Human immune recognition-based multicomponent subunit vaccines against tuberculosis

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ABSTRACT: The cell-mediated immune response, with its shift in favour of type-1 over type-2 T-helper cell immune response, is generally regarded as essential to protection against mycobacterial infections. The aim of this study was to evaluate the protective potential of two multicomponent subunit vaccines (MSV-1 and MSV-2) against tuberculosis (TB) based on human immune recognition.

MSV-1 consisted of five immunodominant antigens (TB10.4, early secretory antigenic target (ESAT)-6, culture filtrate protein (CFP)-8, CFP-10 and CFP-15) selected from a group of polypeptides, which induced a predominant T-cell response in immune human subjects, whereas MSV-2 consisted of antigens (CFP-11, CFP-21, CFP-22.5, *Mycobacterium tuberculosis* protein (MPT)-64 and CFP-31) selected from a group of polypeptides which induced a subdominant T-cell response along with the antibody response.

Both of these sets of polypeptides were extensively recognised in healthy individuals with significant interferon gamma release compared to the diseased population. In C57BL/6J mice, at the level of the lungs, the order of protective efficacy for the test vaccines was: bacille Calmette–Guérin (BCG) > MSV-2 > MSV-1. The protective efficacy of MSV-1 was found to be significantly less than that of MSV-2 and BCG at the level of spleen, whereas that of MSV-2 was comparable to that of BCG.

The results of this study indicate that high T-helper cell type 1 response-inducing polypeptides selected on the basis of human immune recognition do not necessarily impart protection during vaccination experiments.

KEYWORDS: Human immunity, multicomponent subunit vaccine, tuberculosis

A subunit vaccine consisting of key protective antigens of *Mycobacterium tuberculosis* could have advantages over the existing bacille Calmette–Guérin (BCG) vaccine. Since the early 1990s, efforts to develop a subunit vaccine against tuberculosis (TB) have focused on proteins released from the growing mycobacteria into the extracellular medium [1–3]. Many are unique to *M. tuberculosis*, and, to date, only a few have been evaluated for their immunological properties and protective potential in various animal models as subunit vaccine candidates [3–8]. Furthermore, the majority of the antigens used to date for vaccine purposes have been selected on the basis of immunoreactivity in animal models [3, 6, 7]. However, ideal vaccine antigens need to be selected on the basis of immune recognition by a large percentage of different ethnic human populations [9–11]. Moreover, the immunological parameters required for protective

efficacy of an antituberculous vaccine are still not clearly defined. Further, as a vaccine based on a single antigen cannot be consistently protective in a genetically diverse population, experimental vaccines based on the combination of protective antigens also need to be evaluated [8, 12].

The low molecular mass protein fractions isolated from the secretory proteome of <40 kDa of *M. tuberculosis* are known to be predominantly recognised by the peripheral blood mononuclear cells (PBMCs) of healthy TB contacts, a human model of protective immunity to TB [9, 11]. Therefore, in the present study, a group of immunodominant low molecular mass purified polypeptides predominantly recognised by T-lymphocytes and another group of immunodominant antigens recognised by both the T- and B-lymphocytes of immune subjects (TB contacts/memory immune) were evaluated for their recognition by different donor categories of

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human TB and healthy subjects. Two experimental multi-component subunit vaccines (MSVs) constituted using highly immunodominant polypeptides from both groups were evaluated for their protective efficacy against experimental TB in C57BL/6J mice using dimethyldioctadecylammonium bromide (DDA)–monophosphoryl lipid A (MPL) adjuvants.

MATERIALS AND METHODS

Bacterial culture

M. tuberculosis strain H37Rv, originally obtained from the National Collection of Type Cultures (London, UK) and maintained on Löwenstein–Jensen medium, was used in the present study.

Animals

Female 4–5-week-old C57BL/6J (H-2^b) mice weighing 15–20 g (National Centre for Laboratory Animal Sciences, Hyderabad, India) were used in the present study. Mice were housed in cages contained within a negative pressure-regulated animal isolator and were fed on a standard pellet diet and water *ad libitum*.

