264) to confirm the inserted sequence in the cassette. All of these PCR reactions were done according to a program recommended by (JenaBioscience, Germany). Then the products were electrophoresed on 1% agarose gel.

# Evaluation of target protein expression:

Promastigotes from the three strains (wild-type, Lt-asSOD, and Lt-GFP) were washed twice by PBS1x and then subjected to lysis buffer (NP-40 0.1%, NaCl 150mM, Tris-Hcl 10 mM, EDTA 1mM, Aprotinin 0.1U/ml,

pH = 7.5) and chilled on ice with shaking for 1h. The extracts were calibrated directly by Bradford assay, then equal amounts of proteins from wild-type and transfected parasites were electrophoresed by SDS-PAGE. Western blot analysis was performed with polyclonal rabbit antibodies against SODB1at 1/1000 dilution [35]. The secondary antibodies used were alkaline peroxidase-conjugated goat anti-rabbit IgG at 1/3000 dilution, Bromo-4-chloro-3-indolyl-5, and Nitro blue tetrazolium phosphate were the substrates. The blots were analyzed and quantified by ImageJ free software.

# Studying the growth curve of the wild-type and transfected parasites:

Parasite cells in all cultures were counted daily after fixation of 25µl from each culture with an equal volume of formaldehyde 0.1% in PBS, and the growth curves have been plotted for them.

# Macrophage infectivity assay:

Human mononuclear cells were isolated from the peripheral blood of a healthy adult male -with written consent- using Ficoll, Histopaque 1077 (Sigma Aldrich, US) and cultured in two micro slides in IMDM medium containing 10% FBS. Adherent cells were incubated at 37°C to differentiate into macrophages during 12 days. Then the macrophages were infected by the stationary phase of the wild-type and transfected parasites (5 parasites per macrophage) for 4h. Unbound parasites were removed by washing with PBS 1x, then a fresh medium was added and the cells incubated till two-time points: the first for 24h, and the second for 36h. then the cells were fixed and stained with 4% Giemsa stain. the cells were counted at 1000x magnification. This experiment was repeated under the same conditions at a completely distinct time. statistically, $P$-values for the infectivity percentage were detected by the Chi-square test, and the macrophage parasite load was detected by two-way ANOVA using GraphPad prism7 software. Statistical significance was set at p < 0.05. The p-values were reported as: ns (p > 0.05), * (p ≤ 0.05), ** (p ≤ 0.01), *** (p ≤ 0.001), and

**** (p ≤ 0.0001).

# Results

# Parasite sampling and genotyping:

Genotyping was accomplished using PCR reactions and the results were considered positive for *L.tropica* species, the PCR product size was identified with the PCR product size of the reference strains of *L.tropica* (Supplementary Fig. 1).

# Preparing the antisense construct:

The cloning was confirmed by colony PCR reactions. the results were positive for many colonies (Supplementary Fig. 2), also the restriction sites analysis was positive when a band of ~671bp was observed. sequencing results showed 97.5% nucleotide similarity with the ORF of the *sodb1* gene that

belongs to the Syrian strain (KY366316) (Supplementary Fig. 7), And 88.6% when compared with the sequence (ORF + 3'UTR) that belongs to the Afghani strain (CM024318) (Supplementary Fig. 8). On the other hand, The alignment revealed 100% nucleotide identity when comparing the total silencing cassette sequence to another sequence has accession number (OM718775) belonging to the same Syrian *L.tropica* strain early isolated in this study (complete CDS of SODB1 + 340 nucleotides of 3'UTR region), which was submitted to the GenBank of NCBI previously by the authors (Supplementary Fig. 6). After the sequencing result, and when the alignment was performed, it was clear that the silencing sequence of *L.tropica* was 640bp after excluding the adapters of the primers (20bp), .and there is deletion of 11 nucleotides in comparison with *L.major.* consequently (Supplementary Fig. 9), the silencing plasmid construction was ready to use and the silencing cassette was located in the appropriate direction.

**Transfection of** Leishmania:

The linear form was confirmed for the two vectors pLEXSY-GFP-sat2.1 and the cloned vector pLEXSY-neo-sodb1, whereas the bands were observed at the expected size after electrophoresis (Supplementary Fig. 3, A and B).

