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Original article

Arabinosylated lipoarabinomannan modulates the impaired cell mediated immune response in *Mycobacterium tuberculosis* H37Rv infected C57BL/6 mice

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Abstract

Mycobacterium tuberculosis is a facultative intracellular pathogen that flourishes inside the host macrophages. This organism has the ability to deactivate the cell-mediated immune responses involving the down-regulation of pro-inflammatory cytokines, T cell proliferation, apoptosis of CD4+T cells and impairment of the expression of MHC Class II molecules. We observed that Arabinosylated Lipoarabinomannan (Ara-LAM), a glycolipid present in the cell wall of the avirulent *Mycobacterium smegmatis*, could effectively restrict the growth of tubercle bacilli, induced the transcription of Th1 cytokines in alveolar macrophages (AMs) and splenocytes, enhanced the frequency of CD4+T cells secreting IFN-γ and induced the expression of MHC Class II molecules on the splenocyte membrane, compared to that of *Mycobacterium tuberculosis* H37Rv infected C57BL/6 mice. Collectively our findings strongly suggest that Ara-LAM had the potency to restore the impaired cell mediated immune responses in mice infected with *Mycobacterium tuberculosis* H37Rv, and hence could be utilized as an effective immuno-prophylactic tool in the control of tuberculosis.

Keywords: Cellular immunity; Antigen presentation; Tuberculosis

1. Introduction

Tuberculosis, a widespread infectious disease affecting 7.5 billion people per year [1], is caused by *Mycobacterium tuberculosis,* a facultative intracellular pathogen that replicates within the phagosome, evading the fusion with lysosomes [2]. *Mycobacterium tuberculosis* infection initially induces inflammatory responses and granuloma formation within the lungs [3], but gradually exerts its pathogenic effects by suppressing the cell-mediated immune responses [4]. It is known that both CD4+T cells and CD8+T cells, the main effectors of T cell mediated immunity [5], undergo apoptosis under prolonged *M. tuberculosis* infection thus decreasing Th1 cytokine production. Besides, *M. tuberculosis* as well as *M. bovis* inhibits antigen processing by murine macrophages via a mechanism involving decreased synthesis of MHC Class II molecules [6]. Since the commercially available antibiotics have proven to be ineffective in controlling the progression of drug resistant tuberculosis, newer approaches aimed at targeting the immune mechanisms of the host are coming up as alternative medication. Mycobacterial lipid antigens and oligosaccharide protein conjugates are proven to be highly immunogenic against the virulent pathogen [7,8]. Furthermore, *Mycobacterium welchii*, a saprophytic non-virulent organism is reported as an effective vaccine against tuberculosis, leprosy and other diseases including cancer, because of its

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immunomodulatory properties [9–11]. *Mycobacterium smegmatis* vaccine vectors have also proven to induce HIV-1 envelope specific CD8+T cell responses [12].

Ara-LAM, a cell wall glycolipid isolated from avirulent *Mycobacterium smegmatis* is a potent inducer of pro-inflammatory cytokines and iNOS [13,14]. This compound is also recently reported to induce IL-10 that behaves in a pro-inflammatory manner [15]. However, whether Ara-LAM possessing such pro-inflammatory response evoking properties could modulate the host cell mediated immunity has been unexplored yet. Therefore our present investigation emphasizes on the effectiveness and the utilization of Ara-LAM in modulating the impaired cell mediated immunity in a murine model of tuberculosis.

2. Materials and methods

2.1. Animals

Black C57BL/6 mice (National Centre for Laboratory Animal Sciences, Hyderabad, India, 6–8 weeks), 5 mice were placed in each group. Two mice were infected separately and sacrificed 7 days post infection to confirm the establishment of the infection. Since the set of *in vivo* experiments were repeated twice, 30 mice were used to carry out the entire study.

2.2. *Culture of* Mycobacterium tuberculosis *H37Rv*

Mycobacterium tuberculosis H37Rv (ATCC 25177) was grown at 37 °C on Middlebrook 7H10 Agar (Difco) medium supplemented with 10% OADC (Oleic Acid, Albumin, Dextrose, Catalase, Becton Dickinson) and 0.05% Tween-80 (Sigma). Bacteria grown in late log phase were grown in liquid Middlebrook 7H9 media supplemented with 0.2% glycerol, 0.05% Tween-80, and 10% OADC, and frozen at −70 °C. CFU was determined on Middlebrook 7H10 agar, and single cell suspension was prepared as described by Havlir et al. [16].

2.3. Isolation and purification of Ara-LAM

As described in our previous work, Majumder N. et al. [15]. In brief, Ara-LAM was purified from strain LR222 of *M. smegmatis*. All the experiments were performed with a single isolate of the culture.

LAM was isolated and roughly purified according to the process stated by Hunter et al., SDS-PAGE was performed, and the specific 37 kDa band of LAM was visible by Silver Staining, alongside the known Man-LAM isolated from *M. tuberculosis H37Rv*. Coomassie blue staining of the gel showed no bands, and protein estimation of this compound by Bio-Rad protein assay reagent also showed negligible protein content. This LAM was further purified according to the rapid and simple method stated by Hamasur et al. [17]. LPS contamination was checked by *Limulus test* and was found to be <25 ng/mg in both Man-LAM and Ara-LAM.