Study population

Newly diagnosed moderately advanced TB patients (13 males (mean $\pm$ SD aged 45 ± 10 yrs); five females (aged 14 ± 8 yrs)) who were admitted to the Nehru Hospital (Postgraduate Institute of Medical Education & Research, Chandigarh, India) and the Tuberculosis and Chest Diseases Hospital (Patiala, India) formed part of the study population. The diagnosis was based on history and chest radiography, and the extent of the disease was graded by chest radiography according to the criteria of the National Tuberculosis and Respiratory Disease Association (New York, NY, USA) [13]. All of the patients had a history of cough, fever and cachexia lasting >6 months and their chest radiographs were suggestive of TB. The active disease was further confirmed by sputum smear positivity for acid-fast bacilli using Ziehl–Neelsen staining. All patients demonstrated more than two bacteria in 10 fields in repeated smear tests. All of the patients were later found to respond to antituberculous therapy. Healthy medical and paramedical subjects (12 males (aged 34 ± 6 yrs); 11 females (29 ± 5 yrs)), who were in direct contact with TB patients or in contact with biological samples or cultures from these patients for >6 months, were considered to be hospital contacts and were drawn from the above-mentioned hospitals. All TB contacts and endemic controls had no previous history of TB. Active pulmonary TB was excluded from TB contacts and endemic controls by chest radiography and sputum smears for acid-fast bacilli. Moreover, all contacts and endemic controls remained healthy over a period of 3 yrs. The subclinical infection of TB contacts was confirmed by early secretory antigenic target (ESAT)-6-specific *in vitro* lymphoproliferation and tuberculin skin test positivity. Eighty-two per cent of TB contacts showed ESAT-6-specific lymphoproliferation (stimulation index (SI) >3) and all TB contacts were positive for Siebert purified protein derivative of tuberculin (PPD) with a median induration response of 20 mm and minimum induration readings of 15 mm in the Mantoux test. Recently admitted graduate students from different socio-economic strata (nine males (aged 25 ± 2 yrs); eight females (aged 24.5 ± 2 yrs)) served as endemic controls and were all healthy with no known contact with TB

patients. The median induration response of endemic controls was 13 mm, with 29% showing ESAT-6-specific *in vitro* lymphoproliferation and 24% exhibiting induration readings of >15 mm in the Mantoux test. All diseased and healthy subjects used in the present study were adults who had received childhood *M. bovis* BCG vaccination, but without any booster dose, and were also HIV-negative. The Institutional Ethics Committee of the Postgraduate Institute of Medical Education & Research approved the study, and blood samples were drawn with the prior consent of the study subjects.

Antigens

Total culture filtrate proteins (CFPs) of *M. tuberculosis* H37Rv (RvCFP) were prepared by growing *M. tuberculosis* in stationary pellicle culture in modified liquid Youman's medium for 4 weeks [14]. A total of 26 low molecular mass polypeptides identified as immunodominant antigenic targets from *M. tuberculosis* H37Rv culture filtrate and categorised into different groups on the basis of type of immune response induced in humans models of immunity to TB were used as test antigens. The polypeptides were purified using a strategy based on separation of complex mixtures of secretory proteins on the basis of charge using diethylaminoethyl-Sepharose CL-6B anion exchange column chromatography as a first step [15], followed by separation according to size *via* high-resolution preparative sodium dodecylsulphate-polyacrylamide gel electrophoresis and subsequent electroelution [10].

Isolation of PBMCs and lymphocyte proliferation assay

PBMCs were isolated from heparinised venous blood samples by density-gradient centrifugation using Ficoll-Hypaque. The cells were cultured at 1×10^5 cells $\cdot$ well⁻¹ in complete RPMI 1640 medium (Sigma, St Louis, MO, USA) [11]. Purified low molecular mass polypeptides ($2 \mu\text{g} \cdot \text{mL}^{-1}$) were used for *in vitro* stimulation. RvCFP and PPD ($2 \mu\text{g} \cdot \text{mL}^{-1}$) were used for comparison, whereas phytohaemagglutinin ($1 \mu\text{g} \cdot \text{mL}^{-1}$) was used as mitogen to check cell reactivity and viability.

Interferon- γ ELISA

Interferon (IFN)- γ ELISA was carried out to estimate the IFN- γ levels released in lymphocyte culture supernatants on day 5 in response to *in vitro* stimulation with antigens using a commercially available anti-human IFN- γ reagent set (OPT EIA™; Becton Dickinson and Company, Biosciences, Pharmingen, San Diego, CA, USA). The assay was performed according to the manufacturer's instructions. The detection limit of the assay was $2.35 \text{ pg} \cdot \text{mL}^{-1}$.

