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 leishmanisis (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 leishmaniais [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, Hydrabad, 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-lebelled 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.

Table 1. Sequences of the PCR primers.

Gene	Sequences
TLR2	FP 5' – TCTGGGCAGTCTTGAACATTT –3'
	RP 5' – AGAGTCAGGTGATGGATGTCG –3'
IFN-γ	FP 5' – AGCTCTTCCTCATGGCTGTTTC –3'
	RP 5' – TGTTGCTGATGGCCTGATTGT –3'
Perforin	FP 5' – CTGAGCGCCTTTTTGAAGTC –3'
	RP 5' – AAGGTAGCCAATTTTGCAGC –3'
Granzyme-B	FP 5' – CTCTCGAATAAGGAAGCCCC –3'
	RP 5' – CTGACCTTGTCTCTGGCCTC –3'
T-bet	FP 5' – CCTCTTCTATCCAACCAGTATC –3'
	RP 5' – CTCCGCTTCATAACTGTGT –3'
IFN-γ promoter	FP 5' – GAGAAATTCACATTACAAGGGC –3'
	RP 5' – TTAAGATGGTGACAGATAGGTGG –3'
Perforin promoter	FP 5' – GTACTAGCCTGCTCAAACCT –3'
	RP 5' – CTAATCACAGTGTCCCATGAG –3'
Granzyme promoter	FP 5' – ATGCTCCTGATTACCCTCAC –3'
	RP 5' – CAGAGAACCACCACTTACAG –3'
TLR-2 siRNA	FP 5' –AAAGAGAAAGTACTTACTGCACCTGTCTC–3'
	RP 5' – AATGCAGTAAGTACTTTCTCTCCTGTCTC –3'
T-bet siRNA	FP 5' – AAACAAACATCCTGTAATGGCCCTGTCTC –3'
	RP 5' –AAGCCATTACAGGATGTTTGTCCTGTCTC–3'
GAPDH	FP 5' – GTTGTCTCCTGCGACTTCAACA –3'
	RP 5' – TCTCTTGCTCAGTGTCCTTGCT –3'

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CD8+ T-cell proliferation assay

Splenic CD8+ T-cells were cultured with autologus infected macrophages (10:1) for 72h and labellled with [3H]-thymidine (1μCi/10^5 cells, JONAKI, DAE) for 18h before harvesting. [3H]-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 $3 \times 10s$ 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-κB, 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≤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-expresseing CD25 (Fig 1A). Activation of TLR2 in CD8$^+$ T-cells is associated with their enhanced effecter 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
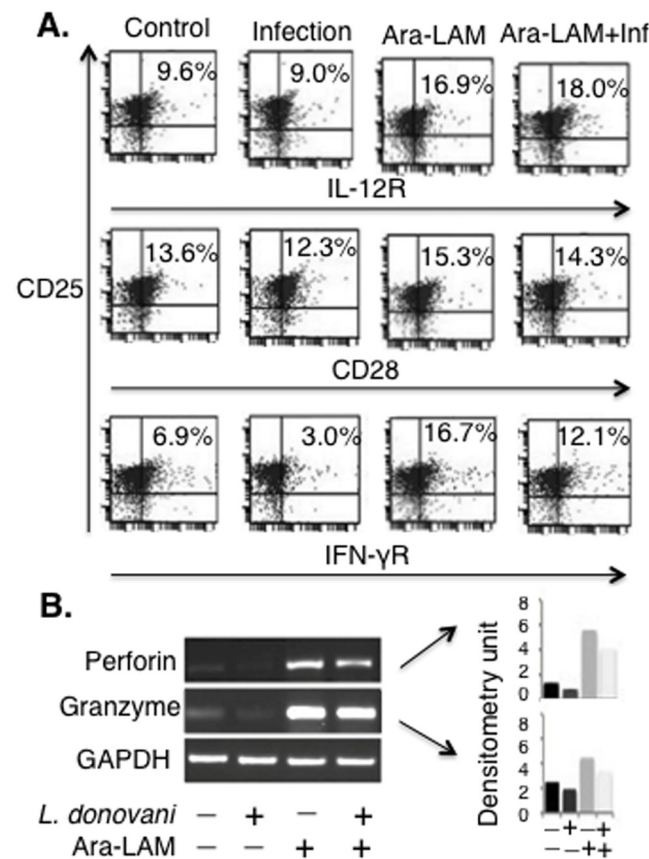

