High Frequency of CD4+ T Cells Specific for the TB10.4 Protein Correlates with Protection against *Mycobacterium tuberculosis* Infection

Sandra Hervas-Stubbs,[1,2]† Laleh Majlessi,[1,2]† Marcela Simsova,[3] Jana Morova,[3] Marie-Jesus Rojas,[1,2] Clémence Nouzé,[1,2] Priscille Brodin,[4] Peter Sebo,[3] and Claude Leclerc[1,2]*

Institut Pasteur, Unité de Biologie des Régulations Immunitaires, Paris F-75015, France[1]; INSERM, E 352, Paris F-75015, France[2]; Laboratory of Molecular Biology of Bacterial Pathogens, Institute of Microbiology of the Academy of Sciences of the Czech Republic, Prague, Czech Republic[3]; and Institut Pasteur, Unité de Génétique Moléculaire Bactérienne, Paris F-75015, France[4]

TB10.4 is a newly identified antigen of *Mycobacterium tuberculosis* recognized by human and murine T cells upon mycobacterial infection. Here, we show that immunization with *Mycobacterium bovis* BCG induces a strong, genetically controlled, Th1 immune response against TB10.4 in mice. BALB/c and C57BL/6 strains behave as high and low responders to TB10.4 protein, respectively. The TB10.4:74–88 peptide was identified as an immunodominant CD4+ T-cell epitope for *H-2^d* mice. Since recent results, as well as the present study, have raised interest in TB10.4 as a subunit vaccine, we analyzed immune responses induced by this antigen delivered by a new vector, the adenylate cyclase (CyaA) of *Bordetella pertussis*. CyaA is able to target dendritic cells and to deliver CD4+ or CD8+ T-cell epitopes to the major histocompatibility complex class II/I molecule presentation pathways, triggering specific Th1 or cytotoxic T-lymphocyte (CTL) responses. Several CyaA harboring either the entire TB10.4 protein or various subfragments containing the TB10.4:20–28 CTL epitope were shown to induce TB10.4-specific Th1 CD4+ and CD8+ T-cell responses. However, none of the recombinant CyaA, injected in the absence of adjuvant, was able to induce protection against *M. tuberculosis* infection. In contrast, TB10.4 protein administered with a cocktail of strong adjuvants that triggered a strong Th1 CD4+ T-cell response induced significant protection against *M. tuberculosis* challenge. These results confirm the potential value of the TB10.4 protein as a candidate vaccine and show that the presence of high frequencies of CD4+ T cells specific to this strong immunogen correlates with protection against *M. tuberculosis* infection.

Tuberculosis (TB) remains a leading cause of mortality, with around 8 million new cases of TB worldwide and at least 1.5 million deaths per year. The current vaccine, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), displays highly variable efficiency (0 to 80%) in preventing adult pulmonary tuberculosis. Thus, a more effective vaccine is urgently needed for control of tuberculosis. Some new mycobacterial antigens (Ag) recently identified have been shown to induce a protective response against *Mycobacterium tuberculosis* in animal models, such as ESAT-6, Ag85A, Ag85B, Rv1196, and Rv0125 (12, 45) and are now entering clinical trials. TB10.4 is a recently identified Ag encoded by the Rv0288 gene located in the *esx* cluster 3 that appears to be essential for the virulence of *M. tuberculosis* (22). Rindi et al. (34) showed that Rv0288 expression is markedly down-regulated in the attenuated H37Ra strain as compared to that in the virulent H37Rv strain. Moreover, newly reported comprehensive identification of essential genes in *M. tuberculosis* includes the *esx* cluster 3 in the list of 600 genes that are essential for in vitro growth (35). The sequence of TB10.4 protein was shown to be highly conserved in the clinical isolates of *M. tuberculosis* (41). All of these data suggest that TB10.4 may play an important role in *M. tuberculosis* pathogenesis, although its function is still unknown.

TB10.4 protein is a target for antimycobacterial immune responses in humans (41, 42). Indeed, TB10.4 is strongly recognized by BCG-vaccinated individuals as well as by around 70% of TB patients and induces in these individuals high levels of gamma interferon (IFN-γ) (41). These observations suggest that TB10.4 represents an attractive candidate to develop a new vaccine against *M. tuberculosis*. However, subunit vaccines are usually poorly immunogenic and require coadministration of adjuvants for the generation of an effective immune response. In addition, Ag delivery by the most common adjuvant formulations induced mostly major histocompatibility complex (MHC) class II molecule-restricted immune responses. Thus, without effective delivery to the MHC class I pathway, subunit vaccines usually stimulate only CD4+ T-cell responses.