2.4. Infection of mice and treatment with Ara-LAM

2.4.1. Intravenous infection of mice

C57BL/6 mice were divided into three separate groups with five mice in each, as follows: the group 1 (control mice) were injected i.v with 100 µl PBS, group 2 (Infected mice) were injected with 10^7 CFU of *M. tuberculosis* H37Rv in 0.05% Tween-80 containing PBS via the lateral tail vein [18]; group 3 (Ara-LAM pretreated group) were injected i.p with 30 µg of Ara-LAM, kept for 2 days, following which they were infected with the bacillus as described before.

2.4.2. Aerosolic infection of mice

M. tuberculosis H37Rv stock was diluted in sterile distilled water, and a stock of 10^6 bacteria/ml was prepared. The mice were placed in an aerosolic infection chamber (Glas-Col). The suspension of 10^6 bacteria/ml was introduced into the nebulizer, kept in the aerosolic chamber for 45 min, so that approximately 90–100 bacteria were delivered into the lung of the mice [19].

Two mice were infected separately at the same time (not from the either of the groups). They were sacrificed 7 days post infection, and infection was confirmed by Ziehl-Neelsen staining of lung and spleen smears. Animal experiments were performed as per permission from the Animal Ethics Committee.

2.5. Quantification of viable mycobacteria by CFU count

Lungs and spleen were homogenized and plated in Middlebrook 7H10 agar media in order to count Colony Forming Units (CFU) 3 weeks after inoculation [20].

2.5.1. Histological study of lung pathology

Lung sections were stained using hematoxylin-eosin to examine the lung pathology under different treated conditions.

2.6. Estimation of SGOT and SGPT

Sera was collected from different groups of mice and SGOT and SGPT activity was measured using kit (Merck) to determine *in vivo* cytotoxicity.

2.7. Isolation of alveolar macrophages (AMs) and splenocytes

C57BL/6 mice after treatments and infection described as above were sacrificed and the trachea was exposed in an aseptical chamber. The trachea was fitted with a narrow 1.7 mm cannula/catheter and BAL (bronchoalveolar lavage) was performed with chilled PBS containing 5 mM EDTA, 500 µl per lavage. The procedure was repeated and the total BAL was pooled in a tube containing RPMI-1640 medium with 10% FBS. Lavaged cells were centrifuged at 300 × *g* for 10 min, counted and plated for 3 h in complete RPMI media till the macrophages attached to the cell plate [21]. These cells were proven to be macrophages since their flow cytometric analysis with biotinylated anti

CD11b antibody and secondary streptavidin-FITC conjugated antibody (BD Pharmingen) showed more than 94% of positive cells (Fig. 3A). The cell viability was also checked by Trypan blue dye exclusion technique and found to be greater than 94% (data not shown).

Spleens from each treatment group of infected C57BL/6 mice were removed aseptically. The spleens were macerated and flushed repeatedly to make a homogenous suspension. Briefly, spleen homogenate was subjected to centrifugation on Histopaque 1077 (Sigma) gradient and splenocytes were collected, washed and resuspended in RPMI-1640 complete medium supplemented with 10% fetal bovine serum.

2.7.1. T cell proliferation assay

T cell proliferation assay was performed as described previously [22]. Cells were plated in triplicate at 10^5 cell/well concentrations in 96-well plates and allowed to proliferate for 72 h at 37 °C in 5% CO_2 incubator in the presence or absence of PPD (Purified Protein Derivative; at a dose of 10 μg/ml; a kind gift from STM, Kolkata). The cells were then pulsed with 1 μCi (6.7 Ci/m mole) [^{3}H] Thymidine/well for 18 h, following which they were harvested. [^{3}H] Thymidine uptake, as an index of proliferation, was measured by liquid scintillation counter (Tri-Carb 2100TR; Packard Instrument).

2.7.1.1. Isolation of RNA and RT-PCR. RNA was isolated according to the standard protocol [23]. RNA was extracted from AMs and splenocytes using TRIZOL™ reagent (SIGMA), that was reverse transcribed using Revert Aid™ M-MuLV Reverse Transcriptase (Fermentas). The cDNA encoding the gene was amplified using specific primers, for IL-12p40 (forward 5′-CAACATCAAGAGCAGTAGCAG-3′; reverse 5′-TACTCC CAGCTGACCTCCAC-3′, Accession no. NM_008352.1, product size 311 bp); for TNFα (forward 5′-GGCAGGTCTA CTTTGGAGTCATTGC-3′; reverse 5′-ACATTCGAGGCTCC AGTGAATTCGG-3′, Accession no. NM_013693.1, product size 300 bp), for IFN-γ (forward 5′-GGATATCTGGAGGAA CTGGC-3′; reverse 5′-CGACTCCTTTTTCCGCTTCCT-3′, Accession no. NM_008337.1, product size, 433 bp) and for iNOS2 (forward 5′-CCCTTCCGAAGTTTCTGGCAGCAG C-3′; reverse 5′-GGCTGTCAGAGCCTCGTGGCTTTGG-3′, Accession no. NM_010927.1, product size 496 bp). PCR amplification was conducted in a reaction volume of 50 μl using a Perkin Elmer Gen Amp PCR system 2400 and 0.5 unit of Taq polymerase set for 35 cycles (denaturation: at 94 °C for 30 s; annealing: at 58 °C for 30 s; extension: at 72 °C for 30 s) for IL-12p40 and IFN-γ. For TNF-α and iNOS2, PCR reactions at 94 °C for 30 s, at 58 °C 30 s and 72 °C for 1 min were carried out for 30 cycles. Glyceraldehyde-3 Phosphate Dehydrogenase (GAPDH) was PCR amplified using, 5′-CAAGGCTGTGGGCAAGGTCA-3′ and 5′-AGGTGGAAGA GTGGGAGTTGCTG-3′ oligos (PCR product size 242 bp). PCR amplified product was subsequently size fractioned on 1% agarose gel, stained with Ethidium Bromide and visualized under UV-light.