Cytotoxicity assay

The *in vitro* cytotoxic T-lymphocyte (CTL) response induced by mycobacterial antigens was measured by means of neutral red assay as described by PARISH and MULLBACHER [16] with some modifications. Monocytes obtained as adherent populations were cultured in 96-well flat-bottomed tissue culture plates in complete RPMI 1640 medium (1×10^4 cells $\cdot$ well⁻¹) to differentiate into macrophages (target cells), whereas total PBMCs (1×10^6 cells $\cdot \text{mL}^{-1}$) were cultured with $20 \mu\text{g} \cdot \text{mL}^{-1}$ RvCFP in 6-well plates in a humidified atmosphere of 5% carbon dioxide at 37°C for 7 days to obtain effector cells. On day 7, differentiated macrophages were gently washed in RPMI 1640 medium and cultured overnight with the optimal concentration of purified

mycobacterial antigens (2 µg purified polypeptides). On day 8, nonadherent effector cells from antigen-stimulated PBMC cultures were harvested and counted. Effector cells were dispensed in a 96-well plate containing macrophages pulsed with antigen at the optimal effector:target cell ratio of 20:1. Effector and target cells were co-cultured in RPMI 1640 medium containing 10% autologous serum for 16 h. At the end of the incubation period, the wells were washed and the remaining adherent cells incubated with 0.04% neutral red in PBS (pH 7.4) for 1 h, and finally washed in RPMI 1640/PBS. A solution of 0.1 M acetic acid in 50% ethanol was added to each well to release neutral red. Absorbance was read at 550 nm, and results were expressed as percentage lysis of target cells at the effector:target cell ratio used.

Immunisation and challenge of mice

Mice were immunised with two sets of cocktail preparations, each containing five immunodominant antigens selected on the basis of human immune recognition studies. Cocktail preparation (0.2 mL) was injected subcutaneously on the back, in three equal doses of 50 µg (containing 10 µg of each antigen) at biweekly intervals, using MPL and DDA (both Sigma) as adjuvants. Antigens were emulsified in DDA (250 µg·dose⁻¹), and MPL was used as coadjuvant (25 µg·dose⁻¹) as described previously [4]. At the time of the last dose of experimental subunit vaccination, a group of mice received a single dose of BCG (BCG Vaccine Laboratory, Guindy, Chennai, India; 1 × 10⁵ colony-forming units (cfu)) injected subcutaneously at the base of the tail. The mice were challenged 8 weeks after the first dose of experimental MSV preparation *via* the lateral tail vein with 1 × 10⁵ cfu *M. tuberculosis* H37Rv suspended in

0.1 mL PBS. Four weeks after the challenge, the animals were sacrificed and *M. tuberculosis* cfu enumerated in target organs [4].

Statistical methods

In the case of human recognition experiments, comparison between different groups of individuals was performed using the Mann–Whitney two-tailed test. For protection experiments, two-way comparison between the test and control group was performed using an unpaired t-test. Multiple comparisons between different groups were performed by means of ANOVA. The statistical analyses were considered significant at the level of *p* < 0.05.

RESULTS

Immune responses of healthy TB contacts and TB patients to mycobacterial antigens

Polypeptides (104 electrophoretic bands) were purified from the low-molecular-mass region (<40 kDa) of the secretory proteome of *M. tuberculosis*. The purified polypeptides were subjected to recognition testing by PBMCs from healthy TB contacts and healed TB patients (data not shown), a population considered as a model of protective immunity against TB [9–11]. The 28 polypeptides selected on the basis of initial screening in the immune population were used to constitute groups I (table 1) and II (table 2). All group-I and group-II antigens were initially subjected to reactivity testing with a panel of known monoclonal antibodies (mAbs) or polyclonal antibodies in order to identify previously defined CFPs of low molecular mass by ELISA. The antibodies CS-18 (directed against superoxide dismutase), α-MPT-53 (*M. tuberculosis*

