Chapter Four

Co-administration of escheriosome entrapped TLR agonist with RD antigen based nanovaccine induces protective T cell memory response and imparts protection against experimental murine tuberculosis
4.1. Introduction

In addition to HIV/AIDS and malaria, TB caused by *M. tb* is another leading infectious disease accounting for a large fraction of global deaths annually (Gowthaman et al. 2012, Ansari et al. 2011). *M. tb* has been reported by World Health Organization (WHO) to infect 9 million people and cause approximately 2 million deaths annually. Thirty percent of the world population has been estimated to be infected with *M. tb* (Gowthaman et al. 2012, Kaufmann et al. 2010, Lonnroth et al. 2010, Kaufmann 2010). The emerging MDR- and TDR- strains of *M. tb*, alarming number of TB cases across the globe and co-infection of *M. tb* along with HIV (Gowthaman et al. 2012, World Health Forum 1993) have instigated WHO to announce TB as a global emergency. Immune system although successfully controls active *M. tb* infection, but fails to obliterate the bacteria which renders the infection to become latent (Russel et al. 2010). The latent *M. tb* can become active later and spread the disease (Gowthaman et al. 2012, Kaufmann et al. 2010, Lonnroth et al. 2010). Drug regimens employing antibiotics are currently in vogue as curatives for TB but they may lead to the emergence of drug resistance, particularly when they are lengthy, complex, and have low rates of compliance (Gandhi et al. 2010). Therefore preventive measures i.e. effective vaccines against TB are a rational approach for its eradication. The only reliable TB vaccine available is BCG which is a live attenuated strain of the related bacterium *M. bovis*. BCG has been estimated to have been administered to more than 4 billion people (Gowthaman et al. 2011). Nonetheless, the ever increasing number of TB cases indicates the reduced efficacy of BCG in preventing TB. In fact, BCG has been found to be effective in newborns and toddlers but it fails to adequately protect adults from TB infection (Ansari et al. 2011). Therefore, an advanced or more effective version of BCG or altogether a newer vaccine against TB is the need of the hour.

The inadequate efficacy of BCG is attributed to the absence of certain genomic loci and its incapability of mounting prolonged memory in the host (Ansari et al. 2011). Sixteen regions absent from BCG (encompassing 129 ORFs) have been found to be present in *M. tb* H37Rv strain (Mahmood et al. 2011). The RD encompassing ORF Rv3620c is absent from all vaccine strains of *M. bovis*. Rv3619c and Rv3620c are members of ESAT-6/CFP-10 family and are secretory proteins of *M. tb* and have been
und to form a heterodimeric complex in a manner similar to ESAT-6 and CFP-10 which also form strong 1:1 heterodimeric stable complex (Mahmood et al. 2011). There are evidences suggesting that the major T-cell Ags of the complex ESAT-6 and FP-10 form a tight, 1:1 complex (Renshaw et al. 2002). Moreover, overlapping synthetic peptide studies have demonstrated that the Rv3619c/Rv3620c subfamily consists of potent T-cell Ags (Alderson et al. 2000). Recently, we have also reported at Rv3619c predominantly activates T cell response in the host (Ansari et al. 2011).

Vaccines were earlier conventionally based on killed, live-attenuated microorganisms attenuated toxins but the safety concerns of reversion of attenuated strains to virulence and risk of excessive inflammation have reduced their use. Recently interest shifted to vaccines based on proteins, peptides or Ag-expressing DNA or RNA. Rather than the use of whole microorganisms, they are, however, lesser immunogenic than whole organisms and therefore require appropriate immunological adjuvants. The adjuvant plays a crucial role in evoking a potent immune response against an Ag. Therefore, it becomes necessary to choose the right adjuvant for Ag delivery so as to develop an effective subunit vaccine.

ease of intracellular pathogens like M. tb, in addition to immunizing with an Ag capable of evoking T cell response, administering Ags along with adjuvants eliciting a CTL response is a pre-requisite since Abs generally fail to impart protection against organisms occupying intracellular abode. Alum (aluminium hydroxide) is the only FDA approved adjuvant but it evokes cell mediated immune response only feebly (Ansari et al. 2011). Archaeosomes, archael lipid based vesicles have been demonstrated to evoke potent cell mediated and humoral response against Ags encapsulated in them. The lipids present in the plasma membrane of chaebacteria are different from the conventional glycerophospholipids present in most of the microorganisms. The polar lipids in plasma membrane of various members of Archaeabacteria share the constant-length phytanyl chains, usually fully saturated, and bonded by ether linkages to sn-2,3 carbon of glycerol backbone (Sprott et al. 1996). The distinct chemical structures of archael lipids confer considerable ability to archaeosomes developed by using total polar lipids (TPL) of various chaebacteria including Halobacterium salinarum (Sprott et al. 1996). The polar
head groups exposed to the outer surface of archaeosomes have the potential to interact with mammalian cell surface molecules, whereas the type and proportion of lipid cores markedly influence the stability (Choquet et al. 1996) and permeability (Mathai et al. 2001) of the vesicular structures. Archaeosomes have been proven to be superior adjuvants, capable of facilitating strong and long lasting, CD4⁺ and CD8⁺ cytotoxic T cell and Ab responses against entrapped proteins in the host (Krishnan et al. 2000a, Sprott et al. 1997, Krishnan et al. 2000b). Recently, in order to improve the protective efficacy of Ags, Toll-like receptor (TLR) agonists are also co-administered with the Ag. They function as adjuvants and initiate inflammatory signals that alert the immune system facilitating a heightened immune response against the Ag. Zymosan is a TLR2/TLR6 agonist. Ligands binding to TLR-2 induce preferred production of IL-12 cytokine (Gowthaman et al. 2012) which leads to Th1 polarization, a crucial player in the generation of CTL response.