After transfection, the GFP fluorescence appeared in Lt-GFP strain in both stages promastigote and amastigote, when examined under a fluorescent microscope, which is evidence of the success of the expression of the green fluorescent protein in wild-type (Fig. 1). After 12 days the wild-type culture was clear due to the death of the non-transfected cells, on the other hand, the transfected parasite cultures were turbid and the cells checked daily with the microscopy detection.

(Fig. 1)
# Confirmation of genomic integration by PCR:

The integration of the cassettes into the *Leishmania* genome was confirmed whereas the PCR products were observed at the expected size by the electrophoresis (Supplementary Fig. 4, A and B).
# Evaluation of target protein expression:

Polyclonal antibodies against SODB1 protein recognized an 18KDa two bands polypeptide as a result of Western blot applied for the cytosolic fractions extracted from *L.tropica* promastigotes of the wild-type and transfected parasites. There was distinct difference between the clarity of the two bands, whereas the expression in the wild-type was 2.4 higher than Lt-asSOD according to ImageJ software that's what indicates the low level of expression in the Lt-asSOD transfected parasites (Fig. 2).

(Fig. 2)
# Studying the growth curve of the wild-type and transfected parasites:

There were no differences among the three strains (Supplementary Fig. 5). And that's what reveals the ineffective role of the transfection on the growth curve, and that confirms that SODB1protein doesn't interfere with the cells' proliferation, growing period, and the parasites' number at each phase.

# Macrophage infectivity assay:

To determine the effectiveness of the silencing system in knocking-down the transfected and the wild-type strains were used to infect human PBMC, and the percentage of infected macrophages was detected by counting the cells and determining the infectivity and the parasite load at 24 and 36h post-infection at two distinct experiments (Fig. 3).

(Fig. 3)

The infectivity of the macrophages with the wild-type strain was 52.5% after 24h, and 59.6% after 36h. the Lt-GFP strain infectivity was 45.8% and 57.7% at 24 and 36h respectively. While the Lt-asSOD strain infectivity was 21.3% and 15.0% after 24 and 36h respectively. The statistical analysis results showed no significant differences when comparing the wild-type strain with the Lt-GFP strain in macrophage infectivity percentage at 24h and 36h ($p$-value = 0.1345, 0.6131 respectively) and in parasite load at 24h and 36h ($p$-value = 0.9994, 0.8121 respectively). While there were very significant differences when comparing the wild-type strain to the Lt-asSOD strain, the experiment reported $p$-values < 0.0001 at 24h and 36h for the percentage of infected macrophages, and for parasite load it was $p$-value = 0.0002 at 24h and $p$-value < 0.0001 at 36h (Fig. 4). Thus SODB1 knocking-down lead to clear decreased in growth and survival of amastigote parasites within macrophages in vitro.

(Fig. 4)

# Discussion

In this study, a Syrian strain of *Leishmania* parasites was isolated from a CL. disease patient attended the Dermatology Hospital in Damascus, after culturing the parasites in vitro and genotyping it was *Leishmania tropica*. The gene silencing construction was prepared by cloning a specific fragment of the SODB1 gene within the expression vector pLEXSY-neo2.1. The parasites of *L.tropica* were transfected with silencing construction (pLEXSY-neo2.1-SOD) and pLEXSY-GFP-sat2.1 plasmid and selected with the appropriate antibiotics. We verified the success of the recombination within the genome of *Leishmania* parasites by detecting the fluorescence of *GFP* and by PCR reactions. The gene expression of *SODB1* protein was studied using western blotting, and an obvious difference was observed between both the wild-type and the mutant parasites. Finally, the infectivity of wild-type and mutant parasites to human macrophages was studied, and very significant differences were obtained, which clearly shows the efficiency of the produced silencing system in reducing the ability of *Leishmania tropica* parasites to survive and resist harsh conditions within macrophages.

Initially, asRNA technology was chosen over others, based on studies that showed that the parasites of *L.tropica* don't possess the major enzymes (Argonaut and Dicer) of small RNA interference system. In

contrast to species belonging to the subgenus *Viannia* [36].

To prepare the recombinant plasmid for the process of gene silencing, the selected plasmid was designed to use an expression system specific to all kinds of *Leishmania* parasites, it offers constitutive integration into the genome of *Leishmania*. This system is one of the most important systems for protein expression in use today, and many studies have relied on it to express different proteins [37, 38]. In this paper, we depended on the constitutive integration to reduce expression by the antisense RNA methodology. some studies relied on the asRNA methodology and benefited from the advantages of other plasmids, but all of those studies relied on transient vectors, and thus the expression was temporary and limited in the transfected generation only [39–41].