The adenylate cyclase (CyaA) of *B. pertussis* has the capacity to deliver its catalytic domain into the cytosol of eukaryotic cells (23). This ability has been exploited to deliver peptides and proteins up to 100 amino acids (aa) to both MHC class I and II pathways (25, 28, 33, 37). Recently, we have shown that CyaA binds specifically to the $\alpha_M\beta_2$ integrin (CD11b/CD18) (18) and thus targets inserted Ag to CD11b+ dendritic cells (DC) (18). In cattle with bovine TB, as well as in human TB patients, fusion of mycobacterial proteins to CyaA improved their in vitro recognition by specific T cells (44, 46). These results extend previous observations made in the mouse model (25, 37), where 100 times higher molar efficiency in T-cell stimulation has been described for Ag delivered as CyaA fusion protein. Importantly, the CyaA vector not only enhances in vitro presentation of Ag but also allows the in vivo induction

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TABLE 1. Recombinant detoxified CyaA used in this study

CyaA toxoid	TB10.4 insert		Flanking sequences of TB10.4 insert[a]	OVA insert[b] (position)
	Fragment	Position		
CyaA-TB10.4:1-96-OVA	1-96 (entire)	233	EATGG^{232}**VRVPRRRRKSL**-TB10.4-**SLRRRRYVH**^{233}LDRER	+ (108)
		336	KEYIG^{335}**VRVPRRRRKSL**-TB10.4-**SLRRRRYVH**^{336}QQRGE	
CyaA-TB10.4:15-33-OVA	15-33	233	EATGG^{232}**VR**-AGDMAGYAGTLQSLGAEIA-**VH**^{233}LDRER	+ (108)
		336	KEYIG^{335}**VR**-AGDMAGYAGTLQSLGAEIA-**VH**^{336}QQRGE	
CyaA-TB10.4:20-28-OVA	20-28	233	EATGG^{232}**VR**-GYAGTLQSL-**VH**^{233}LDRER	+ (108)
		336	KEYIG^{335}**VR**-GYAGTLQSL-**VH**^{336}QQRGE	
CyaA-TB10.4:18-30-OVA	18-30	233	EATGG^{232}**VR**-MAGYAGTLQSLGA-**VH**^{233}LDRER	+ (108)
		336	KEYIG^{335}**VR**-MAGYAGTLQSLGA-**VH**^{336}QQRGE	
CyaA-OVA:257-264				+ (224)
CyaA				

[a] The inserts in CyaA consisted of the entire polypeptide of TB10.4 (residues 1 to 96) or of the indicated TB10.4-derived epitopes, of which the full sequence is underlined. The introduced flanking residues are printed in boldface, and the superscript numbers indicate positions of CyaA residues between which the antigen inserts were introduced.

[b] The OVA insert is made up of peptide residues 257 to 264.

of strong cytotoxic T-lymphocyte (CTL) (10, 13, 14, 33) and Th1 CD4$^+$ (10, 25, 37) immune responses. The simultaneous induction of CTL and Th1 CD4$^+$ T-cell responses is an important aim for inducing protection against intracellular pathogens such as *M. tuberculosis*.

In the present study, we have characterized and compared the immunogenicities of the TB10.4 protein formulated in a cocktail of strong Th1-promoting adjuvants or delivered by CyaA in the absence of adjuvant. We show that both of these strategies induce strong TB10.4-specific T-cell responses. However, the frequency and polyclonality of the TB10.4-specific CD4$^+$ T-cell response were much higher upon vaccination with the adjuvanted TB10.4 protein than following delivery by CyaA. In contrast, TB10.4-specific CD8$^+$ T-cell responses were only induced by immunization with CyaA harboring the TB10.4 insert. When both approaches were evaluated for their capacity to elicit protective immunity against an aerosol challenge with *M. tuberculosis*, only immunization with the TB10.4 protein given in adjuvant induced a protective response, with a protection level comparable to that of BCG. These results confirm the potential value of the TB10.4 protein as a candidate vaccine and demonstrate the correlation between high frequency of anti-TB10.4 Th1 cells and protection against *M. tuberculosis*.

MATERIALS AND METHODS

Mice and cell lines. Specific-pathogen-free 6-week-old female BALB/c (*H-2^b*) or C57BL/6 (*H-2^d*) mice were obtained from Charles Rivers (Arbresle, France). Animals were kept in the Pasteur Institute animal facilities under specific-pathogen-free conditions. Experiments involving animals were conducted according to the institutional guidelines for animal care.

CTLL-2 and P815 cell lines were obtained from the American Type Culture collection (LGC Promochem, Molsheim, France). B3Z (21), a CD8$^+$ specific T-cell hybridoma specific for the K^b-restricted OVA:257–264 epitope, was a generous gift of N. Shastri (University of California) and was maintained in the presence of 1 mg/ml G418 and 400 μg/ml hygromycin B in complete RPMI medium (RPMI 1640 with Glutamax [Invitrogen, Life Technologies, Cergy-Pontoise, France]) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 × 10^{-5} mol/liter

2-mercaptoethanol). YB8 is a CD8$^+$ T-cell hybridoma specific for the K^d-restricted TB10.4:20–28 epitope (26).

Mycobacteria. *M. tuberculosis* (H37Rv) was grown in Middlebrook 7H9-A medium supplemented with bovine albumin, dextrose, and catalase (ADC) (Difco, Becton Dickinson Microbiology Systems, Sparks, MD) and containing 0.05% Tween 80, as previously described (6).

Peptides. TB10.4:20–28 (26), TB10.4:74–88, Ag85A:101–120 (20), ESAT-6: 1–20 (5), and OVA:257–264 (7) peptides were synthesized by Neosystem (Strasbourg, France).

