Toll-Like Receptor 2 Targeted Rectification of Impaired CD8⁺ T Cell Functions in Experimental Leishmania donovani Infection Reinstates Host Protection

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Abstract

Leishmania donovani, a protozoan parasite, causes the disease visceral leishmaniasis (VL), characterized by inappropriate CD8⁺ T-cell activation. Therefore, we examined whether the Toll-like Receptor 2 (TLR2) ligand Ara-LAM, a cell wall glycolipid from non-pathogenic Mycobacterium smegmatis, would restore CD8⁺ T-cell function during VL. We observed that by efficient upregulation of TLR2 signaling-mediated NF-κB translocation and MAPK signaling in CD8⁺ T-cells (CD25⁺CD28⁺IL-12R⁺IFN-γR⁺), Ara-LAM triggered signaling resulted in the activation of T-bet, which in turn, induced transcription favourable histone modification at the IFN-γ, perforin, granzyme-B promoter regions in CD8⁺ T-cells. Thus, we conclude that Ara-LAM induced efficient activation of effector CD8⁺ T-cells by upregulating the expression of IFN-γ, perforin and granzyme-B in an NF-κB and MAPK induced T-bet dependent manner in VL.

Introduction

Visceral leishmaniasis caused by the protozoan parasite, Leishmania donovani, is fatal, if untreated. Dysfunctions of macrophages and T-cells during VL result in severe immunosuppression [1–3]. CD4⁺ T-cells activation is essential for IFN-γ-mediated protection against leishmanias [4] whereas CD8⁺ T-cells confer protection via perforin, granzyme-B-mediated direct killing of Leishmania-infected host cells [5–6]. L. donovani induces functional exhaustion of CD8⁺ T-cells [7]. Activation of Leishmania-specific CD8⁺ T-cells is indispensable for clearance of the pathogen. For naive CD8⁺ T cells’ antigen-specific activation and differentiation, signals from T-cell receptor, CD28, IL-12-induced IFN-γ and TLR2 are required [8–12]. The transcription factor T-bet promotes naive CD8⁺ T-cell differentiation to effector cytotoxic
T lymphocytes (CTL) [13–14], expressing CD25 (IL-2R-α), IL-12 receptor (IL-12R) and IFN-γR.

TLR2, forming heterotypic associations with TLR1 or TLR6, recognizes triacetylated or diacetylated lipopeptides, respectively [15–17]. Ara-LAM, a cell wall glycolipid of *Mycobacterium smegmatis*, has been reported to confer protection against leishmanial pathogenesis via TLR2-dependent induction of the proinflammatory responses [18]. Ara-LAM–induced activation of p38 MAPK signalling in *Leishmania* infected macrophages shifts their Th2 phenotype towards Th1 via chromatin modification at various proinflammatory cytokine gene loci [19]. However, it was unclear if Ara-LAM would modulate TLR2 signalling in CD8+ T-cells, which might play a potential role in the regression of leishmanial pathogenesis.

In this study, we have demonstrated that Ara-LAM drives the activation of CD8+ T-cells, which are CD25+CD28+IL-12R+IFN-γR+ and specifically destroys the *L. donovani*-infected macrophages. Ara-LAM enhances T-bet expression in CD8+ T-cells via upregulation of NF-κB and p38MAPK in a TLR2-dependent pathway. T-bet ultimately enhances the expression of IFN-γ, perforin, and granzyme-B in CD8+ T-cells via histone modifications at their respective promoter regions to restore host-protective CD8+ T-cell responses.

