Twin attributes of tyrosyl tRNA-synthetase of *Leishmania donovani*: a housekeeping protein translation enzyme and a mimic of host chemokine

Sneha Anand and Rentala Madhubala

School of Life Sciences, Jawaharlal Nehru University, New Delhi 110067, India

Running Title: Tyrosyl-tRNA synthetase of *Leishmania donovani*

To whom correspondence should be addressed: Prof. Rentala Madhubala, School of Life Sciences, Jawaharlal Nehru University, New Delhi. Tel and Fax: +91-11-26742630;

Email: rentala@outlook.com

Keywords: Tyrosyl-tRNA synthetase, *Leishmania donovani*, heterozygous mutant, ELR motif, moonlighting protein, chemokine, fisetin, cytokines

ABSTRACT

Aminoacyl-tRNA synthetases (aaRSs) are housekeeping enzymes essential for protein synthesis. Apart from their parent aminoacylation activity, several aaRSs perform non-canonical functions in diverse biological processes. The present study explores the twin attributes of *Leishmania* tyrosyl-tRNA synthetase (*LdTyrRS*) namely, aminoacylation and as a mimic of host CXC chemokine. *Leishmania donovani* is a protozoan parasite. Its genome encodes a single copy of tyrosyl-tRNA synthetase. We first tested the canonical aminoacylation role of *LdTyrRS*. The recombinant protein was expressed, and its kinetic parameters were determined by aminoacylation assay. To study the physiological role of *LdTyrRS* in *Leishmania*, gene deletion mutants were attempted via targeted gene replacement. The heterozygous mutants showed slower growth kinetics and exhibited attenuated virulence. *LdTyrRS* appears to be an essential gene as the chromosomal null mutants did not survive. Our data also highlights the non-canonical function of *L. donovani* tyrosyl-tRNA synthetase. We show that *LdTyrRS* protein is present in the cytoplasm and exits from the parasite cytoplasm into the extracellular medium. The released *LdTyrRS* functions as a neutrophil chemotactant. We further show that *LdTyrRS* specifically binds to host macrophages with its ELR (Glu-Leu-Arg) peptide motif. The ELR-CXCR2 receptor interaction mediates this binding. This interaction triggers enhanced secretion of the pro-inflammatory cytokines TNF-α and IL-6 by host macrophages. Our data indicates a possible immunomodulating role of *LdTyrRS* in *Leishmania* infection. This study provides a platform to explore *LdTyrRS* as a potential target for drug development.

INTRODUCTION

Aminoacyl-tRNA synthetases (aaRSs) are the central enzymes in protein translation, providing charged tRNAs for the appropriate construction of peptide chains. The canonical function of aaRSs is to charge specific tRNAs with their cognate amino acids and thereby contribute to accurate mRNA translation during protein synthesis. Thus, aaRSs are essential components of protein synthesis in every living species.

Apart from their basic function of charging tRNA molecules for protein synthesis, non-canonical functions like ribosomal RNA biogenesis, angiogenesis, apoptosis, transcriptional regulation and cell signaling have also been reported for several aaRSs (1,2). Novel functions of this group of enzymes depend on the addition of one or more new domains or motifs during the course of evolution (3).

Tyrosyl-tRNA synthetase (TyrRS) is one such aaRS which belong to a family of class I synthetases, characterized by a structurally well conserved amino-terminal Rossmann fold domain which contains the signature sequences “HIGH” and “KMSKS”. The mammalian TyrRS contains a closely homologous Endothelial Monocyte Activating Polypeptide II (EMAPII) domain at the C-terminus (4). Under specific conditions, human TyrRS is processed by an elastase enzyme into a free carboxy-terminal EMAPII-like domain and a second amino-terminal part known as mini-TyrRS. Both the released proteins are active in distinct immune signaling pathways (4,5). The carboxy-terminal domain of human TyrRS mimics the
cytokine function of EMAPII. On the other hand, human mini TyrRS, via its ELR (Glu-Leu-Arg) motif, interacts with the CXC-chemokine receptor (CXCR1/2), and like IL-8, functions as a chemoattractant for polymorphonuclear leukocytes (PMNs) (4,5). The ELR motif is a signature motif of CXC chemokines such as IL-8 that are active as PMN chemoattractants (6). This motif is essential for receptor binding and the chemotactic activity of CXC chemokines (7-9). The ELR motif in mini-TyrRS is also important for its chemokine-like activity (4,5). This data on human TyrRS suggests that the twin attributes of chemokine trigger and aminoacylation coexist in TyrRS.

Not much is known about aaRSs in Leishmania sp. Leishmania genus is the causative agent of leishmaniasis, a group of neglected diseases. The clinical symptoms of the disease depend on the species involved. Leishmania has a digenetic life cycle. Infected sand flies inoculate the mammalian host with promastigotes. Within the mammalian host, promastigotes differentiate into amastigotes that replicate in phagolysosomes. Leishmania parasites have the capability of subverting host function, thereby allowing the parasite to thrive within the organism (10,11). The development of resistance to currently available anti-leishmanial drugs has led to an urgent need to discover novel drug targets (12). In this regard, aaRSs constitute ideal targets for drug development.

The crystal structure of L. major TyrRS has been recently solved, and it is known to exist as an asymmetric pseudo-dimer (13). In the present study, we report the catalytic promiscuity and moonlighting function of Leishmania donovani TyrRS (LdTyrRS). The twin attributes of L. donovani tyrosyl-tRNA synthetase, namely, aminoacylation and chemokine trigger have been determined. Our earlier comprehensive bioinformatic analysis led to the identification of a total of 26 aaRSs in Leishmania (14). The Leishmania genome encodes a single copy of TyrRS (tritrypdb ID: LdBPK_141460.1). The present study characterizes the aminoacylation activity of LdTyrRS. To elucidate the physiological role of LdTyrRS, gene deletion mutations were attempted via targeted gene replacement. Heterozygous knockout mutants of LdTyrRS showed reduced growth and were attenuated in their infectivity, indicating the essentiality of this protein. Several attempts to generate homozygous null mutants of LdTyrRS were unsuccessful due to the presence of a single copy of the TyrRS gene. Fisetin, a natural flavonoid compound, was found to inhibit parasite growth by inhibiting the aminoacylation activity of LdTyrRS. Apart from its role in translation, we also report the non-canonical function of LdTyrRS. The most notable and intriguing feature of LdTyrRS is the presence of an “ELR” motif which is the signature motif conserved among IL-8 chemokines (15) and indicates a possible immunomodulating role of this protein. We explored the significance of this ELR motif in the parasite housekeeping enzyme LdTyrRS. Our comprehensive study involving the characterization, localization and immunological attributes of LdTyrRS provides a platform to explore LdTyrRS as a potential target for drug development.

RESULTS

Characterization of Leishmania Tyrosyl-tRNA synthetase (LdTyrRS) - Multiple sequence alignment of LdTyrRS (Uniprot ID: A4HW83, tritrypdb ID: LdBPK_141460.1) with representative sequences from other eukaryotes such as human (Uniprot ID: P54577), Plasmodium (UniProt ID: Q8IAR7), Trypanosoma brucei (UniProt ID: Q57WH7, tritrypdb ID: Tb927.7.3620) and L. major (UniProt ID: Q4QFJ7, tritrypdb ID: LMJF_14_1370) was generated using CLUSTALW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). This multiple sequence alignment showed that LdTyrRS belongs to a family of class I synthetases, characterized by a structurally well-conserved amino-terminal Rossmann fold domain which contains the signature sequences “HIGH” and “KMSKS”. Our earlier bioinformatics analysis has revealed the presence of an “ELR (Glu-Leu-Arg) motif” which is the signature motif conserved among CXC chemokines (14). The ELR motif in LdTyrRS was found to be present at the 22nd amino acid position and indicated a possible immunomodulating role of this enzyme (Fig. 1). The alignment also suggested complete conservation of the ELR motif in parasitic tyrosyl-tRNA synthetases (Plasmodium, T. brucei and L. major). Interestingly, the ELR motif was found to
be evolutionarily absent in TyrRs from lower eukaryotes and prokaryotes (Fig 2).

In order to characterize the recombinant *LdTyrRS* protein, the full-length gene was cloned into a bacterial expression vector pET30a. A histidine-tagged fusion protein with an estimated molecular mass of ~80 kDa was induced. This size correlated with the amino acid composition of *LdTyrRS* protein (~74 kDa) with a His6 tag (~6 kDa) at the N-terminus (Fig. 3A). Recombinant *LdTyrRS* was purified to homogeneity by metal affinity chromatography (Fig. 3A). Purification yielded ~2 mg of purified protein per liter of bacterial culture. The recombinant protein (*tLdTyrRS*) was recognized by an anti-His tag monoclonal antibody (Fig. 3B). To further characterize *tLdTyrRS*, the purified protein was analyzed by MALDI-TOF/TOF mass spectrometry (data not shown). The spectrum of the protein analyzed by BioTools version 2.2 showed an intensity coverage of 46.6% for tyrosyl-tRNA synthetase (*Leishmania infantum*). The expression of the full-length *LdTyrRS* enzyme was confirmed in *Leishmania* cell lysates by immunoblot analysis (Fig. 3C). The anti-*LdTyrRS* antibody detected a ~74 kDa band in the cell extracts of both the promastigotes and the amastigotes (Fig. 3C).

**Enzymatic activity and kinetic parameters for *LdTyrRS* —** To assess the aminoacylation activity of *tLdTyrRS*, a coupled-enzyme assay was performed. The aminoacylation reaction was carried out with *tLdTyrRS* in the presence of PPIase, and the Pi produced was measured with a malachite green solution. Recombinant *LdTyrRS* acylated tRNA_{Tyr} in a time-dependent manner, demonstrating that *L. donovani* TyrRS gene encodes a functional enzyme (Fig 3D). The kinetic parameters and specificity of *tLdTyrRS* were determined with L-tyrosine and tRNA_{Tyr} as substrates in vitro (Fig 3E and F). Enzyme kinetics was performed with varying concentrations of L-tyrosine (from 0.1–20 µM) while other components were kept constant (Fig. 3E). The results showed that the enzyme reaction was dependent on L-tyrosine concentration (Fig. 3E). The *K_m* value of *tLdTyrRS* for L-tyrosine was 0.21 ± 0.0245 µM, which is closer to that reported in the case of human TyrRS (0.3 µM) (16). Since tRNA_{Tyr} is another essential substrate of the aminoacylation reaction, therefore, we also performed tRNA_{Tyr} dependent enzyme kinetics studies (Fig. 3F). The estimated *K_m* of *LdTyrRS*, for tRNA_{Tyr} (1.177 ± 0.2271 µM) was closer to that of humans (0.9 µM) (16) but higher than that of *S. cerevisiae* (0.2 µM) (17).

**Subcellular localization of *LdTyrRS* —** The amino acid sequence analysis of *LdTyrRS* by web-based programs like signalP and pSORT II predicted that *LdTyrRS* does not contain any detectable signal peptide or cleavage site. Moreover, predictions with MARSPred and LocTree3 also indicated a preferentially cytosolic localization. To ascertain the localization of TyrRS in *L. donovani*, immunofluorescence analysis of log phase promastigotes stained with anti-*LdTyrRS* antibody and DAPI was conducted. The kinetoplast and nuclear DNA in these cells were readily identified by their bright staining with DAPI (Fig 4 panel B). *LdTyrRS* was found to be localized only in the cytoplasm of the parasite (Fig 4C & D). Earlier data from mass spectrometry has demonstrated a predominantly cytoplasmic localization of *TyrRS* in *T. brucei* (18). Controls performed with mouse pre-immune sera, non-permeabilized cells, and secondary antibody alone showed no detectable signal (data not shown).