Reckoning with above detailed facts, we were of the opinion that Rv3620c would be a potent inducer of T cell response in the host as evoked by its secretory counterpart Rv3619c. Therefore, in the present study we first evaluated the immune response evoked by Rv3620c in mouse models. Significant augmentation of various Th1 cytokines and IgG2a to IgG1 ratio in Rv3620c immunized animals as compared to saline control group demonstrated that Rv3620c is a potent T cell Ag and if administered along with a suitable adjuvant or immuno-modulator can impart protection against TB. Therefore, considering the adjuvant properties of archaeosomes, we analysed the immune response evoked upon administering Rv3620c encapsulated in archaeosomes. Moreover, reckoning with the Th1 bias induced by zymosan, we assessed the immune response elicited upon administration of archaeosome entrapped Rv3620c along with escherosome (Escherichia coli lipid based vesicles) encapsulated zymosan in Balb/c mouse models of TB.
2. Materials and methods

2.1. Chemicals and reagents

Standard reagents used in the study were purchased from Sigma (USA), unless otherwise mentioned. The following reagents were procured from Difco Laboratories: Middlebrook 7H9 broth; Middlebrook 7H11 medium; and oleic acid, albumin, xtrose, and catalase (OADC). Tissue culture media (RPMI1640), BSA, antimycotic solution, and plastic-wares were purchased from BD Biosciences (USA). pET expression vectors were procured from Novagen (Darmstadt, Germany). Oligonucleotides for gene isolation were bought from BIO Serve (Hyderabad, India). Restriction endonucleases, T4 DNA ligase and DNA size markers were procured from New England Bio-labs (Beverly, MA, USA). Taq polymerase, other reagents for PCR, Plasmid Miniprep kit, and the Gel extraction kit used for plasmid preparations and DNA purification processes, were obtained from Qiagen. Nickel/nitrilotriacetic acid (Ni/NTA) superflow metal-affinity chromatography matrix was obtained from Agenc. To concentrate expressed protein, Amicon-Ultra was used (molecular mass cutoff 3 kDa; Millipore, Bangalore, India). [\(^{3}H\)-thymidine was procured from nersham Pharmacia Biotech. Abs The following Abs were procured from e-osciences: fluorochrome-labeled antimouse Abs; fluorescein isothiocyanate– conjugated CD4 (GK 1.5), CD8 (53.67), PE-conjugated CD44 (IM7), CD80 (B7-1), CD86 (GL1), PerCP conjugated CD62L (MEL-14) IgG2a (R35-95) isotype control. G1, IgG2a isotype kit (550487) and IL-4, IFN-γ, IL-12 Cytokine kits were procured from BD Biosciences.

2.2. *M. bovis* BCG and *M. tb* strain

*B. bovis* BCG (Danish) and *M. tb* H37Rv strains were kindly provided by Dr. V.M. stoch (National JALMA Institute for Leprosy and other Mycobacterial Diseases). *tb* was cultured into Middlebrook 7H9 broth containing 0.2% glycerol and 0.05% veen-80 supplemented with albumin, dextrose and catalase (Zhang et al. 1998). The viability of the bacteria was determined by culturing them on Middlebrook 7H11 medium supplemented with oleic acid, albumin, dextrose, and catalase and counting the number of CFUs.
4.2.3. Cloning, expression and purification of Rv3620c

Genomic DNA of *M. tb* H37Rv was prepared and desired gene Rv3620c was cloned following method as described earlier (Sambrook and Russel 2001). Briefly, genes encoding Rv3620c were PCR amplified using oligonucleotide primers and pfu DNA polymerase, and cloned into pET-NH6 vector with restriction sites EcoRI and HindIII. This cloning strategy added an additional 30 residues at N-terminal including six residues of His-tag. The vectors containing Rv3620c were then transformed into BL21 (λDE3) *E. coli* cells and grown in Luria-Bertani medium supplemented with ampicillin (100 µg/mL).

For expression of protein, BL21 (λDE3) cells, containing the plasmid pETNH6-Rv3620c, were grown in Luria-Bertani medium supplemented with ampicillin (100 µg/mL) and induced at *A*₆₀₀ = 1.0 with a final concentration of 0.5 mM isopropyl-β-d-thiogalactopyranoside. Culture was further grown for 12–14 h at 27°C. The protein of interest was purified over Ni/NTA matrix using a standard protocol under the denaturing conditions, as per the manufacturer’s instructions except that NaCl and guanidine hydrochloride were excluded from the buffer. The eluted fractions were checked for purity by SDS-PAGE (15% gel) as well as Western blot analysis following the standard method (Mahmood et al. 2011). The protein was refolded by dialysing it against refolding buffer containing 25 mM NaH₂PO₄, 100 mM NaCl and 1 mM 20 mM NaH₂PO₄, 50 mM NaCl, and 0.1% NaN₃, pH 6.5. The construct pET-NH6-Rv3620c encoded protein contained 30 extra N-terminal residues with His-tag.