**Materials and Methods**

**Reagents and chemicals**

RPMI-1640 medium, penicillin and streptomycin, SB203580 (p38MAP Kinase inhibitor), SN50 (NF-κB inhibitor) were from Sigma (St. Louis, MO, USA). dNTPs, Revert Aid M-MuLV Reverse Transcriptase, oligo dT, RNase inhibitor and others for cDNA synthesis were from Fermentas (Ontario, Canada). Phospho-H3 and acetyl-H3 Abs were from Abcam (Cambridge, UK) and chromatin immunoprecipitation (ChIP) assay kit was from Millipore (Bedford, MA). TLR2, MyD88, IRAK 1, NF-κB, p38, phospho-p38, ERK1/2, phospho-ERK1/2, T-bet, β-Actin antibodies were from Santa Cruz Biotechnology (Texas, USA). Ara-LAM was isolated as previously described [20]. Lipopolysaccharide contamination (<25 ng/mg) was checked by the Limulus test. All antibodies for FACS were from BD Biosciences (San Diego, USA).

**Animals and parasites**

*L. donovani* strain AG-83 (MHOM/IN/1983/AG-83) was maintained in vitro in Medium-199 (Sigma, St. Louis, MO) with 10% FCS (Gibco-BRL) and virulence was maintained by passage through BALB/c mice. Stationary-phase promastigotes obtained by suitable transformation were used for experiments [21]. BALB/c mice were infected with stationary phase *L. donovani* promastigotes (i.v., 2×10^7/mouse). BALB/c mice (6–8 weeks, NCLAS, Hyderabad, India) were divided into the following experimental groups: (1) control (receiving PBS); (2) infected (receiving *L. donovani*); (3) Infected and Ara-LAM–treated infected (Ara-LAM 30μg/kg body weight-injected 2 days prior to infection); (4) control shRNA and (5) TLR2 shRNA (TLR2 shRNA or control shRNA [bearing scrambled sequence] treatment 72h before Ara-LAM treatment). Mice were sacrificed on 14 and 28 days after infection by cervical dislocation method as mentioned by Institutional Animal Ethical Committee (Bose Institute), bearing a registration number: 1796/PO/ERe/S/14/CPCSEA. This study followed the Institutional Animal Ethical Committee approval. *L. donovani* infection was expressed in Leishman-Donovan units.

**Isolation and purification of macrophages and CD8+ T-cells**

Thioglycolate-elicited (i.p., 4% w/v, 1.0 ml/mouse) macrophages from different experimental groups of BALB/c mice were infected with stationary phase *Leishmania* promastigotes at a
ratio of 1:10 [22]. Splenic CD8+ T-cells (purity >99% as ascertained by FACS) from the indicated mice were isolated by positive selection using CD8+ IMag beads, according to the manufacturer’s instructions (BD Biosciences). CD8+ T-cells were cultured in RPMI-1640 with plate-bound anti-CD3ε (5μg/mL) and CD28 (1μg/mL).

**Preparation of TLR2 and T-bet-specific siRNA**

TLR2 and T-bet-specific siRNA were synthesized using the Silencer siRNA Construction kit (Ambion). Scrambled siRNA was synthesized with the similar GC content. Silencing primers are listed in the Table 1.

**Flow cytometry**

CD8+ T-cells from differently treated mice groups were stained with PE-labeled TLR2, IFN-γ, IFN-γR, IL-12R, CD28 or IL-10, APC-Cy7 labelled CD25, FITC-labelled IFN-γ. For intracellular cytokine staining, brefeldin A (10μg/mL) was added 4h prior to harvest, fixed, and permeabilized (0.1% saponin) and stained with anti-IFN-γ-PE, anti-perforin-PE and anti-granzyme-B-PE antibodies. Cells were analyzed using a FACS Verse flow cytometer.