**Gene deletion studies of tyrosyl-tRNA synthetase —** Since TyrRS is an important component of protein translation, we explored whether its depletion from the cell would affect aminoacylation and impact parasite growth and infection. The essentiality of *LdTyrRS* was assessed by classical gene replacement experiments where attempts were made to replace both the alleles of *LdTyrRS* by drug resistance genes. This was achieved by the generation of inactivation cassettes with hygromycin phospho-transferase (*HYG*) or neomycin phospho-transferase (*NEO*) as selection markers along with 5′UTR and 3′UTR of the *TyrRS* gene, as described in Experimental procedures. Linear replacement cassettes made by fusion PCR reaction were electro-transfected into wild-type (WT) *L. donovani* promastigotes leading to the generation of heterozygous parasites (*TyrRS/HYG* or *TyrRS/NEO*) in which one allele of the *LdTyrRS* gene was replaced with either the hygromycin or neomycin drug resistance gene. The replacement of a single allele of the *LdTyrRS* gene by drug resistance gene cassette was confirmed by a PCR-based analysis. After 3-4 passages, DNA from
heterozygous mutant parasites (TyrRS/HYG or TyrRS/NEO) was isolated and subjected to a PCR-based analysis using primers external to the inactivation cassette of LdTyrRS gene (Fig. 5A). The PCR analysis demonstrated the correct integration of HYG and NEO replacement cassettes at the TyrRS locus in heterozygotes (TyrRS/HYG or TyrRS/NEO), as indicated by the appearance of 1.2 (Fig. 5B, lane-1) and 1.3 Kb (Fig. 5B, lane-2) bands in the case of HYG cassette and 1.1 (Fig. 5B, lane-1) and 1.3 Kb (Fig. 5B, lane-2) bands in the case of NEO cassette, along with the 1.0 (Fig. 5B, lane-3) and 1.3 Kb (Fig. 5B, lane-4) bands corresponding to the WT LdTyrRS gene. This data confirmed that a single allele of the LdTyrRS gene had been replaced in heterozygous mutant parasites (TyrRS/HYG or TyrRS/NEO). Several attempts to replace both the alleles of LdTyrRS gene to generate homozygous gene deletion mutants failed. Although few clones resistant to both the drugs were obtained, PCR analyses demonstrated that the LdTyrRS gene was still present in the genome of these parasite lines (data not shown), thus indicating that LdTyrRS is an essential gene.

The effect of disruption of a single allele of the TyrRS gene at protein level was studied by Western blot analysis. Densitometric analysis was performed to evaluate the levels of TyrRS protein across different parasite lines. Comparative densitometry of the bands revealed a ~1.8-fold decreased expression of TyrRS protein in heterozygous mutants (TyrRS/HYG) (Fig 5C, lane-4) as compared to that in WT parasites. Complementation of the heterozygous mutant parasites (TyrRS/HYG) with an episomal copy of the TyrRS gene (TyrRS/HYG[pTyrRS']) restored protein expression to levels comparable to that of WT parasites (Fig 5C, lane-3). The overexpression of LdTyrRS protein in overexpressing mutants (WT[pTyrRS']) was also confirmed by a Western blot analysis. A ~1.5-fold increase in TyrRS protein level was observed in TyrRS overexpressors (WT[pTyrRS']) (Fig 5C, lane-1) as compared to that in WT parasites.

To further establish that the elimination of a single allele of the TyrRS gene in L. donovani conferred TyrRS enzyme deficiency, the aminoaacylation activity of TyrRS was determined in genetically manipulated parasites and compared to that in WT L. donovani parasites. A ~2-fold decrease in the aminoaacylation activity of TyrRS was observed in heterozygous parasites (TyrRS/HYG) as compared to that in WT parasites (Fig. 5D). Similar results were obtained with TyrRS/NEO parasites (data not shown). The WT and the ‘add-back’ lines (TyrRS/HYG[pTyrRS']) exhibited comparable TyrRS activity levels (Fig. 5D).

To assess if the reduced expression of TyrRS compromised the cellular growth of heterozygous mutant parasites, growth kinetic studies were undertaken. Heterozygous parasites (TyrRS/HYG) consistently showed growth delay as compared to WT parasites (Fig. 5E). The complementation of TyrRS/HYG mutants with an episomal copy [pTyrRS'] rescued the growth of these parasites similar to that of the WT control. Thus, it is reasonable to assume that a gene dosage effect resulted in the production of lesser TyrRS protein, and such a conjecture would suggest that TyrRS is involved in optimal cell proliferation.

We also examined the survival of heterozygous mutant parasites (TyrRS/HYG) inside murine macrophages in vitro. Virulence studies in a mouse macrophage cell line were carried out to determine the effects of genetic deficiency of TyrRS and to characterize LdTyrRS enzyme further as a potential therapeutic target. To this end, murine macrophage cell line was infected with WT, TyrRS heterozygous mutant (TyrRS/HYG) and ‘add-back’ (TyrRS/HYG[pTyrRS']) parasites at an MOI of 20:1. WT parasites were capable of infecting and sustaining robust infection in murine macrophages, whereas the parasitemia of the heterozygous mutants was reduced by ~50% relative to WT parasites 24 h post-infection (Fig 5F). Similar results were obtained with TyrRS/NEO parasites (data not shown). Complementation with an episomal copy of the TyrRS gene restored the infectivity of the heterozygous (TyrRS/HYG) mutants similar to that of the WT parasites. Taken together, our data suggests that LdTyrRS gene has a significant role in the growth and intramacrophage survival of amastigotes.

**Leishmanicidal activity of TyrRS inhibitors -** Resveratrol is a natural phenolic compound which has recently been shown to inhibit the activity of human TyrRS (19). A crystal structure of resveratrol bound to the active
site of human TyrRS has been reported (19). Another flavanoid compound fisetin has also been identified to bind TyrRS of L. major (13). To test the efficacy of these compounds on L. donovani, log phase promastigotes were cultured in the presence of increasing concentrations of fisetin and resveratrol. Both the compounds were found to inhibit the growth of promastigotes in a dose-dependent manner (Fig. 6A). The effective concentration which caused 50% inhibition of growth (IC50) after 72 h of drug addition was 31.07 µM for fisetin and 47.21 µM for resveratrol (Fig. 6A). The sensitivities of amastigotes were also tested in an intracellular amastigote-macrophage model. The IC50 of fisetin and resveratrol for amastigotes after 3 days of drug treatment was 1.53 µM and 9.3 µM respectively (Fig. 6B). At these concentrations, both fisetin and resveratrol did not affect the viability of the macrophage cell line J774A.1, with the IC50 being >400 µM after 48 h of drug treatment.

The effect of these compounds on the aminoaetylation activity of LdTyrRS (Fig 6C) was also tested. Fisetin inhibited the enzymatic activity of recombinant LdTyrRS with an IC50 of ~14.23 µM (Fig 6C), while a concentration of resveratrol as high as 1 mM failed to inhibit the enzymatic activity of LdTyrRS (Fig 6C). Thus, our results suggest that the anti-leishmanial effect of resveratrol is not due to the inhibition of LdTyrRS.

In order to ascertain whether the anti-leishmanial activity of fisetin is mediated through the inhibition of LdTyrRS, we also evaluated the effect of fisetin on the growth of genetically manipulated parasites (Fig 6D). WT, overexpressors (WT[pTyrRS]), heterozygous mutants (TyrRS/HYG) and ‘add-back’ (TyrRS/HYG[pTyrRS]) parasites were treated with fisetin at a concentration of 35 µM. In the absence of drug treatment (untreated), the growth of each parasitic line was normalized to a value of 1.0. After 72 h of treatment with fisetin, the growth rate of each parasitic line was calculated relative to the untreated control. Parasites overexpressing LdTyrRS (WT[pTyrRS]) were found to be more resistant to growth inhibition by fisetin as compared to WT parasites (Fig. 6D). In contrast, heterozygous mutant parasites (TyrRS/HYG) were found to be more susceptible to inhibition by fisetin when compared to WT parasites (Fig. 6D). Complementation of Tyrosyl-tRNA synthetase of Leishmania donovani
measured the amount of the cytosolic marker glucose 6-phosphate dehydrogenase (G6PD) in the parasite (promastigote) culture supernatants by an enzymatic assay (20). The G6PD activity in culture supernatants was found to be negligible (data not shown). As a control, parasite culture supernatants were also analyzed for the presence of mitochondrial tryparedoxin peroxidase (mTXNPx), which has been reported to be a parasitic non-secretory protein (21) (Fig. 7F). No secretion of mTXNPx was observed in promastigote culture supernatants (Fig. 7F). Taken together, our immunofluorescence, immunoprecipitation, and Western blotting data provide compelling evidence indicating the secretion of LdTyrRS from both promastigotes and amastigotes.

Tyrosyl-tRNA synthetase of Leishmania possesses an immunologically active ELR motif - Previously, human "mini" TyrRS has been shown to display potent neutrophil chemotaxis activity owing to the presence of an immunologically active ELR motif (4). A transwell migration assay was performed to evaluate the chemotactic activity of LdTyrRS towards mouse neutrophils (Fig 8A). The neutrophils which migrated into the lower chamber were quantitated by measuring their myeloperoxidase activity (Fig 8B) as described in Experimental Procedures. N-formyl-Met-Leu-Phe (fMLP) is a chemotactic peptide which was used as a positive control. The addition of both fMLP and tLdTyrRS stimulated the chemotactic migration of neutrophils (Fig 8B & C). Recombinant LdTyrRS stimulated the chemotactic activity of neutrophils at a concentration of 0.1 nM (6 ng), which is equivalent to the physiological amount of protein present in ~2X10⁵ parasites. Since ‘ELR’ motif is critical for chemotactic activity, we also prepared LdTyrRS mutant in which the ‘ELR’ motif was mutated to ‘AAA’. The mutant LdTyrRS (ELR/AAA) did not show any chemotactic activity towards neutrophils (Fig 8B & C). Also, under these experimental conditions, spontaneous migration of neutrophils was negligible as indicated by the media control (Fig 8B & C).

Overall, these results suggest that owing to its ELR motif, LdTyrRS functions as a chemoattractant for neutrophils. It is also known that the Leishmania parasite exploits neutrophils as Trojan horses before they enter their definitive host cells, i.e. macrophages (22). In light of the role of neutrophils as host cells for Leishmania, it may be hypothesized that secreted LdTyrRS functions as a virulence factor to induce an inflammatory recruitment of neutrophils at the site of infection.

LdTyrRS interaction with host macrophages – In addition to attracting neutrophils, another important function of the ELR motif is to mediate the interaction of CXC chemokines with host immune cells like macrophages. This interaction further triggers the release of proinflammatory cytokines from host immune cells (23-25). Direct binding of tLdTyrRS to host macrophages was visualized by immunofluorescence analysis. The specific interaction of tLdTyrRS with mouse macrophages was observed by using anti-LdTyrRS (Fig 9A) and anti-his antibodies (Fig 9E). On the other hand, the mutant LdTyrRS (ELR/AAA) did not show any binding to host macrophages (Fig 9B & F). Also, no binding could be detected in macrophages which were not incubated with any protein and pre-immune sera controls (Fig 9C & D). This data indicated an ELR-motif-mediated interaction of LdTyrRS with host macrophages.