2.7.1.1.1. Real-time quantitative PCR. Real-time RT-PCR was performed on iCycler (Bio-Rad Laboratories) using SYBR green reagent (Sigma). The PCR mixture (25 μl) contained 10 pmole of each primer (same combination of forward and reverse primers were used as in semi-quantitative RT-PCR), 8 μl of water, 12.5 μl of a commercial SYBR Green PCR master mixture and 2.0 μl of cDNA. The samples were placed in 96-well plates (Bio-Rad) that were sealed with optical sealing tape (Bio-Rad). PCR amplifications were performed by using the iCYCLER iQ Multi-color real-time PCR detection system. The thermal cycling conditions were as follows: initial activation step (5 min at 95 °C) and cycling step (denaturation for 30 s at 94 °C, annealing for 30 s at 58 °C and then extension for 1 min at 72 °C × 40 cycles) followed by melt curve analysis. An internal control GAPDH was amplified in separate tubes. We have used the Comparative cycle threshold method (ΔΔCt method) for relative quantitation of gene expression [24]. Briefly, the Ct for the target amplicon and the Ct for the internal control (GAPDH) were determined for each sample. The ΔCt for each experimental sample was subtracted from the calibrator. This difference was called the ΔΔCt value. Finally, the arithmetic calibrator ($2^{-\Delta\Delta Ct}$) was used to calculate the amount of target normalized to the amount of an internal control and relative to the amount of the calibrator.

2.8. Intracellular cytokine staining

Splenocytes isolated after 3 weeks post infection, were plated aseptically (10^6/ml) and stimulated with PPD (10 μg/ml) for 48 h. Cells were then incubated with Brefeldin A (10 μg/ml) (Sigma) for 4 h, washed in PBS containing 1% FBS and fixed with 1% paraformaldehyde (Sigma) following which permeabilization with 0.1% saponin was done and stained with anti mouse CD4-FITC and anti mouse IFN-γ-PE Ab (Santa Cruz Biotech.) [1]. Cells were analyzed on a FACS caliber cytofluorometer using the Cell Quest software (BD Bioscience).

2.8.1. Confocal microscopy

Splenocytes were permeabilized with 0.1% saponin, resuspended in cold wash buffer (PBS/0.1% NaN_3/1% FBS), then centrifuged at 1000 × *g* for 5 min. After fixing with 1% paraformaldehyde, the cells were incubated at 4 °C for 1 h with anti mouse MHC class II diluted (1:500) (Santa Cruz Biotech) in 50 μl of wash buffer. After washing thoroughly, anti mouse IgG-FITC was added to the cells (1:500), and incubated in dark at 4 °C for 45 min. Finally the cells were washed and mounted with 90% glycerol on a glass slide, and observed under a laser scanning microscope (LSM 510; ZEISS). MHC Class II-FITC positive cells were counted if the protein was found to be expressed on the cell surface, and the expression was assessed by quantification of confocal microscopy pictures (3 fields for each counted for approximately 100 cells for each set) as described by Kitatani et al. [25].

2.9. Statistical analysis

The data, represented as mean ± standard deviation (SD), is from one experiment, which was performed at least three times. Student's *t*-test was employed to assess the significance of the differences between the mean values of control and experimental groups. A P value of less than 0.05 was considered significant and less than 0.001 was considered highly significant.

3. Results

3.1. Effect of Ara-LAM on the viable mycobacterial count and lung pathological response of M. tuberculosis H37Rv infected C57BL/6 mice

Based on the previous reports about the immunomodulatory properties of Ara-LAM, we studied the effect of pre-treatment of Ara-LAM on the lung pathology of *Mycobacterium tuberculosis* H37Rv infected (aerosolically) C57BL/6 mice by hematoxylin-eosin staining of lung sections of different groups of mice. As clearly shown, lung section of control mice showed a normal morphology (Fig. 1A), while that in *M. tuberculosis* H37Rv infected mice showed granulomas lacking focus and disarranged tissue structure, with few or no infiltrating lymphocytes surrounding them (Fig. 1B). However, distinct granulomas with surrounding infiltrating lymphocytes were visible in Ara-LAM pretreated group of infected mice (Fig. 1C).

Our findings were supported by the estimation of the number of viable bacilli (in the form of $\log_{10}$ CFU) from both the lungs and spleen of *M. tuberculosis* H37Rv infected C57BL/6 mice. A significant inhibition in $\log_{10}$ CFU of *Mycobacterium tuberculosis* were observed in both lung and spleen (Fig. 1D) of Ara-LAM pre-treated group of *M. tuberculosis* infected mice ($\nabla P < 0.05$, $^{*}P < 0.01$).