Based on the published sequence of TB10.4 (42), a set of 82 overlapping 15-mer peptides covering the entire TB10.4 protein in a single-amino-acid step was synthesized on polyethylene pins according to standard PEPSCAN procedures (Mimotope, Claytin, Victoria, Australia). The scale of synthesis for the peptides was 1 to 3 mg per peptide. The peptides were supplied in the synthesized form and diluted in H$_2$O containing 0.5% dimethyl sulfoxide to reach a concentration of 1 to 3 mg/ml.

Recombinant proteins and CyaA toxoid construction. The constructed recombinant CyaA toxoids, carrying TB10.4 protein/epitopes inserted in the catalytic domain at position 233 or 336, are listed in Table 1. The plasmid constructs were generated by insertion of appropriate synthetic oligonucleotides and PCR products encoding the given antigen, which were introduced into unique BsrGI sites located between codons 232 and 233 or 335 and 336 of the CyaA open reading frame carried on pT7CACT1-BsrGI (16). All inserts were systematically controlled by DNA sequencing for the absence of undesired mutations.

To construct CyaA carrying the entire TB10.4 antigen, a synthetic HindIII adaptor was first introduced into the BsrGI sites of pT7CACT1-BsrGI plasmids, introducing also a sequence encoding eight positively arginine residues (VPRR RRKSL/SLRRRRY), to compensate for the negatively charged residues present in the TB10.4 antigen (Table 1).

The oligonucleotides encoding inserted epitopes were designed (i) to introduce a restriction site for rapid identification of the insertion mutants, (ii) to stop CyaA synthesis if inserted in the inverted orientation, and (iii) to destroy the original insertion site on ligation. To allow monitoring of the delivery of the AC domain with the inserted Ag into MHC class I pathway, CyaA constructs were further tagged by insertion of an OVA:257-264 CD8$^+$ T-cell epitope at position 108 or 224 of the AC domain (Table 1). All generated CyaA fusion proteins were genetically detoxified by ablating their catalytic adenylate cyclase activity by placing dipeptide inserts disrupting the ATP-binding site between residues 188 and 189, as described previously (14). The genetically detoxified control CyaA and the recombinant CyaA constructs carrying the inserted Ag were produced from plasmids derived from pT7CACT1-BsrGI (16) in *Escherichia coli* XL1-Blue (Stratagene); purified from inclusion bodies in a mixture of 8 M urea, 50 mM Tris-Cl, pH 8, 2 mM EDTA; and characterized as previously described (31).

The free control Ag, ESAT-6 and TB10.4, were produced in *E. coli* BL21(λDE3) from the pET28 expression vector. A double-stranded DNA adap-

tor carrying BsrGI site (ad-BsrGI-a, 5′-CATGACTGTACAAC; and ad-BsrGI-b, 5′-TCGAGTTGTACAGT) was inserted as an NcoI and XhoI fragment into pET28b expression vector (Novagene, Darmstadt, Germany) to allow subsequent insertion of the PCR fragment encoding the entire open reading frame of ESAT-6 and a synthetic adaptor sequence introducing a unique restriction site for HindIII for TB10.4 gene incorporation, respectively. The absence of undesired mutations in all cloned sequences was verified by DNA sequencing. Bacteria transformed with appropriate plasmids were grown at 37°C in Luria-Bertani medium supplemented with 60 μg/ml of kanamycin. The proteins were produced by induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and purified from inclusion bodies by single-step metal affinity chromatography on TALON matrix (Clontech Laboratories, Inc. Mountain View, CA), which was equilibrated with a mixture of 8 M urea, 300 mM NaCl, and 50 mM Tris-Cl, pH 8. The proteins were eluted with a mixture of 8 M urea, 300 mM NaCl, 50 mM Tris-Cl, pH 8, and 150 mM imidazole. The purified proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting with antihistidine monoclonal antibody (MAb; Sigma, Saint Louis, Mo.).

Ag presentation assays. Bone-marrow (BM)-derived DC (BM-DC) were generated in complete RPMI medium supplemented with 2 ng/ml of granulocyte-macrophage colony-stimulating factor. The nonadherent and semiadherent cells were recovered at day 7 or 8 by flushing the plates with EDTA (5 mM). These cells usually contained 70% CD11c$^+$ CD11b$^+$ cells. To study OVA:257–264 presentation, 10^5/well BM-DC from C57BL/6 mice were preincubated for 4 h with various concentrations of peptides or recombinant proteins. After washing, BM-DC were cocultured with 10^5/well B3Z cells for 18 h. For TB10.4:20–28 presentation, 10^5/well BM-DC from BALB/c mice and 10^5/well YB8 cells were cocultured during 18 h with various concentrations of Ag. Activation of B3Z or YB8 T-cell hybridomas was determined by measuring the amount of interleukin-2 (IL-2) in the supernatant by a standard CTLL-2 bioassay.