Triggering of cytokine secretion by LdTyrRS – To check if LdTyrRS triggers cytokine activity of, murine macrophage cell line was incubated with rLdTyrRS, mutant ELR/AAA-LdTyrRS, lipopolysaccharide (LPS) (as a positive control), and rLdLeuRS (as a negative control). The culture supernatants were analyzed for the presence of proinflammatory cytokines such as TNF-α, IL-6, IL-12, and IFN-γ. Time kinetic analysis by ELISA revealed maximal production of the inflammatory cytokine TNF-α (Fig. 10A) during 12 h of culture and IL-6 within 24 hours of culture (Fig. 10B). Interestingly, the mutant ELR/AAA- LdTyrRS did not trigger cytokine release from macrophages and behaved similarly to the control protein rLdLeuRS (Fig. 10A & B). Moreover, the release of TNF-α and IL-6 by rLdTyrRS was mediated in a concentration-dependent manner (Fig. 10C & D). Other proinflammatory cytokines like IL-12 and IFN-γ were not induced by tLdTyrRS (Fig. 10E). We also checked the induction of an important anti-inflammatory cytokine IL-10, which is involved in disease progression in leishmaniasis (26). Recombinant LdTyrRS failed to trigger IL-10
secretion (Fig. 10E) by host macrophages. This data indicates that \textit{Ld}TyrRS specifically triggers the release of IL-6 and TNF-\(\alpha\) in a time- and dose-dependent manner.

We also corroborated our findings with the native \textit{Ld}TyrRS that was immunoprecipitated from promastigote culture supernatants (PDTyrRS). Native \textit{Ld}TyrRS was equally capable of triggering TNF-\(\alpha\) and IL-6 release from murine macrophages (Fig. 11A & B). It was also observed that pre-incubation of native \textit{Ld}TyrRS (PDTyrRS) with anti-\textit{Ld}TyrRS antibodies (PDTyrRS + \(\alpha\textit{Ld}TyrRS\)) substantially blocked TNF-\(\alpha\) (\(P < 0.001\)) and IL-6 production (\(P < 0.001\)) from macrophages (Fig. 11A & B), thereby suggesting a specific motif-based \textit{Ld}TyrRS interaction with host macrophages.

\textit{Potential receptor(s)} of \textit{Ld}TyrRS on host macrophages - ELR-motif-based binding of CXC chemokines to a CXCXR2 receptor on immune cells has been shown to activate NF-\(\kappa\)B and induce proinflammatory cytokine expression (23). To determine whether the binding of \textit{Ld}TyrRS is also mediated through ELR-CXCR2 receptor interaction, we pre-incubated human macrophages with anti-CXCR2 and anti-\textit{Ld}TyrRS antibodies and tested whether antibody-mediated receptor or protein blockades inhibited \textit{rLd}TyrRS-macrophage interaction. The interaction was analyzed by FACS analysis. Recombinant \textit{Ld}TyrRS bound to mouse macrophages was detected by an anti-\textit{Ld}TyrRS antibody (Fig. 12A). On the other hand, specific antibody-mediated blocking of the CXCR2 receptor on murine macrophages reduced \textit{rLd}TyrRS binding (Fig. 12B). The blocking of \textit{rLd}TyrRS protein with anti-\textit{Ld}TyrRS antibodies also reduced binding of the protein to macrophages (Fig. 12C). As expected, mutant \textit{Ld}TyrRS (ELR/AAA) protein did not show any binding to host macrophages (Fig 12D). Furthermore, the blockage of \textit{rLd}TyrRS-macrophage interactions using either anti-CXCR2 or anti-\textit{Ld}TyrRS antibodies significantly reduced the release of TNF-\(\alpha\) (\(P < 0.001\)) and IL-6 (\(P < 0.001\)) from murine macrophages (Fig. 12E & F). Our data strongly highlights the role of ELR motif in mediating \textit{Ld}TyrRS interactions with host macrophages. We, therefore, conclude that ELR-motif-based \textit{Ld}TyrRS binding \textit{in-vitro} likely occurs using specific receptors on host macrophages and that this binding might lead to very specific host immune system activation.

\section*{DISCUSSION}

Aminoacyl-tRNA synthetases (aaRSs) are key enzymes that drive the protein translational machinery. Apart from their translational functions, aaRSs are implicated in various non-canonical functions such as gene transcription, mRNA translation, inflammation and immune response (1). Therefore, aaRSs constitute a significant subset of proteins, and inhibition of their enzymatic activity can be deleterious to the organism. Hence, experimental dissection of critical translation components like aaRSs is of high priority as one of the avenues of novel target discovery in pathogen biology.

Tyrosyl-tRNA synthetases (TyrRS) are yet to be experimentally investigated as drug targets in parasites. The few parasite-specific studies available on TyrRS have largely focused on the structural aspects of the enzyme. A recent comprehensive study highlighted the structural and functional aspect of TyrRS from \textit{Plasmodium falciparum} (24). The structural analysis of TyrRS orthologue from \textit{Leishmania major} reveals several crucial differences between the host and pathogen tyrosyl-tRNA synthetase active sites (13). These differences could potentially be exploited for the design of structure-based inhibitors of parasite TyrRSs. The present study highlights the functional attributes of tyrosyl-tRNA synthetase of \textit{Leishmania} as a housekeeping protein translation enzyme and also as a mimic of host CXC chemokine.

In the present study, we, for the first time, report the characterization of tyrosyl-tRNA synthetase from \textit{L. donovani} (\textit{Ld}TyrRS). The overall \(K_m\) values deduced by kinetic analysis for \textit{Ld}TyrRS appear to be closer to the \(K_m\) values reported for other mammalian TyrRS. \textit{TyrRS} appears to be an essential gene as attempts to delete both copies of the \textit{TyrRS} gene from the parasite genome failed. Heterozygous parasites (\textit{TyrRS/HYG}) were found to have slower growth kinetics and exhibited attenuated virulence. Because \textit{L. donovani} \textit{TyrRS} appears to be an essential gene for parasite survival, we also analyzed the efficacy of known tyrosyl-tRNA synthetase inhibitors- resveratrol and fisetin on parasite survival and the aminoacylation activity
of LdTyrRS. Fisetin was found to inhibit the parasite growth by inhibiting the LdTyrRS aminoacylation activity. This data is in agreement with a previous report which suggests binding of fisetin to the active site of trypanosomal TyrRS (13). Several subtle yet crucial differences between Leishmania and human TyrRS (13) could potentially be exploited for the design of structure-based inhibitors for LdTyrRS.

Leishmania has evolved sophisticated mechanisms to evade or subvert host immune responses and establish chronic infection. Leishmania parasites have the capacity to subvert phagocytosis (27) and modulate cytokine secretion (11,28), thus allowing the parasite to thrive within phagocytic cells and within the host organism as a whole. From previous studies, it has become clear that a range of effector molecules secreted by the Leishmania parasite play a vital role in this process (20,29). In this context, the expression of a mimic of a human cytokine could contribute to the modulation of host signaling pathways to the parasite’s advantage. This report highlights the moonlighting activity of a parasite tyrosyl-tRNA synthetase (LdTyrRS), to function as a mimic of host CXC chemokine. Aminoacyl-tRNA synthetases have a vital role in translating the genetic code. Numerous studies have shown that the members of this enzyme family are quite adept at ‘moonlighting’ (1,3,30). Non-canonical roles have been suggested for several parasite tRNA synthetases. Studies have indicated that human tyrosyl-, tryptophanyl- and lysyl-tRNA synthetases can be secreted extracellularly and can mimic cytokines (4,31,32). TyrRS of Plasmodium and LysRS of Entamoeba also mimic the functions of human cytokines (24,33).

Our immunofluorescence and immunoblotting data reveal that both promastigotes and amastigotes secrete LdTyrRS. Interestingly, the secreted tyrosyl-tRNA synthetase from L. donovani was found to lack a classical N-terminal secretion signal peptide. Previous reports have identified secretion by exosomes as a major mechanism by which Leishmania exports secreted virulence factors which help in communication with the host (29, 34). Due to the absence of any defined secretory signal, it may be hypothesized that the release of LdTyrRS also occurs by a non-classical secretion mechanism. Understanding the exact mechanism by which the secretion of LdTyrRS occurs remains to be explored and is the focus of ongoing work.

We further investigated the capacity of LdTyrRS to modulate neutrophil recruitment. Our data shows that by using its ELR motif, secreted LdTyrRS can induce direct chemotaxis of neutrophils. A recent study has highlighted the role of neutrophils as host cells for Leishmania (35-37). Although parasite culture supernatants have been found to have chemotactic activity towards neutrophils (35), parasite-secreted protein(s) that specifically trigger the initial recruitment of neutrophils are poorly understood. The present study identifies LdTyrRS present in the parasite culture supernatant, as a parasite secretory protein having the capacity to modulate neutrophil recruitment. Thus, it may be hypothesized that secretion of LdTyrRS is a pro-parasitic response which is likely to bring about the recruitment of neutrophils to the site of infection.

Because ELR motif is also involved in a ligand-based interaction with CXCR2 receptors on immune cells, we also analyzed the binding of LdTyrRS to host macrophages. Our confocal and FACS data show that via its ELR motif, released LdTyrRS interacts with a specific receptor on host macrophages. Our data also indicates that the LdTyrRS-macrophage interaction triggers the release of the pro-inflammatory cytokines TNF-α and IL-6. Previous studies have shown that Leishmania infection triggers the release of TNF-α and IL-6 (38-41). A central function of these two pro-inflammatory cytokines is to help in the recruitment of phagocytes like neutrophils and monocytes (42-44). Both these cell types are infection targets and essential for the establishment of Leishmania infection (45-47). In the case of Leishmania infections, the release of TNF-α and IL-6 by the parasite secretory protein GP63 has been directly associated with inflammatory phagocyte (neutrophil and monocyte) recruitment (40). Thus, it may be hypothesized that the secretion of TNF-α and IL-6 by LdTyrRS also contributes towards the accrual of neutrophils at the site of Leishmania infection. These results clearly indicate that the secreted LdTyrRS enables Leishmania to elicit pro-inflammatory cytokine release and neutrophil recruitment, both of which contribute to the establishment of infection. In our present set of

Tyrosyl-tRNA synthetase of Leishmania donovani
experiments, we observed that 0.1 nM (6 ng) of \( \text{LdTyrRS} \) was sufficient to initiate neutrophil chemotaxis and cytokine release from host cells. This concentration was found to correlate with the amount of protein present in \( \sim 2 \times 10^5 \) parasites.

During \textit{Leishmania} infection, most sand flies transmit \( 10^3 - 10^5 \) parasites per blood meal (48). Earlier reports indicate that tissue damage to the skin caused by the infected sand fly bites is important to induce neutrophil recruitment within the first 90 minutes, further indicating that the initial neutrophil recruitment in the case of a natural infection is parasite-independent (46). However, in the case of an experimental infection in mice, a needle injection of \( 10^5 \) \textit{Leishmania} parasites is required to observe parasite-dependent neutrophil recruitment at later time points (6–24 h post-infection) (49, 50). Thus, in a natural \textit{Leishmania} infection, apart from parasitic proteins other factors derived from the sand fly also contribute to neutrophil chemotaxis in the infected dermis. Since all these chemotactic factors (both parasite and sandfly derived) work in a synergistic manner, it would be interesting to observe the cooperation between the \textit{LdTyrRS} and other chemotactants. Though in the present study, the amount of \textit{LdTyrRS} used was within the physiological range, the exact amount of protein secreted during natural infection, and the minimal effective concentration required for its chemotactic activity needs to be evaluated by further experimentation.

In summary, our data indicates a possible immunomodulating role of \textit{LdTyrRS} in \textit{Leishmania} infection. \textit{LdTyrRS} functions as a direct chemotactant for neutrophils. \textit{LdTyrRS} was also found to induce TNF-\( \alpha \) and IL-6 release which is known to be associated with inflammatory phagocyte recruitment in \textit{Leishmania} infections. Improved knowledge of \textit{Leishmania}-induced inflammation will further our understanding of how the parasite establishes infection, modulates the immune response, metastasizes, and causes pathology.