3.2. In vivo cytotoxicity of Ara-LAM: effect on SGOT and SGPT activity

Ara-LAM being known as a cytotoxic molecule [26] we checked the *in vivo* cytotoxicity of a standardized non-lethal dose of this molecule (30 μg/ml) by studying Serum Glutamate Oxaloacetate Transferase (SGOT) and Serum Glutamate Pyruvate Transferase (SGPT) levels from serum collected from the different groups of mice. SGOT and SGPT are the enzymes that are indicators of liver function and *in vivo* cytotoxicity, the levels of which are significantly enhanced under pathogenic conditions. We observed that both SGOT and SGPT activity levels (Fig. 2) were significantly ($^{*}P < 0.05$ and $^{**}P < 0.01$) diminished in sera from Ara-LAM pre-injected mice compared to the *M. tuberculosis* infected group, while that in *M. tuberculosis* infected group of mice were much enhanced compared to that in control group ($\nabla P < 0.02$, $^{*}P < 0.05$, $^{**}P < 0.01$).

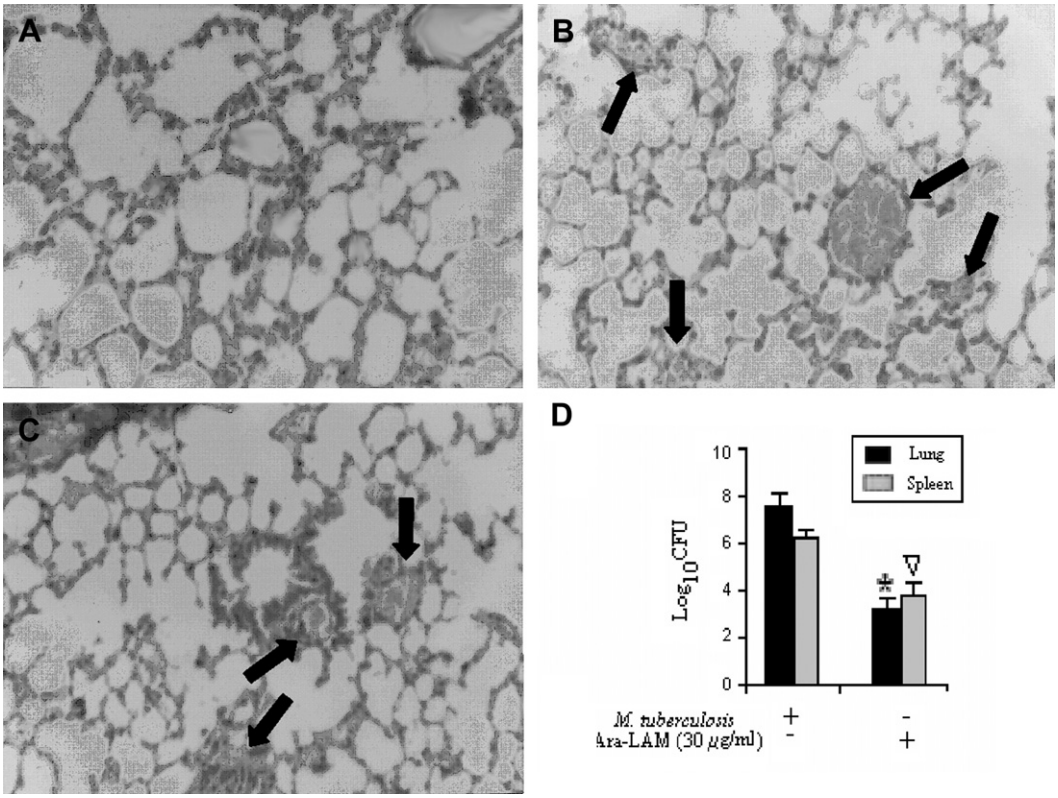

Fig. 1. Effect of Ara-LAM treatment in restricting *M. tuberculosis* growth. Hematoxylin-eosin staining of lung section of C57BL/6 mice (A) control (B) *M. tuberculosis* H37Rv infected (C) Ara-LAM treatment prior to *M. tuberculosis* infection. (D) CFU of *Mycobacterium tuberculosis* from murine lung and spleen homogenate. Magnification shown here is 40× 10×. CFU count is expressed as mean ± SD from 5 mice per set ($\nabla P < 0.05$, $^{*}P < 0.01$).

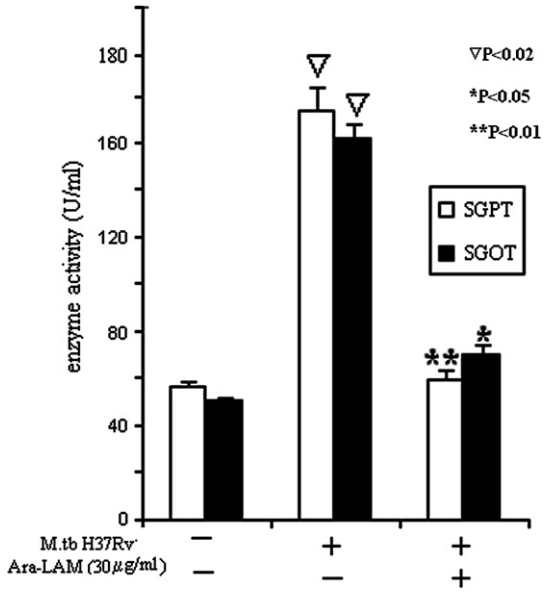

Fig. 2. *In vivo* cytotoxicity test. SGOT and SGPT activity levels in U/ml. The data is expressed as mean ± SD from 5 mice. (*$P < 0.05$, **$P < 0.01$, Ara-LAM treated mice compared to infected mice) and ($\nabla P < 0.02$) infected mice compared to control mice.