**Isolation of RNA and Reverse Transcriptase polymerase chain reaction**

Total RNA from purified CD8+ T-cells were extracted using TRI reagent using standard protocol [23]. The total RNA was reverse transcribed using Revert Aid M-MuLV reverse transcriptase (Fermentas). GAPDH was used as a loading control. Sequences of the PCR primers are given in the Table 1.

| Gene          | Sequences                                      |
|---------------|-----------------------------------------------|
| TLR2          | FP 5’ = TCTGGGCAGTCTTGAAACATT -3’             |
|               | RP 5’ = AGACTCCATTGGATGATGTCG -3’             |
| IFN-γ         | FP 5’ = AGCTCTTCTCATGGCTATTT -3’              |
|               | RP 5’ = TGTGCTGATGGCTGATTGTG -3’              |
| Perforin      | FP 5’ = CTGAGCGCTTTTTGAAATGTC -3’             |
|               | RP 5’ = AAGTAGCCTATTGGGAGCC -3’               |
| Granzyme-B    | FP 5’ = CCTCTCAGAAAGGAGGCCCC -3’              |
|               | RP 5’ = CTGACCTTCTCTGCGCTATCT -3’             |
| T-bet         | FP 5’ = CTCCTCTATCAAACCGATATG -3’             |
|               | RP 5’ = CTCGCTTTCTAAAATCTGTG -3’              |
| IFN-γ promoter| FP 5’ = GAGATATTACATACATCCGAGG -3’            |
|               | RP 5’ = TTAAGATGTTGAGATAGTGGG -3’             |
| Perforin promoter | FP 5’ = GTGATGCTGCTCAACTACCT -3’        |
|               | RP 5’ = CTAATGGATGCAGGATCTGG -3’              |
| Granzyme promoter | FP 5’ = ATGCCTCTGATTACCTACAC -3’         |
|               | RP 5’ = CAGAGAACCACGCATTACAG -3’              |
| TLR-2 siRNA   | FP 5’ = AAAGAGAAAGTACTTACTGACCTGTCCTC -3’    |
|               | RP 5’ = AATTGAGAAGTACTTTCTGCTGTCCTC -3’      |
| T-bet siRNA   | FP 5’ = AAAAAAAATGCTATGGAGGCTGCTC -3’        |
|               | RP 5’ = AAGAGAGGATGTTGATCGCTGTCCTC -3’       |
| GAPDH         | FP 5’ = GTGATGCTCCTGAACTACACA -3’             |
|               | RP 5’ = TCTGCTGCTGCTGACCTTGGCTC -3’          |

Table 1. Sequences of the PCR primers.

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**TLR2 Mediated CD8+ T-Cell Activation**

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CD8\(^+\) T-cell proliferation assay
Splenic CD8\(^+\) T-cells were cultured with autologous infected macrophages (10:1) for 72h and labelled with \([^{3}H]\)-thymidine (1μCi/10\(^5\) cells, JONAKI, DAE) for 18h before harvesting. \([^{3}H]\)-thymidine incorporation was determined using a liquid scintillation counter (Tri-Carb 2100TR; Packard Instrument) [24].

Chromatin immunoprecipitation (ChIP) assay
ChIP assays were conducted using the ChIP Assay kit following the manufacturers protocol. Purified CD8\(^+\) T-cells (1×10\(^6\)) from the indicated mice were co-cultured with autologous \(L.\) donovani–infected macrophages (1×10\(^5\)) for 45 min, paraformaldehyde(1%)-fixed for 10 min at 37°C and washed with ice-cold PBS containing 1mM PMSF, harvested and lysed in SDS lysis buffer. DNA was sheared by ultrasonication using a High Intensity Ultrasonic Processor (Hielscher, Teltow, Germany) for 3 × 10s pulses at 20% amplitude. Lysates were cleared by centrifugation and diluted in ChIP dilution buffer. Lysates were pre-cleared using protein A-agarose and a sample of “input DNA” was collected at this point. Protein-DNA complexes were immunoprecipitated with 5μg of antibodies (phospho H3, acetyl H3, T-bet) overnight at 4°C. Antibody-protein-DNA complexes were then captured using protein A-agarose for 1 h at 4°C. After washing beads with low and high salt, LiCl, and TE buffers, the protein/DNA complexes were eluted in buffer (1%SDS, 0.1M NaHCO\(_3\) ). DNA was then extracted and precipitated. PCR was conducted using promoter specific primers (Table 1).