Mouse immunization. Mice received a single subcutaneous (s.c.) injection of either BCG (10^6 CFU) or TB10.4 protein (10 μg) in adjuvant or adjuvant alone. The adjuvant used was a cocktail composed of dimethyl dioctadecyl ammonium bromide (DDA) (250 μg), monophosphoryl lipid A (MPL) (25 μg), and trehalose dicorynomycolate (TDM) (25 μg). This cocktail will be referred to as DMT. The DDA and the MPL-TDM emulsion were purchased from Sigma-Aldrich (Saint Louis, Mo.). To prepare the adjuvant, a solution of 10 mg/ml of DDA in distilled H$_2$O was heated at 80°C for 30 min and vortexed every 5 min to obtain a fine emulsion. The lyophilized MPL-TDM mixture was reconstituted in 1 ml of phosphate-buffered saline (PBS) to reach a concentration of 0.5 mg/ml of each compound. Immediately before use, the room temperature-cooled DDA and the MPL-TDM emulsions were mixed with the appropriate amount of TB10.4 Ag. For immunization with recombinant CyaA, mice received two intraperitoneal (i.p.) injections (at days 0 and 15) of recombinant CyaA (50 μg) in alum.

T-cell assays. Twenty-one days after priming, splenocytes of immunized mice were removed and analyzed by in vitro assays. In some assays, CD4$^+$ or CD8$^+$ T cells were depleted by negative selection using an Automacs (Miltenyi Biotech). To measure T-cell proliferation, splenocytes were cultured (10^6/well) in 96-well flat-bottom plates in synthetic HL-1 medium (BioWhittaker, Walkersville, MD) supplemented with 2 mM Glutamax, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5×10^{-5} mol/liter 2-mercaptoethanol (Invitrogen, Life Technologies, Cergy-Pontoise, France) in the presence of various concentrations of Ag. Cultures were pulsed 72 h later with 1 μCi/well of [methyl-^{3}H]thymidine (ICN, Orsay, France) for 18 h, and the incorporated cpm were counted in a Wallac Microbeta counter (Perkin-Elmer, Cortaboeuf Cedex, France). To assay IL-2, IL-4, IL-5, and IFN-γ production, cells were cultured (10^6/well) in RPMI complete medium. The levels of IL-2 were determined in culture supernatants, after 24 h of incubation, by a CTLL-2 bioassay. Amounts of IL-4, IL-5, and IFN-γ were quantified in culture supernatants, after 72 h of incubation, by standard sandwich enzyme-linked immunosorbent assay (ELISA) using purified anti-IL-4, anti-IL-5, or anti-IFN-γ MAbs (BVD4-1D11, TRFK5, and R4-6A2 clones, respectively; BD Pharmingen) for the capture and corresponding biotinylated MAbs (BVD6-24G2, TRFK4, and XMG1.2 clones; BD Pharmingen) for the detection. In some experiments, the assay was standardized with recombinant murine cytokines (BD Pharmingen). Ag-specific cytokine production was calculated after background subtraction.

The IFN-γ enzyme-linked immunospot (ELISPOT) assay was performed by incubation of serial dilutions of splenocytes on anti-IFN-γ MAb (R4-6A2)-coated 96-well Multiscreen filtration plates (Millipore, Saint-Quentin-en-Yvelines, France). Splenocytes from immunized mice were added to wells previously filled with gamma-irradiated (3,000 rads) syngeneic naive splenocytes (5×10^5/well), either with or without the Ag. Cells were then incubated for 40 h at 37°C, and plates were washed extensively with distilled H$_2$O and PBS containing 0.025% Tween 20. Biotinylated anti-IFN-γ MAb (XMG1.2) and streptavidin-

alkaline phosphatase (BD Pharmingen) were used to detect the spots. Spots were revealed by the addition of the 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium (Sigma-Aldrich). Frequency of IFN-γ-producing cells was determined by counting the number of spots per well with a computer-assisted ELISPOT image analyzer (Bioreader-3000 Pro). The results were expressed as the number of spot-forming cells (SFC) per million splenocytes.

For the in vitro cytotoxicity assay, splenocytes (5×10^7) from immunized mice were stimulated in vitro for 5 days with TB10.4:20–28 peptide (10 μg/ml) in RPMI complete medium. The cytotoxic activity was determined in a 4-h in vitro ^{51}Cr release assay using P815 (H-2^d) tumor cells loaded with 50 μM of TB10.4: 20–28 peptide. ^{51}Cr release was determined by use of a Microbeta Trilux liquid scintillation counter (Wallac, Turku, Finland). The percentage of specific lysis was calculated as 100 × [(experimental release − spontaneous release)/(maximal release − spontaneous release)]. Maximum release was evaluated by adding 10% Triton X-100 to P815 cells, whereas spontaneous release was determined by incubating P815 in medium. The percentage of specific lysis was calculated after deduction of the nonspecific lysis (obtained for unloaded P815) from the total lysis of TB10.4:20-28-loaded P815 cells at multiple effector-to-target cell (E:T) ratios. Lysis of unloaded P815 usually ranges from 0 to 15%.

Protection assays against infection with *M. tuberculosis*. At the time points indicated, unvaccinated or immunized mice were challenged via the aerosol route using a home-made nebulizer. Two milliliters of a suspension containing 5×10^6 CFU/ml was aerosolized to obtain an inhaled dose of ≈100 ± 10 CFU/mouse. Infected mice were placed in isolator in P3 high-level-security animal house facilities. One month postchallenge, lungs and spleen of mice were homogenized by use of an MM300 organ homogenizer (QIAGEN, Courtaboeuf, France) and 2.5-mm-diameter glass beads. Serial fivefold dilutions of homogenates were cultured on 7H11 agar supplemented with ADC (Difco, Becton Dickinson, Sparks, MD). CFU were counted after 15 to 18 days of incubation at 37°C.