4.2.4. Isolation of total polar lipids of Archaeabacteria

The total polar lipids of *H. salinarum* were isolated following Bligh and Dyer method as modified in our lab (Kumar and Gupta 1983). In brief, the cells were cultured on nutrient agar plates containing 1 M NaCl. After 72 h, culture was harvested and washed thrice with phosphate buffer containing 150 mM NaCl. Subsequently, the cells were sonicated with the help of probe sonicator for 5 min (30 seconds pulse) and finally dispersed in mixture of 1:2 V/V ratios of methanol and chloroform. The cell suspension was overnight stirred on magnetic stirrer at 4°C; and the solution was filtered to remove any solid residue. The filtrate was washed by gentle mixing with one fifth volume of 150 mM NaCl. The two phases (organic vs aqueous) were
allowed to separate using separating funnel. Finally, the organic phase was evaporated using rota evaporator (Laborota-4000, Heidolph, Germany) to obtain the lipid.

4.2.5. Isolation of *E. coli* lipids

*E. coli* was cultured in nutrient broth (1% peptone, 0.3% Beef extract, 0.3% yeast extract and 1% sodium chloride; pH 7.4). The cells were harvested from mid-log phase (18–20 h). Phospholipids were isolated by the method of Bligh and Dyer, as modified by Ames (Chauhan et al. 2011).

4.2.6. Preparation of liposome and assessment of Ag entrapment efficiency

To increase vaccine potential Rv3620c, we developed an archaeosome based Ag delivery system, where membrane lipids isolated from *H. Salinarum* were used for archaeosome preparation. Archaeal lipid based unilamellar vesicles were prepared by sonication method as standardized in our lab (Ansari et al. 2011). Briefly, dry lipid film was hydrated with normal saline to form lipid suspension. To get homogenous population of unilamellar liposome, hydrated lipid was sonicated for 1 h in bath type sonicator at 4°C (Power sonics, South Korea). Lipid vesicles were centrifuged for 10 min at 11,500 g at 4°C to remove large lipid aggregates (Kirby and Gregoriadis 1984). The unilamellar liposomes were mixed with equal volume of protein solution (10 mg/ml stock). Several freeze thaw cycles were executed to increase the efficiency of entrapment. Un-entrapped protein was separated using sepharose 6B column. The Rv3620c bearing liposomes were eluted out in void volume. Entrapment efficiency was determined by BCA method after lysing the liposomes with 1% Triton X-100.

4.2.7. Preparation of escheriosomes

The zymosan bearing liposomes were prepared using *E. coli* lipid essentially by following the published procedure as standardized in our lab (Chauhan et al. 2011). Briefly, *E. coli* lipids (total lipid 20 mg) were reduced to thin dry film under N2 atmosphere. The film was hydrated, followed by sonication in a bath-type sonicator for 1 h at 4°C under N2 atmosphere. The liposomes thus formed were mixed at this stage with an equal volume of zymosan (30 mg/ml stock). The mixture was flash frozen and thawed (3 cycles), and then lyophilized. The free flowing, dried powder thus obtained was rehydrated with distilled water (120μl) and finally re-constituted
with PBS. The preparation was centrifuged at 14,000×g and the pellet was further washed at least 3 times with PBS to remove the traces of the un-entrapped solute. The liposomes (given volume) were lysed with 10% Triton X-100 solution (the final concentration of Triton X-100 was maintained 1%) and the amount of zymosan entrapped was estimated by HPLC.

4.2.8. Animals and Immunization schedule
Female BALB/c mice (6–8 weeks old) were procured from JALMA institute for leprosy and other mycobacterial diseases, Agra, India. Mice were quarantined in the biosafety level 3 animal facility, in accordance with guidelines from the CPCSEA (Committee for the purpose of control and supervision of experiments on animals, Govt. of India), and kept on pellet feed diet and water ad libitum. Mice were immunized with various forms of Ag viz. free Ag, BCG, archaeosome entrapped Ag, combination of archaeosome entrapped Ag and escherisome encapsulated zymosan along with appropriate control groups of animals such as saline (no adjuvant). The animals were immunized by subcutaneous route at the base of their tail in lower abdominal region with 25 μg of Ag in a volume of 100 ml of vehicle per animal corresponding to lipid concentration in range of 1.5–2.0 mg/injection. On day 21, the animals were boosted with matching formulation of Ag using the same route of administration. In BCG group single dose of 1X10^6 bacilli was administered intradermally.

4.2.9. Ethics Statement. All animal experiments were approved by the Institutional Animal Ethics Committees of IBU-AMU and NJIL & OMD, India. All animal experiments were performed according to the National Regulatory Guidelines issued by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals). Our approval ID was 332/CPCSEA, Ministry of Environment and Forest, Govt. of India.