TABLE 1 Characterisation of group-I purified polypeptides

Serial No.	Protein No.	mAb reactivity	H37Rv annotation	App M _r , kDa	Theor M _r [#] , kDa	pI	Identity
1	6	Nil	Unknown	4.5	Unknown	Unknown	Unknown
2	35	PV-2++	Unknown	4.0	Unknown	Unknown	ESAT-6 family member? ⁺
3	36	PV-2++++	Rv0288	4.5	10.391	4.50	TB10.4 ⁺
4	38	HYB 76-8++++	Rv3875	6.0	11.75	3.52	ESAT-6 ^{+,§}
5	39	HYB 76-8++	Unknown	7.0	Unknown	Unknown	ESAT-6 family member? ⁺
6	40	Nil	Rv0496	8.0	34.80	6.32	CFP-8 (conserved hypothetical protein) ^{*,§}
7	41	K8493++	Unknown	9.0	Unknown	Unknown	ESAT-6 family member? ⁺
8	42	K8493+++	Unknown	9.5	Unknown	Unknown	ESAT-6 family member? ⁺
9	43	K8493++++	Rv3874	10.0	10.79	4.31	CFP-10 ⁺
10	44	SA-12/IT-3++	Unknown	11.0	Unknown	Unknown	Unknown
11	47	α-MPT-53++	Rv0164	15.0	20.18	5.90	CFP-15 ^{*,§}
12	65	Nil	Unknown	5.5	Unknown	Unknown	Unknown
13	66	Nil	Unknown	6.0	Unknown	Unknown	Unknown
14	69	SA-12/IT-3+++	Unknown	9.0	Unknown	Unknown	Unknown
15	70	SA-12/IT3++++	Rv3418C	10.0	14.45	4.93	GroES (hsp10/MPT-57) ^{+,§}
16	76	α-MPT-53++	Rv2445C	14.5	14.47	5.30	CFP-14.5 (ndkA) ^{*,§}
17	77	Nil	Rv1827	16.0	17.21	4.30	CFP-16 ^{*,§}
18	79	Nil	Rv2185C; Rv1827	18.0	16.292; 17.21	4.80; 4.30	CFP-18 [*] (mixed sample) [§]

These polypeptides induced a predominant T-cell response in human subjects with protective immunity to tuberculosis (TB). mAb: monoclonal antibody; App: apparent; M_r: relative molecular mass; Theor: theoretical; pI: isoelectric point; Nil: no reactivity; ESAT: early secretory antigenic target; CFP: culture filtrate protein; GroES: 10-kDa heat shock protein (hsp10); MPT: *Mycobacterium tuberculosis* protein; ndk: nucleoside diphosphate kinase. ++: optical density (OD) 0.20–0.30; +++: OD 0.30–0.40; ++++: OD > 0.50. #: from [17]; *: novel T-cell antigen; +: by mAb/polyclonal antibody reactivity; §: by liquid chromatography–tandem mass spectrometry.

TABLE 2 Characterisation of group-II purified polypeptides

Serial No.	Protein No.	mAb reactivity	H37Rv annotation	App M _r , kDa	Theor M _r , [#] kDa	pI	Identity
1	1	K8483++	Rv0632C; Rv1626	22.5	24.35; 22.66	5.50; 5.00	CFP-22.5 [†] (mixed sample: enoyl-CoA-hydratase; component response regulator) ⁺
2	2	K8483+++++	Rv1984C	21.0–22.0	18.67	4.60	CFP-21 (cutinase precursor) [‡]
3	25	Nil	Unknown	19.0	Unknown	Unknown	Unknown
4	28	L24b4+++++	Rv1980C	24.0	26.15	4.83	MPT-64 ^{†,§,f}
5	31	IT-49+++++	Rv1886C	29.5	29.85	5.10	Ag85B (MPT-59; mycolyl transferase) ^{†,§,f}
6	32	IT-49+++++	Rv3804C	30.5	31.44	5.38	Ag85A (MPT-44; mycolyl transferase) ^{†,§,f}
7	33	Nil	Rv0831C	31.0	30.18	5.10	CFP-31 (hypothetical protein) ^{†,†}
8	37	PV-2; HYB 76-8++	Unknown	5.5	Unknown	Unknown	ESAT-6 family member? [‡]
9	45	IT-3/SA-12++	Rv3874; Rv3592; Rv3914	12.5	10.00; 11.00; 12.50	5.11 (Rv3914)	CFP-12.5 [†] (mixed sample: 10 and 11 kDa conserved hypothetical protein; 12.5 kDa thioredoxin/MPT-46) [†]
10	46	Nil	Rv2433C	13.5	11.00	10.93	CFP-11 (conserved hypothetical protein) ^{†,f}

These polypeptides induced both T- and D-cell responses in human subjects with protective immunity to tuberculosis. mAb: monoclonal antibody; App: apparent; M_r: relative molecular mass; Theor: theoretical; pI: isoelectric point; Nil: no reactivity; CFP: culture filtrate protein; CoA: coenzyme A; MPT: *Mycobacterium tuberculosis* protein; Ag: antigen; ESAT: early secretory antigenic target. ++: optical density (OD) 0.20–0.30; +++++: OD>0.50. #: from [17]; †: novel T-cell antigen; ‡: by liquid chromatography–tandem mass spectrometry; §: by mAb/polyclonal antibody reactivity; f: by N-terminal sequencing.