Preparation of nuclear and cytoplasmic extracts
The nuclear extracts were prepared from CD8\(^+\) T-cells as described previously [25]. Briefly, cells were resuspended in ice-cold hypotonic buffer [10 mM HEPES (pH 7.9), 1.5 mM MgCl\(_2\), 10 mM KCl, 0.2 mM PMSF, and 0.5 mM DTT] for 10 min, homogenized and the nuclei were precipitated (3300×g, 5 min, 4°C). The supernatant was used as the cytoplasmic extract. The nuclear pellet was extracted in ice-cold nuclear extraction buffer [20mM HEPES (pH 7.9), 0.4M NaCl, 1.5mM MgCl\(_2\), 0.2mM EDTA, 25% glycerol, 0.5mM PMSF and 0.5mM DTT] for 30min (12,000×g for 30 min, 4°C). The supernatant was used as nuclear extract.

Preparation of cell lysate and immunoblot analysis
CD8\(^+\) T-cells were lysed using lysis buffer for isolation of protein [26]. Equal amounts of protein (30μg) were subjected to 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked overnight with 3%BSA in Tris-saline buffer (pH 7.5), and immunoblotting was performed to detect MyD88, IRAK 1, NF-xB, T-bet, β-Actin, GAPDH, and phosphorylated or total p38MAPK and ERK1/2, as described previously [27].

Coimmunoprecipitation
In coimmunoprecipitation studies, the lysates of differently treated CD8\(^+\) T-cells were incubated with specific antibody (TLR2, MyD88). The complexes were captured with immobilized Protein A-agarose beads, washed, resolved by 10% SDS-PAGE and developed with antibodies to MyD88 and IRAK 1 to detect TLR2-MyD88, MyD88-IRAK 1 interactions [28].

Statistical analysis
All experiments were performed in triplicate and a minimum of 4 mice was used per group. The data, represented as mean values ± SD, are from one experiment that was performed at
least 3 times. One-way ANOVA test (using a statistical package, Instat3) was employed to assess the significance of the differences. \( p \leq 0.001 \) was considered significant.

**Results**

**Ara-LAM upregulates IL-12R and IFN-γR in CD8\(^+\) T-Cells in *L. donovani* infection**

We studied the effect of Ara-LAM on BALB/c mice-derived CD8\(^+\) T-cells in indicated groups. Naïve CD8\(^+\) T cells proliferate in response to TCR and CD28 signals, but require IFN-γ and IL-12 to develop effector functions [29–30]. We investigated the status of CD28 on CD8\(^+\) T cells expressing CD25, receptor for IL-12 (IL-12R) and IFN-γ (IFN-γR) [31–32]. 28 days after infection, compared to the splenic CD8\(^+\) T cells of untreated infected mice, Ara-LAM strongly induced the expression of IL-12R and a moderate induction of IFN-γR on splenic CD8\(^+\) T cells, co-expressing CD25 (Fig 1A). Activation of TLR2 in CD8\(^+\) T-cells is associated with their enhanced effector functions [18–19]. Therefore, we tested whether Ara-LAM, being a TLR2 ligand, could activate the CD8\(^+\) T-cells by upregulating the transcription of perforin and granzyme-B through the activation of the CD8\(^+\) T-cells by upregulating the transcription of perforin and granzyme-B.

![Fig 1. Characterization of CD8+ T cells at 28 days postinfection upon Ara-LAM treatment in *Leishmania donovani* infected BALB/c mice.](https://doi.org/10.1371/journal.pone.0142800.g001)
granzyme-B. We observed a significant enhancement in both perforin and granzyme-B expression in CD8⁺ T-cells isolated from Ara-LAM treated *L. donovani* infected mice compared to that of untreated infected mice (Fig 1B).