Considering that drug resistance is a major concern in anti-parasitic chemotherapy, there is continuous pressure to identify new drug targets. Our comprehensive analyses on the twin abilities of \textit{Leishmania} parasite tyrosyl-tRNA synthetase provide a platform to explore \textit{LdTyrRS} as a potential target for drug development.

**EXPERIMENTAL PROCEDURES**

**Materials** - All restriction enzymes and DNA modifying enzymes were obtained from New England Biolabs. Paromomycin and hygromycin were obtained from Sigma. Plasmid pET-30a was obtained from Novagen. \textit{E. coli} DH10\( \beta \) and Rosetta were used as hosts for plasmid cloning and protein expression, respectively. \textit{Ni}\(^{2+}\)-NTA agarose was purchased from Qiagen. DNA and protein markers were acquired from New England Biolabs. Resveratrol and fisetin were obtained from Sigma-Aldrich. ELISA kits and antibodies were obtained from BD Bioscience. The rabbit anti-tubulin-\( \alpha \) antibody was obtained from Neomarker (Fremont, CA). Other materials used in this study were of analytical grade and were commercially available.

**Strains and culture conditions** - \textit{L. donovani} \textit{Bob} (\textit{LdBob}/strain/MHOM/SD/62/15C2LD) was obtained from Dr. Stephen Beverley (Washington University, St. Louis, MO). Wild-type (WT) promastigotes were cultured at 22°C in M199 medium (Sigma) supplemented with 100 units/ml penicillin (Sigma), 100 \( \mu \)g/ml streptomycin (Sigma) and 5% heat-inactivated fetal bovine serum (Gibco). Wild-type (WT) parasites were routinely cultured in media with no drug supplementation, whereas genetically manipulated \textit{TyrRS} heterozygotes (\textit{TyrRS}/\textit{HYG}, \textit{TyrRS}/\textit{NEO}) were maintained in either 200 \( \mu \)g/ml hygromycin or 300 \( \mu \)g/ml paromomycin, respectively. The \textit{TyrRS} overexpressing (\textit{WT}/\textit{pTyrRS}\(^+\)) parasites were maintained in 800 \( \mu \)g/ml zeocin. The ‘add-back’ line \textit{TyrRS}/\textit{HYG}/\textit{pTyrRS}\(^+\), was grown in 800 \( \mu \)g/ml zeocin and 150 \( \mu \)g/ml hygromycin. For characterization of mutant parasites phenotypically, the cells were sub-cultured without selection markers prior to experiments.

Axenic amastigotes were generated by using the standard protocol as described earlier (51,52). Briefly, late-log phase promastigote cultures (\( \sim 2 \times 10^7/\)ml) were adapted to grow at 26°C in an acidic amastigote media (RPMI-1640/25 mM 2-(N-morpholino)ethanesulfonic acid (MES)/pH 5.5). Once established, these parasites were subsequently grown in acidic medium (RPMI-1640/MES/pH 5.5) at 37°C in a humidified atmosphere containing 5% \( \text{CO}_2 \). Throughout this \textit{in-vitro} adaptation process, the cellular morphology of the parasites was examined by both
phase-contrast light microscopy and Giemsa-stained preparations. The mouse macrophage-like cell line J774.A1 obtained from ATCC was cultured in RPMI 1640 media (Sigma) supplemented with 10% FBS and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37°C with 5% CO₂.

Ethics Statement - All animal experiments were performed according to the guidelines approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India. The protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Jawaharlal Nehru University (JNU), New Delhi (IAEC Code Number: 11/2013). All mice used for the experiments were ethically sacrificed by asphyxiation with carbon dioxide according to the institutional and the CPCSEA (Govt. of India) regulations.

Cloning, expression, and purification of recombinant LdTyrRS protein - The gene for LdTyrRS (tritrypDB ID: LdBPK_141460.1) was amplified by PCR using a forward primer with a flanking BamHI site (5’ TTTTGGATCCATGAAACACGGACGACCCTAC C 3’) and reverse primer with a flanking HindIII site (5’ TTTTAAGCTTTACCTCTTCTTTGCCCATCT TTCC 3’) from L. donovani genomic DNA. The 2049 bp amplification product encompassing the LdTyrRS open reading frame (ORF) was cloned into a pET30a vector (Novagen) using BamHI and HindIII restriction sites. This construct (LdTyrRS-pET30a) containing a His₆-tag at the N-terminus was transformed into the E. coli Rosetta strain (Novagen). The expression of recombinant LdTyrRS (rLdTyrRS) was induced with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 18°C for 16 h. The protein was purified by a Ni²⁺-nitrilotriacetic acid-agarose resin (Qiagen) by eluting with increasing concentrations of imidazole. The purified protein was found to be > 95% pure as judged by SDS-PAGE.

Site-directed mutagenesis of the ELR peptide motif of LdTyrRS - The ELR motif of LdTyrRS was replaced with AAA by site-directed mutagenesis. The mutation in the LdTyrRS gene was performed using the QuikChange II site-directed mutagenesis kit (Agilent Technologies, USA) following the manufacturer’s instructions. Subsequently, a DNA sequence analysis was performed to confirm the mutations. The mutated recombinant protein (ELR/AAA) was expressed and purified using the same protocol as specified above for the recombinant WT LdTyrRS.

Aminoacylation spectrophotometric assay – The aminoacylation assays were performed as previously described (53). Briefly, the substrate L. donovani tRNA_Tyr was synthesized by in-vitro transcription from a PCR product template that contained a T7 promoter followed by the L. donovani tRNA_Tyr sequence (tritrypDB ID: LinJ.34.tRNA7) and the terminal CCA sequence. The in vitro transcription reaction was performed with the MEGAScript T7 polymerase kit (Ambion; Life Technologies) according to the manufacturer’s instructions. The reaction mixtures were then extracted with phenol-chloroform-isooamyl alcohol (25:24:1, vol/vol; Sigma-Aldrich), and the tRNAs were precipitated with isopropanol (Sigma-Aldrich). The tRNA was refolded by heating at 70°C for 10 min, followed by the addition of 10 mM MgCl₂ and slow cooling to room temperature. The aminoacylation reaction was performed as described earlier (53) in 30 mM Hepes (pH 7.5), 150 mM NaCl, 30 mM KCl, 50 mM MgCl₂, 1 mM DTT, 200 μM ATP, 10 mM L-tyrosine, 8 μM tRNA_Tyr, 2 units/ml inorganic pyrophosphatase (PPiase) (Sigma) and 0.2 μM rLdTyrRS protein at 37°C. The reaction was stopped at different time-points by the addition of 10 mM EDTA and developed by malachite green (Echelon Bioscience). The absorbance was then measured at 620 nm by a Spectramax M2 reader (Molecular Devices). The determination of $K_m$ and $V_{max}$ for L-tyrosine and tRNA_Tyr was achieved by varying the concentration of L-tyrosine or tRNA_Tyr in the reaction mix while maintaining the other components in excess. To determine the effect of the inhibitors on the aminoacylation activity of rLdTyrRS, reactions were performed in the presence of inhibitors as described previously (53). Briefly, a reaction mixture containing rLdTyrRS (0.2 μM) was incubated with different concentrations of fisetin/resveratrol (0.1 nM to 1000 μM) for 30 min at 37°C. The reactions were stopped and quantitated as described above. To determine the 50% inhibitory concentration (IC₅₀), the dose-response data was fitted to the log
Tyrosyl-tRNA synthetase of Leishmania donovani

(Molecular constructs for replacement of TyrRS alleles - For inactivation of the LdTyrRS gene, a targeted gene replacement strategy based on PCR fusion was employed (54). Briefly, the flanking regions of the TyrRS gene (5′UTR and 3′UTR) were amplified and fused by PCR to the hygromycin phospho-transferase gene (HYG) or neomycin phospho-transferase gene (NEO). The 5′UTR (599 bp) of the LdTyrRS gene was obtained from WT L. donovani genomic DNA by PCR amplification with either primer A and B_hyg or primers A and B_neo (Table 1). The NEO gene was amplified from pX63-neo with primers C_neo & D_neo. The HYG gene was amplified from pX63-HYG with primers C_hyg and D_hyg (Table 1). The 3′UTR (580 bp) of LdTyrRS gene was obtained from L. donovani WT genomic DNA by PCR amplification using primers E_hyg/E_neo and reverse primer F (Table 1). The 5′UTR of L. donovani TyrRS gene was then ligated to either of the antibiotic resistance marker genes (HYG/NEO) by PCR using primers A and D_hyg or A and D_neo. This fragment (5′UTR-marker gene) was then fused with 3′UTR using primers A & F, yielding the linear replacement cassette, 5′UTR-Hyg-3′UTR or 5′UTR-Neo-3′UTR.

To generate the episomal construct, the full-length LdTyrRS coding sequence was amplified with a forward primer harboring XbaI site (primer G) and a reverse primer with HindIII site (primer H) (Table 1). The amplified LdTyrRS gene was then cloned into the pSP72α-zeo-α vector to get pSP72α-zeo-α-TyrRS episomal construct. All the fragments and constructs were sequenced for confirmation.

Generation of genetically manipulated parasites - After PCR amplification and purification, ~2 μg of the linear replacement cassette (5′UTR-Hyg-3′UTR or 5′UTR-Neo-3′UTR) was individually transfected by electroporation in WT L. donovani promastigotes (55). The transfecants were subjected to antibiotic selection depending on the marker gene. The cells resistant to antibiotic selection were further subjected to PCR-based analysis to check for the correct integration of replacement cassettes using primers shown in Table 2. Thereafter, the second round of transfection was initiated to knock out the other allele of the TyrRS gene.

To generate the episomal complementation mutants, episomal construct (pSP72α-zeo-α-TyrRS) was transfected into the heterozygous TyrRS/HYG parasites to get the ‘add-back’ line (TyrRS/HYG[pTyrRS′ ]). The wild type promastigotes were also transfected with the episomal construct (pSP72α-zeo-α-TyrRS) to generate the overexpressing (WT[pTyrRS′]) mutant parasites. The correct integration was confirmed by a PCR (data not shown) and a Western blot analysis.

Growth and infectivity assays - Growth rate experiments were conducted by inoculating stationary phase parasites at a density of 1 x 10⁶ cells/ml in M199 medium with 5% FBS in 25 cm² flasks without respective selection drug at 22°C. The growth rate of each culture was determined at 24 h intervals with a Neubauer hemocytometer. Growth studies with individual cell lines were done at least three times, and similar results were consistently obtained.

For the infectivity assay, J774.A1 murine macrophage cell line was plated at a cell density of 5 x 10⁵ cell/well in a 6-well flat bottom plate. The adherent cells were infected with stationary-phase promastigotes, at a ratio of 20:1 for 6 h. Excess non-adherent promastigotes were then removed by incubating the cells for 30 s in phosphate buffer saline (PBS). These were then subsequently maintained in RPMI 1640 media containing 10% FBS at 37°C with 5% CO₂. Giemsa staining was performed to visualize the intracellular parasite load.

Drug sensitivity assay – To determine the effect of inhibitors (resveratrol and fisetin) on the viability of L. donovani promastigote cells a colorimetric MTT assay was performed as described previously (56). Briefly, log phase promastigotes (5 x 10⁴ cells/well) were seeded in a 96-well flat-bottomed plate (Nunc) and incubated with different inhibitor concentrations at 22°C. Because the inhibitors were dissolved in DMSO, a sample without inhibitors but with an equal volume of DMSO served as an additional control. After 72 h of incubation, 10 μL of MTT (5 mg/ml) was added to each well and the plates were further incubated at 37°C for 4 h. The reaction was stopped by the addition of 50 μL of 50% isopropanol and 20% SDS followed by gentle shaking at 37°C for 30 min to 1 h. The absorbance was measured at 570 nm in a microplate reader.
Tyrosyl-tRNA synthetase of *Leishmania donovani*

(SpectraMax M2 from Molecular Devices). The percentage of parasite growth at different inhibitor concentration was determined relative to untreated control cells and 50% inhibitory concentration (IC$_{50}$) was calculated.