RESULTS

BCG induces a strong and genetically controlled Th1 immune response against the TB10.4 protein. In order to study the immunogenicity of the TB10.4 protein in the context of BCG vaccination, mice from two different strains, BALB/c (H-2^d) and C57BL/6 (H-2^b), were immunized s.c. with BCG. At day 21 postimmunization, the frequency of TB10.4-specific IFN-γ-producing splenocytes was determined by ELISPOT assay. As shown in Fig. 1A, the response to the TB10.4 protein induced by BCG immunization was strongly genetically controlled. Indeed, a high number of TB10.4-specific IFN-γ-producing cells was found in BALB/c mice, whereas no response was detected in C57BL/6 mice. This genetic difference in the response against the TB10.4 protein was further confirmed by mapping the immunodominant TB10.4 T-cell epitopes. Splenocytes from BCG-primed or naïve mice were stimulated in vitro with overlapping 15-mer peptides covering the entire TB10.4 protein, and IFN-γ levels in culture supernatant were determined by ELISA. As shown in Fig. 1B, at least three highly immunodominant regions containing H-2^d-restricted TB10.4 T-cell epitopes were identified in BALB/c mice, expanding from amino acids 16 to 40, 44 to 65, and 63 to 96. Very low IFN-γ levels were detected when splenocytes from BCG-primed C57BL/6 mice were stimulated either by the TB10.4 protein or by overlapping 15-mer peptides. Only peptides belonging to the 1-9 region, were weakly recognized, suggesting that this region may contain TB10.4 T-cell epitopes for the H-2^b haplotype. These data identify BALB/c and C57BL/6 strains as high and low responders to the TB10.4 protein, respectively.

The TB10.4:74–88 peptide was chosen for a detailed study of the TB10.4-specific T-cell response induced in BALB/c mice. This peptide was selected because it is central in the 63–96 region, which gives rise to the most intense IFN-γ response in
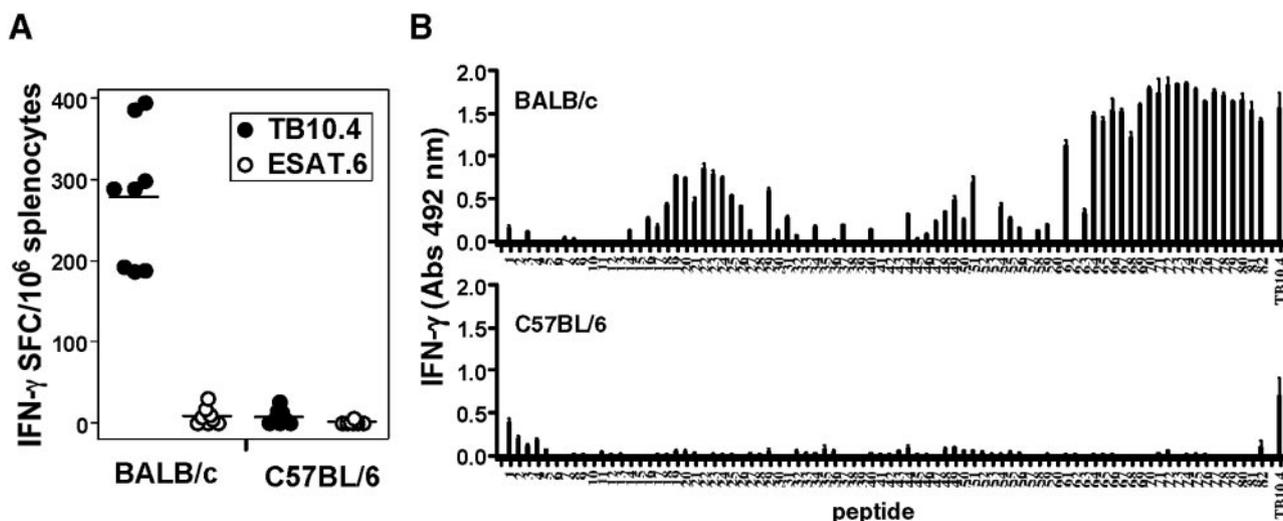
FIG. 1. BCG immunization induces a genetically controlled T-cell response against TB10.4 protein. BALB/c and C57BL/6 mice were immunized by a single s.c. BCG injection (10^6 CFU), and 21 days later, T-cell responses were analyzed using total splenocytes. (A) Frequency of TB10.4-specific IFN-γ-producing cells. Splenocytes from BCG-primed mice were stimulated with TB10.4 or ESAT-6 protein (100 nM), and production by IFN-γ-producing cells was measured by ELISPOT assay. (B) Mapping of the TB10.4 dominant T-cell determinants in BALB/c and C57BL/6 mice. Splenocytes from BCG-primed or naïve mice were stimulated in vitro with the TB10.4 protein (100 nM) or with overlapping 15-mer peptides (1 to 3 μg/ml) covering the entire TB10.4 protein. Peptides are referred to in terms of the NH₂-terminal amino acid of each peptide. IFN-γ levels were determined in culture supernatant by ELISA and are shown as the A_{492}. The absorbance (Abs) values depicted correspond to the difference between the absorbance corresponding to supernatants from naïve and primed mice. The absorbance corresponding to supernatants from naïve mice never exceeded an optical density of 0.1.