4.2.10. Challenge with Mycobacterial infection and assessment of residual bacterial burden in vital organs
Twenty six weeks post last booster, 10 BALB/c mice from each group were challenged with virulent M. tb H37Rv through aerosol route. Suspension having a
bacterial count of $5 \times 10^7$/ml in a volume of 10 ml was added to the venture nebulizer unit of the middle brook Aerosol Generator device (Glas-ool, USA) to deposit approx. 100 bacilli in each mouse. To evaluate the protective efficacy of various in-house developed vaccine candidates, bacterial load in lungs and spleens of experimental animals was determined at various time points. After a stipulated time period (four and eight weeks post challenge), three animals from each group were sacrificed; their spleens and lungs were taken out aseptically and homogenized in 7H9 media. Different dilutions of prepared homogenate were plated onto 7H11 agar plates supplemented with oleic acid, albumin, dextrose and catalase. In BCG (Danish) immunized group, thiophene carboxylic hydrazide (TCH) at concentration of 2 mg/ml, was added to inhibit the growth of BCG and incubated for 3 weeks at 37°C. After stipulated incubation period, colonies were counted to calculate the bacterial load.

4.2.11. Determination of Ab isotype in sera of immunized mice
Mice were bled at different time intervals and their sera were analyzed for the presence of Ag specific Abs. Subsequently, the Abs were analyzed for their isotypes using following method. Briefly, ninety-six well microtitre plates were incubated overnight with 200ng Ag/well in carbonate-bicarbonate buffer (0.05 M, pH 9.6) at 4°C. After washing and blocking steps, the plates were incubated with serially diluted test and control sera at 37°C for 2 h. After excessive washing of the plates, 100 ml of (1:5000 dilution of stock) goat anti-mouse anti-IgG1, and IgG2a Abs were added in each well and incubated for 1 h at 37°C. After washing steps, 100 ml of (1:5000 dilution of stock) HRP conjugated rabbit anti-goat Ab was added to each well and each plate was incubated at 37°C for 1 h. The plates were washed again followed by adding 100 ml of substrate solution (6 mg OPD in 12 ml of substrate buffer with 5 µl of 30% H$_2$O$_2$) and were finally incubated at 37°C for 40 min. The reaction was stopped by the addition of 50 µl of 1 M H$_2$SO$_4$. The absorbance was read at 490 nm with a microtitre ELISA plate reader (Bio-Rad, USA).

4.2.12. Isolation of T lymphocytes from spleens of immunized mice
Mice belonging to various immunized groups were sacrificed at different time points both post booster as well as post challenge with infection. Single cell suspension of
the spleen was prepared as described elsewhere (Mallick et al. 2007). Briefly, spleens isolated from animals belonging to various groups were macerated and suspension was treated with ACK lysis buffer (0.15 mol/L ammonium chloride, 10 mmol/L potassium bicarbonate, and 88 mmol/L edetic acid) for lysis of the red blood cells. The cell suspension was centrifuged at 1500 g for 5 min and cell pellet was washed with HBSS solution 3 times and resuspended in RPMI 1640 medium containing 10% FBS and 0.1% antimycotic cocktail.

4.2.13. Lymphocyte proliferation assay

Lymphocyte proliferation assay was performed as described elsewhere (Mallick et al. 2007). Briefly, lymphocytes isolated from the spleens of mice belonging to various immunized groups were incubated in round bottomed 96-well plates (2x10^5 cells per well) in 200 µL of RPMI 1640 medium with 10% FBS. To determine the effect of concentration of Ag on T cell proliferation, varying concentrations (1-50 µg/well) of Rv3620c were used for priming of target cells. In the next set, splenic cells from various immunized groups were incubated with a known amount of corresponding matching formulations of Rv3620c. Cells incubated with the medium alone (without Ags) were used as controls. After 72 h, the cultures were pulsed with 0.5 mCi of[^3]H]-thymidine. After 16 h, the plates were harvested onto glass-fiber filter mats by the use of Tomtec-Harvester-96 (Tomtec). The incorporated radioactivity was measured with liquid scintillation spectroscopy (Wallac-1450 Microbeta Trilux; Perkin Elmer).

4.2.14. Cytokine assay: Determination of IFN-γ, IL-4 and IL-12

Both type I and type II cytokines induced by splenocytes upon their co-culture in the presence of various forms of Rv3620c were estimated using appropriate and specific biotinylated Ab pairs according to the manufacturer's protocols. Briefly, 50 µl of the purified capture Abs were adsorbed overnight on polystyrene microtitre plates at 4°C in carbonate buffer of pH 9.5. Plates were washed five times with PBST and blocked with 5% skimmed milk. After the usual steps of washing, 50 µl of the supernatant (isolated from cultured splenocytes after 48 h) was dispensed in each well to determine its cytokine content. After stipulated incubation time, the plates were thoroughly washed and incubated with biotinylated polyclonal goat anti-mouse cytokine detection Ab. Afterward, the plates were washed three times with PBST.
Subsequently, 100 μl of streptavidin-HRP conjugate was added to each well and plate was incubated for 30 min at room temperature. The plates were again washed three times with PBST and finally colored complex was developed with tetra methyl benzidine. The absorbance was read at 450 nm with a microtitre plate reader (Bio-Rad). A known specific recombinant cytokine was used as standard for calculating level of given cytokine in the samples tested and concentration was expressed as pg/mL.