Fig 1. Characterization of CD8+ T cells at 28 days postinfection upon Ara-LAM treatment in *Leishmania donovani* infected BALB/c mice. (A) CD8$^+$ T from differently treated BALB/c mice 28 days postinfection were subjected to FACS analyis to check the expression of CD25$^+$IL12R$^+$, CD25$^+$CD28$^+$, CD25$^+$IFN-γR$^+$ cells. Data are from one of three representative experiments. (B) In separate set of experiment, CD8$^+$ T cells from differently treated mice group were isolated and cultured in presence of plate-bound anti-CD3ε mAbs (5μg/mL) and CD28 (1μg/mL) and expresion of perforin and granzyme-B was done by conventional RT PCR. Data are from one of three representative experiments.

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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 28days 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
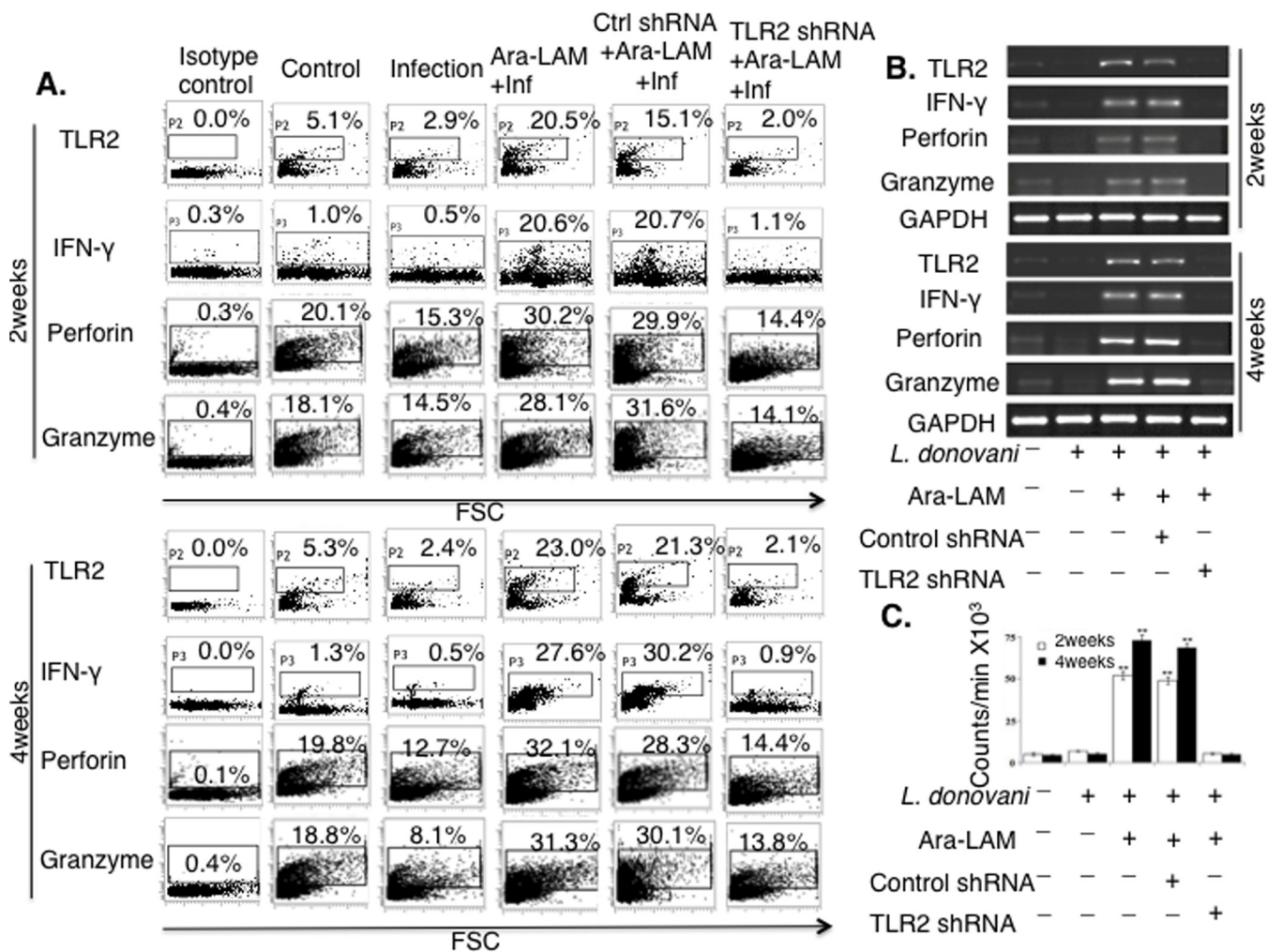