protein (MPT) 53), IT-3 (10-kDa heat shock protein (hsp 10)), IT-4 (16-kDa α -crystallin), IT-20 (14-kDa α -crystallin), IT-10 (20.5-kDa uncharacterised protein), IT-12 (19-kDa lipoprotein), IT-23 (phosphate transport subunit S), IT-44 (CFP-32), IT-49 (antigen (Ag) 85 complex), IT-52 (MPT-51), IT-59 (33-kDa uncharacterised protein), IT-69 (CFP-20), mc9246 (28-kDa uncharacterised protein), PV-2 (TB10.4), HYB 76-8 (ESAT-6), L24b4 (MPT-64), K8483 (CFP-21) and K8493 (CFP-10) were used as probes. Subsequently, selected immunodominant antigens were further characterised by either N-terminal sequencing or liquid chromatography–tandem mass spectrometry (tables 1 and 2). These immunodominant polypeptides were subjected to recognition testing by peripheral blood lymphocytes of various donor categories in the present study. Among all the donors, healthy contacts demonstrated high proliferative and IFN- γ responses to the mycobacterial antigens tested, whereas TB patients gave exceedingly low responses. Considering an SI of 3.0 as the positive cut-off value for determining percentage recognition, all of the polypeptides of the two groups (I and II) were found to be predominantly recognised by PBMCs from healthy TB contacts (fig. 1a and b). In group I, the maximum lymphocyte proliferation was observed in response to polypeptide 42 (median SI 16.87 (interquartile range (IQR) 5.46–25.19); 78.26% recognition), whereas, in group II, the maximum lymphocyte proliferation was observed in response to polypeptide 45 (median SI 10.04 (IQR 4.26–14.77); 78.26% recognition). The maximum IFN- γ response was induced by polypeptide 43 (median [IFN- γ] 178.0 pg·mL⁻¹ (IQR 44.00–295.00 pg·mL⁻¹)) in group I, whereas, in group II, polypeptide 2 (median [IFN- γ] 170.0 pg·mL⁻¹ (IQR 39.70–298.00 pg·mL⁻¹)) was found to induce high IFN- γ levels (fig. 1c and d).

When the PBMC responses of moderately advanced TB patients (fig. 2) were compared to those of healthy TB contacts (fig. 1), suppressed responses towards mycobacterial antigens

were observed. Analysis of lymphocyte proliferative responses obtained with group-I and group-II polypeptides indicated that only polypeptide Nos. 39 and 41 (median SI >3) were recognised by PBMCs of TB patients (fig. 2a and b). The lymphocyte proliferation response, as well as IFN- γ release, was maximum for polypeptide 39 (median SI 3.92 (IQR 2.18–5.54); 55.55% recognition; median IFN- γ 64.50 pg·mL⁻¹ (IQR 18.25–153.75 pg·mL⁻¹)). The lymphocyte proliferative responses of all group-I polypeptides except for polypeptide 6 were significantly high in healthy TB contacts compared to moderately advanced TB patients. Conversely, except for the Ag85A and B complex, all group-II polypeptides were found to induce significantly pronounced lymphocyte proliferative responses in healthy TB contacts (fig. 1a and b; fig. 2a and b). The IFN- γ responses of all group-I polypeptides except for polypeptides 6, 42, 66 and 70 were significantly high in healthy TB contacts, whereas all of the group-II polypeptides except for polypeptide 25 demonstrated significantly high IFN- γ responses in healthy TB contacts compared to moderately advanced TB patients (fig. 1c and d; fig. 2c and d).

According to prevailing concepts of TB vaccination, polypeptides predominantly recognised by T-lymphocytes of healthy TB contacts, but not by TB patients are implicated in protective immunity [9–11]. When group-I polypeptides were analysed for inclusion in an experimental MSV, polypeptides 35, 36, 38, 39, 40, 41, 42, 43, 47 and 65 were identified as the top 10 polypeptides predominantly recognised by T-lymphocytes of healthy TB contacts (when arbitrary cut-offs of median SI of >5.0 and median IFN- γ level of >50.0 pg·mL⁻¹ were employed). Polypeptide 42 induced marked lymphocyte proliferation and IFN- γ induction in healthy TB contacts. However, it was also found to be recognised in TB patients, and was considered unsuitable for inclusion in experimental vaccine preparation. Polypeptides 35, 39, 41 and 42 cross-reacted with mAbs/polyclonal antibodies used to identify