Choosing a two-parts sequence which includes part of the SODB1 ORF and part of 3' UTR was based on some studies that indicated that the target region of the mRNA molecule is not restricted on the translation initiation region (eg. Ribosome binding site, RBS), but also includes parts of the non-coding regions which located at 3'UTR [42, 43], due to the importance of this region as it contains specific sites at the formation of the polyadenylation tail in pre-mRNA maturating. These sites include two important sites of Polyadenylation Signals: The first is the AUAAA character or its less frequent variant form AUUAAA. The second site is the downstream sequence elements (DSE) site, which is represented by G/U rich region or U rich region only. These sites are responsible for the assembly of polyprotein complexes that ensure the maturation of the pre-mRNA molecule into mature mRNA. The formation of the polyadenylation tail affects the export of the mRNA molecule outside the nucleus, as well as ensures correct translation of the mRNA molecule [44, 45]. Another study confirmed the importance of targeting the 3' UTR of mRNA to achieve silencing [46]. During the sequencing process. When comparing the silencing cassette, it was found that there was a high similarity between the ORF part of the cassette and the corresponding sequence of the SODB1 gene belonged to a Syrian isolate, where a slight difference was noticed. Conversely, when comparing the whole cassette with the corresponding sequence of the *L.tropica* Afghan isolate the similarity decreased to 88.6%, and we can return this difference due to the possibility of different genetic sequences within the strains belonging to the same species according to their distribution sites. if we take a closer look, it can be distinguished that most differences were concentrated in the 3' UTR when compared to the afghan *L.tropica* isolate, which includes deletions of some nucleotides. Subsequently, Lt-GFP parasites were selected using the antibiotic Nourseothricin, and Lt-asSOD were selected using the LEXSY Neo which was consistent with a resistance gene included in the corresponded vector.

In both cases, we add the antibiotics twice after the death of the wild-type cells to ensure that the non-transfected cells will not survive. After that, verifying the recombination at the molecular level occurred by using primers hybridize with two regions, the first is located on the genome and the other located on the inserted cassette, that allows verifying the success of the recombination when obtaining the expected PCR reaction product that recommended by the manufacturer. The similar growth phases of cells indicate that LEXSY plasmids, the transfection process, and the studied protein SODB1 don't interfere in cell proliferation, division, and growth in an artificial medium.

western blotting was performed and showed that the expression of SODB1 protein decreased in the transfected parasites compared to the wild-type parasites. Remarkably, each band identified during the western blotting analysis consisted of two bands placed exactly on top of each other. and this could be interpreted by the possibility of polyclonal antibodies to bind proteins that have combined epitopes like the known protein SODB2, whose gene is more than 90% similar to that of the SODB1 [47]. This may allow the silencing to include SODB1 and SODB2 proteins, as they belong to the family of FeSODs enzymes and are very similar. Accordingly, sequencing of the silencing cassette was compared with its ORF only with the same region of SODB2 gene isolated from a Syrian strain and the similarity ratio was 91.4%, and by comparing the coding region sequence with the sequence of SODB2 gene of the Afghan isolate, the identity percentage was 88.9%. While comparing the entire sequence with its coding and non-coding regions with the same gene in the Afghan isolate, the similarity percentage decreased to 68.7%, and this could indicate the possibility of this silencing system affecting both mRNA molecules of the SODB1 and SODB2 genes, especially in strains like the Syrian one where the alignment percentage was higher than the Afghani one, that the asRNA don't need a 100% matching to achieve the knocking-down.