The sensitivity of amastigotes to fisetin and resveratrol was tested in an intracellular amastigote-macrophage model as described earlier (57). Briefly, the J774A.1 cell line (1x10$^5$ cells/well) was cultured in eight-chamber Lab-Tek tissue culture slides (Nunc, USA) and infected with stationary phase promastigotes at an MOI of 20:1 as described above. The infected macrophages were then incubated for 72 h with different concentrations of inhibitors (0.1 nM-10000 μM). The slides were fixed and stained with Giemsa. The number of amastigotes per cell was counted in 100 macrophages at different drug concentrations. The 50% inhibitory concentration (IC$_{50}$) was obtained by determining the reduction in parasite burden relative to the untreated infected controls.

**Antibody generation and immunofluorescence microscopy** – Antibodies against the *tLd*TyrRS were raised commercially in rabbits (Merck). Briefly, the purified *tLd*TyrRS protein (50 μg) was subcutaneously injected in rabbit using Freund’s complete adjuvant (Sigma), followed by three booster doses of the recombinant protein (20 μg) in Freund’s incomplete adjuvant (Sigma), at a 2-week interval. The sera were then collected after the last booster. Protein A purified IgG fractions (anti-*Ld*TyrRS antibody) were utilized for further immunological assays.

For the intracellular localization of *Ld*TyrRS, *L. donovani* promastigotes were immobilized on poly-L-lysine-coated coverslips. The cells were fixed and permeabilized followed by incubation with the anti-*Ld*TyrRS antibody (1:1000) for 1 h at RT. Subsequently, the cells were washed and then incubated for 45 min with the Alexa 546-conjugated goat anti-rabbit IgG antibody (Thermo Fisher Scientific catalog no. A-11071). The nuclear and the kinetoplastid DNA was stained with DAPI (Invitrogen). The fluorescence of the parasites stained with the anti-*Ld*TyrRS antibody was visualized by a confocal laser scanning microscope (Olympus FluoViewTM FV1000 with objective lenses PLAPON 60× O, NA- 1.42) at an excitation wavelength of 556 nm. The cellular DNA stained with DAPI was visualized at an excitation wavelength of 405 nm.

Immunofluorescence analysis of the infected macrophages was performed as previously described (58). Briefly, 7774 cells (1 × 10$^5$) were grown on coverslips in RPMI 1640 supplemented with 10% FBS. The adherent cells were then infected with *L. donovani* stationary-phase promastigotes at an MOI of 20:1 or left uninfected. After 24 h of infection, the cells were fixed and permeabilized followed by incubation with the primary (anti-*Ld*TyrRS and secondary (Alexa 488-conjugated goat anti-rabbit IgG) antibodies. All antibody incubations were of 50 min duration and were followed by six to eight washes in PBS. The images were then visualized by the confocal laser scanning microscope (Olympus FluoViewTM FV1000 with objective lenses PLAPON 60× O, NA- 1.42) at an excitation wavelength of 495 nm. DAPI was used to stain the nuclei of both the parasite and host macrophages.

**Immunoprecipitation and Western blot analysis** - Immunoprecipitation of the *Ld*TyrRS was performed using the culture supernatants as previously described (58). Briefly, the culture supernatants from uninfected macrophages, infected macrophages, and promastigote cultures were collected by centrifugation. The culture supernatants were neutralized with 5N NaOH and concentrated using an Amicon ultra-15 centrifugal filter unit (Millipore). The concentrated culture supernatants were incubated overnight at 4°C with anti- *Ld*TyrRS antibodies. Protein A-sepharose beads (Sigma) were then added to each sample for 30 min, following which the beads were washed with *Leishmania* lysis buffer [50 mM Tris pH 8, 137 mM NaCl, complete protease inhibitor cocktail (Roche)]. The immunoprecipitated proteins were eluted from the beads using 0.2 M glycine buffer. SDS-PAGE was used to separate the eluted immunoprecipitated proteins from the culture supernatant which were then detected by a Western blot analysis.

Protein samples for Western blot analysis were fractionated on a 10% SDS-PAGE gel and blotted onto a nitrocellulose membrane using electrophoretic transfer cell (Bio-Rad). After blocking with 5% skimmed milk, the membrane was incubated for 2 h at room temperature (RT) with the anti-*Ld*TyrRS antibody (1:1000). The
membrane was then washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) and incubated with the horseradish peroxidase (HRP) conjugated anti-rabbit antibody (Cell Signaling Technology, catalog no. 7076S; 1:5000). The blot was developed by the enhanced chemiluminescence (ECL®) kit (Amersham Biosciences) according to the manufacturer’s protocol.

The quantification of \( \text{LdTyrRS} \) in the immunoblot was carried out as described earlier (59,60). Briefly, different concentrations of \( \text{rLdTyrRS} \) were analyzed using the anti-\( \text{LdTyrRS} \) antibody by Western blotting. Band intensities for \( \text{rLdTyrRS} \) were measured densitometrically. A standard curve of protein load versus band intensity was then produced (data not shown). The band intensity of \( \text{LdTyrRS} \) from the parasite lysate was calibrated against the standard curve generated for the recombinant protein. 30 ng of \( \text{LdTyrRS} \) was estimated to be present in ~10\(^6\) parasites. In our present set of experiments with mammalian cells, the minimum quantity of \( \text{LdTyrRS} \) used was 6 ng which is equivalent to the amount of protein present in ~2X10\(^5\) \( \text{Leishmania} \) parasites. Since the experimental infections in mice are done using 10\(^5\)-10\(^7\) \( \text{Leishmania} \) parasites (49, 61), it would appear that the amount of \( \text{LdTyrRS} \) (6 ng) used is within the physiological range.

Chemotaxis assays – For the neutrophil chemotactic assay, mice neutrophils were isolated with Ficoll-Paque (Sigma) centrifugation method, as previously described (62). The final preparation consisted of >98% neutrophils, as determined by Wright-Giemsa staining. The cells were resuspended in chemotaxis media (RPMI 1640 containing 10% FBS) at a concentration of 5 x 10\(^5\) cells/ml. 200 µl of this cell suspension (1 x 10\(^6\) cells) was plated in the upper well of a 24-well transwell Boyden chemotaxis plate (Costar). Recombinant \( \text{LdTyrRS} \) (0.1 nM or 6 ng), N-Formylmethionyl-leucyl-phenylalanine (fMLP) (10\(^{-8}\) M) and mutant \( \text{LdTyrRS} \) protein (ELR/AAA) (0.1 nM or 6 ng) were diluted in 600 µl of the chemotaxis medium and placed in the lower well. The filled chemotaxis transwell plates were then incubated at 37°C in a humified CO\(_2\) incubator for 4 h. After the incubation, non-migratory cells on the upper surface of the filter were removed by wiping with a cotton swab. The number of neutrophils which migrated into the lower chamber was determined. For quantification, the myeloperoxidase activity of neutrophils was measured as previously described (63). Briefly, neutrophils sedimented by light centrifugation were solubilised in 0.1% Triton-X 100. To this lysate, 300 µl of the o-dianisidine solution containing 0.003% sodium perborate was added. The color of the reaction was developed for 30 min and terminated by the addition of 50 µl 0.5 N HCl. The absorbance of the reaction was measured at 405 nm. The number of neutrophils which migrated to the lower chamber was calculated from a standard curve obtained with the lysates of a known number of neutrophils. Migration was plotted either as the mean number of neutrophils migrated in the lower chamber or as migration index (MI = number of migrating cells in each condition/ number of migrating cells in basal medium).

Cell binding assays - \textit{In vitro} binding of the \( \text{rLdTyrRS} \) to mouse macrophages was performed by immunofluorescence analysis using standard protocols. The murine macrophage cell line J774 was cultured on coverslips in 6-well plates and maintained in RPMI 1640 medium containing 10% FBS. Recombinant \( \text{LdTyrRS} \) (0.1 nM or 6 ng) and mutant \( \text{LdTyrRS} \) protein (0.1 nM or 6 ng) (ELR/AAA) were incubated with the cells for 2 h at 4°C. The cells were then washed and incubated with 2% paraformaldehyde (Sigma) for 15 min. Following this, the primary (anti-\( \text{LdTyrRS} \); 1:1000) and fluorescently conjugated secondary (Thermo Fisher Scientific catalog no. A-11071; 1:3000) antibodies were added. Fluorescence microscopy was performed with confocal laser scanning microscopy (Olympus FluoViewTM FV1000 with objective lenses PLAPON 60× O, NA- 1.42).

\textit{In-vitro} binding of the \( \text{rLdTyrRS} \) to J774 cells was also analyzed by Fluorescence-activated cell sorting (FACS) using standard protocols. Analytical flow cytometry was performed by indirect staining of the macrophages with the anti-\( \text{LdTyrRS} \) antibody (1:1000) and the fluorescently conjugated secondary antibodies (Thermo Fisher Scientific catalog no. A-11071; 1:3000). The data obtained on BD FACS Calibur (BD Biosciences) was analyzed using cell quest software.

Cytokine secretion assays - The concentrations of IL-6, TNF-α, IL-12, IFN-γ and
Tyrosyl-tRNA synthetase of Leishmania donovani

IL-10 in culture supernatants were determined by ELISA. Briefly, J774 murine macrophages (5 \times 10^5) were cultured in RPMI 1640 medium with 10% FBS in 24-well plates. LPS, rLdTyrRS and control proteins (rLdTyrRS mutant protein (ELR/AAA) & rLdLeuRS) were added to the cells at different concentrations. After 6, 12 and 24 h, the culture supernatants were collected, serially diluted and the cytokine concentrations were quantified by ELISA. The assay was performed using a BD Pharmingen Opt EIA kit according to the manufacturer's instructions. To remove endotoxin contamination, recombinant proteins (rLdTyrRS, rLdTyrRS mutant protein (ELR/AAA) and rLdLeuRS) were passed through polymyxin beads (Sigma) before the assay. To confirm that the proteins were endotoxin free LAL (Limulus Amebocyte Lysate) assay (Thermo Fisher Scientific catalog no. 88282) was performed.

Statistical analysis - Results for the aminoacylation activity, parasitemia, and the neutrophil migration index were entered as column data in Graph Pad Prism and analyzed using the Student’s t-test. Results for cytokine analysis were entered as grouped data in Graph Pad Prism 5 and analyzed by two-way ANOVA followed by Tukey’s multiple comparison post-test. The data is represented as mean ± SD. A P value of < 0.05 was accepted as an indication of statistical significance.

Acknowledgments: We thank the Central Instrumentation Facility at the School of Life Sciences, Jawaharlal Nehru University for MALDI-TOF analysis and for providing the imaging facility. We are grateful to Dr. Chandrima Saha for providing the Leishmania mitochondrial tryparedoxin peroxidise (mTXNPX) antibody.