FIGURE 1. a, b) Lymphocyte proliferative and c, d) interferon (IFN)- γ responses of healthy tuberculosis contacts ($n=23$) after *in vitro* stimulation with: a, c) group-I, and b, d) group-II low molecular mass purified polypeptides. The median induration response in tuberculin skin tests carried out in the study subjects was 20 mm. Horizontal bars represent medians and each symbol represents one individual. The median counts per minute of cultures without antigen was 873. The median stimulation index (SI) in response to phytohaemagglutinin (PHA), total culture filtrate proteins of *Mycobacterium tuberculosis* H37Rv (RvCFP) and Siebert purified protein derivative of tuberculin (PPD) was 24.91 (interquartile range (IQR) 13.35–50.49), 8.56 (IQR 4.23–16.21) and 9.49 (IQR 5.49–15.66), respectively. The median IFN- γ response of cultures without antigen was 12.0 pg·mL⁻¹. The IFN- γ levels released into lymphocyte culture supernatants in response to *in vitro* stimulation with PHA, RvCFP and PPD were 3,010.00 (IQR 1,257.50–4,110.00), 610.00 (IQR 123.25–1,925.00) and 200.00 (IQR 42.80–1,580.00) pg·mL⁻¹, respectively.

known ESAT-6 family proteins. These polypeptides were considered to be isoforms or homologous heteroforms of various ESAT-6 family proteins and were excluded. Thus, only polypeptides 36 (TB10.4), 38 (ESAT-6), 40 (CFP-8), 43 (CFP-10) and 47 (CFP-15) were considered as five immunodominant polypeptides, predominantly recognised by healthy TB contacts on the basis of lymphoproliferative and IFN- γ responses ($p<0.5$ – <0.001) with respect to moderately advanced TB patients, suitable for inclusion in an MSV.

Conversely, when group-II polypeptides were analysed for utility in constituting experimental MSV, the order of recognition was 45>37>46>2>1>33>28, considering both median SI (>3.0) and IFN- γ responses (>50 pg·mL⁻¹) induced by PBMCs of healthy TB contacts. However, polypeptide 45 reacted with IT-3/SA-12 mAb and showed cross reactivity with hsp 10, and was excluded. Similarly, polypeptide 37, which cross-reacted with mAbs used to identify known ESAT-6 family proteins, was also not considered suitable for inclusion. Thus, polypeptides 46 (CFP-11), 2 (CFP-21), 1 (CFP-22.5), 33 (CFP-31) and 28 (MPT-64) from group II were considered suitable for development of an MSV. The characteristic feature of these

polypeptides was that they all demonstrated subdominant lymphocyte proliferative responses (SI 3.06–5.36) compared to those from group I (SI 5.98–12.29). Barring polypeptide 2, the IFN- γ responses of group-II polypeptides were also subdominant.

Recognition of polypeptides in a healthy endemic population

Group-I and group-II polypeptides were further analysed for recognition by T-lymphocytes of the general Indian population (fig 3). When the lymphoproliferative and IFN- γ responses of immunodominant group-I antigens (polypeptides 36, 38, 40, 43 and 47) in healthy TB contacts (Fig. 1a and c) were compared to those observed in healthy individuals from the endemic population (fig. 3a and c), the differences were found to be significant for all of these polypeptides ($p<0.05$ – $p<0.001$ versus healthy endemic controls). These results demonstrate predominant recognition of polypeptides 36, 38, 40, 43, 47 in healthy TB contacts compared to noncontacts. Subsequently, when the lymphoproliferative response of predominantly group-II antigens (*i.e.* 1, 2, 28, 33 and 46) in healthy TB contacts (fig. 1b) were compared with those observed in healthy individuals

FIGURE 2. a, b) Lymphocyte proliferative and c, d) interferon (IFN)- γ responses of moderately advanced tuberculosis (TB) patients ($n=18$) after *in vitro* stimulation with: a, c) group-I, and b, d) group-II low molecular mass purified polypeptides. Horizontal bars represent medians and each symbol represents one individual. The median counts per minute of cultures without antigen was 562. The median stimulation index (SI) in response to phytohaemagglutinin (PHA), total culture filtrate proteins of *Mycobacterium tuberculosis* H37Rv (RvCFP) and Siebert purified protein derivative of tuberculin (PPD) was 8.48 (interquartile range (IQR) 5.67–22.98), 3.22 (IQR 1.10–7.54) and 2.36 (IQR 1.42–3.64), respectively. The median IFN- γ response of cultures without antigen was 8.0 $\text{pg}\cdot\text{mL}^{-1}$. The IFN- γ levels released into lymphocyte culture supernatants in response to *in vitro* stimulation with PHA, RvCFP and PPD were 490.00 (IQR 160.00–2,510.00), 10.00 (IQR 10.00–46.00) and 26.00 (IQR 10.00–52.00) $\text{pg}\cdot\text{mL}^{-1}$, respectively. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$ versus healthy TB contacts.

from the endemic population (fig. 3b), the differences were nonsignificant for all of the above polypeptides. However, the IFN- γ responses of all of the above group-II polypeptides were significantly high in healthy TB contacts compared to noncontacts (fig. 1d and 3d).