**Ara-LAM-induced CD8⁺ T-cells activation in *L. donovani* infection is TLR2-dependent**

We examined the effect of Ara-LAM treatment on TLR2 surface expression in CD8⁺ T-cells from different groups of BALB/c mice. Ara-LAM treatment significantly augmented the expression of TLR2 in splenic CD8⁺ T-cells on 14 and 28 days post infection (Fig 2A). Because we observed significantly enhanced expressions of IFN-γ, perforin and granzyme-B in CD8⁺ T-cells isolated from Ara-LAM treated *L. donovani* infected mice compared to that of

![Fig 2](image-url)
untreated infected mice (Fig 2A), we tested if TLR2 silencing could abrogate these effector functions. TLR2 silencing abrogated the Ara-LAM induced generation of IFN-γ, perforin, granzyme-B molecules in CD8+ T-cells isolated from the infected mice (Fig 2A and 2B).

It has been noted earlier that *Leishmania* infection of the susceptible host results in apoptosis of T-cells, leading to impairment of cell-mediated immunity [33]. Therefore, we investigated whether Ara-LAM could restore the impaired CD8+ T-cell proliferation in *Leishmania*-infected mice. Ara-LAM treatment resulted in significant enhancement of splenic CD8+ T-cell proliferation compared to the splenic CD8+ T-cells from untreated infected mice. The Ara-LAM–induced CD8+ T-cell proliferation was significantly attenuated during TLR2 silencing condition (Fig 2C). These results suggested that Ara-LAM stimulation leads to the enhanced effector function as well as the proliferation of CD8+ T-cells via TLR2 dependent pathway.

### Ara-LAM triggers T-bet recruitment and histone modification at the IFN-γ, Perforin and Granzyme-B promoter regions in CD8+ T-cells

The expressions of IFN-γ, perforin and granzyme-B in CD8+ T cells is principally regulated at the level of transcription, which, in turn, is strictly dependent upon the favourable histone modifications at their respective promoter regions [19]. Therefore, we performed ChIP assays to investigate whether the Ara-LAM-mediated enhancement of IFN-γ, perforin and granzyme-B expression in CD8+ T-cells was due to chromatin modification at their respective promoter regions. We observed a significantly higher level of phosphorylated (Fig 3A) and acetylated (Fig 3B) histones at the promoter regions of IFN-γ, perforin, granzyme-B in the splenic CD8+ T-cells of Ara-LAM treated *L. donovani* infected BALB/c mice relative to the splenic CD8+ T-cell from untreated infected mice. These Ara-LAM mediated histone modifications at the IFN-γ, perforin and granzyme-B promoter regions in CD8+ T-cells were significantly attenuated in TLR2 silenced condition (Fig 3A and 3B). Therefore, Ara-LAM induced transcription favourable histone modifications at the IFN-γ, perforin, and granzyme-B loci of CD8+ T-cells in a TLR2 dependent pathway.

The effector functions of CD8+ T-cells are predominantly regulated by the transcription factor T-bet [34]. Therefore, we investigated the accumulation of T-bet in the splenic CD8+ T-cells of different experimental mice groups. Immunoblot analysis showed that accumulation of T-bet was significantly higher in the splenic CD8+ T-cells of Ara-LAM treated *L. donovani* infected mice compared to that of the untreated infected mice (Fig 3C).

T-bet binding at the promoter regions of IFN-γ, perforin, granzyme-B is a crucial event for the optimal induction of these effectors molecules in CD8+ T-cells [13]. Therefore, we performed ChIP-on-ChIP assay to investigate the interaction of T-bet with the promoter regions of IFN-γ, perforin, and granzyme-B in CD8+ T-cells from Ara-LAM treated and untreated *L. donovani* infected BALB/c mice. Ara-LAM treatment induced a strong association of T-bet with the IFN-γ, perforin, and granzyme-B promoter regions in CD8+ T-cells compared with that of the untreated *L. donovani* infected mice; TLR2 silencing decreased Ara-LAM induced T-bet recruitment to the promoter regions of IFN-γ, perforin, granzyme-B in CD8+ T-cells (Fig 3D). Collectively, Ara-LAM-induced TLR2 dependent activation of T-bet augmented the transcription of target effector genes in CD8+ T-cells of infected mice.