Fig 2. Ara-LAM facilitates TLR2 dependent activation and expansion of CD8+ T-cells in *Leishmania donovani* infected BALB/c mice. (A) Purified CD8+ T-cells were subjected to FACS analysis for TLR2 expression. Separately, purified CD8+ T-cells from differently treated mice were co-cultured with autologous infected macrophages (10:1) for 48hrs and IFN-γ, perforin, granzyme-B expression were determined by intracellular FACS. (B) CD8+ T-cells from differently treated mice groups were stimulated as described previously and conventional RT PCR was done after RNA extraction. (C) Purified CD8+ T-cells from differently treated mice and autologous *L. donovani*–infected macrophages were co-cultured for 72 hours. Proliferation was determined by an 18 h [3H] thymidine incorporation assay. Data were presented as count/million (×10^3). Results were mean value ±SD. from triplicate wells. The asterisk indicated a statistically significant induction (**$P<0.001$) of T-cell proliferation, compared with that in infected mice.

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 accumulaion of T-bet in the splenic CD8+ T-cells of different experimental mice groups. Immunoblot analysis showed that accmulation 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
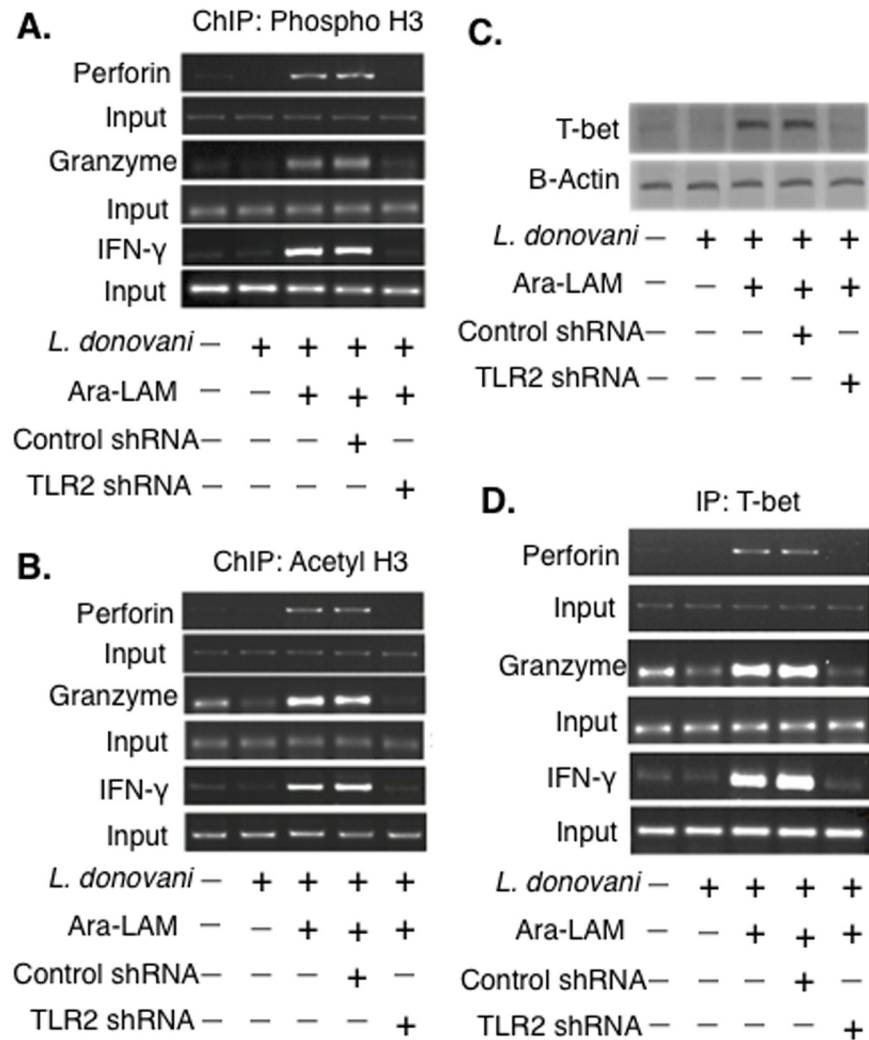
Fig 3. **Histone H3 modifications at the IFN-γ, Perforin, Granzyme-B promoter of CD8+ T-cells in different groups of BALB/c mice.** (A-B) CD8+ T cells from differently treated mice groups were co-cultured with autologous infected macrophages for 45 min and chromatin immunoprecipitation (ChIP) assays were conducted. Immunoprecipitations were performed using Abs specific to phosphorylated H3 histone (IP: phospho-H3), acetylated H3 histone (IP: acetyl-H3) and conventional RT PCR was performed using primers specific to the *IFN-γ*, *perforin* and *granzyme-B* promoter region. (C) CD8+ T-cells were co-cultured with infected macrophages, lysed and the nuclear protein extracts were analyzed for the activation of T-bet by Western blot. (D) CD8+ T-cells from differently treated mice group were co-cultured with autologous *L. donovani*–infected macrophages, Immunoprecipitations were conducted using T-bet (IP: T-bet) specific Abs. Conventional RT-PCR was performed for amplifying the putative T-bet binding sites of the *IFN-γ*, *perforin*, *granzyme-B* promoter. Data represented were one of the three indepenedent experiments with similar results performed in the same way.