3.3. Ara-LAM administration induces the expression of pro-inflammatory cytokines and iNOS2 in C57BL/6 mice infected with Mycobacterium tuberculosis *H37Rv*

Finding that Ara-LAM at the specified non-lethal dose did not exert *in vivo* cytotoxicity in mice, we investigated whether Ara-LAM administration could induce the pro-inflammatory cytokines and iNOS, essential for generation of an anti-mycobacterial immune response [13,14,27]. After 3 weeks of *M. tuberculosis* H37Rv infection, we quantified the induction of different pro-inflammatory cytokine mRNA expression in AMs and splenocytes of Ara-LAM injected, *M. tuberculosis* infected mice by using Real Time quantitative PCR (the purity of macrophages are mentioned in Section 2, Fig. 3A). We observed a significant inhibition in TNF-α, IL-12p40, IFN-γ and iNOS2 expressions in infected mice compared to control. However, the Ara-LAM injected group exhibited 14.7 fold, 10.2 fold, 8.3 fold and 11.6 fold increases in the expression levels of TNF-α, IL-12p40, IFN-γ and iNOS2 respectively in the AMs, compared to uninfected mice (Fig. 3B). RNA from splenocytes also showed a similar profile of pro-inflammatory cytokines; as depicted by a strong induction of

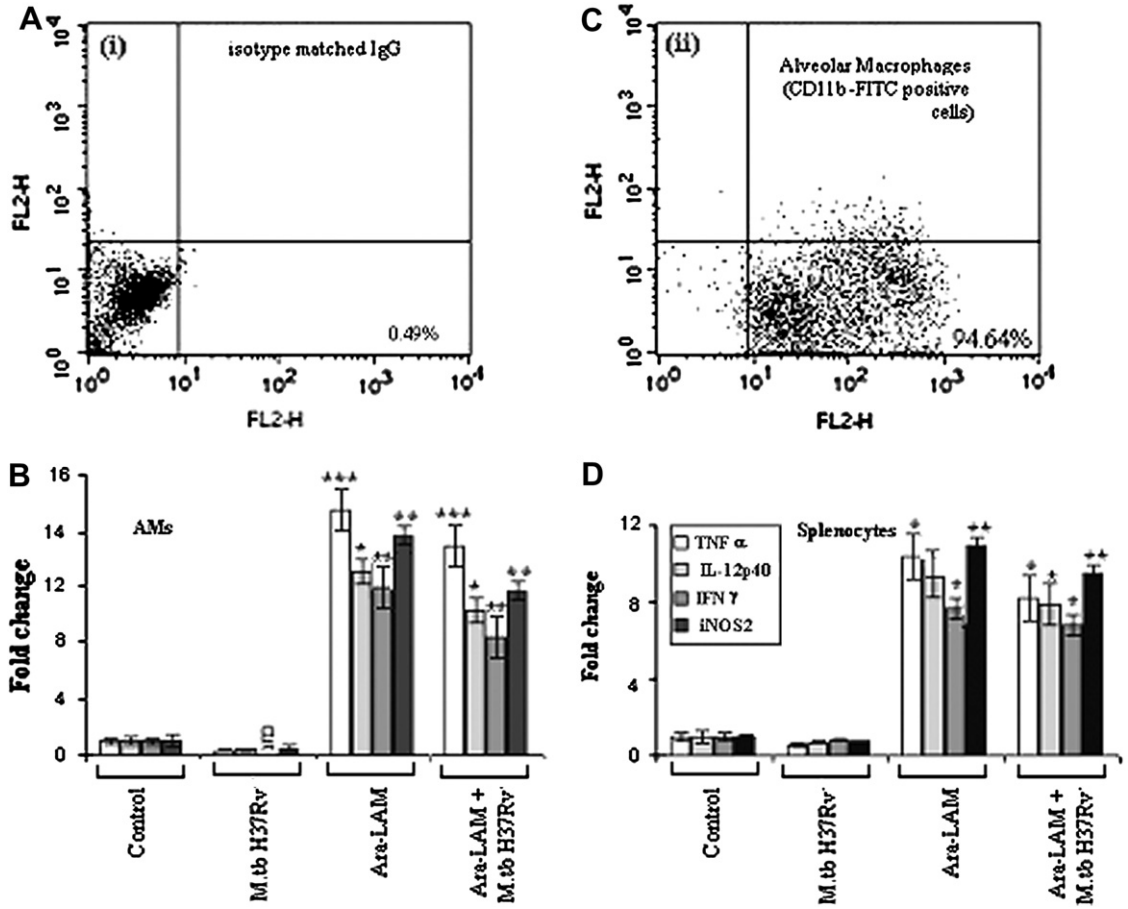

Fig. 3. Ara-LAM induced Th1 cytokine expression in *M. tuberculosis* infected mice. (A) (i) AMs incubated with isotype matched IgG (ii) Percentage of CD11b-FITC positive cells. Real Time PCR showing the fold change of the mRNA products of TNF-α, IL-12p40, and IFN-γ in (B) AMs and (C) splenocytes respectively. The data are expressed as mean ± SD from three independent experiments giving similar results (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). ND = not detectable.