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

Author contributions: SA conducted the experiments. RMB designed the study, supervised the experiments and edited the manuscript with contributions from SA. Both the authors reviewed the manuscript.
Tyrosyl-tRNA synthetase of Leishmania donovani

REFERENCES
1. Guo, M., and Schimmel, P. (2013) Essential nontranslational functions of tRNA synthetases. Nat. Chem. Biol. 9, 145-153
2. Park, S. G., Ewalt, K. L., and Kim, S. (2005) Functional expansion of aminoacyl-tRNA synthetases and their interacting factors: new perspectives on housekeepers. Trends Biochem. Sci. 30, 569-574
3. Guo, M., Yang, X. L., and Schimmel, P. (2010) New functions of aminoacyl-tRNA synthetases beyond translation. Nat. Rev. Mol. Cell Biol. 11, 668-674
4. Wakasugi, K., and Schimmel, P. (1999) Two distinct cytokines released from a human aminoacyl-tRNA synthetase. Science 284, 147-151
5. Wakasugi, K., and Schimmel, P. (1999) Highly differentiated motifs responsible for two cytokine activities of a split human tRNA synthetase. J. Biol. Chem. 274, 23155-23159
6. Strieter, R. M., Polverini, P. J., Kunkel, S. L., Arenberg, D. A., Burdick, M. D., Kasper, J., Dzuiba, J., Van Damme, J., Walz, A., Marriott, D., and et al. (1995) The functional role of the ELR motif in CXC chemokine-mediated angiogenesis. J. Biol. Chem. 270, 27348-27357
7. Addison, C. L., Daniel, T. O., Burdick, M. D., Liu, H., Ehler, J. E., Xue, Y. Y., Buechi, L., Walz, A., Richmond, A., and Strieter, R. M. (2000) The CXC chemokine receptor 2, CXCR2, is the putative receptor for ELR+ CXC chemokine-induced angiogenic activity. J. Immunol. 165, 5269-5277
8. Stillie, R., Farooq, S. M., Gordon, J. R., and Stadnyk, A. W. (2009) The functional significance behind expressing two IL-8 receptor types on PMN. J. Leukoc. Biol. 86, 529-543
9. Graves, D. T., and Jiang, Y. (1995) Chemokines, a family of chemotactic cytokines. Crit. Rev. Oral Biol. Med. 6, 109-118
10. Kaye, P., and Scott, P. (2010) Leishmaniases: complexity at the host-pathogen interface. Nat. Rev. Microbiol. 9, 604-615
11. Cecilio, P., Perez-Cabezas, B., Santarem, N., Maciel, J., Rodrigues, V., and Cordeiro da Silva, A. (2014) Deception and manipulation: the arms of leishmania, a successful parasite. Front Immunol. 5, 480
12. Croft, S. L., Sundar, S., and Fairlamb, A. H. (2006) Drug resistance in leishmaniases. Clin. Microbiol. Rev. 19, 111-126
13. Larson, E. T., Kim, J. E., Castaneda, L. J., Napuli, A. J., Zhang, Z., Fan, E., Zucker, F. H., Verlinde, C. L., Buckner, F. S., Van Voorhis, W. C., Hol, W. G., and Merritt, E. A. (2011) The double-length tyrosyl-tRNA synthetase from the eukaryote Leishmania major forms an intrinsically asymmetric pseudo-dimer. J. Mol. Biol. 409, 159-176
14. Gowri, V. S., Ghosh, I., Sharma, A., and Madhubala, R. (2011) Unusual domain architecture of aminoacyl tRNA synthetases and their paralogs from Leishmania major. BMC Genomics 13, 621
15. Baggiolini, M., and Clark-Lewis, I. (1992) Interleukin-8, a chemotactic and inflammatory cytokine. FEBS Lett. 307, 97-101
16. Jia, J., Li, B., Jin, Y., and Wang, D. (2003) Expression, purification, and characterization of human tyrosyl-tRNA synthetase. Protein Expr. Purif. 27, 104-108
17. Kijima, S., Ohta, T., and Imahori, K. (1968) Purification and characterization of tyrosyl-RNA synthetase from baker's yeast. J. Biochem. 63, 434-445
18. Cestari, I., Kalidas, S., Monnerat, S., Anupama, A., Phillips, M. A., and Stuart, K. (2013) A multiple aminoacyl-tRNA synthetase complex that enhances tRNA-aminoacylation in African trypanosomes. Mol. Cell. Biol. 33, 4872-4888
19. Sajish, M., and Schimmel, P. (2015) A human tRNA synthetase is a potent PARP1-activating effector target for resveratrol. Nature 519, 370-373
20. Silverman, J. M., Chan, S. K., Robinson, D. P., Dwyer, D. M., Nandan, D., Foster, L. J., and Reiner, N. E. (2008) Proteomic analysis of the secretome of Leishmania donovani. Genome Biol. 9, R35
21. Gadelha, F. R., Goncalves, C. C., Mattos, E. C., Alves, M. J., Pineyro, M. D., Robello, C., and Peloso, E. F. (2013) Release of the cytosolic tryptophan peroxidase into the incubation medium and a different profile of cytosolic and mitochondrial peroxiredoxin expression in H2O2-treated Trypanosoma cruzi tissue culture-derived trypomastigotes. Exp. Parasitol. 133, 287-293

22. Laskay, T., van Zandbergen, G., and Solbach, W. (2003) Neutrophil granulocytes—Trojan horses for Leishmania major and other intracellular microbes? Trends Microbiol. 11, 210-214

23. Chandrasekar, B., Melby, P. C., Sarau, H. M., Raveendran, M., Perla, R. P., Marelli-Berg, F. M., Dulin, N. O., and Singh, I. S. (2003) Chemokine-cytokine cross-talk. The ELR+ CXC chemokine LIX (CXCL5) amplifies a proinflammatory cytokine response via a phosphatidylinositol 3-kinase-NF-kappa B pathway. J. Biol. Chem. 278, 4675-4686

24. Bhatt, T. K., Khan, S., Dwivedi, V. P., Banday, M. M., Sharma, A., Chandele, A., Camacho, N., Ribas de Pouplana, L., Wu, Y., Craig, A. G., Mikkonen, A. T., Maier, A. G., Yogavel, M., and Sharma, A. (2011) Malaria parasite tyrosyl-tRNA synthetase secretion triggers pro-inflammatory responses. Nat. Commun. 2, 530

25. Gupta, G., Bhattacharjee, S., Bhattacharyya, S., Bhattacharya, P., Adhikari, A., Mukherjee, A., Bhattacharyya Majumdar, S., and Majumdar, S. (2009) CXC chemokine-mediated protection against visceral leishmaniasis: involvement of the proinflammatory response. J. Infect. Dis. 200, 1300-1310

26. Kane, M. M., and Mosser, D. M. (2001) The role of IL-10 in promoting disease progression in leishmaniasis. J. Immunol. 166, 1141-1147

27. Gupta, G., Oghumu, S., and Satoskar, A. R. (2013) Mechanisms of immune evasion in leishmaniasis. Adv. Appl. Microbiol. 82, 155-184

28. Matte, C., and Olivier, M. (2002) Leishmania-induced cellular recruitment during the early inflammatory response: modulation of proinflammatory mediators. J. Infect. Dis. 185, 673-681

29. Silverman, J. M., Clos, J., de’Oliveira, C. C., Shrivani, O., Fang, Y., Wang, C., Foster, L. J., and Reiner, N. E. (2010) An exosome-based secretion pathway is responsible for protein export from Leishmania and communication with macrophages. J. Cell Sci. 123, 842-852

30. Brown, M. V., Reader, J. S., and Tzima, E. (2010) Mammalian aminoacyl-tRNA synthetases: cell signaling functions of the protein translation machinery. Vascul. Pharmacol. 52, 21-26

31. Wakasugi, K., Slike, B. M., Hood, J., Otani, A., Ewalt, K. L., Friedlander, M., Cheresh, D. A., and Schimmel, P. (2002) A human aminoacyl-tRNA synthetase as a regulator of angiogenesis. Proc. Natl. Acad. Sci. U.S.A 99, 173-177

32. Park, S. G., Kim, H. J., Min, Y. H., Choi, E. C., Shin, Y. K., Park, B. J., Lee, S. W., and Kim, S. (2005) Human lysyl-tRNA synthetase is secreted to trigger proinflammatory response. Proc. Natl. Acad. Sci. U.S.A 102, 6356-6361

33. Castro de Moura, M., Miro, F., Han, J. M., Kim, S., Celada, A., and Ribas de Pouplana, L. (2011) Entamoeba lysyl-tRNA synthetase contains a cytokine-like domain with chemokine activity towards human endothelial cells. PLoS Negl. Trop. Dis. 5, e1398

34. Lambertz, U., Silverman, J. M., Nandan, D., McMaster, W. R., Clos, J., Foster, L. J., and Reiner, N. E. (2012) Secreted virulence factors and immune evasion in visceral leishmaniasis. J Leukoc. Biol. 91, 887-899

35. van Zandbergen, G., Hermann, N., Lauš, H., Solbach, W., and Laskay, T. (2002) Leishmanial promastigotes release a granulocyte chemotactic factor and induce interleukin-8 release but inhibit gamma interferon-inducible protein 10 production by neutrophil granulocytes. Infect. Immun. 70, 4177-4184

36. Lauš, H., Muller, K., Fleischer, J., Reiling, N., Jahnke, N., Jensenius, J. C., Solbach, W., and Laskay, T. (2002) Intracellular survival of Leishmania major in neutrophil granulocytes after uptake in the absence of heat-labile serum factors. Infect. Immun. 70, 826-835