### Ara-LAM promotes NF-κB activation and MAPK signaling in CD8+ T-cells

Ara-LAM triggers the TLR2 mediated downstream signaling in host macrophages [18–19]. However, Ara-LAM induced modulation of TLR2 signaling in CD8+ T-cells has not been explored till date. The association between TLR2 and MyD88 is a crucial event for the initiation
Therefore, we carried out co-immunoprecipitation studies to investigate the TLR2-MyD88 interaction in Ara-LAM–treated CD8+ T-cell. We observed a strong association between TLR2 and MyD88 in Ara-LAM–treated CD8+ T-cells compared with that of the untreated CD8+ T-cells. IRAK-1, crucial for activation of TLR2 downstream signaling [18], was found to be intricately associated with MyD88 in Ara-LAM–treated CD8+ T-cells compared to the untreated CD8+ T-cells (Fig 4A). Furthermore, nuclear translocation of NF-κB were significantly augmented in Ara-LAM treated CD8+ T-cell compared to the...
untreated CD8⁺ T-cells. Ara-LAM induced TLR2 downstream signaling mediated NF-κB translocation was completely abrogated under TLR2 silenced condition (Fig 4B).

In addition to the NF-κB activation, TLR2 signaling can also modulate p38 and ERK-1/2 MAPK signaling cascades [19]. Therefore, we investigated whether Ara-LAM treatment could induce MAPK phosphorylation in CD8⁺ T-cells. Ara-LAM treatment resulted in significantly higher level of p38 MAPK phosphorylation (Fig 4C), however; it failed to induce significant ERK1/2 phosphorylation in CD8⁺ T-cells (Fig 4C). TLR2 silencing abrogated the Ara-LAM induced p38 phosphorylation (Fig 4D). Taken together, Ara-LAM activated p38MAPK and NF-κB activation in infected CD8⁺ T-cells.

Fig 4. Ara-LAM activates TLR2 signalling via activation of NF-κB and p38 MAPK in naive CD8⁺ T-cells. (A) Purified CD8⁺ T-cells (1×10⁶/mL) were stimulated with plate-bound anti-CD3ε mAbs (5μg/mL) and CD28 (1μg/mL) for 24 hrs and transfected with control siRNA or TLR2 siRNA, followed by Ara-LAM (3μg/mL) treatment for 24 hr. The cells were then lysed and subjected to immunoprecipitation with anti-TLR2 antibody, and the blots were probed with anti-MyD88 antibody. (B) Cells were lysed and immunoprecipitated with anti-IRAK1 antibody; the blots were probed with anti-IRAK1 antibody. Cytosolic and nuclear protein extracts were analyzed for nuclear translocation of NF-κB. (C-D) In yet separate experiments, CD8⁺ T cells were treated by Ara-LAM for 5, 15, 30, and 60 min, and lysed. The lysate was subjected to Western blot analysis for the expression of p38MAPK, phospho-p38MAPK and ERK1/2, phospho-ERK1/2. Data represented were one of the three independent experiments with similar results performed in the same way.

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Ara-LAM enhances T-bet expression in CD8+ T-cells via upregulation of NF-κB and p38MAPK signaling

Since, Ara-LAM induced efficient activation of T-bet in CD8+ T-cells (Fig 3C) and enhanced the activation of NF-κB and p38MAPK in CD8+ T-cell (Fig 4), we hypothesized that T-bet activation in CD8+ T-cells might be regulated either by the NF-κB or MAPK signalling or by both of these signalling molecules. Ara-LAM mediated activation as well as mRNA expression of T-bet was attenuated in the presence of SB203580 and SN50, the pharmacological inhibitors of p38MAPK and NF-κB, respectively (Fig 5A and 5B), suggesting that the activation of T-bet in Ara-LAM treated CD8+ T-cells was regulated by TLR2 triggered NF-κB and p38MAPK signalling cascade. Besides, the enhanced IFN-γ, perforin and granzyme-B expression in Ara-LAM stimulated CD8+ T-cells were significantly decreased during T-bet silencing, as well as, NF-κB and the p38MAPK inhibition (Fig 5B). Therefore, the upregulation of IFN-γ, perforin and granzyme-B expression in Ara-LAM stimulated CD8+ T-cells were due to the NF-κB and the p38MAPK dependent activation of T-bet.