of TLR2 downstream signaling [18]. 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-cell 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
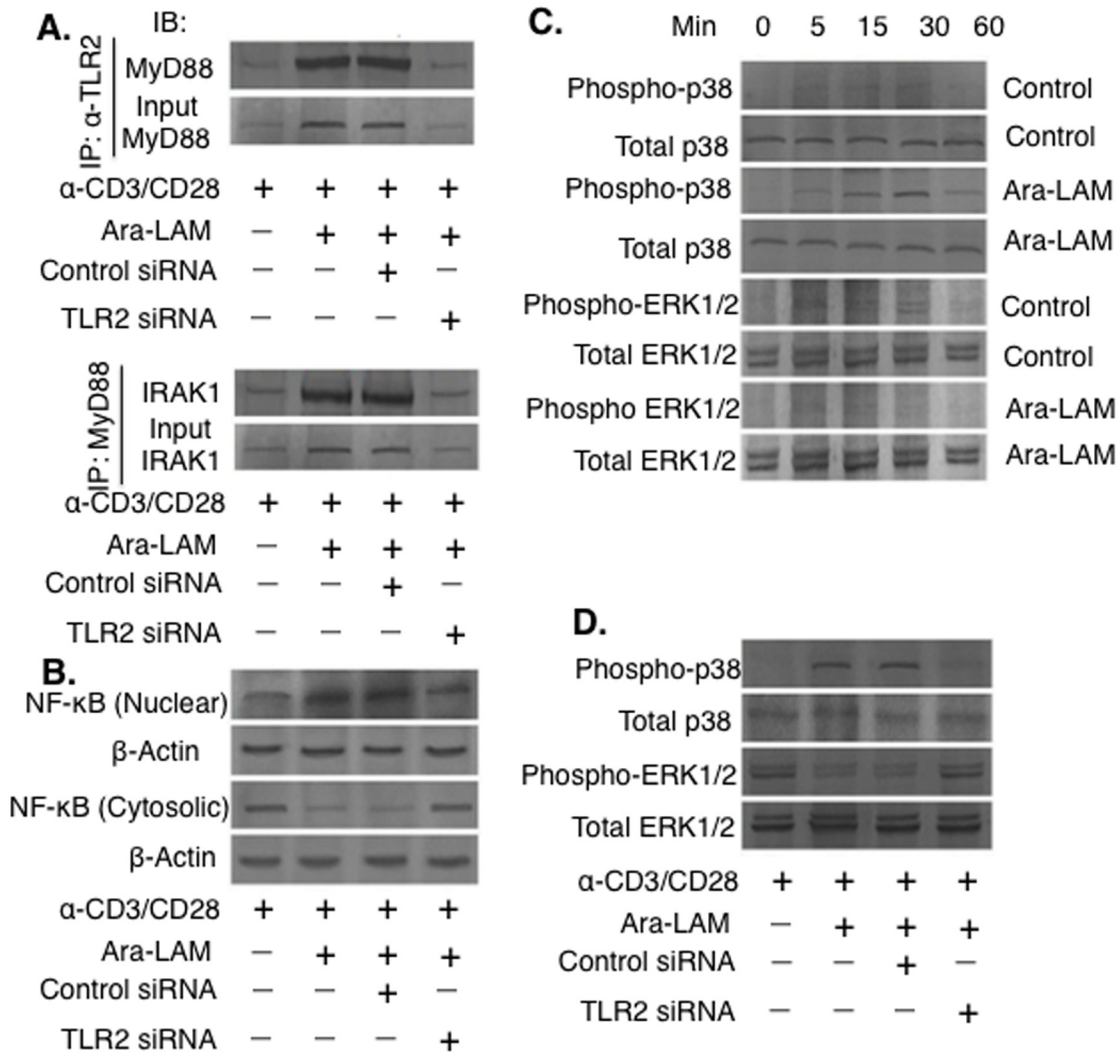
Fig 4. Ara-LAM activates TLR2 signalling via activation of NF-κB and p38 MAPK in naive CD8+ T-cells. (A) Purified CD8+ T-cells (1×10^6/mL) were stimulated with plate-bound anti-CD3ε mAbs (5μg/mL) and CD28 (1μg/mL) for 24 hrs and transfected with control siRNA or TLR2 siRNA, followed by Ara-LAM (3μg/mL) 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-MyD88 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 independenet experiments with similar results performed in the same way.