Cytotoxic T-cell response induced by mycobacterial antigens

Antigens were also analysed for their ability to induce *in vitro* cytotoxic T-cell responses in healthy TB contacts (fig. 4). Amongst group I, only polypeptides 36 and 38 were recognised predominantly by CTLs (cytotoxicity $>30\%$). Conversely, amongst immunodominant group-II polypeptides (*i.e.* 1, 2, 28, 33 and 46), predominant cytotoxicity was observed when the macrophages were pulsed with polypeptides 2, 28, 33 and 46.

Protective efficacy of experimental subunit vaccines

TB10.4, ESAT-6, CFP-8, CFP-10 and CFP-15 were used to constitute experimental MSV-1, whereas CFP-21, CFP-22.5, MPT-64, CFP-31 and CFP-11 constituted MSV-2. Investigation of their protective efficacy, by determining the number of cfu

in the lungs and spleens of mice, revealed significantly lower $\log_{10}\text{cfu}$ in both of the experimental vaccine groups and the BCG-immunised group than in unvaccinated controls (table 3). MSV-2 imparted better protection than MSV-1, and the protection imparted was found to be significant at the level of spleen ($p<0.05$). The protective efficacy of MSV-2 was, however, found to be comparable to that of BCG. These results demonstrate the utility of MSV-2 as a prospective MSV and needs further evaluation.

DISCUSSION

According to the existing notion, mycobacterial antigens inducing dominant cellular immune responses should be considered to be important for inclusion in future subunit antituberculous vaccines. However, this concept has been challenged by some recent reports indicating higher protective efficacy of T-cell-subdominant antigens compared to immunodominant antigens [18]. Further, in order to develop an ideal subunit vaccine, selected candidate antigens should be able to be recognised by a genetically diverse population, representing a broad spectrum of major histocompatibility complex molecules [12]. Therefore, selected mycobacterial proteins, inducing

FIGURE 3. a, b) Lymphocyte proliferative and c, d) interferon (IFN)- γ responses of healthy tuberculosis (TB) noncontacts ($n=17$) after *in vitro* stimulation with: a, c) group-I, and b, d) group-II low molecular mass purified polypeptides. The median induration response in tuberculin skin tests carried out in the study subjects was 13 mm. Horizontal bars represent medians and each symbol represents one individual. The median counts per minute of cultures without antigen was 726. The median stimulation index (SI) in response to phytohaemagglutinin (PHA), total culture filtrate proteins of *Mycobacterium tuberculosis* H37Rv (RvCFP) and Siebert purified protein derivative of tuberculin (PPD) was 24.80 (interquartile range (IQR) 20.57–33.90), 4.22 (IQR 2.80–5.95) and 3.70 (IQR 2.03–5.60), respectively. The median IFN- γ response of cultures without antigen was 4.00 $\text{pg}\cdot\text{mL}^{-1}$. The IFN- γ levels released into lymphocyte culture supernatants in response to *in vitro* stimulation with PHA, RvCFP and PPD were 3,840.00 (IQR 3,800.00–4,085.00), 282.00 (IQR 144.00–660.00) and 79.50 (IQR 19.35–133.00) $\text{pg}\cdot\text{mL}^{-1}$, respectively. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$ versus healthy TB contacts.

T-cell-immunodominant (group-I) and -subdominant (group-II) responses, were evaluated for their recognition by different donor categories. The results of the present study clearly demonstrate that healthy TB contacts show a greatly enhanced response to the mycobacterial antigens tested, whereas TB patients exhibit depressed immune responses. These observations are in good agreement with the results of various previous studies [9–11]. Furthermore, observations of enhanced recognition of mycobacterial antigens in healthy contacts compared to noncontacts, as well as observations in healthy TB noncontacts (endemic controls) compared to TB patients, were also consistent with those reported earlier from endemic countries [19, 20]. These recognition studies in different donor categories helped in the selection of suitable candidates from group I and group II for constitution of experimental MSVs. MSV-1 included five polypeptides inducing dominant T-cell-mediated immune responses, whereas MSV-2 included five polypeptides having subdominant T-cell immune responses along with antibody response.