Ara-LAM reduces hepatic and splenic parasitic burden in BALB/c mice

We have observed that Ara-LAM activates the CD8+ T cells via upregulation of IFN-γ and the cytotoxic molecules perforin and granzyme-B. These activated T cells have the ability to kill the infected macrophages to reduce the parasite burden [5–6]. In accordance with this phenomenon, we observed a significant reduction of the hepatic as well as splenic parasite burden in the Ara-LAM treated infected mice groups compared to the untreated infected mice groups (Fig 6) on 14 and 28days postinfection. However, Ara-LAM mediated clearance of parasites was significantly attenuated in TLR2 silenced condition. Collectively, the data clearly suggest that Ara-LAM plays a very important role to clear the L. donovani infection via a TLR2 dependent mechanism.

Fig 5. Ara-LAM promptly regulates effector functions in CD8+ T-cells through NF-κB and p38MAPK mediated T-bet signalling. (A) CD8+ T-cells were isolated by MACS from the spleen BALB/c mice. Purified CD8+ T cells were stimulated as described previously and allowed to transfect with control siRNA or T-bet siRNA, or treated with SB203580 (SB) (5μg/ml), or SN50 (SN) (20μg/ml), subsequently followed by Ara-LAM (3μg/mL) treatment for 24 hr. The cells were then lysed and nuclear protein extracts were prepared, followed by subjected to Western blot with anti-T-bet. (B) The blot shown is representative of triplicate experiments that yield similar type of results. In a separate set of experiments, after the treatment schedule, the cells were collected in Trizol for RNA extraction, and conventional RT PCR analysis was performed to determine the expression of T-bet, IFN-γ, perforin, granzyme-B. Data represented were one of the three independent experiments with similar results performed in the same way.

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Reversion of the Th subset expansion in *L. donovani* infected mice

We checked the effect of Ara-LAM on IFN-γ and IL-10 producing-CD8+ T-cells in both spleen and liver by flow cytometry. In both uninfected and infected mice, Ara-LAM up-regulated the IFN-γ secreting CD8+ T-cells in spleen as well as liver. IL-10 producing CD8+ T-cells were suppressed by Ara-LAM pretreatment in both spleen and liver of infected as well as uninfected mice. Although in case of hepatic CD8+ T-cells, the effect of Ara-LAM on both IFN-γ and IL-10 secretion was not very significant (Fig 7).

**Discussion**

Leishmanial pathogenesis is associated with abrogated pro-inflammatory responses, resulting in immune suppression of the host [9–11]. The persistence of the infection during VL was due to the impaired cell-mediated immunity which in turn was intricately associated with the
severe dysfunction of cytotoxic CD8+ T-cells [7]. Therefore, activation of CD8+ T cells is very much important to eradicate L. donovani mediated infection. Recently, it has been reported that activation of TLR signalling could restore the impaired effector function of CD8+ T-cells in various models of infectious diseases [35]. Therefore, we intended to study whether Ara-LAM, so far known to be a TLR2 ligand [18], could restore the functional capacity of the effector CD8+ T-cells during VL.