BCG-immunized mice, and, as shown later in this study, it is the best TB10.4-derived peptide recognized upon immunization with TB10.4 delivered by CyaA. To determine if this epitope is a CD4⁺ or a CD8⁺ T-cell epitope, splenocytes from BCG-vaccinated BALB/c mice were depleted in vitro for CD4⁺ or CD8⁺ T cells and stimulated with the TB10.4:74–88 peptide. As shown in Fig. 2A, IFN-γ production in response to the TB10.4:74–88 peptide was abolished after CD4⁺ but not after CD8⁺ T-cell depletion. Moreover, CD4⁺ T-cell-depleted splenocytes from BCG-immunized BALB/c mice did not release IFN-γ in response to the other TB10.4 peptides tested in Fig. 1B, showing that the identified regions only contain CD4⁺ T-cell epitopes (data not shown).

Ag85A has been shown to be a highly immunodominant Ag in the context of M. tuberculosis infection and BCG vaccination (20). Nevertheless, as shown in Fig. 2B, upon BCG vaccination of BALB/c mice, the frequency of IFN-γ-producing cells specific for TB10.4 was markedly higher than for the strongly immunogenic Ag85A, as assessed by using equimolar amounts of the TB10.4:74–88 and Ag85A:101–120 peptides, the latter containing the immunodominant H-2ᵈ-restricted CD4⁺ T-cell epitope from Ag85A (20). It is noteworthy that Th1 responses were highly reproducibly stronger to TB10.4 than to Ag85A from days 15 to 30 post-BCG immunization (data not shown). To further characterize the T-helper response induced, splenocytes from BCG-primed or naïve BALB/c mice were in vitro stimulated with various concentrations of the TB10.4:74–88 peptide or the negative control ESAT-6:1–20 peptide. As shown in Fig. 2C, a strong proliferative response specific for the TB10.4:74–88 epitope was detected in BCG-primed mice. This strong proliferative response was accompanied by a high production of Th-1 cytokines (IL-2 and IFN-γ) and by low

(IL-5) or undetectable (IL-4) levels of Th2 cytokines (Fig. 2C) (data not shown). Together, these data show that upon immunization with BCG, a Th1-polarized T-helper response specific for the TB10.4 protein is induced in the high-TB10.4-responder BALB/c strain.