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 p38MAPK 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.

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.
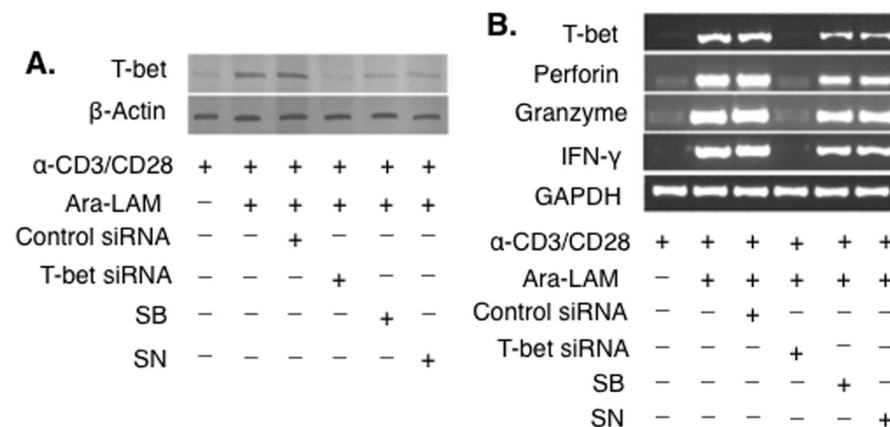

Fig 5. Ara-LAM promptly regulates effector functions in CD8⁺ T-cells through NF-κB and p38MAPK mediated T-bet signalling. (A) CD8⁺ T-cells were isolated by MACS from the spleen BALB/c mice. Purified CD8⁺ T cells were stimulated as described previously and allowed to transfect with control siRNA or T-bet 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 independendent experiments with similar results performed in the same way.