TNF-α by 8.2 fold, IL-12 p40 by 7.9 fold, IFN-γ by 6.8 fold and iNOS2 by 9.5 fold in Ara-LAM treated infected mice compared to infected group (Fig. 3C).

3.4. Effect of Ara-LAM on T cell proliferation in M. tuberculosis infected C57BL/6 mice

The above findings prompted us to study whether Ara-LAM could induce T cell proliferation, essential for the cell mediated immune responses and the secretion of Th1 cytokines as well. Splenocytes were isolated 3 weeks post infection and the experiment was performed as described in Section 2. Fig. 4 showed there was 4.5 fold enhanced T cell proliferation in the splenocytes of Ara-LAM administered mice infected with *M. tuberculosis* H37Rv, compared with infected C57BL/6 mice.

3.5. Effect of Ara-LAM on IFN-γ production by CD4+T cells

Proliferation of T cells, mainly CD4+T cells producing IFN-γ [5], are essential for protection against tuberculosis [5]. Splenocytes isolated from infected mice with or without pre-treatment with Ara-LAM were stimulated with PPD (10 μg/ml) for 72 h. The frequency of IFN-γ secreting CD4+T cells was determined by intracellular cytokine staining. FACS analysis showed more than 7 fold greater frequency of CD4+T cells producing IFN-γ in *M. tuberculosis* infected C57BL/6 mice pre-treated with Ara-LAM (Fig. 5E) compared to that in infected mice (Fig. 5B). Increased frequency of

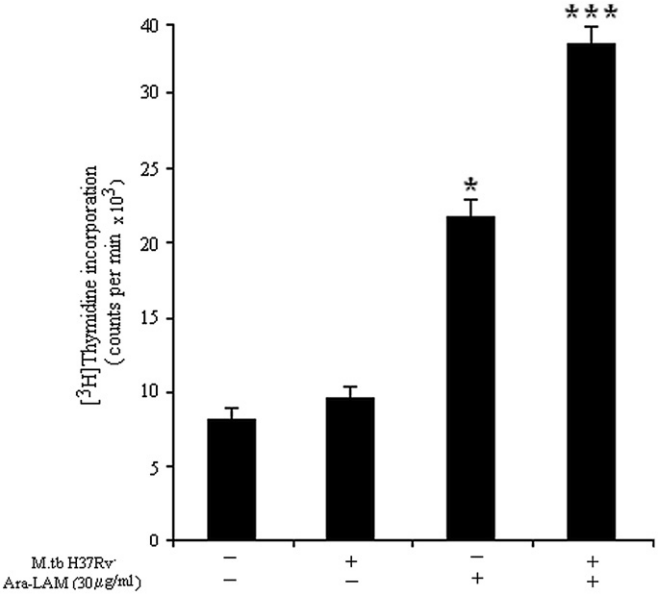

Fig. 4. Effect of Ara-LAM on T-cell proliferation in *M. tuberculosis* infected mice. Proliferative responses to PPD (10 μg/ml) by splenocytes by [^{3}H] thymidine incorporation. The data represent the mean ± SD from triplicate wells (***$P < 0.001$) from three individual experiments.

CD8+T cells was also observed under Ara-LAM treatment in infected mice, but the effect was not so significant (data not shown). Fig. 5F depicts a bar diagram showing the percentage of CD4+T cells producing IFN-γ from 5 mice in each group; and the splenocytes from Ara-LAM treated group of mice showed a significantly higher (∼4 times) the

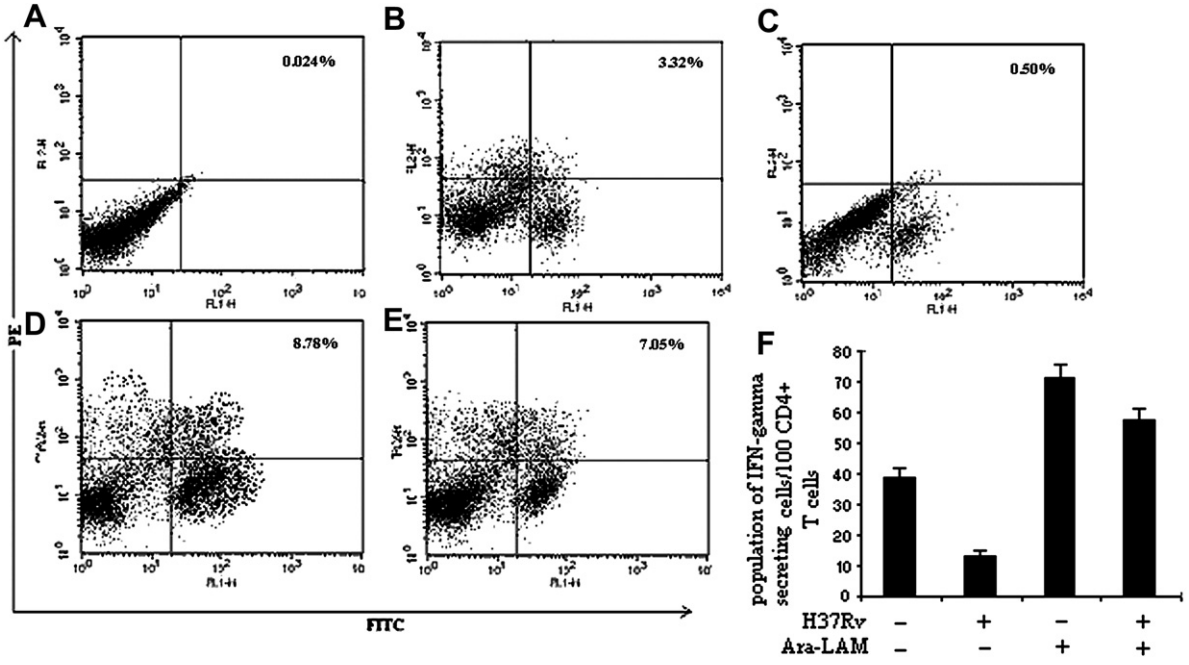

Fig. 5. Effect of Ara-LAM on the frequency of IFN-γ producing CD4+T cells in infected mice. (A) negative control (B) Splenocytes from control mice. (C) Splenocytes from *M. tuberculosis* H37Rv infected mice (D) Splenocytes from Ara-LAM injected mice (E) Splenocytes from Ara-LAM pretreated and infected mice. Numbers in the upper right quadrant represent the percentage of CD4+T secreting IFN-γ. These data are from one of five experiments showing similar results. X-axis represents the population of FITC positive cells, while Y-axis corresponds to that of PE positive cells. (F) A bar diagram showing the percentage of CD4+T cell population secreting IFN-γ (from 5 mice) along with their mean ± SD (*$P < 0.05$).

population of CD4+T cells producing IFN-γ compared to those from *Mycobacterium tuberculosis* infected mice.

3.6. Ara-LAM induces MHC Class II expression in M. tuberculosis *H37Rv infected C57BL/6 mice*

CD4+T cells producing IFN-γ are MHC Class II restricted, and are critical to the control of *Mycobacterium tuberculosis* infection [28]. Hence we studied whether Ara-LAM could restore the impaired MHC Class II expression on the splenocytes of *M. tuberculosis* H37Rv infected C57BL/6 mice. We observed that *M. tuberculosis* infected mice showed inhibition in the expression of MHC Class II on the cell surface compared to that of the control mice (Fig. 6B) However, Ara-LAM pre-treatment of both uninfected as well as *M. tuberculosis* infected mice could prominently restore the expression of MHC Class II molecules on the cell surface compared to that in infected mice (Fig. 6D), as shown by confocal microscopy. The bar diagram (Fig. 6E) depicts that splenocytes from Ara-LAM pretreated group of mice exhibited increased fluorescence compared to *Mycobacterium tuberculosis* infected group.

4. Discussion

Mycobacterium tuberculosis infection is associated with an impairment of the cell-mediated immune response of the susceptible host, marked by apoptosis of the CD4+ and CD8+T cells [5] and decrease in the production of Th1 cytokines. Since several mycobacterial lipid antigens and *Mycobacterium welchii* (*Mw*) as an effective immunomodulator have been reported to be effective in boosting the immune response against pathogenesis [13,14], we investigated whether Ara-LAM from avirulent mycobacteria could regulate the impaired cell mediated immunity in *Mycobacterium tuberculosis* H37Rv infected C57BL/6 mice.

Ara-LAM was observed to have a direct inhibitory effect on the growth of viable *M. tuberculosis* H37Rv *in vivo*, as indicated

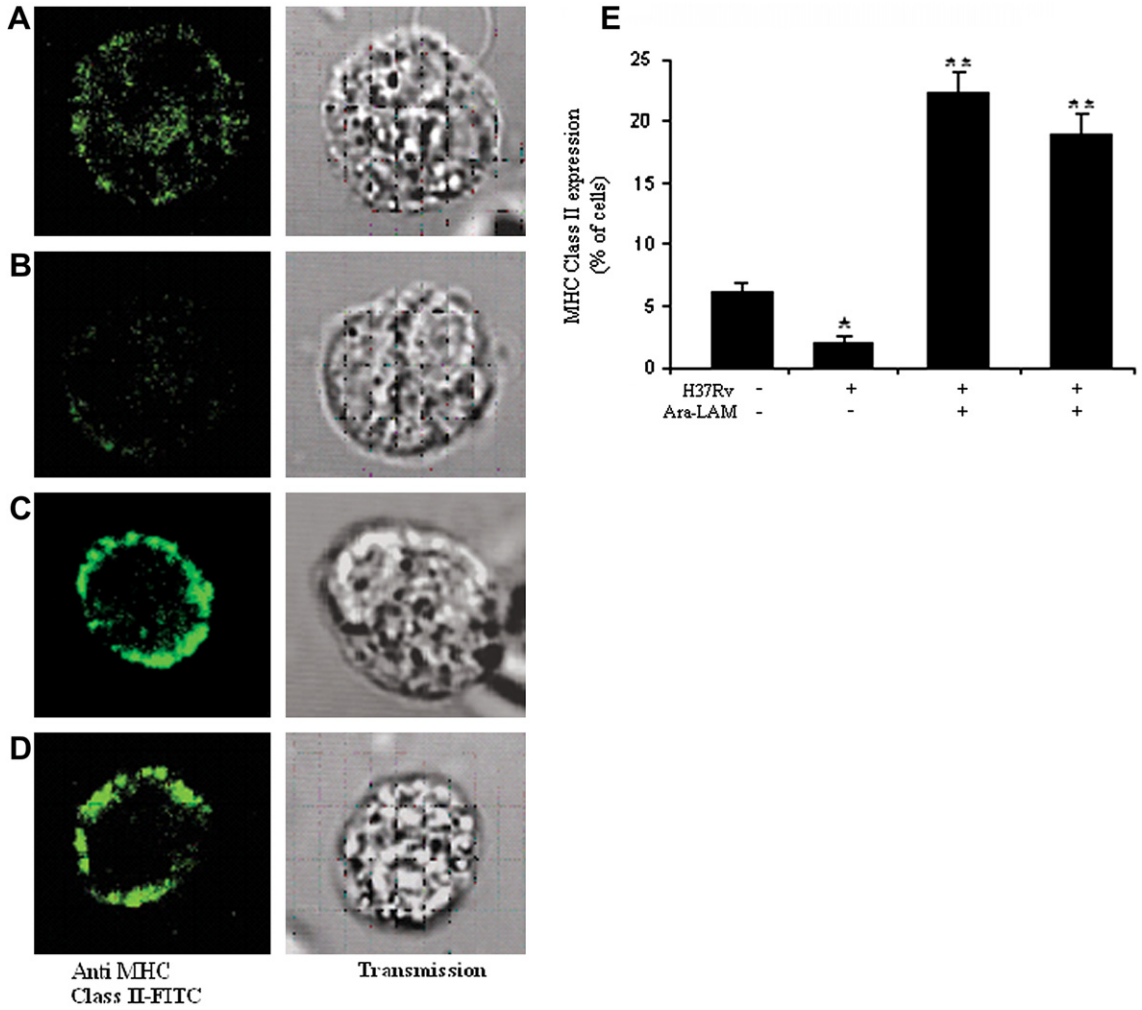

Fig. 6. Ara-LAM restores the expression of MHC Class II on the infected murine splenocyte. (A) Control (B) Infected with *M. tuberculosis* (C) Ara-LAM treated mice followed by *M. tuberculosis* infection. (D) A bar diagram showing the MHC Class II–FITC positive cells, counted from 3 fields each, counting up to approximately 100 cells per set $^{**}P < 0.01$.

by a reduction in the number of viable bacilli (as $\log_{10}$ CFU) in the lungs and spleen of *Mycobacterium tuberculosis* H37Rv infected C57BL/6 mice (Fig. 1D). Ara-LAM pretreated group of infected mice also displayed a visibly prominent granuloma surrounded with infiltrating lymphocytes in hematoxylin-eosin stained histological slides (Fig. 1C) compared to infected mice (Fig. 1B) where the granulomas lacked focus and showed very few infiltrating lymphocytes. Ara-LAM at a non-cytotoxic dose of 30 μg/ml showed normal SGOT and SGPT levels as detected from the sera collected from different groups of mice. This dose of Ara-LAM was capable of inducing pro-inflammatory cytokines TNF-α, IL-12p40, IFN-γ and iNOS2 expression in the AMs and splenocytes of Ara-LAM treated group of infected mice, essential for host immunity against Mycobacterial infection (Fig. 3). The induction of Th1 cytokines was due to a 4.5 fold enhancement of T cell proliferation (Fig. 4), and a significantly higher frequency of CD4+T cells producing IFN-γ in Ara-LAM pre-treated group of infected mice (Fig. 5). Since the abrogation of Th1 cytokine production during *Mycobacterium tuberculosis* infection is associated with apoptosis of T cells [5], Ara-LAM could exhibit an effective anti-mycobacterial effect by the enhancement of T cell proliferation and the population of CD4+T cells that are the main effectors of cell-mediated immunity.

The CD4+T cells recognize MHC Class II molecules on the surface of the antigen presenting cells, and MHC class II expression on the cell surface is essential for an effective immune response against *M. tuberculosis* infection [28]. We also observed a significantly higher expression of MHC Class II on the splenocyte membrane of Ara-LAM pre-treated group of infected mice compared to the infected group (Fig. 6).

Collectively our findings establish that a non-cytotoxic dose of Ara-LAM, with its immuno-modulatory properties, could restrict the growth of viable tubercle bacilli in lungs and spleen of infected mice by a mechanism involving up-regulation of pro-inflammatory cytokines, enhanced T cell proliferation, increased frequency of CD4+T cell producing IFN-γ and higher expression of MHC Class II on the cell surface- essential for pathogen clearance. Ara-LAM has the potency to induce inflammatory reactions within the lungs by the induction of Toll like receptor-2 (TLR-2) signaling [29], which is an essential arm of innate immunity. Besides, the enhancement of CD8+ and mainly CD4+T cell population that act as carriers of the mycobacterial antigens to the surface receptors provide an effective means of protection against tuberculosis via the adaptive immune response [30]. Therefore, to our knowledge, this is a novel depiction of Ara-LAM as a regulator of cell mediated immune response in a murine model of tuberculosis even after 3 weeks of prolonged infection, thus, could be utilized as a potent immuno-prophylactic tool in restricting the disease.

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