Efficient in vitro MHC class I delivery of the TB10.4:20–28 CD8⁺ T-cell epitope by CyaAs harboring different TB10.4 fragments. We have previously shown that CyaA from B. pertussis is a powerful vaccine vector, able to carry in its AC domain foreign polypeptides of up to several hundred amino acid residues in length and able to facilitate the delivery of the inserted CD8⁺ or CD4⁺ T-cell epitopes into the MHC class I or II presentation pathways (10, 25, 28, 33, 37). Previously, we have identified a new CD8⁺ T-cell epitope, referred to as TB10.4:20–28 (H-2Kᵈ), that is shared by TB10.4 and TB10.3 proteins (26). CD8⁺ T cells from M. tuberculosis-infected mice efficiently lysed target cells pulsed with the TB10.4:20–28 peptide, indicating that this epitope is naturally processed and presented by MHC class I molecules in M. tuberculosis-infected mice (26). It is noteworthy that in vitro stimulation of splenocytes from such mice with this MHC class I TB10.3/4 epitope does not lead to production of IFN-γ. To study the ability of the CyaA vector to promote induction of TB10.4-specific T-cell responses, we constructed different detoxified CyaA harboring the entire sequence of the TB10.4 protein or short sequences containing the TB10.4:20–28 epitope, with or without its natural flanking regions. Insertion sites at residue 233 or 336 of CyaA were used in parallel, in order to compare their potential for delivery of the inserted TB10.4 polypeptides. All CyaA further harbored the reporter OVA:257–264 CD8⁺ T-cell epitope (H-2Kᵇ) inserted at position 108. As a negative control, we used a detoxified CyaA without any inserted epitope (re-
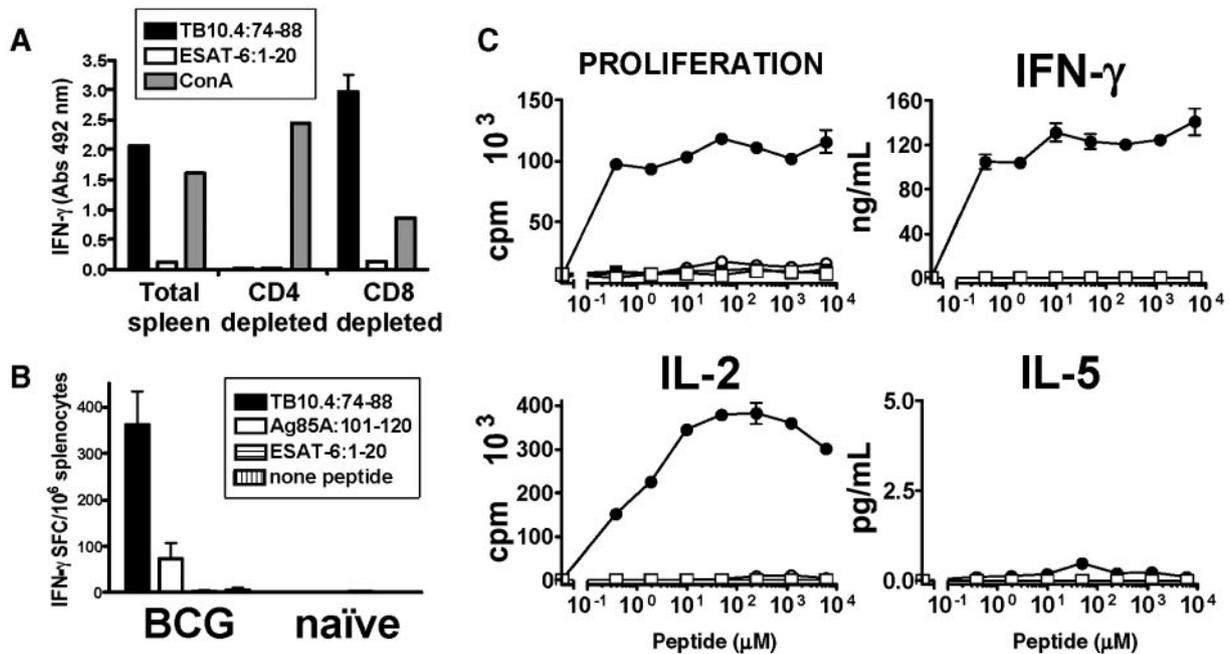
FIG. 2. Immunization with BCG induces a strong TB10.4-specific Th1 immune response in BALB/c mice. BALB/c mice were immunized by a single s.c. BCG injection (10^6 CFU), and 21 days later, T-cell responses were analyzed using total splenocytes. (A) Recognition of the TB10.4:74–88 peptide by $CD4^+$ T cells. Splenocytes were depleted in vitro of $CD4^+$ or $CD8^+$ T cells by negative selection. Total, $CD4^+$- or $CD8^+$-depleted splenocytes were stimulated in vitro with TB10.4:74–88 or the negative control ESAT-6:1–20 peptide (1 µg/ml) or with concanavalin A (ConA; 2 µg/ml), and IFN-γ levels in culture supernatant were determined by ELISA. (B) Frequency of TB10.4 and Ag85A T-cell epitope-specific IFN-γ-producing cells determined by ELISPOT assay. Splenocytes from BCG-primed or naïve BALB/c mice were stimulated with TB10.4:74–88, Ag85A:101–120, or ESAT-6:1–20 peptide (6 µM). (C) TB10.4-specific T-helper response induced after immunization with BCG. Splenocytes from naïve (squares) or BCG-primed (circles) BALB/c mice were stimulated with TB10.4:74-88 (solid symbols) or the control ESAT-6:1-20 peptide (open symbols). Proliferation was determined by [^{3}H]thymidine incorporation. Cytokine levels were determined in culture supernatant by CTLL-2 assay (IL-2) or by ELISA (IFN-γ and IL-5). Results are representative of two to four experiments (three mice per group).

ferred to as control CyaA). In some experiments, we also used a detoxified CyaA harboring the OVA:257–264 epitope inserted at position 224 (CyaA-OVA). These different recombinant CyaA constructs are listed in Table 1.

We first used an in vitro presentation assay to examine whether the insertion of the TB10.4 sequences would affect the delivery of the reporter OVA:257–264 epitope. Various recombinant CyaA were incubated with BM-DC from C57BL/6 mice and B3Z T-cell hybridoma, specific for the OVA:257–264 epitope. As shown in Fig. 3A, the OVA:257–264 epitope was delivered with comparable efficiency by the CyaA harboring only this epitope at position 224, as well as by the various CyaA constructs harboring this epitope at position 108 and the TB10.4:20–28, TB10.4:15–33 or TB10.4:18–30 sequences at site 233 or 336. Similar efficacy in the delivery of the OVA: 257–264 epitope was found regardless of the position within CyaA of these short TB10.4 inserts (site 233 or 336). In contrast, the delivery of the OVA:257–264 epitope was strongly affected when the entire TB10.4 protein (TB10.4:1–96) was inserted into CyaA, leading to a total lack of B3Z stimulation when this protein was inserted at position 336 (Fig. 3B).