37. Aga, E., Katschinski, D. M., van Zandbergen, G., Lauš, H., Hansen, B., Muller, K., Solbach, W., and Laskay, T. (2002) Inhibition of the spontaneous apoptosis of neutrophil granulocytes by the intracellular parasite Leishmania major. J. Immunol. 169, 898-905
38. Arena, A., Capozza, A. B., Delfino, D., and Iannello, D. (1997) Production of TNF alpha and interleukin 6 by differentiated U937 cells infected with Leishmania major. New Microbiol. 20, 233-240
39. Wenzel, U. A., Bank, E., Florian, C., Forster, S., Zimara, N., Steinacker, J., Klinger, M., Reiling, N., Ritter, U., and van Zandbergen, G. (2012) Leishmania major parasite stage-dependent host cell invasion and immune evasion. FASEB J. 26, 29-39
40. Arango Duque, G., Fukuda, M., Turco, S. J., Stager, S., and Descoteaux, A. (2014) Leishmania promastigotes induce cytokine secretion in macrophages through the degradation of synaptotagmin XI. J. Immunol. 193, 2363-2372
41. Karam, M. C., Hamdan, H. G., Abi Chedid, N. A., Bodman-Smith, K. B., Eales- Reynolds, L. J., and Baroody, G. M. (2006) Leishmania major: low infection dose causes short-lived hyperalgesia and cytokines upregulation in mice. Exp. Parasitol. 113, 168-173
42. Griffin, G. K., Newton, G., Tarrio, M. L., Bu, D. X., Maganto-Garcia, E., Azcutia, V., Alcaide, P., Grabie, N., Luscinskas, F. W., Croce, K. J., and Lichtman, A. H. (2012) IL-17 and TNF-alpha sustain neutrophil recruitment during inflammation through synergistic effects on endothelial activation. J. Immunol. 188, 6287-6299
43. Fielding, C. A., McLoughlin, R. M., McLeod, L., Colmont, C. S., Najdovska, M., Grail, D., Ernst, M., Jones, S. A., Topley, N., and Jenkins, B. J. (2008) IL-6 regulates neutrophil trafficking during acute inflammation via STAT3. J. Immunol. 181, 2189-2195
44. Vieira, S. M., Lemos, H. P., Grespan, R., Napimoga, M. H., Dal-Secco, D., Freitas, A., Cunha, T. M., Verri, W. A., Jr., Souza-Junior, D. A., Jamur, M. C., Fernandes, K. S., Oliver, C., Silva, J. S., Teixeira, M. M., and Cunha, F. Q. (2009) A crucial role for TNF-alpha in mediating neutrophil influx induced by endogenously generated or exogenous chemokines, KC/CXCL1 and LIX/CXCL5. Br. J. Pharmacol. 158, 779-789
45. Ribeiro-Gomes, F. L., and Sacks, D. (2012) The influence of early neutrophil-Leishmania interactions on the host immune response to infection. Front Cell Infect. Microbiol. 2, 59
46. Peters, N. C., Egen, J. G., Secundino, N., Debrabant, A., Kimblin, N., Kamhawi, S., Lawyer, P., Fay, M. P., Germain, R. N., and Sacks, D. (2008) In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. Science 321, 970-974
47. Ribeiro-Gomes, F. L., Roma, E. H., Carneiro, M. B., Doria, N. A., Sacks, D. L., and Peters, N. C. (2014) Site-dependent recruitment of inflammatory cells determines the effective dose of Leishmania major. Infect. Immun. 82, 2713-2727
48. Hurrell, B. P., Regli, I. B., and Tacchini-Cottier, F. (2016) Different Leishmania Species Drive Distinct Neutrophil Functions. Trends Parasitol. 32, 392-401
49. Hurrell, B. P., Schuster, S., Grun, E., Coutaz, M., Williams, R. A., Held, W., Malissen, B., Malissen, M., Yousefi, S., Simon, H. U., Muller, A. J., and Tacchini-Cottier, F. (2015) Rapid Sequestration of Leishmania mexicana by Neutrophils Contributes to the Development of Chronic Lesion. PLoS Pathog. 11, e1004929
50. Thalhofer, C. J., Chen, Y., Sudan, B., Love-Homan, L., and Wilson, M. E. (2011 ) Leukocytes infiltrate the skin and draining lymph nodes in response to the protozoan Leishmania infantum chagasi. Infect. Immun. 79, 108-117
51. Mittra, B., Cortez, M., Haydock, A., Ramasamy, G., Myler, P. J., and Andrews, N. W. (2013) Iron uptake controls the generation of Leishmania infective forms through regulation of ROS levels. J. Exp. Med. 210, 401-416
52. Debrabant, A., Joshi, M. B., Pimenta, P. F., and Dwyer, D. M. (2004) Generation of Leishmania donovani axenic amastigotes: their growth and biological characteristics. Int. J. Parasitol. 34, 205-217
53. Cestari, I., and Stuart, K. (2013) A spectrophotometric assay for quantitative measurement of aminoacyl-tRNA synthetase activity. J. Biomol. Screen. 18, 490-497
54. Darveau, A., Pelletier, A., and Perreault, J. (1995) PCR-mediated synthesis of chimeric molecules. Methods in Neurosciences 26, 77–85
Tyrosyl-tRNA synthetase of Leishmania donovani

55. Kapler, G. M., Coburn, C. M., and Beverley, S. M. (1990) Stable transfection of the human parasite Leishmania major delineates a 30-kilobase region sufficient for extrachromosomal replication and expression. *Mol. Cell. Biol.* 10, 1084-1094

56. Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 65, 55-63

57. Kansal, S., Tandon, R., Dwivedi, P., Misra, P., Verma, P. R., Dube, A., and Mishra, P. R. (2012) Development of nanocapsules bearing doxorubicin for macrophage targeting through the phosphatidylserine ligand: a system for intervention in visceral leishmaniasis. *J. Antimicrob. Chemother.* 67, 2650-2660

58. McCafferty, L. I., and Matlashewski, G. (2010) Localization and induction of the A2 virulence factor in Leishmania: evidence that A2 is a stress response protein. *Mol. Microbiol.* 77, 518-530

59. Krobitsch, S., Brandau, S., Hoyer, C., Schmetz, C., Hubel, A., and Clos, J. (1998) Leishmania donovani heat shock protein 100. Characterization and function in amastigote stage differentiation. *J. Biol. Chem.* 273, 6488-6494

60. Taylor, S. C., and Posch, A. (2014) The design of a quantitative western blot experiment. *Biomed. Res. Int.* 2014, 361590

61. Rolao, N., Melo, C., and Campino, L. (2004) Influence of the inoculation route in BALB/c mice infected by Leishmania infantum. *Acta Trop.* 90, 123-126

62. Luo, Y., and Dorf, M. E. (2001) Isolation of mouse neutrophils. *Curr. Protoc. Immunol.* Chapter 3, Unit 3 20

63. Kawa, S., Kimura, S., Hakomori, S., and Igarashi, Y. (1997) Inhibition of chemotactic motility and trans-endothelial migration of human neutrophils by sphingosine 1-phosphate. *FEBS Lett.* 420, 196-200

**FOOTNOTES**

This work was supported by a grant from Department of Biotechnology, Government of India (102/IFD/SAN/3321/2014-15) to Rentala Madhubala. Rentala Madhubala is a JC Bose National Fellow. Sneha Anand is a recipient of funding from the University Grants Commission, India.

The abbreviations used are: TyrRS, Tyrosyl tRNA synthetase; *Ld*TyrRS, *Leishmania donovani* TyrRS; tRNA<sup>Tyr</sup>, Tyrosyl-<sup>Tyr</sup>tRNA; EMAPII, Endothelial Monocyte Activating Polypeptide II domain; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide; fMLP, Formylmethionyl-leucyl-phenylalanine; ELR/AAA, Mutant *Ld*TyrRS protein in which ELR motif has been mutated to AAA; r*Ld*LeuRS, Recombinant *Leishmania donovani* Leucyl tRNA Synthetase.
Tyrosyl-tRNA synthetase of *Leishmania donovani*

**FIGURE LEGENDS**

**Figure 1:** Multiple sequence alignment of representative TyrRS sequences from kinetoplastids, human and *Plasmodium* species generated using CLUSTALW. The key residues present in the aminoacylation and catalytic domains are highlighted in a gray color background. The ELR motif is highlighted in bold font.

**Figure 2:** Domain organization of TyrRS from eukaryotes and prokaryotes. The catalytic and anticodon binding domains are indicated. The ELR motif is present at the N-terminus and is shown in red. The HIGH and KMSKS active site motifs are common to all class I catalytic domains. The AIDQ motif is characteristic of the ATP binding site in TyrRS. The AC1 motif corresponds to the anticodon-binding domain that interacts with the anticodon stem of tRNA<sup>TYr</sup>. The AC2 motif specifically recognizes the anticodon bases G34 and U/W35.

**Figure 3:** Purification and enzymatic characterization of recombinant *LdTyrRS*. **A:** Purification of recombinant *LdTyrRS* protein on Ni<sup>2+</sup>-NTA affinity resin. M, molecular weight marker; Lane 1, uninduced cell lysate; Lane 2, induced cell lysate; Lane 3, *rLdTyrRS*. **B:** Western blot analysis of the recombinant *LdTyrRS* protein using anti- His-tag mouse antibody (1:3000). M- Molecular weight marker, rTyrRS - purified *rLdTyrRS*. **C:** Immunoblotting analysis of the *Leishmania* promastigote (P) and amastigote (A) cell lysate (~40 µg) with the anti- *LdTyrRS* antibody. **D:** Time course of tRNA<sup>TYr</sup> aminoacylation by recombinant *LdTyrRS*. Reactions were performed with L-Tyrosine and tRNA<sup>TYr</sup> as the substrates. The data show an average of three experiments performed in duplicate ± SD. **E and F:** Aminoacylation kinetics of *LdTyrRS* as a function of L-Tyrosine concentration (E) or tRNA<sup>TYr</sup> concentration (F). The results represent mean ± SD with *n* = 3.

**Figure 4:** Subcellular localization of *LdTyrRS* in *L. donovani*. Immunofluorescence analysis by confocal micrograph of wild-type log phase promastigotes. **Panel A:** phase contrast image **Panel B:** promastigotes stained with DAPI **Panel C:** anti-*LdTyrRS* antibody detected using Alexa 542 (red)-conjugated secondary antibody **Panel D & E:** merged micrographs. ‘k’ and ‘n’ indicate kinetoplastid and nuclear DNA respectively. The scale bar represents 10 µm.

**Figure 5:** Generation and characterization of heterozygous knockout mutants of *LdTyrRS* **A:** Map of *LdTyrRS* genomic locus and location of the primers used for confirmation by PCR-based analysis along with the expected band sizes. Primer 4 was designed as a forward primer to match the upstream region of *LdTyrRS* gene, and primers 8, 3 and 6 were designed internal to *TyrRS*, *HYG*, and *NEO* coding regions, respectively. Primer 2 was designed as a reverse primer to match the downstream region of *LdTyrRS* gene, and primers 7, 1 and 5 were designed as forward primers, internal to *TyrRS*, *HYG*, and *NEO* coding regions, respectively. **B:** Genomic DNA from heterozygous *TyrRS/HYG* and *TyrRS/NEO* mutant parasites was used as a template for PCR analysis. The specific integration of the replacement cassette was checked with *HYG*, *NEO* and *LdTyrRS* (WT) gene specific primers. M indicates the molecular size marker in kb. **C:** Western blot analysis of equal protein quantities (~30 µg) from whole cell lysates prepared from wild-type (WT), *TyrRS* overexpressors (WT[<sup>pTyrRS</sup>]), ‘add-back’ (*TyrRS/HYG[<sup>pTyrRS</sup>]*), and heterozygous mutant (*TyrRS/HYG*) parasites. The loading was normalized with α-Tubulin (50 kDa) antibody. **D:** Comparison of the aminoacylation activity of *TyrRS* in cell lysate of wild-type (WT), heterozygous (*TyrRS/HYG*) mutant and ‘add-back’ (*TyrRS/HYG[<sup>pTyrRS</sup>]*). **E:** Growth curve of *L. donovani* WT, ‘add-back’ (*TyrRS/HYG[<sup>pTyrRS</sup>]*), and heterozygous mutant (*TyrRS/HYG*) promastigotes in M199 media. The results represent Mean ± SD with *n* = 3. **F:** Comparison of infectivity of *L. donovani* wild-type (WT), heterozygous mutant (*TyrRS/HYG*) and ‘add-back’ (*TyrRS/HYG[<sup>pTyrRS</sup>]*). The results represent mean ± SD with *n* = 3. Student’s t-test was performed, and the *P*-values are indicated.
Figure 6: Effect of TyrRS inhibitors on parasite growth and enzyme activity. A: Inhibition of promastigote growth in the presence of resveratrol and fisetin. The assay was done in 96-well plates and growth was estimated by MTT assay. Percentage growth of parasite was plotted against different concentrations of inhibitors B: Effect of resveratrol and fisetin on amastigote growth progression was studied by observing Giemsa-stained infected macrophages under a microscope. C: Inhibition of rLdTyrRS aminoclaylation activity by resveratrol and fisetin. D: Comparison of the effect of fisetin activity on wild-type (WT) and genetically manipulated parasites. WT, overexpressors (WT[pTyrRS’]), heterozygous mutants (TyrRS/HYG) and ‘add-back’ (TyrRS/HYG/[TyrRS’]) parasites were treated with fisetin at a concentration of 35 µM. The cell growth was determined after 72 hours. In the absence of drug treatment (untreated), the growth of each parasitic line was normalized to 1.0. After treatment with fisetin, (treated) growth was calculated relative to the corresponding untreated control. The bar graph represents the mean ± SD with n = 3. Student’s t-test was performed, and P-values are indicated.