We confirmed that Ara-LAM pre-treatment in L. donovani infected BALB/c mice significantly augmented the expression of TLR2 along with the concomitant increase in IFN-γ, perforin, and granzyme-B in the splenic CD8+ T-cells (Figs 1 and 2). Because knowing phenotype is important for its functional significance, we examined the phenotype of these T cells, which are IL-12R+IFN-γ+CD28+ co-expressing CD25 (Fig 1A). Moreover, Ara-LAM pre-treatment was found to be associated with significant enhancement of CD8+ T-cell proliferation (Fig 2C), a prerequisite for its effector function. TLR2 silencing significantly attenuated such Ara-LAM mediated enhanced expression of these effector molecules in CD8+ T-cells (Fig 2).

The optimal transcriptional induction of the IFN-γ, perforin, granzyme-B genes in CD8+ T-cells requires histone modification at their respective promoter regions [19]. Our study revealed that Ara-LAM pretreatment led to transcription favourable histone H3 phosphorylation and acetylation specifically at the promoter region of IFN-γ, perforin, granzyme-B in the splenic CD8+ T-cells of L. donovani-infected BALB/c mice (Fig 3A and 3B). In line with the fact that the activation of the transcription factor T-bet is a crucial event for the expression of IFN-γ, perforin, granzyme-B in CD8+ T-cells [13, 34], we observed an increased T-bet accumulation (Fig 3C) along with enhanced T-bet binding to the promoter regions of IFN-γ, perforin, granzyme-B in the Ara-LAM treated splenic CD8+ T-cells of L. donovani infected BALB/c mice (Fig 3D). Hence, our study revealed that Ara-LAM induces T-bet dependent activation of effector molecules in CD8+ T-cells to hinder leishmanial pathogenesis.
Further, we intended to investigate the underlying molecular mechanism of Ara-LAM induced CD8+ T-cells activation during VL. Since, Ara-LAM confers its protective functions against VL via activation of the TLR2 downstream signaling in host macrophages [18–19], we investigated Ara-LAM mediated modulation of TLR2 signaling in CD8+ T-cells. Ara-LAM treatment led to successful initiation of TLR2 signalling in naive CD8+ T-cells via the TLR2-MyD88 association (Fig 4A), resulting in the selective activation of intermediate signalling molecules, IRAK1 and ultimately leading to nuclear translocation of NF-κB (Fig 4B). Ara-LAM treatment also led to increased p38MAPK phosphorylation along with concomitant attenuation of ERK1/2 phosphorylation in splenic CD8+ T-cells (Fig 4C and 4D). These observations indicate that Ara-LAM induced activation of TLR2 downstream signalling molecules leads to enhanced effector function of CD8+ T-cells during VL.

We observed that inhibition of NF-κB and p38MAPK, by their respective pharmacological inhibitors, significantly abrogated the Ara-LAM–induced T-bet expression and activation in CD8+ T-cells (Fig 5A and 5B). Our results indicated the active involvement of NF-κB and p38MAPK signalling in the regulation of Ara-LAM driven T-bet activation. Further, Ara-LAM treatment significantly reduced the parasite burden in the liver and spleen 14 and 28 days after the infection (Fig 6) accompanied with the expansion of IFN-γ+CD8+ T-cells (Fig 7). It is therefore feasible to conclude that Ara-LAM mediated reduced parasitic burden may be due to the CD8+ T-cell driven killing of the infected macrophages. It is noteworthy to mention that Ara-LAM works well when utilized as an immunotherapeutic agent administered 2days prior to L. donovani infection [18–20, 28].

In summary, Ara-LAM confers significant protection through activation of CD8+ T-cells in L. donovani infected BALB/c mice. The novelty of our work lies in the fact that Ara-LAM-induced TLR2 signaling leads to the activation of the transcription factor T-bet which plays a pivotal role in restoring the effector functions of CD8+ T-cells in L. donovani infected susceptible host. Thus, we are one the way to devise a strategy in near future so that Ara-LAM can be used as a suitable vaccine during L. donovani infection.

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Author Contributions
Conceived and designed the experiments: SB SKM SM. Performed the experiments: SB SKM BPC MKJ KH SD SBM. Analyzed the data: SB BS SM. Wrote the paper: SB SKM BS SM.

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