4.2.15. Expression of cell surface markers on immune cells as revealed by FACS analysis

The splenocytes were harvested and stained for flowcytometric analysis as described elsewhere (Ansari et al. 2011). Briefly, 1X10⁶ splenocytes were washed twice with FACS buffer (PBS with 1% BSA and 0.1% sodium azide). Cells were incubated with Fc block (2.4G2) or with FITC/PE/PerCP tagged monoclonal Abs (CD4, CD8, CD44, CD62L, CD80, CD86 and isotype control) for 30 min at 4°C. After washing, cells were fixed with 1% paraformaldehyde. The cytometry data was acquired using a fluorescence activated cell sorter (GUAVA). Data was analyzed with Express-Plus software. The total number of cells of a definite phenotype (CD⁴⁺CD44⁴⁺⁴⁺CD62L⁴⁺⁴⁺, CD⁸⁺CD44⁴⁺⁴⁺CD62L⁴⁺⁴⁺) were calculated by taking the percentage of the gated cell population, as determined by flow cytometry, multiplying them with the total number of cells obtained per mouse, and finally dividing the furnished result by the number of events.

4.2.16. Statistical analysis

Data were analyzed and two groups were compared employing the Student’s t test as well as one way ANOVA (Holm-Sidak method) to compare all groups, using Sigma-Plot version 10.0 and 11.0 software. The p values 0.05, 0.01, 0.001 were considered as significant for analysis of the data.
4.3. Results

4.3.1. SDS-PAGE and Western blot analysis of Rv3620c

Rv3620c was cloned, expressed and finally characterized by electrophoresis and western blot assay. SDS-PAGE profile of the recombinant protein, Rv3620c (Figure 4.1A) exhibits its size to be 11kDa as reported elsewhere (Mattow et al. 2003). The immuno-dominance of Rv3620c in terms of Ag-specific Ab production was assessed by Western blot analysis. The sera were found to recognize Rv3620c Ag as elucidated by Figure 4.1B.

![SDS-PAGE and Western blot profile of Rv3620c](image)

**Figure 4.1.** SDS-PAGE and Western blot profile of Rv3620c. SDS-PAGE profile of recombinant Rv3620c using Commassie Brilliant Blue staining (A) and evidence of immunodominance of Rv3620c in terms of Ab response as enumerated by Western blot assay (B).

4.3.2. Characterization of liposomes

The Rv3620c encapsulating archaeosomes and zymosan bearing escherisomes were characterized. The TEM image in addition to ascertaining the formation of nanoparticles reveals their size to be in the nano-range (approx. 100nm) (Figure 4.2A). Figure 4.2B shows the uptake of calcein loaded archaeosomes by macrophages as revealed by fluorescence microscopy.
4.3.3. Cytokine assay

Splenocyte culture supernatants belonging to various immunized groups were examined to quantitate the levels of Th1 (IFN-\(\gamma\) and IL-12) as well as Th2 (IL-4) cytokines. Free Rv3620c was found to evoke significantly higher Th1 polarization in immunized animals as compared to saline control. The archaeosome entrapped Rv3620c (Archae-Rv3620c) and the combination of archaeosome entrapped Rv3620c and zymosan encapsulated escheriosomes (EC-Z) were found to induce significantly higher levels of Th1 cytokines in the immunized animals as compared to free Ag (Figure 4.3) and physical mixture of archaeosomes and Rv3620c (data not shown). However, the EC-Z and Archae-Rv3620c combination exhibited the highest Th1 polarization amongst all the preparations tested. EC-Z alone exhibited insignificant levels of Th1 cytokines (data not shown). The animals immunized with BCG exhibited significantly less Th1 cytokine production post booster than groups immunized with Archae-Rv3620c alone or mixture of Archae-Rv3620c and EC-Z. On the contrary, cytokine levels induced upon BCG immunization boosted and crossed the levels evoked by free Ag at 4 and 8 weeks post challenge time points. Insignificant levels of Th2 cytokine (IL-4) were detected at all time points post booster although augmentation in the IL-4 levels was observed by 4th and 8th week post challenge with *Mycobacterium* infection in all groups presumably due to onset of the disease (Figure 4.4A). Control group (saline) exhibited no significant expression of either Th1 or Th2 cytokines at either post booster or post challenge time points when compared to immunized groups (p,0.001).
Figure 4.3. Cytokine response. Th1/Th2 bias was ascertained by determining cytokine response in splenocyte culture supernatant belonging to various immunized groups at different time points; (A) IFN-γ, (B) IL-12. To activate splenocytes belonging to group of animals immunized with free Rv3620c, free form of 50μg Rv3620c was used while splenocytes isolated from animals immunized with archaeosome entrapped Rv3620c either alone or in combination with escheriosome encapsulated zymosan were co-cultured with archaeosome entrapped Rv3620c (50 μg
Ag). The data represent mean of three determinants ± S.D. and are representative of two different experiments with similar observation.

4.3.4. IgG2a and IgG1 class switching
Ab isotype class switching was determined in various immunized animals by analyzing the ratio of IgG2a and IgG1 in their sera. A significant difference between IgG2a Abs produced upon free Rv3620c immunization as compared to saline control is seen. Archae-Rv3620c as well as mixture of Archae-Rv3620c and EC-Z induced significantly higher levels of IgG2a/IgG1 ratio than free Ag (Figure 4.4B). Archae-Rv3620c when given along with EC-Z was found to generate a slightly higher IgG2a/IgG1 ratio indicating a synergistic response. The IgG2a and IgG1 isotype ratio in BCG immunized group was found to be lower than all the Rv3620c preparations (free Rv3620c, Archae-Rv3620c and EC-Z+ Archae-Rv3620c) at post booster as well as post challenge time points (Figure 4.4B).