Figure 7: Localization of the LdTyrRS in infected macrophages. A-C: Confocal microscopy of the uninfected (A) and L. donovani infected macrophages (B-C) showing the secretion of LdTyrRS protein in the macrophage cytoplasm. White arrows indicate intracellular amastigotes. Parasite and macrophage nuclei were visualized with DAPI (blue). Anti-LdTyrRS antibody (green) was used to visualize the parasitic LdTyrRS . Staining of infected macrophages with rabbit pre-immune sera was used as a negative control (B). Scale bar corresponds to a size of 10 µm. D: Western blots of the parasite culture supernatants using antibodies against LdTyrRS. The culture supernatant of the infected (Lane-1) and uninfected (Lane-2) macrophages was electroblotted onto nitrocellulose membrane and probed with the anti-LdTyrRS antibody & HRP conjugated anti-rabbit antibody. The blot was then developed with commercially available ECL reagent. Western blot analysis of the promastigote culture supernatants to check for the presence of LdTyrRS (E) and mTXNpX (F). Lane-1: L. donovani promastigote cell lysate (20 µg); Lane-2: promastigote culture supernatant. The parasites were suspended at a concentration of ~10⁶ parasites per ml in RPMI-1640 media without FBS and incubated for 8 h under normal culture conditions. The LdTyrRS protein was immunoprecipitated from a minimum of ~80 ml of culture supernatants containing a total of ~8 x 10⁶ promastigotes and detected by a western blot analysis as described in Experimental procedures.

Figure 8: The effect of LdTyrRS on neutrophil chemotaxis was studied by a transwell assay. Wildtype rLdTyrRS, mutated rLdTyrRS (ELR/AAA), media (negative control) and fMLP (positive control) were used as chemoattractants. A: Shows a representative photograph of the cell migration in each condition. B: The number of cells that migrated due to the effect of different chemoattractants were quantitated using a myeloperoxidase assay. C: Migration is also plotted as Migration Index (MI; the number of cells migrating in each condition/number of cells migrating in basal medium). Data (i.e., the number of neutrophils migrated and the migration index) is represented as mean ± SD with n = 4. Student’s t-test was performed, and P-values are indicated.

Figure 9: LdTyrRS interaction with macrophages in-vitro. A-B: Confocal images representing the binding of rLdTyrRS (green) (A) and mutant LdTyrRS (ELR/AAA) to mouse macrophages (B). C-D: Represent controls for primary/secondary antibodies alone (that is, no added LdTyrRS) (C) and pre-immune sera (D). For nucleus visualization, DAPI (blue) localization is shown. The interaction of LdTyrRS with the mouse macrophage cell line was visualized by staining with the anti-LdTyrRS antibody (A-D). E-F: The binding of rLdTyrRS (E) and ELR/AAA mutant LdTyrRS (F) to mouse macrophages was also detected using anti-his monoclonal antibody. The scale bar corresponds to a size of 10 µm.

Figure 10: Effect of rLdTyrRS on mouse macrophages. A-B: The secretion profile of TNF-α (A) and IL-6 (B) was measured at different time points using ELISA. C-D: Dose-dependent increase in the secretion of cytokines like TNF-α (C) and IL-6 (D) from mouse macrophages was also measured. Media alone, rLdLeuRS enzyme and ELR-AAA mutant of LdTyrRS were used as negative controls. LPS was used as a positive control in all the experiments. E: The secretion profile of other cytokines like IFN-γ, IL-10, and
Tyrosyl-tRNA synthetase of Leishmania donovani

IL-12 that are secreted from mouse macrophages on exposure to rLdTyrRS was also analyzed. The results represent mean ± SD with n = 3. Tukey’s test was performed, and P-values are indicated.

Figure 11: Effect of native LdTyrRS on the cytokine secretion profile of mouse macrophages A-B: Secretion profile of TNF-α (A) and IL-6 (B) using the L. donovani promastigote culture supernatants alone (Cult. Sup), promastigote culture supernatants neutralized with the anti-TyrRS antibody (Sup + α-TyrRS Ab), native LdTyrRS immunoprecipitated from promastigote culture supernatant (PD-TyrRS) and finally native LdTyrRS (PD-TyrRS) neutralized with its own antibody (PD-TyrRS+ α-TyrRS Ab). Recombinant LdTyrRS was used as a positive control. TNF-α and IL-6 secretion are clearly enhanced significantly when immunoprecipitated native LdTyrRS (PD-TyrRS) is used. The triggering activity of native LdTyrRS (PD-TyrRS+ α-TyrRS Ab) is reduced when pre-incubated with anti-LdTyrRS antibodies (1:3000), indicating specific activation of macrophages with native LdTyrRS (PD-TyrRS). The results represent mean ± SD with n = 3. Tukey’s test was performed, and P-values are indicated.

Figure 12: Potential LdTyrRS receptor on mouse macrophages. A-D: FACS analysis to assess the binding of rLdTyrRS to mouse macrophages. rLdTyrRS bound to mouse macrophages was analyzed by indirect staining with the anti-TyrRS antibody. Overlay histograms show binding of rLdTyrRS to the surface of mice macrophages (solid line) with unstained controls (purple solid) as background. Histogram (solid line) shows positive binding of rLdTyrRS to mouse macrophages (A). rLdTyrRS binding to macrophages is reduced when macrophages are pre-incubated anti-CXCR2 (B) or when rLdTyrRS is neutralized with anti-LdTyrRS antibodies (C). The binding is also significantly reduced with the mutant LdTyrRS (ELR/AAA) protein (D). E-F: Cytokine secretion assays for IL-6 (E) and TNF-α (F) using mice macrophages and anti-CXCR2 and anti-LdTyrRS antibodies. The secretion of these pro-inflammatory cytokines is reduced when macrophages and LdTyrRS protein were preincubated with the anti-CXCR2 (TyrRS + α-CXCR2 Ab) and anti-LdTyrRS (TyrRS + α-TyrRS Ab) antibodies, respectively. The results represent mean ± SD with n = 3. Tukey’s test was performed, and P-values are indicated.
Table 1. Primers used for generation of the Hyg and Neo specific linear replacement cassette fragments.

| S.No | L. donovani Primers | Sequences |
|------|---------------------|-----------|
| 1.   | A                   | 5’ AACATGACGCAGTGAGGTTGCTCCGTT 3’ |
| 2.   | B<sub>Hyg</sub>     | 5’ GGTGAGTTACGGCTTTTCATATGCGCAGTCTCCGTT 3’ |
| 3.   | C<sub>Hyg</sub>     | 5’ CTGTTACGGCTGACAGGCCATATGAAAGCGCTGAACCTCA 3’ |
| 4.   | D<sub>Hyg</sub>     | 5’ GCCGCCCTCTCTCACCTATACCCTATTCTCTGCCACGA 3’ |
| 5.   | E<sub>Hyg</sub>     | 5’ CTCGTCGAGGGCGAGTGATAGTGAGAGGCGGC 3’ |
| 6.   | B<sub>Neo</sub>     | 5’ CAATCCATCTTGATCATCATATGCGGCAGTCTCCGTT 3’ |
| 7.   | C<sub>Neo</sub>     | 5’ CTGTTACGGCTGACAGGCCATATGAAAGCGCTGAACCTCA 3’ |
| 8.   | D<sub>Neo</sub>     | 5’ GCCGCCCTCTCTCACCTATACCCTATTCTCTGCCACGA 3’ |
| 9.   | E<sub>Neo</sub>     | 5’ CTTCTTGACGAGTTCTCTGAGTTAGTGAAGAGGCGGC 3’ |
| 10.  | F                   | 5’ GTGGAAGCAGGGCGACCAACACA 3’ |
| 11.  | G                   | 5’ TTTTTCTAGAATGACACGGACACCGCTAC 3’ |
| 12.  | H                   | 5’ TTNTAAGCTTACCTCTTTGACATCTCTTCC 3’ |
Table 2. Primers used for the molecular characterization of the genetically manipulated parasites by PCR-based analysis.

| S.No | \textit{L. donovani} Primers | Sequences |
|------|-----------------------------|-----------|
| 1.   | Primer 1                    | 5' TGTAGAAGTACTCGCCGATAGTGG 3' |
| 2.   | Primer 2                    | 5' AGATCGCATTTGCAGCACG 3' |
| 3.   | Primer 3                    | 5' CGCAGCTATTTACCCGCAGGACAT 3' |
| 4.   | Primer 4                    | 5' CGTCGTCATTTCCGCCTTACG 3' |
| 5.   | Primer 5                    | 5' ATAGCGTTGGCTACCCGTGAATTGC 3' |
| 6.   | Primer 6                    | 5' AACACGGGCAGCATCAGAGCAGCCGATTG 3' |
| 7.   | Primer 7                    | 5' GAAGAAGATGGCAAAGAAGGTA 3' |
| 8.   | Primer 8                    | 5' GCGGTCGTCCGTTAGTTCAT 3' |
| Protein      | Sequence                                                                 | Length |
|--------------|---------------------------------------------------------------------------|--------|
| H. sapiens   | LKPKKVFKQDASKLISECIA- QWKQTNFMTKLGSISCKSLK                                | 523    |
| P. falciparum|                                                                           | 373    |
| T. brucei    | LQ---RVEEVC-GDLKNAAGVVAAMLYVADAAMLKATHAICTSHDRGCEIATDFEGKL               | 545    |
| L. donovani  | LS---HIEELYGEGRLNAGGVIALMRVATAMLSVHVISLDSGHINAFAREYTKERI                 | 537    |
| L. major     | LS---HVEELYGGEVRAGGVIALMRVATAMLSVHVISLDSGHINAFAREYTKERI                 | 537    |
| H. sapiens   |                                                                           | 523    |
| P. falciparum|                                                                           | 373    |
| T. brucei    | RVIPALGEGVPPSNELPPSETLSAGPVNDLDLDDDDRMWRRKIKRAYCAPNEDAN                  | 605    |
| L. donovani  | ECVQFLEGRIPALHRPG-------AAPAVLGADDVYLDNDMDIRKIKKAYSAPNEEAN              | 591    |
| L. major     | DCVQTLGPIALHRPG-------AAPAVLGADDVYLDNDMDIRKIKKAYSAPNEEAN               | 591    |
| H. sapiens   |                                                                           | 528    |
| P. falciparum|                                                                           | 373    |
| T. brucei    | PVLSIAATWLMREQGGALLIERTEANGGDYHKEQGLRADALSAGLHPADLQKVAVSKLGILD         | 665    |
| L. donovani  | PVISVAGHLNAQVGNVSYNTPEALVADCGSGALHPADLKAQVQLLLD                        | 651    |
| L. major     | PVISVAGHLNAQVGNVSYNTPEALVADCGSGALHPADLKAQVQLLLD                      | 651    |
| H. sapiens   |                                                                           | 528    |
| P. falciparum|                                                                           | 373    |
| T. brucei    | KCAAAKAVLSTAGKKAQTLKNAEKLSSK                                            | 697    |
| L. donovani  | RSAQARALLNGEL-KKNMTALRNAEKMAKRR                                       | 682    |
| L. major     | RSAQARALLNGEL-KKNMTYLRNAEKMAKRR                                         | 682    |
Fig. 4
Fig. 8

(A) Images showing the number of neutrophils migrated: Media, ELR/AAA, TyrRS, fMLP.

(B) Bar graph showing the number of neutrophils migrated per condition: Media, ELR/AAA, TyrRS, fMLP.

(C) Bar graph showing the migration index: Media, ELR/AAA, TyrRS, fMLP.

P < 0.05, P < 0.01, P < 0.05.
Fig. 10
Twin attributes of tyrosyl tRNA-synthetase of Leishmania donovani: a housekeeping protein translation enzyme and a mimic of host chemokine

Sneha Anand and Rentala Madhubala

J. Biol. Chem. published online July 5, 2016

Access the most updated version of this article at doi: 10.1074/jbc.M116.727107

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts