Biotechnology and Bioprocess Engineering (2024) 29:589–600
https://doi.org/10.1007/s12257-024-00089-3

RESEARCH PAPER

Engineering potyvirus-like particles to display multiple copies of tuberculosis antigens

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Received: 14 December 2023 / Revised: 17 February 2024 / Accepted: 21 February 2024 / Published online: 11 March 2024

Abstract
Elicitation of antibody and cell-mediated immune responses are crucial for successful vaccine development against tuberculosis (TB). *Mycobacterium tuberculosis* (Mtb) antigens CFP10 and ESAT6, potent and proven vaccine candidates require appropriate adjuvants to trigger better immune response. Virus-like particles carrying repetitive copies of foreign antigens can induce both T and B cell-mediated immunity required for conferring protection against intracellular pathogens. In this study, we developed hybrid potyvirus-like particles (PVLPs) displaying mycobacterial antigens on their surface by translationally fusing the coat protein (CP) gene derived from Johnson grass mosaic virus with CFP 10 or/and ESAT 6 gene(s). The recombinant plasmids carrying fusion constructs were transformed into *Escherichia coli*, the fusion proteins, viz. ESAT6-CP, CP-CFP10 and ESAT6-CP-CFP10, were expressed and purified using Ni-NTA^{2+} affinity chromatography under denaturing conditions. The chimeric CP fusion proteins were self-assembled in vitro into PVLPs by the gradual removal of denaturing conditions. The purified hybrid PVLPs carrying Mtb antigens when injected into mice showed enhanced immunogenicity for both ESAT6 and CFP10 antigens compared to the same antigens immunized without any adjuvant. In vitro stimulation of splenocytes derived from mice immunized with chimeric PVLPs upregulates the expression of cytokines involved in TB immune response.

Keywords Tuberculosis · CFP10 · ESAT6 · Johnson grass mosaic virus · Potyvirus-like particles

1 Introduction

Tuberculosis (TB), a ubiquitous infectious disease caused by *Mycobacterium tuberculosis* (Mtb), remains one of the leading causes of death in humans. Recently, the incidence and mortality rate of TB was aggravated due to the COVID-19 pandemic undermining the global efforts to bring this debilitating disease under control. Lack of effective vaccines, point of care diagnosis, emergence of multi-drug-resistant strains, expensive and prolonged treatment regime hampers the target set by WHO to eradicate TB by the year 2035 [1]. Though Bacille Calmette-Guerin (BCG) is a promising whole cell-based vaccine for TB, it was unsuccessful to prevent pulmonary TB in adolescents and adults [2]; further, it causes BCGosis in immunocompromised individuals [3]. Therefore, attempts have been made to improve the efficacy of BCG vaccination by augmenting them with either booster vaccines or replacing BCG with novel subunit vaccines. Since Mtb is found in extracellular as well as intracellular compartments of infected cells, any rational vaccine design should take cognizance of both cell and humoral-mediated immunity that is essential for control and eradication of TB [4, 5].

Compared to traditional vaccines like whole cell-based or live attenuated, subunit vaccines are advantageous as it contains disease-specific antigens, safer but their intrinsic limitation is poor immunogenicity; hence, they need immunostimulatory adjuvant to enhance immune response. The development of subunit vaccines relies on careful selection of appropriate antigens, adjuvants, and suitable delivery systems. Among various Mtb antigens investigated, two major secretory proteins culture filtrate protein (CFP10) and early secretory antigenic target-6 (ESAT6) expressed by genes existing in RD1 genomic region of Mtb are absent in BCG

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strains but present in virulent strains of Mtb [6, 7]. Furthermore, ESAT6 and CFP10 can be strongly recognized by both $CD4^+$ and $CD8^+$ cells inducing cytokine production, thereby offering protective immune response signifying them as a suitable vaccine candidate [8–10]. These antigens are being exploited in subunit vaccines, viz. GamTBvac [11] and H56/IC31 [12], presently under clinical trials with different types of adjuvants to enhance their immunogenicity.

Self-assembled virus-like particles (VLPs) are gaining importance in vaccine formulation owing to their particulate nature as they can be easily internalized by antigen presenting cells such as dendritic cells and macrophages and stimulate innate and humoral immune responses. Hence, coat proteins (CP) of plant and animal viruses that form VLPs when expressed in heterologous hosts are being investigated as an exquisite platform for antigen presentation, delivery and adjuvant [13]. VLPs are attractive system for protein/peptide presentation because of its highly organized structure, biocompatibility, flexibility, assembly/disassembly process, stability, non-infectious and feasibility of its expression in different heterologous system. Currently, VLPs derived from hepatitis B virus, human papilloma virus and hepatitis E virus are approved for use in humans. Plant virus derived VLPs score over animal viruses since they are less immunogenic and remains in human circulatory system for a while because of the absence of pre-existing virus specific antibodies [14, 15]. Plant viruses such as cowpea mosaic virus [16], bamboo mosaic virus [17], papaya mosaic virus [18] have also been investigated as antigen/peptide presentation system, and it was observed that assembled CP of these plant viruses are capable of stimulating both T and B cell response similar to animal virus derived VLPs.

CP of Johnson grass mosaic virus (JGMV), a potyvirus, assembles into potyvirus-like particles (PVLPs) when expressed in heterologous system such as *Escherichia coli*, yeast and insect cell. Multivalent antigens can be displayed at both C and N termini of JGMV CP as they do not infringe upon the protein–protein interaction between CP monomers and consequent assembly into PVLPs. PVLPs have high aspect ratio rendering large spatial configuration for displaying foreign antigens, and hence, they induce strong immune response [19]. Earlier studies with JGMV PVLPs presenting antigenic peptides derived from intracellular pathogens like *Plasmodium falciparum* and Japanese encephalitis virus showed enhanced protection by producing neutralizing antibodies [20, 21]. Therefore, JGMV PVLP is likely to be a promising delivery/adjuvant system for the Mtb antigen presentation. In the present study, feasibility of JGMV PVLPs to present repetitive copies of TB antigens, ESAT6 and CFP10 at N and/or C termini and their enhancement of immunogenicity was investigated.

2 Materials and methods

2.1 Development of recombinant JGMV CP fusion constructs

The DNA sequence of CFP10 and ESAT6 genes was retrieved from NCBI database. Primers were designed using DNA sequences of CFP10 (GenBank accession no. FJ014498.1) and ESAT6 (GenBank accession no. FJ014499.1) open reading frames and nucleotides encoding polyhistidine tag included in the sense/antisense primer for protein purification. Primers designed for developing fusion constructs were included with nucleotides encoding polyglycine linkers (Table 1). The genomic DNA of Mtb H37Rv strain was obtained from Dr. Amit Singh, Indian Institute of Science, India, used as template to PCR amplify CFP10 and ESAT6 genes (Bio-Rad). CFP10 PCR product was cloned into Nco I and EcoR I sites of pET33b, whereas ESAT6 PCR product was cloned into Nde I and EcoR I sites of pET25b (Invitrogen). JGMV CP gene present in the clone $pTTQ19^{N}CP2$ (kind gift of Dr. Mittur Jagadish,

Table 1 Oligonucleotide primers used for PCR amplification of ESAT6 and CFP10 genes

Gene constructs	Nucleotide sequence of primers
ESAT6	Sense: GACGCG**CATATG**ACAGAGCAGCAGTGG Antisense: AT**GAATTC**GCTAGTGGTGGTGGTGGTGGTGTGCGAACATCCCAGTGAC
CFP10	Sense: CATGCATG**CCATGG**GGATGGCAGAGATGAAGACC Antisense: CGC**GAATTC**CTAGTGGTGGTGGTGGTGGGGAGAAGCCCATTTGCGAGG
ESAT6-CP	Sense: GACGCG**CATATG**CACCACCACCACCACCACATGACAGAGCAGCAGTGG Antisense: TACA**GGATCC**TTT*TCCTCCTCC*AGATGCGAACATCCCAGTGAC
CP-CFP10	Sense: **CTGCAG**TA*GGAGGAGGA*AAAATGGCAGAGATGAAGACC Antisense: TAT**GGTACC**ATTCAGTGGTGGTGGTGGTGGTGAAGCCCATTTGCGAGGA
ESAT6-CP-CFP10	Sense: GACGCG**CATATG**ACAGAGCAGCAGTGG Antisense: TACA**GGATCC**TTT*TCCTCCTCC*AGATGCGAACATCCCAGTGAC

Restriction sites are indicated in bold, 6X his tag encoding nucleotide sequences are underlined, whereas codons of polyglycine linkers are italicized in the given primers

CSIRO, Melbourne, Australia) was subcloned into BamH I and EcoR I sites of pET25b to obtain pET25b:CP. For developing fusion constructs, ESAT6 gene was PCR amplified from Mtb genomic DNA and cloned into 5' end of CP using Nde I and BamH I sites of pET25b:CP resulting in pET25b:ESAT6-CP. Similarly, PCR amplified CFP10 gene was cloned into 3' end of CP gene at Pst I and Kpn I sites of pRSETb:CP and subsequently subcloned into pET25b vector at BamH I and EcoR I sites to generate pET25b:CP-CFP10. To develop double fusion construct, ESAT6 gene was cloned into Nde I and BamH I sites of pET25b:CP-CFP10 to derive pET25b:ESAT6-CP-CFP10 (Fig. 1). Recombinant plasmids were extracted from the transformants using QIAGEN plasmid mini kit (QIAGEN), and presence of gene of interest in the plasmids was confirmed by appropriate restriction enzymes. Nucleotide sequences present in the recombinant constructs were deduced by performing DNA sequencing using T7 promoter and terminator primers (Scigenome).

2.2 Expression and extraction of recombinant proteins

Recombinant plasmids were transformed into *E. coli* BL21(DE3) strains, and transformants were inoculated into LB broth and kept overnight for shaking at 37 °C. After subculturing, bacterial culture was induced with 1 mM IPTG at mid log phase and incubated at 37 °C for 4 h in a shaker. The bacterial cells from culture broth were harvested by centrifugation at 4 °C, 6,000 g for 10 min. The cell pellet was resuspended in lysis buffer (20 mM Tris, pH 8.0, 100 mM NaCl) and homogenized by ultrasonication. The cell lysate was centrifuged at 20,000 g for 15 min at 4 °C followed by a wash with lysis buffer. Samples of both supernatant and pellet fraction were analyzed by SDS PAGE, and it was observed that recombinant protein was present as inclusion bodies. The inclusion bodies were solubilized in lysis buffer containing 8 M urea, allowed to remain at room temperature for 2 h, centrifuged at 20,000 g for 20 min and passed through 0.2-µm filter before loading it onto chelating metal affinity column.

2.3 Purification of JGMV PVLPs

JGMV PVLP was purified according to the protocol described previously with modifications [20]. IPTG-induced *E. coli* cell pellet comprising recombinant JGMV CP protein was resuspended in 20 mM Tris buffer (pH 8.0) containing 20% sucrose, incubated with lysozyme for 20 min at room temperature and centrifuged at 12,000 g for 10 min. Then the pellet was resuspended in 20 mM tris buffer (pH 8.0), sonicated at 60 Hz with 10 cycles of 30 s strike followed by centrifugation. Supernatant was loaded onto discontinuous 10–40% sucrose gradient and centrifuged at 30,000 g for 3 h at 15 °C using SW41 rotor (Beckman Coulter). Fractions were collected and run on SDS PAGE, and fractions containing JGMV CP were pooled for further purification. Pooled fractions were loaded onto HiPrep 16/60 Sephacryl S-300 HR column (GE Healthcare) equilibrated with 20 mM

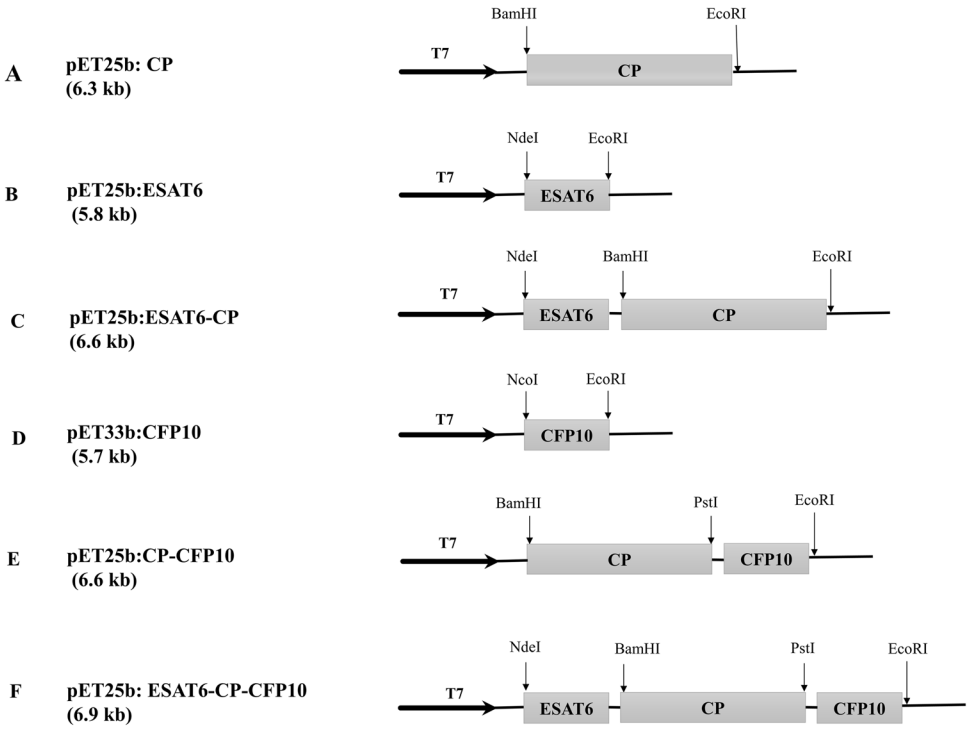

Fig. 1 Recombinant DNA constructs developed for the expression of CFP10, ESAT6 and their fusion with JGMV CP (A-F). For fusion at 5' region of CP gene, PCR product of the ESAT6 gene amplified with the sense and antisense primers containing Nde I and BamH I sites, respectively. The antisense primer designed without termination codon to derive translation fusion between ESAT6 and CP ORFs. Fusion at 3' region of CP gene performed by using an internal Pst I site adjacent to 3' end of CP gene and EcoR I in plasmid vector. Termination codon present at the CP ORF removed while using Pst I and EcoR I sites to derive translation fusion with CFP10 ORF. JGMV: Johnson grass mosaic virus, CP: coat protein, ORF: open reading frame

Tris buffer (pH 8.0). The peak fractions were collected and checked for the presence of JGMV CP followed by transmission electron microscopy (TEM) analysis to confirm PVLP formation.

2.4 Purification of recombinant proteins

HiTrap™ chelating HP 1 mL column (GE Healthcare) activated using 0.1 M nickel sulfate and pre-equilibrated with 8 M urea containing lysis buffer. Urea-solubilized protein lysate was loaded on to column at flow rate of 0.5 mL/min and flowthrough from column collected. The column was washed with lysis buffer to remove unbound proteins. The denatured protein was refolded on column by gradually decreasing urea concentration from 8 to 0 M at a constant flow rate of 0.5 mL/min for 2 h, thereby gradually facilitating renaturation of recombinant protein. The renatured protein was eluted using linear gradient of 0–500 mM imidazole. Purity of recombinant protein was confirmed by resolving samples of peak fractions in 12% SDS PAGE. Eluted peak fractions containing recombinant protein were pooled and dialyzed (MWCO, 2 kDa) against buffer (10 mM phosphate buffer, pH 7.2, 100 mM NaCl) for 72 h at 4 °C for removal of imidazole. Furthermore, dialyzed protein samples were concentrated using centrifugal filter (Amicon, MWCO, 3 kDa) and loaded onto buffer (10 mM phosphate buffer, pH 7.2, 100 mM NaCl) equilibrated HiPrep 16/60 Sephacryl S-300 HR column. Peak fractions were examined for the presence of recombinant protein followed by TEM analysis. Protein concentrations of the purified samples were estimated by Bradford method [22]. The identity of protein ESAT6, CFP10 and JGMV CP was confirmed using mass spectrometry (MALDI-7090, Shimadzu) before immunizing mice.

2.5 Immunization of mice

Healthy Swiss albino mice were purchased from Tamilnadu Veterinary and Animal Sciences University, Chennai, India. Animal experimental protocol was approved by Institutional Animal Ethics Committee, Mepco Schlenk Engineering College, Sivakasi, India (Registration number: MSEC/BT/P2/Myco 2019). Ten groups of 6–8-week-old Swiss albino mice segregated into three mice per group were immunized intramuscularly with 25 µg of purified recombinant JGMV PVLP, CFP10, ESAT6 and ESAT6 + CFP10 with and without Freund's incomplete adjuvant, CP-CFP10, CP-ESAT6 and ESAT6-CP-CFP10 protein antigens. A group of mice immunized with phosphate buffer saline (PBS) alone was maintained as control. Each group administered two booster doses with an interval of two weeks between them. The mice were bled by retro-orbital sinus puncture after 42 days of immunization, and spleen was collected to assess cell-mediated response. Sera were separated from the blood samples and assessed for antigen-specific immune response.

2.6 Western blot analysis

Proteins resolved in the gel were blotted on to nitrocellulose membrane by wet electrophoretic transfer using western blot apparatus (Genei). The membrane was incubated with blocking solution containing 3% skim milk and 0.05% Tween 20 prepared in Tris-buffered saline (TBST) followed by incubation with primary antibody (1/1,000 dilution) raised in mice against recombinant protein. Then membrane was washed and incubated in TBST buffer containing anti-mouse antibody conjugated with alkaline phosphatase (ALP) (Sigma). The membrane was washed again, soaked in substrate buffer and developed using BCIP/NBT substrate to detect purified recombinant proteins.

2.7 ELISA

Purified antigenic protein of 200 ng was prepared in carbonate buffer (pH 9.6) and coated on 96-well microtitre plate and incubated at 37 °C for 1 h. The ELISA plate was washed with PBST (phosphate buffer saline containing 0.05% Tween 20). Then the wells were blocked using 1% bovine serum albumin (BSA) prepared in PBST and incubated at 37 °C for 1 h. The plates were washed using PBST and incubated with different dilutions of mice sera at 37 °C for 1 h. After washing with PBST, plates were incubated with 100 µL of anti-mouse antibody conjugated with horseradish peroxidase (Sigma) for 1 h at 37 °C. Then it was washed twice with PBST, the substrate 3, 3', 5, 5'-tetra-methyl benzidine (Sigma) added, and the reaction was allowed to proceed for 10 min. The reaction was terminated by adding 1 N sulfuric acid (Merck Millipore), and absorbance was read at 450 nm in ELISA reader (Perkin-Elmer). Antibody titers were defined as inverse log10 of the highest dilution corresponding to absorbance value greater than that of PBS immunized control mice obtained at 1:100 dilution.

2.8 Transmission electron microscopy

Glow-discharged carbon-coated copper grids (Quorum Technologies) were coated with 10 µL of hybrid JGMV PVLPs displaying TB antigens at concentration of 0.1 µg/µL for 30 s. Excess solution was removed, and the grids were then negatively stained with 1% uranyl acetate for another 30 s. The grids were air dried and examined at room temperature using Talos L120C transmission electron microscope (Thermo Fisher Scientific) equipped with Ceta camera.

2.9 Cytokine assay

Splenocytes were isolated and seeded at a density of 5×10^5 cells/well in a 24-well plate. The splenocytes obtained from the mice groups immunized with ESAT6, CFP10, ESAT6 + CFP10 antigens with and without adjuvant were left unstimulated or stimulated with either ESAT6 or CFP10 (10 µg/mL) or 0.4 µg/mL of the standard PPD RT23 (Arkray Healthcare Pvt. Ltd.). Similarly, above stimulation protocol was also followed for splenocytes obtained from mouse immunized with CP-ESAT6, CP-CFP10 and CFP10-CP-ESAT6. All the stimulations were performed in triplicate at 37 °C in 5% CO_2. After 72 h of incubation, supernatants were collected and screened for the release of INF-γ by sandwich ELISA. Microtiter plate was coated with mouse IFN-γ monoclonal antibody (clone XMG1.2, Thermo Fisher Scientific) and incubated at 37 °C for 1 h followed by washing with PBST and blocking the unbound sites with 1% BSA. Then the culture supernatants of stimulated and unstimulated cells were added into the wells, incubated for 1 h at 37 °C and the remaining steps followed as described previously. The amount of IFN-γ released from stimulated and unstimulated spleenocytes were quantified by extrapolating experimental values on IFN-γ (Biolegend) standard curve.

2.10 ELISPOT assay

Microtiter plate coated with IFN-γ monoclonal antibody (Thermo Fisher Scientific) was seeded with 1×10^5 splenocytes per well in triplicate and stimulated with 10 µg of recombinant protein for 24 h at 37 °C as described in Sect. 2.9. PPD (0.4 µg/mL) was used as a positive control and Dulbecco's modified eagle medium alone as a negative control. Then the plates were washed with PBST, and IFN-γ monoclonal antibody was added and incubated for an hour. After washing, anti-rabbit ALP conjugate (Sigma) was added and incubated for 1 h at 37 °C. The plate was developed by BCIP/NBT substrate, and the spots were manually counted using dissection microscope. Antigen-specific spots were estimated by subtracting the values of negative control and unstimulated cell.

2.11 Statistical analysis

Statistical analysis was performed to evaluate significance of experimental values using Graphpad Prism 8.0 software (GraphPad Software Inc.) and the data represented as mean ± SD. Variables are compared using nonparametric testing (Tukey's multiple comparisons test) for the assessment of antibody and cytokine response.

3 Results

3.1 Expression, purification and assembly of recombinant proteins into PVLPs

In the present study, low molecular weight secretory Mtb antigens CFP10 and ESAT6 are genetically fused to N- and/or C-terminal of JGMV CP to enhance their immunogenicity. The JGMV CP gene was derived from the original clone pTTQ19^{N}CP and subcloned into BamH I and EcoR I sites of pET25b vector to develop fusion constructs of Mtb antigens. CFP10 gene was amplified from the genomic DNA of Mtb and translationally fused to 3' end of CP sequence to obtain pRSET25b:CP-CFP10, and further CP-CFP10 construct was subcloned into pET25b to obtain pET25b:CP-CFP10. Similarly, ESAT6 gene was amplified and fused into 5' end of CP sequence to derive pET25b:ESAT6-CP. These two fusion constructs could express the recombinant proteins that present the immunodominant epitopes of ESAT6 and CFP10 antigens on the surface exposed N and C termini of CP. The recombinant proteins resolved on SDS PAGE reveal predominant bands at 48 kDa and 45 kDa corresponding to CP-CFP10 and ESAT6-CP, respectively, which was consistent with the predicted molecular weight of those proteins. In the case of CP-CFP10, two predominant bands migrating closer to each other were observed in which lower molecular weight protein could be attributed to proteolytic degradation of protruding N terminus of CP. To display the mycobacterial antigens at both the ends of CP, double fusion construct, viz. pET25b:ESAT6-CP-CFP10, was generated by translationally fusing ESAT6 gene at 5' end and CFP10 at 3'end of CP gene. On expression, pET25b:ESAT6-CP-CFP10 produced a fusion protein, ESAT6-CP-CFP10 of 55 kDa when resolved on SDS PAGE. Flexible amino acid linker, GGGK, was engineered between Mtb antigen and CP monomer to facilitate assembly without impacting PVLP formation. In order to perform purification of recombinant proteins from *E. coli* extract, his tag was engineered at either N or C termini of all the proteins used in this study. JGMV CP, ESAT6 and CFP10 genes were also cloned into pET vectors separately and expression of the clones analyzed by SDS PAGE to confirm respective molecular weight of those proteins as 37, 11 and 12 kDa. Mass spectrometric identification of these proteins was carried out by tryptic digestion, acquisition of mass spectral data and searching the peptide sequence with Mascot engine confirmed the identity of JGMV CP, ESAT6 and CFP10 proteins.

High-level expression of induced proteins was obtained from all the constructs generated in this study, and it was observed that predominant recombinant proteins

partitioned into insoluble fraction as inclusion bodies except CP protein. The soluble CP protein was purified using sucrose gradient centrifugation followed by size exclusion chromatography. The insoluble recombinant proteins were solubilized by clarifying with 8 M urea and purified adopting metal affinity chromatographic technique under denaturation condition. Aliquots of samples representing peak fractions collected from Ni^{2+}-NTA column fixed in Akta FPLC (GE Healthcare) were resolved on SDS PAGE to identify purified proteins with expected molecular weight (Fig. 2A). Peak fractions containing recombinant proteins without contaminants were pooled and dialyzed against 10 mM phosphate buffer (pH 7.2) with 100 mM NaCl to necessitate the polymerization of monomeric proteins into PVLPs. Immunoblot analysis of recombinant proteins with anti-CFP10 (Fig. 2B), anti-ESAT6 (Fig. 2C) or anti-CP antibodies (Fig. 2D) showed a band of 11, 12, 37, 45, 48 and 55 kDa corresponding to ESAT6, CFP10, CP, ESAT6-CP, CP-CFP10 and ESAT6-CP-CFP10 proteins, respectively. The polymerization of monomers into chimeric PVLPs (Fig. 3) was verified by staining purified proteins with 1% uranyl acetate and subjected to TEM analysis to obtain electron micrograph. Flexuous rod shaped 700 ± 100-nm-long and 11–15-nm-wide PVLP particles were observed for CP (Fig. 3A). Both ESAT6-CP and CP-CFP10 assembled into PVLPs that are approximately 200 ± 30 nm in length and 11–16 nm in diameter similar to the CP expressed and purified from

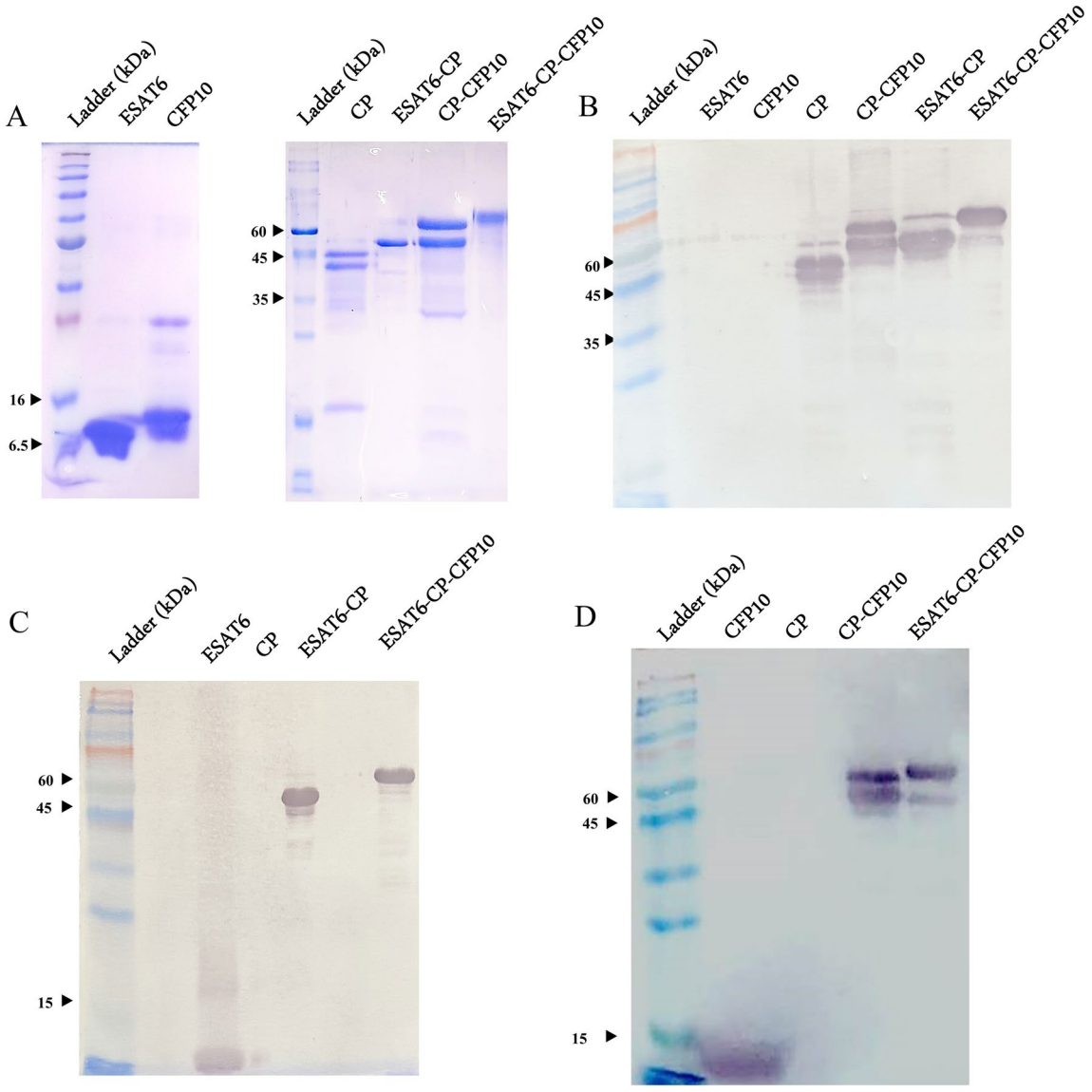

Fig. 2 Characterization of purified recombinant proteins: **A** Purified recombinant proteins resolved on SDS PAGE and Coomassie stained. Immunoblot analysis of fusion proteins probed with primary antibody raised against CFP10 **B**, ESAT6 **C** and CP **D** proteins. CP: coat protein

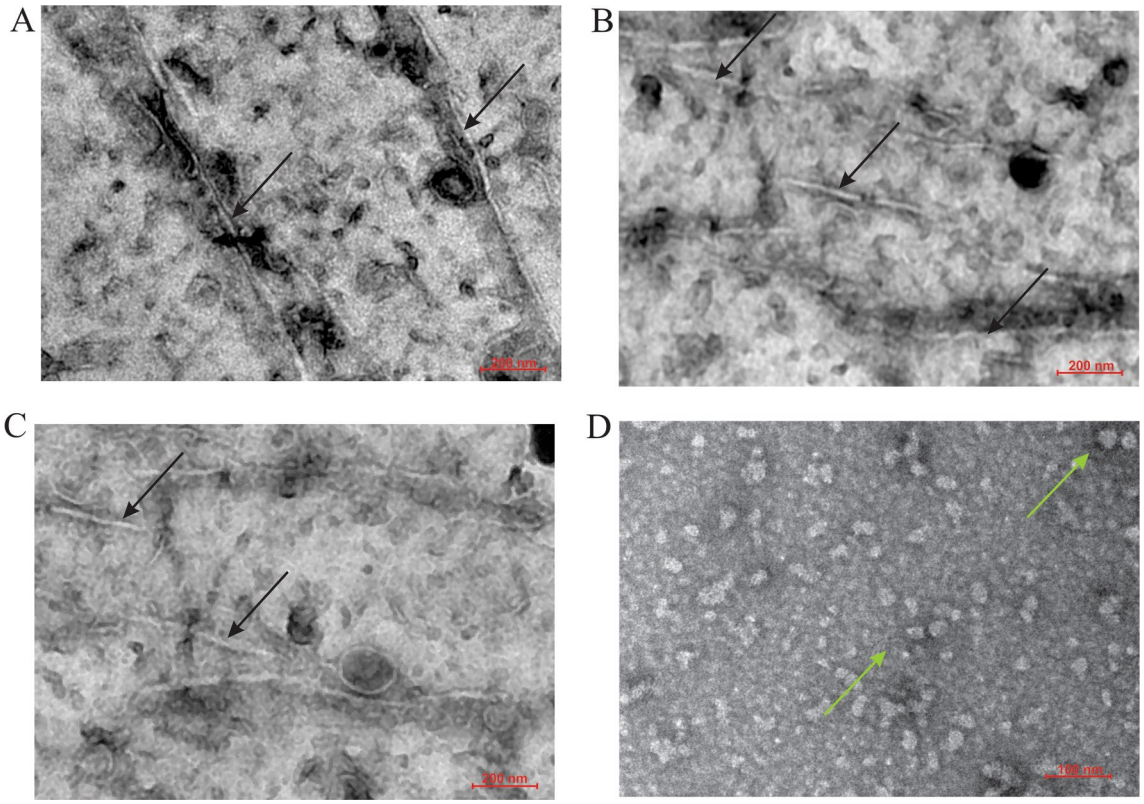

Fig. 3 Transmission electron micrographs of negatively stained CP **A**, CP-CFP10 **B**, ESAT6-CP **C** and ESAT6-CP-CFP10 **D**. Scale bars represent 200 nm. Black and green arrowheads indicate PVLPs and ring like intermediates, respectively. PVLPs: potyvirus-like particles

E. coli (Fig. 3B and C). But these PVLPs appear shorter in contrast to CP, and they were similar in diameter. In contrast, assembly of PVLP was not visualized for ESAT6-CP-CFP10 protein revealing the formation of irregular ring-like intermediate structures (Fig. 3D).

3.2 Evaluating the efficacy of chimeric PVLPs for enhanced immunogenicity

To examine the adjuvanticity of JGMV CP, nine groups of Swiss albino mice were immunized with recombinant CFP10, ESAT6, CFP10 + ESAT6 with and without adjuvant, PVLPs presenting multiple copies of CFP10 or/and ESAT6. Sera were collected after 42 days of immunization and the $\log_{10}$ antibody titers of CFP10 and ESAT6 specific antibodies evaluated by ELISA (Fig. 4). The titers of antibody raised against CFP10 with and without adjuvant were found to be 4.25 and 2.82, respectively ($p < 0.0001$). Similarly, the titers of ESAT6 + CFP10 with (4.41) and without (3.60) adjuvant are significantly ($p < 0.0001$) higher than CFP10 without adjuvant ($p < 0.0001$). When compared to CFP10 with adjuvant, CP-CFP10 showed increased antibody titer of 4.65 ($p < 0.05$). But the difference between ESAT6 + CFP10 with adjuvant, ESAT6-CP-CFP10 (4.28) and CP-CFP10 in having CFP10 specific titer was marginal and statistically insignificant. Discernable difference was observed between antibody titers of ESAT6 with (4.24) and without (2.82) adjuvant, whereas ESAT6-CP (4.30), ESAT6 + CFP10 with adjuvant (4.37) and ESAT6-CP-CFP10 (4.12) showed almost similar response to that of ESAT6 with adjuvant. Also, ESAT6 + CFP10 with adjuvant showed increased antibody titer compared to ESAT6 and ESAT6 + CFP10 without adjuvant (3.45). However, it is observed that there is significant difference in terms of antibody titers between CP-CFP10 and CFP10 with adjuvant, whereas the difference between ESAT6-CP and ESAT6 with adjuvant was observed to be insignificant. These results revealed that chimeric PVLPs are more immunogenic and it produced higher antibody response than CFP10 and ESAT6 immunized without adjuvant.

3.3 Quantification of IFN-γ release induced by chimeric PVLPs

Splenocytes isolated from mice group immunized with recombinant antigens were stimulated with either ESAT6 or CFP10 to analyze whether chimeric PVLPs can enhance the T cell response (Fig. 5). On stimulation with ESAT6, mice

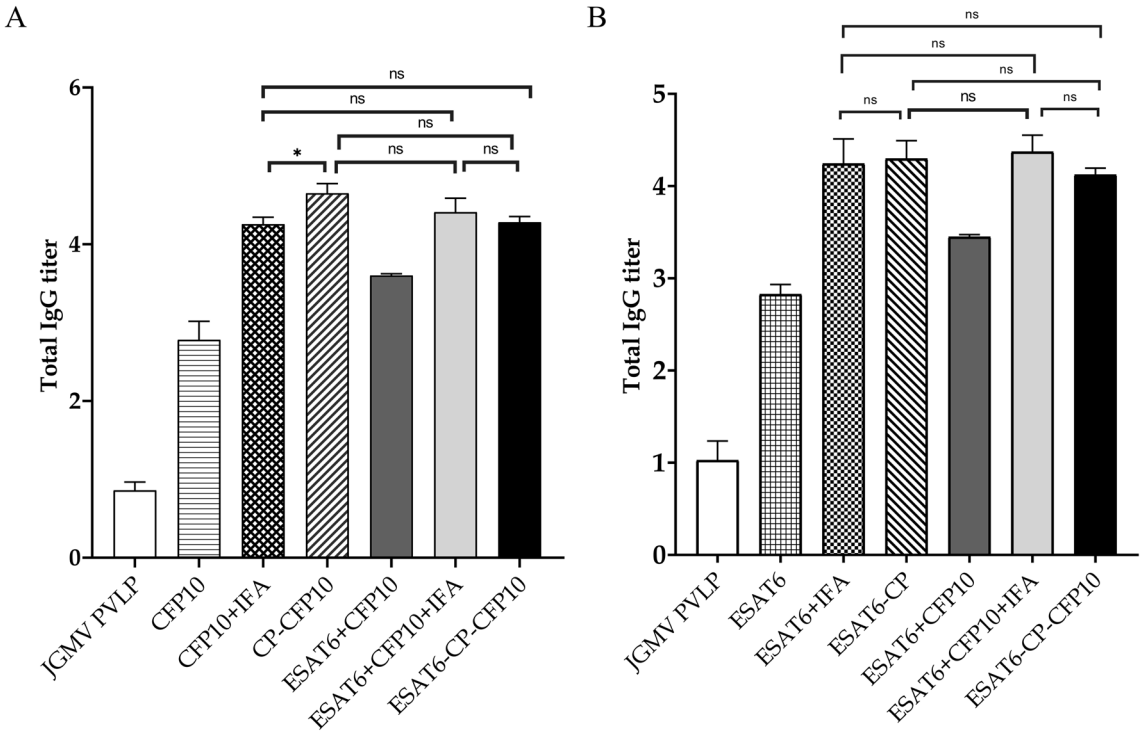

Fig. 4 Evaluation of mycobacterial antigen-specific immune response in mouse. CFP10 **A** and ESAT6 specific **B** antibody titers evaluated in the sera collected from mice immunized with recombinant antigens. Differences between groups were assessed by Tukey's multiple comparisons test; $*p < 0.05$. Data represent the mean ± SD ($n = 3$). JGMV: Johnson grass mosaic virus, PVLP: potyvirus-like particle, CP: coat protein, ns: not significant ($p > 0.05$)

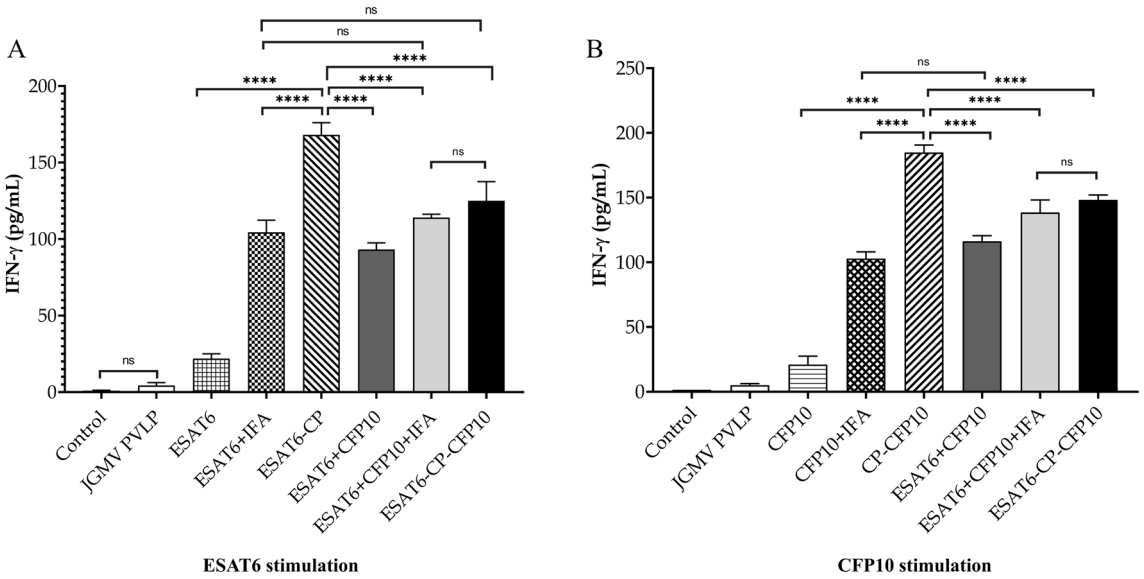

Fig. 5 Production of IFN-γ upon in vitro stimulation of splenocytes with ESAT6 **A** or CFP10 **B**. IFN-γ release by induced splenocytes were quantified by ELISA. IFN-γ response was calculated by subtracting basal level release of unstimulated splenocytes. Differences in IFN-γ response were assessed by Tukey's multiple comparisons test. $****p < 0.0001$. Data represent the mean ± SD ($n = 3$). JGMV: Johnson grass mosaic virus, PVLP: potyvirus-like particle, CP: coat protein, ns: not significant ($p > 0.05$)

groups immunized with PVLPs displaying ESAT6 exacerbated IFN-γ (168 pg/mL) release compared to other groups indicating cell-mediated response. Intriguingly, significant difference was not observed between mice groups immunized with ESAT6 with adjuvant (104 pg/mL), ESAT6-CP-CFP10 (125 pg/mL), ESAT6 + CFP10 with (114.1 pg/mL) and without adjuvant (93.1 pg/mL) for the amount of IFN-γ released. On the other hand, increased release of IFN-γ was observed with mice immunized with CP-CFP10 (184.75 pg/mL) on stimulation of splenocytes with CFP10 as ESAT6-CP. In contrast to ESAT6 stimulation groups, increased response was obtained from ESAT6 + CFP10 with adjuvant (138.4 pg/mL) and ESAT6-CP-CFP10 (148.2 pg/mL) immunized groups when compared to CFP10 with adjuvant (102.8 pg/mL). In addition, the amount of IFN-γ released by ESAT6-CP-CFP10 was not significantly different from that of ESAT6 + CFP10 with adjuvant. Increased release of IFN-γ was observed with mice immunized with CP-CFP10 compared to ESAT6-CP and ESAT6-CP-CFP10 proteins. These results reveal that chimeric PVLPs displaying either ESAT6 or CFP10 showed increased IFN-γ release response in comparison with PVLPs displaying both antigens together.

3.4 Induction of IFN-γ-secreting cells by recombinant antigens

Relative numbers of IFN-γ-expressing cells in splenocytes of mice immunized with different recombinant antigens were quantified by ELISPOT assay, and their numbers are shown as spot-forming units (Fig. 6). Splenocytes obtained from mice vaccinated with recombinant antigens were secreting increased amount of IFN-γ when compared to PBS and PVLP control groups. Both CP-CFP10 (253 SFU/10^5 splenocytes) and ESAT6-CP (238 SFU/10^5 splenocytes) immunized groups exhibited elevated response when compared to all other groups. Similarly, mice group immunized with both antigens (ESAT6 + CFP10) showed better response than single antigen groups (ESAT6 or CFP10). On parity with IFN-γ release assay, PVLPs displaying ESAT6 or CFP10 performed better in comparison with ESAT6-CP-CFP10 groups.

4 Discussion

An efficacious vaccine is required to control all disseminating forms of TB in children and adults. Although BCG is widely accepted prophylactic, subunit vaccines offer better protection against TB but the limitation is requirement of suitable adjuvants that enhance TB-specific protective response. Aluminum salts are the most commonly used adjuvant for humans in vaccine formulations but often cause adverse inflammatory side reactions [23]. Moreover, they exhibited poor cellular immune response with predominant Th2 response insufficient for ideal TB vaccine formulations as they require other supplementary adjuvants to stimulate required T cell response [24–26]. To circumvent problems

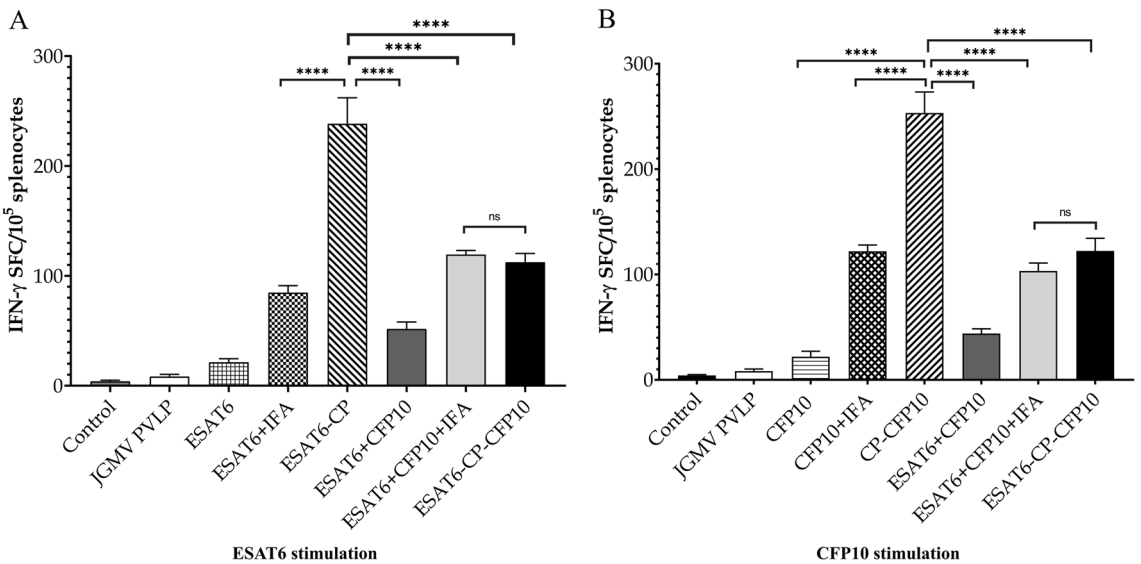

Fig. 6 Antigen-specific IFN-γ-secreting cells elicited by ESAT6 and CFP10. Splenocytes were stimulated with either ESAT-6 or CFP10 for 24 h and IFN-γ-secreting lymphocytes were then quantified by ELISPOT. The frequency of IFN-γ-secreting splenocytes following TB antigen ESAT-6-specific **A** and CFP10-specific **B** stimulation in vitro (SFC: spot-forming units). Differences in IFN-γ response were assessed by Tukey's multiple comparisons test. ****$p < 0.0001$; Data represent the mean ± SD (n = 3). TB: tuberculosis, JGMV: Johnson grass mosaic virus, PVLP: potyvirus-like particle, CP: coat protein, ns: not significant ($p > 0.05$)

associated with aluminum-based adjuvants, many alternatives like lipids, polysaccharides and microbial components have been investigated [27]. Likewise, plant- and animal virus-derived VLPs emerge as an effective immune stimulatory adjuvant expediting cell-mediated immune response [28]. Multivalent antigen display is possible with VLPs due to the presence of surface exposed N and C termini in each CP monomer. Repetitive copies of foreign antigen displayed on VLPs might trigger instant humoral response by cross linking receptors present on B cells for antibody production [29]. ESAT6 and CFP10, key secretory antigens, are suitable candidates for TB vaccine development as repertoire of immunodominant epitopes present on them are proven to induce sustained Mtb-specific immune response. Multiple epitopes spanning the entire amino acid sequence for HTL, CTL and B cell induction are present on ESAT6 and CFP10 and the predicted epitopes are possessing substantial antigenicity [30, 31]. As these antigens encompass multiple T cell and B cell epitopes throughout their amino acid sequences, presentation of entire antigens in their native conformation might elicit better immune response.

In the present study, fusion constructs were designed for the expression of hybrid JGMV CP displaying ESAT6 and CFP10. A short peptide linker was engineered between CP and Mtb antigens so that hybrid CP monomers could assemble into right conformation without causing steric hindrance to each other during the formation of PVLP [32]. Jagadish et al. [19] reported that fusion of shorter peptides and larger polypeptides to the maximum of 26 kDa with N or C termini of JGMV CP without hindering the assembly of PVLP. In our study, hybrid CP monomers expressed by the constructs pET25b:ESAT6-CP and pET25b:CP-CFP10 could assemble into PVLPs confirming that antigen containing 90–100 amino acids could be genetically fused with JGMV CP to produce hybrid PVLPs.

A versatile vaccine formulation is primarily required to induce both humoral and cell-mediated responses to achieve significant protection against TB. Animal immunization studies performed in our study revealed that PVLPs displaying ESAT6 and CFP10 without any adjuvant exhibited better humoral response than Mtb antigens with adjuvant substantiating the self-adjuvanting role of JGMV CP. Further to decipher the role of hybrid PVLPs in stimulating cell-mediated immune response, splenocyte from mice were isolated, stimulated with antigens, and their cytokine response was examined. As IFN-γ-producing T cells are important in eliciting immune response against TB [33], we examined the ability of PVLPs displaying TB antigens to induce IFN-γ production from splenocytes. Upon stimulation of splenocytes with ESAT6 or CFP10, both CP-CFP10 and ESAT6-CP containing PVLPs could induce upregulation of IFN-γ response when compared to antigen with adjuvant injected mice groups. Mice groups immunized with JGMV PVLP alone did not induce any remarkable cell-mediated response on its own but the TB antigens genetically fused to them can enhance the antigen-specific cell-mediated response. Interestingly, mice groups immunized with both antigens showed enhanced T cell response when compared to ESAT6 or CFP10 immunized groups which could be attributed to its synergistic effect as reported in earlier studies [10].

To further enhance the immune response, we developed a double fusion where CFP10 was fused to C terminus and ESAT6 at N terminus of CP to derive ESAT6-CP-CFP10 protein. PVLP formation was not observed for ESAT6-CP-CFP10 albeit the expression, purification and refolding protocols were identical to other fusion proteins generated in this study. Unlike the flexuous rod-shaped PVLPs of ESAT6-CP and CP-CFP10, molten globule-like structures were observed for ESAT6-CP-CFP10. This could be due to either formation of heterodimer complex formed between CFP10 and ESAT6 present on individual ESAT6-CP-CFP10 monomers [34] or internal perturbations generated within fusion partners possibly disrupting polymerization. Amino acid composition and structural configuration of the foreign protein reportedly have repercussion on assembly of PVLPs. Bamboo mosaic virus engineered with foreign proteins containing complete random coil structure coupled with predominant helical composition assembles into PVLPs but the blend of helix and turns in equal frequency abrogated PVLP formation [35]. Most of the earlier studies reported the fusion of complete antigens either at N/C termini or epitopes from antigenic proteins at N and/or C termini of surface exposed potyvirus CP [19, 35–38], and the current study explored the possibility of fusion of different antigens at both termini.

In spite of constrains in PVLP formation, immunization of ESAT6-CP-CFP10 antigen exhibited significant level of Mtb antigen-specific antibody response equivalent to that of ESAT6-CP and CP-CFP10 antigens. But double fusion-derived splenocytes stimulated with ESAT6 or CFP10 antigen failed to show elevated T cell response than ESAT6-CP and CP-CFP10 antigens. The inferior T cell response could be due to structural heterogeneity of refolded ESAT6-CP-CFP10 protein because of which potential Mtb epitopes might have been masked from accessing T cell receptors. To further explore JGMV PVLPs as multivalent antigen carrier and deploy it as an ideal vaccine scaffold for TB and other infectious diseases, the impediments encountered in the polymerization of ESAT6-CP-CFP10 monomers into PVLPs should be adequately addressed.

5 Conclusion

The characteristics of PVLPs to dissociate into monomeric CPs and reassociate into highly ordered particulate structure in vitro offer the possibility of fusing diverse antigens at its

N and C termini. Mtb antigen-carrying hybrid PVLPs developed in this study is capable of stimulating both B cell and T cell. Follow-up protection studies need to be performed in animal challenge models to test the efficacy of hybrid PVLPs in resisting Mtb infection would help in assessing the full potential of JGMV PVLPs as antigen delivery system.

Acknowledgements The authors thank Mepco Schlenk Engineering College, Sivakasi, for the support and facility to carry out this work. We thank DBT (Department of Biotechnology), IISc (Indian Institute of Science, Bangalore) partnership program—Phase II IISc and Tamilnadu Agricultural University, Coimbatore, for providing facility to carry out TEM analysis. We thank Mrs. M. Indiraleka, Assistant Professor, Mepco Schlenk Engineering College, Sivakasi, for assisting us in mice immunization.

Data availability Raw data for this manuscript is available upon reasonable request to the corresponding author.

Declarations

Conflict of interest The authors declare no conflict of interest.

Informed consent Neither ethical approval nor informed consent was required for this study.

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