In the western blotting, we noticed the band of protein at 18KDa, and that's different from the expected size in silico which is 22KDa. This result was confirmed in another study where the molecular weight of SODs in *L.tropica* was 15KDa and 22KDa in *L.donovani*. that's may indicate the complex process of protein post-translation modifications and the role of proteases at different levels and different types of *Leishmania* [41]. When studying the ability of the transfected parasites to resist human macrophages, it was found that there were no significant differences between the wild-type strain and Lt-GFP when compared them in the times applied in two separate experiments. This reveals logically why the transfection process did not interfere with infection, as well as the absence of the effect of LEXSY plasmids on the infectivity, and the continuation of the normal and usual work of *SOD* enzymes in wild-type parasites. While we had a significant decrease in the infectivity of Lt-asSOD, this decrease effect increased with the time at a very significant differences p-value < 0.00001 compared to the wild-type parasites. This performs the importance of the *SODB1* protein in the growth and survival of amastigotes within the macrophage host and the parasites' resistance to harsh conditions inside phagocytes, starting at a highly acidic medium, and ending with various oxidizing agents. This explains the weakened ability of Lt-asSOD parasites to get rid of the superoxide radical released by macrophages, due to the insufficient *SODB1* enzymes to confront these oxidizing factors. These results agree with a study that used asRNA method in both *Leishmania tropica* and *L.donovani*, using a temporary expression plasmid and an inverted sequence of a coding part of the target gene and 3'UTR region. It also showed a decrease in the parasite load within macrophages with very significant differences[41].

## Conclusion

Our study conducted the first using of LEXSY plasmids expression vectors at gene knocking-down in *Leishmania* parasites. we can suggest there was an effective pLEXSY system for silencing target genes in *Leishmania tropica* and encourage use in another *Leishmania* species. The results of this research support what other studies have shown about the importance of the presence of SODB1 proteins in both

promastigote and amastigote stages in the life cycle, helping the parasites to adapt to the midgut environment of the sand fly, which is fatal to the parasite when it moves from one stage to another [48], and protecting the parasite inside macrophages. It appears that the possession by *Leishmania* parasites of several members of the family of SODs enzymes, such as SODB1 and SODB2 would be beneficial for the parasite's survival in unsuitable conditions by controlling high levels of endogenous oxidants, and the loss of these enzymes would attenuate the parasite. These organisms could be considered a preliminary form of an attenuated vaccine and need more studies in vitro and in vivo, especially with the failure of attempts to produce a vaccine from attenuated *Leishmania* by irradiation[49], heat [50], and chemical inhibition [51].

# Abbreviations

SODB1
Superoxide Dismutase B1
GFP
Green Fluorescent Protein
asRNA
antisense RNA
LEXSY
*Leishmania* Exprission System
ssu rRNA
small subunit ribosomal RNA
RBS
Ribosomal Binding Site
ROS
Reactive Oxygen Species
WT
Wild-Type
PBMC
Peripheral Blood Mononuclear Cells.

# Declarations

## Acknowledgments

## Author contributions

CS designed and supervised the study. FH and MK conducted the experiments, FH performed the experiments, FH and MK wrote and revised the manuscript. All authors read and approved the final manuscript.

## Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the National Center for Biotechnology Information (NCBI) repository, under these GenBank accession numbers: OM718775.

## Ethics approval and consent to participate

This study was approved by the local Ethics Committee at Damascus University and Health Ministry and complied with the Helsinki Declaration of the World Medical Association (2013), and informed consent was obtained from individuals prior to recruitment and collection of blood samples for monocytes isolation and prior to isolate the parasites from the patient lesion.

## Consent for publication

Not applicable

## Competing interests

The authors declare that they have no competing interests.

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# Figures



-A- Promastigote *L. tropica*-GFP

[400 X] [200 X]
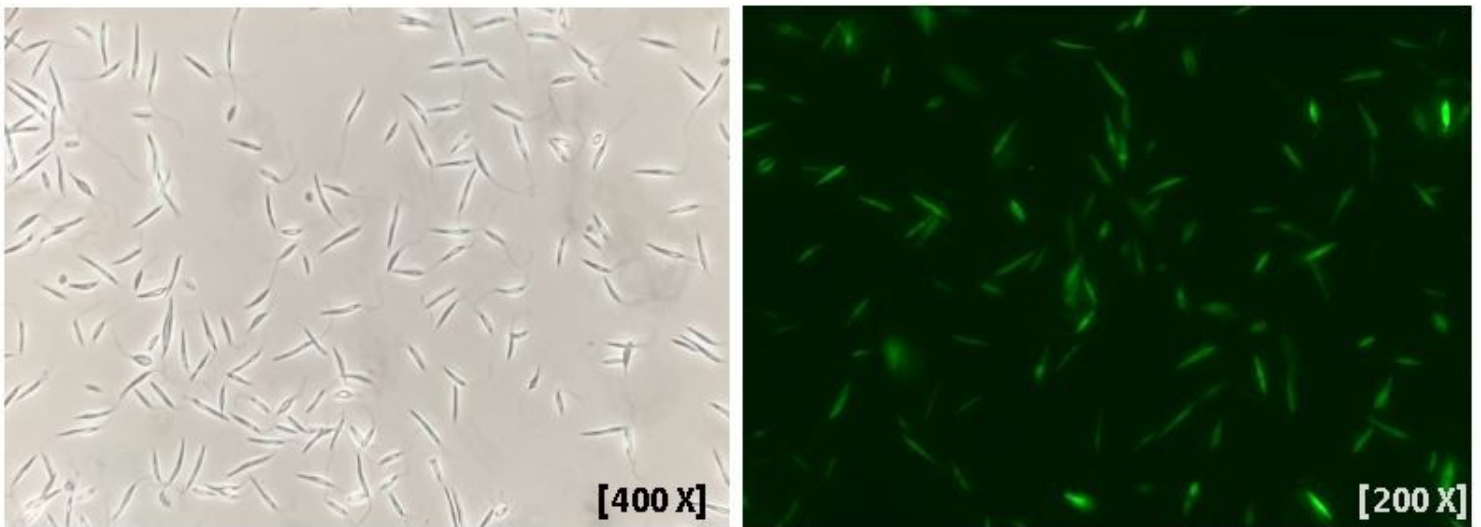
-B- Amastigote *L. tropica*-GFP

[400 X] [400 X]
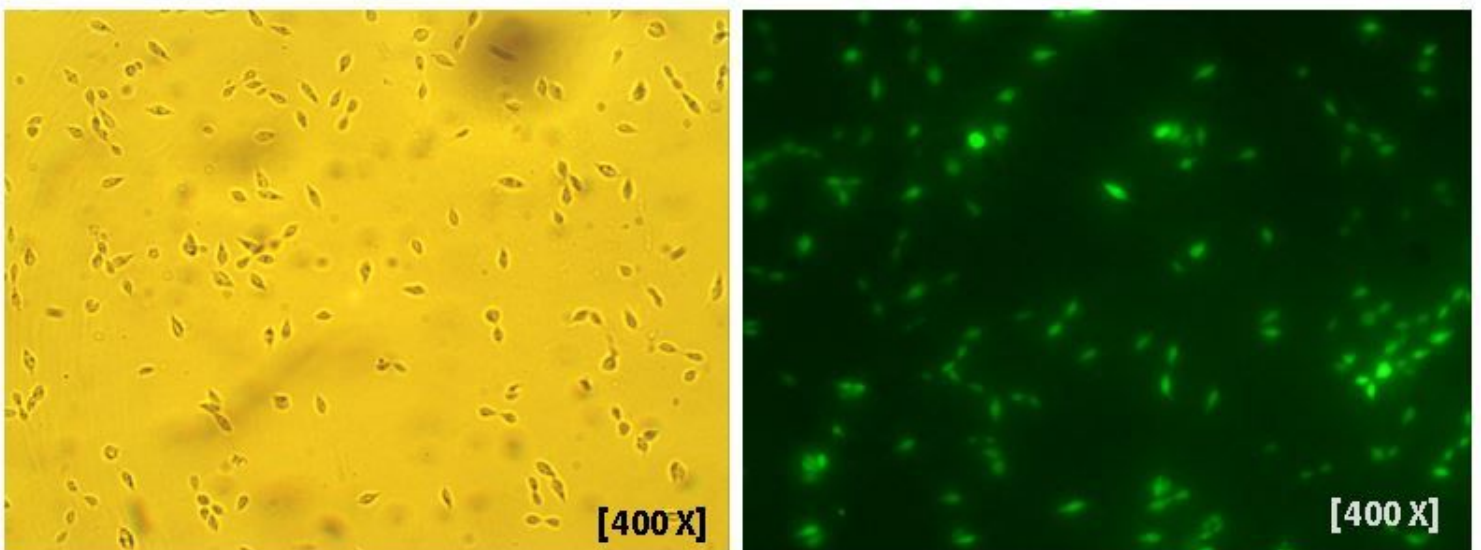
**Figure 1**

transfected *L.tropica* parasites(Lt-GFP) expressing GFP, (A) promastigote, (B) amastigote, were imaged in light microscopy on the left and fluorescence microscopy on the right.
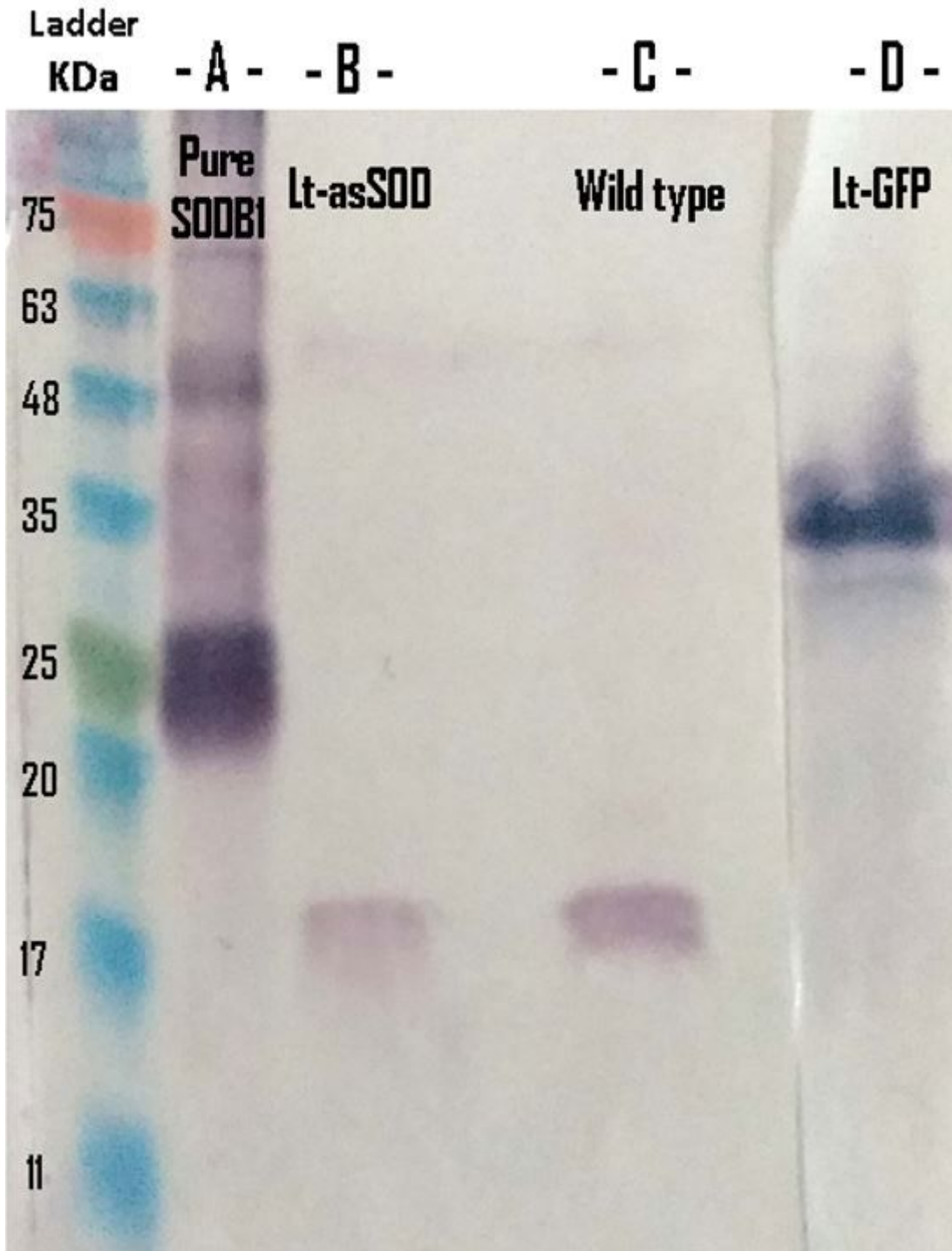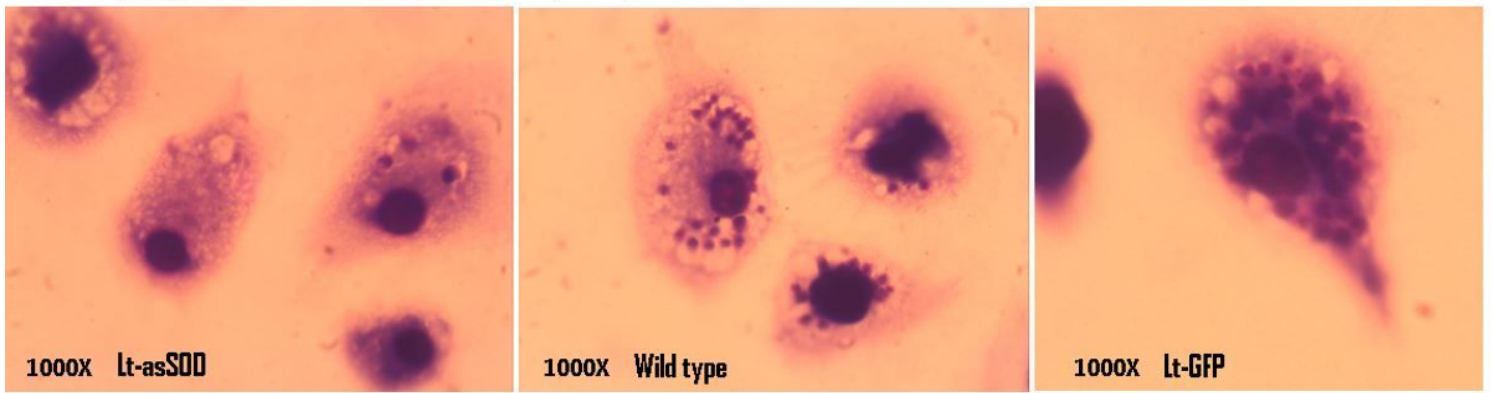
**Figure 2**

the western blot analysis shows the SODB1 protein concentrations. (A) SODB1 protein produced by bacteria BL21(DE3) and purified by affinity chromatography, with Mw = 26 KDa. (B) SODB1 from Lt-asSOD. (C) SODB1 from the wild-type. (D) GFP protein from Lt-GFP with Mw = 27KDa.

**Macrophage cells infected with *L. tropica* parasites after 24 hours of incubation.**



1000X Lt-asSOD
1000X Wild type
1000X Lt-GFP

**Macrophage cells infected with *L. tropica* parasites after 36 hours of incubation.**



1000X Lt-asSOD
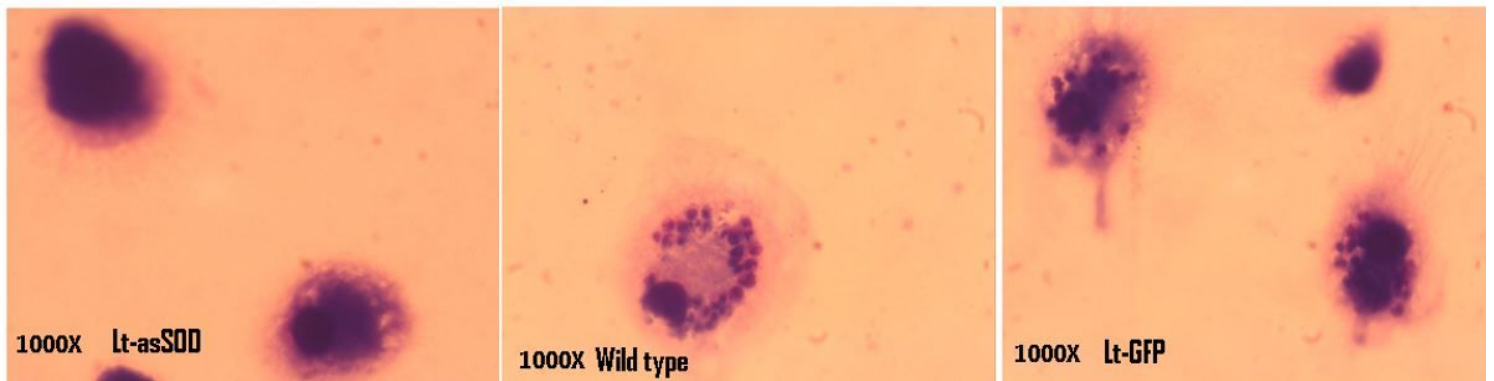1000X Wild type
1000X Lt-GFP