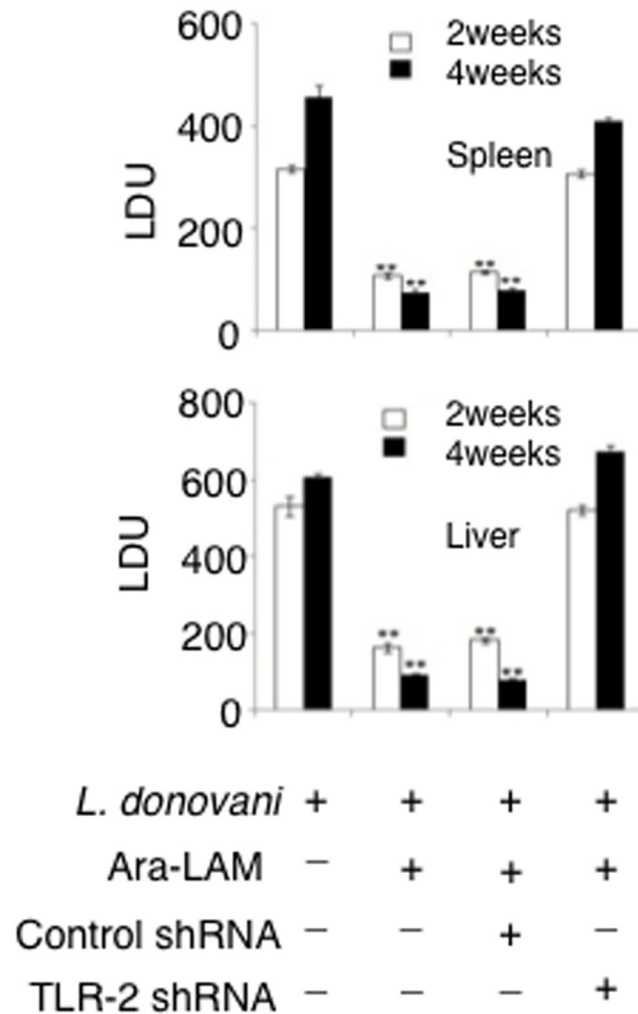doi:10.1371/journal.pone.0142800.g005

Fig 6. Ara-LAM treatment in _L. donovani_ infected BALB/c mice showed reduced parasite burden. Mice were treated as described in Fig 1 legend. Mice from different groups were sacrificed on day 14 and 28 post-infection. The parasite burden in liver and spleen were expressed in Leishman-Donovan units (LDUs). Results were for 3 independent experiments and represented the mean values ± SD for four animals per group. **$P<0.001$ for the comparison with infected mice.

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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
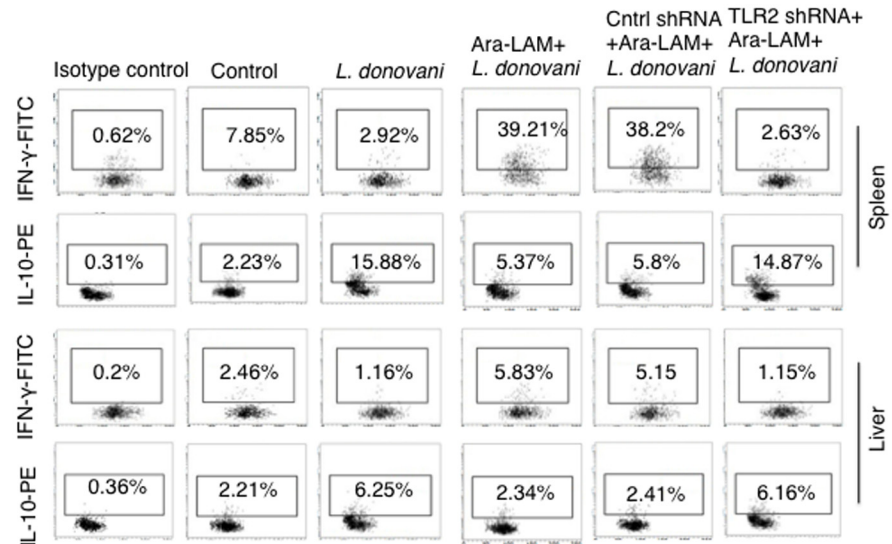
Fig 7. Ara-LAM up-regulates IFN-γ secreting CD8+ T-cells in an organ-dependent manner during *L. donovani* infection. 28days post-infection, different groups of mice were sacrificed. Splenocytes and hepatocytes were stimulated with soluble leishmanial antigen (SLA, 10 μg/mL) for 48hrs. Before harvesting, cells were incubated with brefeldin A (10 μg/mL) for 4 hrs, CD8+ T cells were MACS sorted (see Materials and Methods), permeabilized (0.1% saponin) and stained with anti-mouse IFN-γ-FITC and anti-mouse IL-10-PE antibodies and were analyzed by flow cytometry. Data are from 1 of 3 experiments conducted in the same way with similar results.

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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 accumulaion (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 immunotherapiutic 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.

Acknowledgments

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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