4.3.5. Lymphocyte proliferation
To analyse the dose dependence of T cell proliferation, splenocytes isolated from various immunized groups were co-cultured in the presence of increasing amounts of free Rv3620c (1-50μg) as well as matching formulations of Rv3620c (data not shown). Lymphocytes isolated from various immunized groups were found to proliferate in a dose dependent manner although the groups immunized with Archae-Rv3620c (Archae-Rv3620c alone as well as given in combination with EC-Z) exhibited significantly higher dose dependence than free Ag as well as BCG. The lymphocytes isolated from the animals belonging to control group (administered saline) failed to induce considerably significant proliferation even at higher dose of the Ag (Figure 4.5) as compared to free Rv3620c. Lymphocytes isolated from animals immunized with Archae-Rv3620c exhibited a significantly higher T cell proliferation as compared to animals receiving free Ag but the proliferation response was lower than lymphocytes isolated from animals administered with mixture of Archae-Rv3620c and EC-Z in the group primed with free Ag or with matching formulation of Ag (Figure 4.6A and 4.6B respectively). Activation with PPD was found to induce higher T cell proliferation in BCG immunized group as compared to free Ag activation as reported earlier (Ansari et al. 2011) (Figure 4.6A and 4.6B). At
4\textsuperscript{th} and 8\textsuperscript{th} week post challenge time points, an increase in T cell proliferation was observed although the lymphocytes were primed with same dose of Ag (50\textmu g). Figure 7 clearly depicts that lymphocytes isolated from animals immunized with Archae-Rv3620c formulations (both Archae-Rv3620c alone and mixture of Archae-Rv3620c and EC-Z) proliferate well-off even after 8 weeks of challenge.

4.3.6. Expression of co-stimulatory and memory markers as revealed by flow cytometric analysis

The purified CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells were characterized for the expression of co-stimulatory markers CD80 (B7-1) and CD86 (B7-2). Animals vaccinated with free Rv3620c revealed expression significantly higher levels of co-stimulatory and memory markers as compared to saline control animals. The group of animals immunized with Archae-Rv3620c and combination of Archae-Rv3620c and EC-Z exhibited significantly higher expression of co-stimulatory molecules as compared to control animals and animals vaccinated with free Rv3620c and BCG. Although animals immunized with BCG showed higher expression co-stimulatory markers than those administered free Ag and saline. Amongst the groups immunized with Archae-Rv3620c alone and mixture of Archae-Rv3620c and EC-Z, the animals immunized with the combination showed better induction of co-stimulatory molecules on their surface comparatively (Figure 4.7). Both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells obtained from animals immunized with Archae-Rv3620c alone or given in combination with EC-Z exhibited typical effector memory (CD44\textsuperscript{high}CD62L\textsuperscript{low}) as well as central memory phenotype (CD44\textsuperscript{high}CD62L\textsuperscript{high}) although the effector memory phenotype was found to be more pronounced. BCG and free Ag immunized animals also showed memory phenotypes to some but the expression was significantly lower than Archae-Rv3620c administered groups. The population of effector memory phenotype in BCG immunized group was relatively less for CD8\textsuperscript{+} T cells (Figure 4.8).
Figure 4.4. Levels of IL-4 and IgG2a to IgG1 ratio (A) Similar to the determination of IFN-γ and IL-12, IL-4 was estimated in the culture supernatants of splenocytes belonging to various immunized groups. (B) To further confirm the Th1/Th2 polarization upon immunization with archaeosome-based formulations of Rv3620c, the sera of immunized animals were analysed for the presence of IgG2a and IgG1 isotypes by sandwich ELISA method as detailed in Materials and Methods section.
Figure 4.5. Dose dependent proliferation of lymphocytes isolated from various immunized groups. Increasing amounts (1.5 to 50 µg) of Rv3620c Ag were taken to study the effect of increasing dose on T cell proliferation.

4.3.7. Protective efficacy

The potential of various vaccine preparations was evaluated on the basis of the depletion of bacterial burden in lungs and spleen of immunized animals. As enumerated by Figure 4.9, Archae-Rv3620c vaccine preparation exhibited significant reduction in bacterial burden in immunized mice as compared to BCG and free Rv3620c. Animals immunized with free Rv3620c exhibited a significant reduction in bacterial burden in various organs as compared to control animals. The BCG vaccination was found to be more effective in clearing bacterial burden in either lungs or spleen at 4 week as well as 8 week post challenge time points when compared to immunization with free form of Rv3620c. The Archae-Rv3620c vaccine preparations (Archae-Rv3620c only and combination of Archae-Rv3620c and EC-Z) induced significantly higher reduction in bacterial burden as compared to free Rv3620c as well as BCG. On 8th week post challenge, the mycobacterial load in groups immunized with Archae-Rv3620c alone and mixture of Archae Rv3620c and EC-Z was found to be quite similar. The residual bacterial load data clearly establish superiority of
archaeosome entrapped Ag in eliminating TB than BCG as well as free Ag immunized groups.

Figure 4.6. T cell proliferation response. (A) Proliferation of Rv3620c specific T lymphocytes isolated from immunized animals at various time points post booster and also at 4 and 8 weeks post challenge upon stimulation with a fixed amount (50 μg) of free Rv3620c. While (B) represents T cell proliferation in various immunized groups at various time intervals upon activation with corresponding matching formulations of
Rv3620c. For stimulation of cells belonging to BCG immunized group, PPD at concentration of 50 μg was used, while maintaining rest of the incubation conditions same to that of archaosome based cell groups. Data represent the mean of three determinants ± S.D. Figures are representative of four independent experiments.

Figure 4.7. Expression of costimulatory molecules on APCs. The expression of costimulatory molecules, CD80 (B7-1) and CD86 (B7-2), on macrophages was determined by staining target cells with specific Abs and subsequent analysis by flow cytometry at 8 weeks post-challenge. The histograms for (A) CD80 and (B) CD86 represent various immunized groups: (i) Saline as control; (ii) BCG; (iii) Free Rv3620c; (iv) Archaeosome-entrapped Rv3620c and (v) Combination of archaosome entrapped Rv3620c and escherosome encapsulated zymosan. Data are representative of three independent experiments with similar observations.
Figure 4.8. CD8⁺/CD4⁺ T cell effector and central memory response upon immunization with archaeosome-entrapped Rv3620c. The CD4⁺ and CD8⁺ T cells were harvested and their purity was depicted as described in the ‘Materials and methods’ section. The (A) CD4⁺CD44⁺CD62L⁻/low and (B) CD8⁺CD44⁺CD62L⁻/low phenotypes were analyzed using flow cytometry at 8 weeks post-challenge, representing various immunized groups: (i) BCG; (ii) Free Rv3620c; (iii) Archaeosome-entrapped Rv3620c; and (iv) Mixture of archaeosome entrapped Rv3620c and escheriosome encapsulated zymosan. Data are representative of three independent experiments with similar observations.
Figure 4.9. Protective efficacy. Mycobacterial load, in the lungs (A) and spleens (B) of vaccinated mice, belonging to various groups of animals, was enumerated by plating tissue homogenates of lungs and spleens of vaccinated mice followed by counting the numbers of CFUs at 4 and 8 weeks post challenge following method as described in methodology section of the text. The data were expressed as means of three determinants ± S.D. and are representative of 3 independent experiments.
4.4. Discussion

In our previous study, we have proposed and provided evidence in support of the concept that proteins belonging to the RD region of BCG are potent immunoprotective molecules against TB (Ansari et al. 2011). We demonstrate in the present study that Rv3620c, a protein encoded by RD9 region of BCG, can evoke potent T cell response in immunized animals therefore providing additional support to the hypothesis. Encapsulation of Rv3620c in archaeosomes was found to amplify the generated immune response against this Ag and impart protection against TB. Since signalling via TLRs has been reported to improve the immune response and TLR agonists function as adjuvants (Gowthman et al. 2012). We selected TLR2/TLR6 agonist zymosan and encapsulated in escheriosomes. Zymosan was encapsulated in escheriosomes so as to facilitate delivery of zymosan in the host at a pace similar to Rv3620c entrapped in archaeosomes. The escheriosome encapsulated zymosan when administered along with archaeosome entrapped Rv3620c was found to augment the immune response generated against Rv3620c in comparison to that generated upon immunization of animals with archaeosome entrapped Rv3620c alone.

Encapsulation of Rv3620c may provide a depot effect, leading to slow and sustained release of the Ag ensuing in higher efficacy of archaeosome encapsulated Rv3620c preparation in comparison to free Rv3620c. Moreover, the specific composition of archael lipid has been reported to facilitate phagocytosis of Ag encapsulated archaeosomes by macrophages and dendritic cells in addition to helping in slow and steady release of Ag from archaeosomes which might assist in biodistribution of Ag to various lymphoid organs (Ansari et al. 2011). Mice immunized with archaeosome encapsulated Rv3620c exhibited a CTL response for over a period of six months which is in concordance with earlier reports (Krishnan et al. 2000b, Ansari et al 2011), favouring the development of archaeosome based Rv3620c formulation as a potential vaccine candidate.

In order to rule out the possibility that not the Ag loaded liposomal structure of delivery system but the archaeosomal total polar lipid contents were entirely responsible for various observed immune responses evoked in the animals immunized with Archae-3620c, we included plain archaelipid based vesicles that were not loaded.
with Rv3620c and found that they elicit very weak immune responses similar to saline control (data not shown). This has also been demonstrated in our previous report (Ansari et al. 2011). Similarly, animals administered zymosan entrapped escherisomes alone exhibited a feeble immune response similar to saline control and empty archaeosomes (data not shown).

Signaling and targeting TLR-2 has been successful in several disease models and has also been implicated in controlling TB (Bafica et al. 2005, Reiling et al. 2002). TLR-2 restricts the intracellular survival of M. tb in murine macrophages principally by NO release (Thoma-Uszynski et al. 2001). In the case of human macrophages, TLR-2 triggering upregulates the expression of vitamin D receptor and vitamin D-1-hydroxylase gene leading to induction of the antimicrobial peptide cathelicidin and the killing of intracellular M. tb (Liu et al. 2006). Signaling through TLR-2 can also enhance the long-term T cell memory response. Memory T cells are indispensable in imparting long-term protection against any infection (Chandran et al. 2009). These facts might be the reasons for the enhanced Th1 cell memory response and better protection offered by EC-Z and Archae-Rv3620c combination as the adjuvants properties of archaeosomes and zymosan may have worked synergistically to evoke an overall better immune response than Archae-Rv3620c given alone.

Cytokine responses in the present study were indicative of a predominantly cell-mediated immune response when animals were vaccinated with Archae-Rv3620c either alone or in combination with EC-Z (Figures 4.3 and 4.4). The Ag-specific increase in IFN-γ and IL-12 with minimal IL-4 responses and increased levels of IgG2a suggested that Th1-type immunity rather than Th2-type immunity was induced. The Th1 response to the Archae-Rv3620c and EC-Z combination was greater than that to the Archae-Rv3620c immunized solitarily. This suggests that the effect of vaccinating with the Archae-Rv3620c and EC-Z could be additive compared to vaccinating with Archae-Rv3620c alone. BCG was found to evoke immune responses similar to the free Rv3620c. IFN-γ and IL-12 have been reported to bear a role in vaccination-induced immunity against TB, which concurs with the increased levels of these cytokines measured in the current study. Corroborating results enumerating Th1 polarization were obtained for lympho-proliferation studies. Archae-Rv3620c along
with EC-Z induced highest T cell proliferation followed by Archae-Rv3620c given alone. BCG and free Rv3620c induced more or less similar responses although they induced significantly higher lymphocyte proliferation than saline control (Figures 4.5 and 4.6). Similar to our previous study (Ansari et al. 2011), BCG inspite of lacking RD gene products including Ag Rv3620c was found to evoke Rv3620c specific immune response in the host. The observed T cell proliferation might be a consequence of the fact that some proteins in BCG have overlapping peptide sequences to that of Rv3619c/Rv3620c which might have been successful in inducing production of cross reactive T lymphocytes in the immunized animals.

Apart from the increased Th1 cytokine and lymphocyte proliferation, animals immunized with Archae-Rv3620c exhibited significant augmentation in co-stimulatory molecules and memory markers as compared to free Rv3620c although it was lesser than that induced by Archae-Rv3620c and EC-Z combination (Figure 4.7 and 4.8). Activation of naive T cells requires signaling through the Ag specific TCR and co-stimulatory molecules. The best-characterized co-stimulatory pathway is due to interaction of CD28 on T cells with CD80 (B7-1), CD86 (B7-2) on APCs (Lenschow et al. 1996). It is a well-established fact that enduring T-cell memory response is a cardinal feature of any successful vaccine (Gowthaman et al. 2011). One of the reasons for BCG failure is that it fails to generate long-lasting memory T cells (Andersen and Doherty 2005). Notably, immunization with Archae-Rv3620c and EC-Z combination significantly improved T-cell memory response as compared to that generated upon vaccination by Archae-3620c alone. Interestingly, it has been reported that TLR2 agonists induce T-cell memory. There was a substantially greater percentage of effector memory phenotype (CD44^{high}CD62L^{low}) as well as central memory phenotype (CD44^{high}CD62L^{high}) on both CD4^+ and CD8^+ T cells belonging to animals immunized with archaesosome encapsulated Ag alone as well as given in combination with escheriosome entrapped zymosan in comparison to group immunized with free Rv3620c. In general, central memory persists after rapid clearance of acute infections, and is more effective in controlling secondary infections involving intracellular pathogens (Wherry et al. 2003). On the other hand, the effector memory was reported to be induced by chronic infection (Appay et al. 2002, Champagne et al. 2001). This fact is clearly suggestive of the continued low-level
presentation of Ag to both the CD4$^+$ and CD8$^+$ T cells by APCs at later time points and thus predicts an Ag-depot effect offered by archaeosomes, which in turn results in producing a balanced central and effector memory in the host. The higher expression of memory phenotypes in Archae-Rv3620c and EC-Z combination immunized animals can be owed to the parallel release of zymosan which is TLR2 agonist from escheriosomes and Rv3620c from archaeosomes enabling development of an immunological imprint in the form of memory.

The increased Th1 cytokine responses, lymphocyte proliferation, expression of co-stimulatory and memory markers correlated with decreased bacterial counts observed in the lungs and spleens of immunized animals. Figure 4.9 enumerates that Archae-Rv3620c immunization renders a significant reduction in bacterial burden in lungs and spleen of immunized mice and when immunized in combination with EC-Z, a further depletion of bacterial load is observed. The significant reduction in spleen bacterial counts in vaccinated animals indicates that extra-pulmonary spread of TB has been minimized. The data of the present study clearly suggest that archaeosome based Rv3620c subunit vaccine confers long term protection against TB in model animals. Moreover, the combination of Archae-Rv3620c with escheriosome encapsulated zymosan augments the protective efficacy of the Ag. Remarkably, encapsulation of only a single recombinant M. tb protein in archaeosomes provided a substantial increase in its protective efficacy. Whether entrapment of additional M. tb proteins in archaeosomes will further boost the protective immune response remains to be determined.

Cumulatively, the present study enumerates three major findings i) Rv3620c being inducer of T cell response is a T cell Ag ii) Encapsulation of Rv3620c in archaeosomes significantly enhances the cellular immune response evoked by Rv3620c and imparts protection against TB by inducing long term memory iii) Administering archaeosome entrapped Rv3620c along with escheriosome entrapped zymosan brings about a synergistic effect and further boosts the immune response elicited by Rv3620c.