When the two experimental MSVs, *i.e.* MSV-1 and MSV-2, selected on the basis of human recognition were compared for

protective efficacy, MSV-2 imparted better protection at the level of both lungs and spleen. These findings indicate that no correlation exists between the magnitude of the *in vitro* T-helper cell (Th) type 1 response induced by an antigen during natural processing in healthy TB contacts and the extent of protection imparted by the same antigen after immunisation in a mouse model, comparable with the earlier observations of OLSEN *et al.* [18]. ESAT-6, CFP-10 and TB10.4, which constituted MSV-1, are predominantly expressed in virulent mycobacterial species and are considered virulence factors [21–23]. The possibility of these polypeptides acting as decoy antigens, diverting the immune response towards a biased Th1-type response, resulting in pathology, cannot be ruled out [24]. As many as 29% of healthy household contacts in whom *in vitro* ESAT-6-specific responses are induced were previously suspected to develop active TB [25]. Recently ESAT-6 and CFP-10 have been shown to form a 1:1 complex [26], leading to tissue destruction [27]. This might be one of the reasons why MSV-1 imparted less protection. Moreover, all of the polypeptides used in MSV-1 had a molecular mass of <15 kDa and were found to exhibit less inherent immunogenicity compared to the

FIGURE 4. Evaluation of the ability of: a) group-I, and b) group-II low molecular mass purified polypeptides to induce autologous macrophage cytotoxicity in healthy tuberculosis contacts ($n=8$). The median induration response in skin tests carried out in the study subjects was 23 mm. The median percentage cytotoxicity of cultures without antigen was 7.08. Cytotoxicity was determined using the formula: percentage lysis = $100((C-B)-(E-B))/(C-B)$, where C is the mean optical density (OD) of macrophages without effector cells, B is the mean OD of wells without cells and E is the mean OD of macrophages plus effector cells.

polypeptides used to constitute MSV-2, as determined in mice immunised with RvCFP-DDA-MPL (data not shown). Further, the increased protection imparted by MSV-2 might be due to an increased CTL response to the constitutive polypeptides (fig. 4). Another interesting feature of MSV-2 polypeptides is that all have previously been observed to produce a significant antibody response (data not shown). These results suggest a possible role of antibodies in protection against *M. tuberculosis*. It should be noted that Ag85B, previously defined as protective antigen, was among the group-II polypeptides in the present study and was found to augment both the cell-mediated and humoral immune response with Th1 and Th2 cytokine induction [28]. Moreover, the protective ESAT-6⁵¹⁻⁷¹ peptide [18] has previously been shown to contain both T- and B-cell epitopes [29]. Recently, antibodies have also been shown to exhibit protective effect against TB [30, 31]. These results strengthen the emerging view that the most effective immune response is one that combines both humoral and cellular components for intracellular pathogens such as *M. tuberculosis* [30].

TABLE 3 *Mycobacterium tuberculosis* H37Rv density 4 weeks after infection in the lungs and spleen of vaccinated[#] C57BL/6J mice

	Lung log ₁₀ cfu	Spleen log ₁₀ cfu
Unvaccinated	5.901 ± 0.217	5.744 ± 0.193
PBS-DDA/MPL	5.764 ± 0.223	5.628 ± 0.294
BCG	4.949 ± 0.341**	4.863 ± 0.166**
MSV-1	5.240 ± 0.174**	5.213 ± 0.360*
MSV-2	4.978 ± 0.384**	4.704 ± 0.229***†

Data are presented as mean ± SD ($n=5-8$ animals per group). cfu: colony-forming unit; DDA: dimethyldioctadecylammonium bromide; MPL: monophosphoryl lipid A; BCG: bacille Calmette-Guérin; MSV: multicomponent subunit vaccine. [#]: with BCG or experimental subunit vaccines based on *M. tuberculosis* H37Rv culture filtrate purified polypeptides. ANOVA was performed for comparison of all groups ($p<0.001$ for both target organs). *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$ versus unvaccinated controls; †: $p<0.05$ versus MSV-1-immunised mice (unpaired t-test).

In the present study, contrary to the existing notion, significant protection is imparted by the polypeptides inducing subdominant interferon- γ levels and substantial levels of antibody compared to that imparted by dominant interferon- γ -inducing polypeptides selected on the basis of human recognition during subclinical infection. The present observation, therefore, emphasises the need for careful selection of antigens for the constitution of experimental subunit vaccines against tuberculosis.

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