## Figure 3

The microscopic examination of the human macrophages infection by the wild-type and transfected strains Lt-asSOD, and Lt-GFP at 24 and 36 h post-infection.
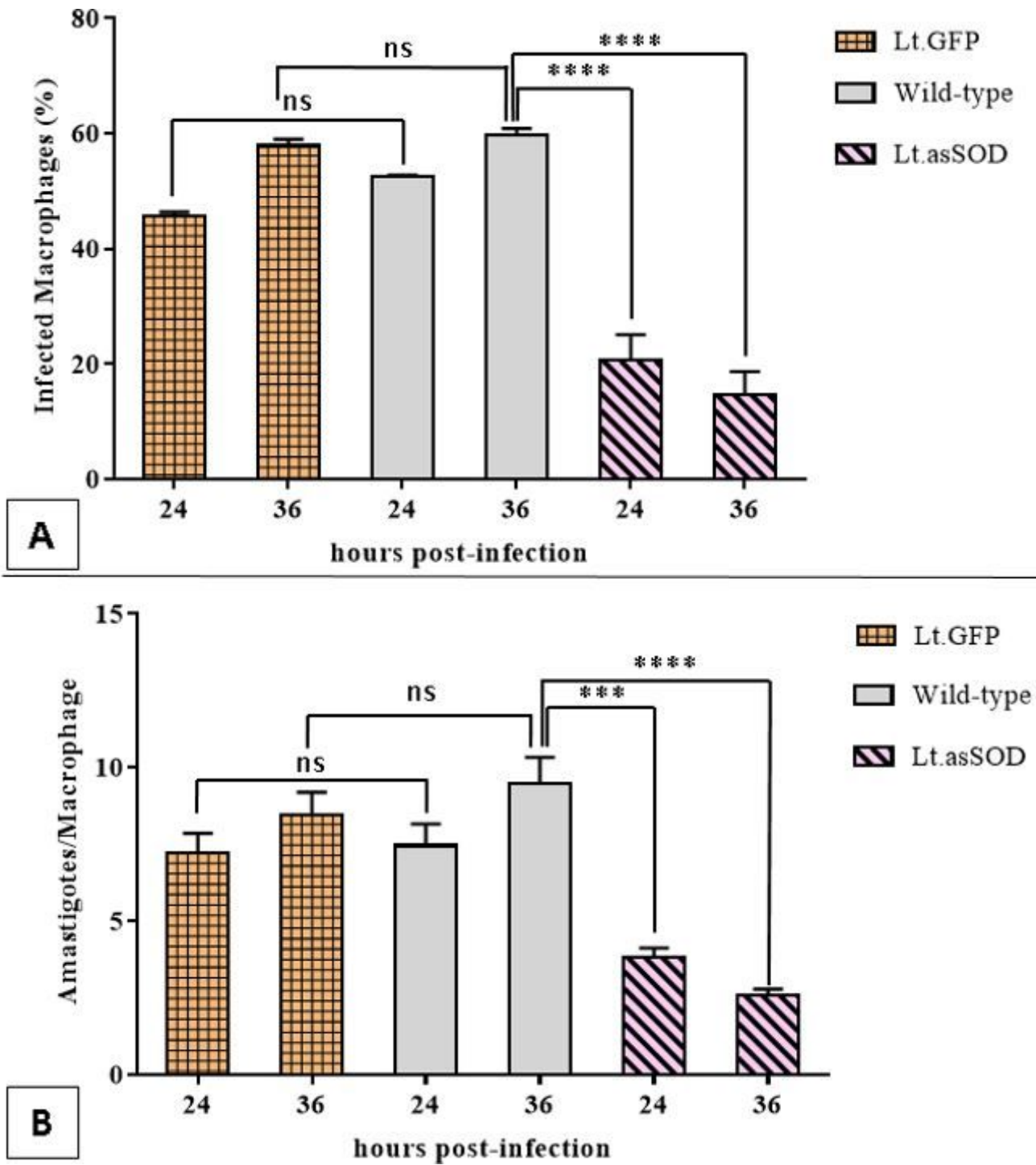
## Figure 4

Analysis of the infection effect of wild-type and transfected strains within human macrophages: *represents significant differences in comparing with the wild-type (* $p < 0.05$; ** $p < 0.01$; ***$p < 0.001$; **** $p \leq 0.0001$). (ns) represents non-significant differences in comparing with the wild-type. (A) Percentage of infected macrophages at 24 and 36h after infection ($p = 0.0001$). (B) average of Number of intracellular amastigotes per macrophage at 24 and 36h after infection.

# Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1.pdf
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- Additionalfile3.xlsx