To study the presentation of the TB10.4:20–28 $CD8^+$ T-cell epitope (H-$2K^d$), a similar in vitro presentation assay was performed. Recombinant CyaA were incubated with BM-DCs from BALB/c mice together with YB8 T-cell hybridoma specific for the TB10.4:20–28 epitope (26). As shown in Fig. 3D to F, most of the CyaA harboring the TB10.4 sequences were able

to stimulate efficiently the YB8 hybridoma, with a comparable or higher efficacy than the free TB10.4:20–28 peptide (Fig. 3F). Except for CyaA-TB10.4:20-28-OVA, insertion at site 233 allowed a more efficient Ag presentation than insertion at position 336. Interestingly, CyaA bearing the entire TB10.4 protein (Fig. 3E) delivered the TB10.4:20–28 $CD8^+$ T-cell epitope as efficiently as CyaA harboring the minimal or the flanked epitope (Fig. 3D). However, as already noted above, despite their efficiency in delivery of the TB10.4:20–28 epitope, the CyaA construct carrying the TB10.4 protein essentially failed to deliver the OVA:257–264 epitope. This reduced delivery of the OVA epitope might be due to competition for binding to an MHC class I molecule between potential H-2^b $CD8^+$ T-cell epitopes from the TB10.4 protein and the OVA:257–264 epitope, as well as it might be due to less efficient processing of the AC region surrounding the position 108 resulting from structural changes due to the insertion of the entire TB10.4 protein at site either 233 or 336. As shown in Fig. 3E, the presentation of the TB10.4:20–28 epitope was strictly dependent on the targeting of BM-DC by the CyaA vector, since the recombinant TB10.4 protein alone was totally unable to stimulate YB8 cells. Parallel in vitro stimulation assays performed with control CyaA (Fig. 3A, B, D, and E) or with the unrelated Ag85A:101–120 peptide (Fig. 3C and F) did not result in any hybridoma activation, showing the specificity of the Ag presentation. Based on this in vitro assay, the CyaA-TB10.4:15-33-OVA (TB10.4 insert at position 233) was chosen for further in
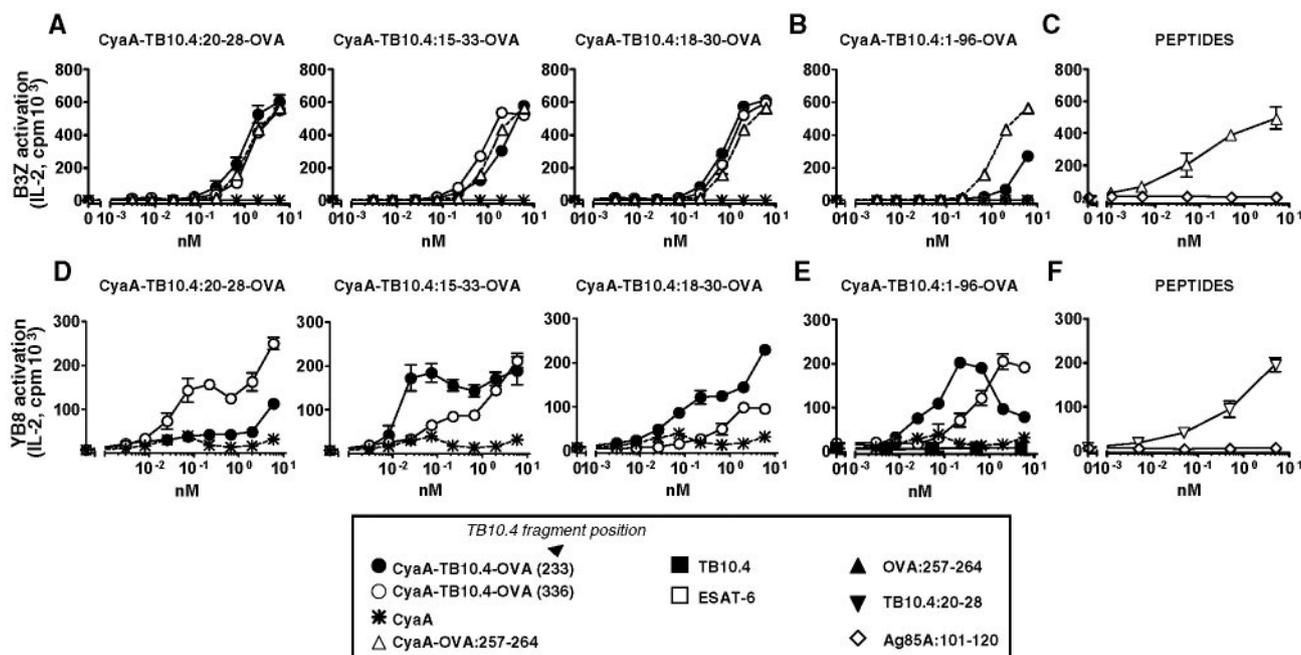
FIG. 3. Efficient in vitro MHC class I delivery of the TB10.4:20–28 CD8^{+} T-cell epitope by CyaA harboring different TB10.4 fragments. Various CyaA harboring different TB10.4 fragments and the reporter OVA:257–264 epitope were constructed. The exact TB10.4 fragment inserted in the CyaA constructs is depicted in each panel (A, B, D, and E). TB10.4 inserts in the CyaA constructs were located at either position 233 or 336, whereas the OVA:257–264 epitope was inserted at position 108. (A to C) To study OVA:257–264 presentation, BM-DC from C57BL/6 mice were preincubated for 4 h with various concentrations of TB10.4-recombinant CyaA, control CyaA, or CyaA-OVA:257–264 (harboring the OVA:257–264 epitope at position 224) (A and B) or with OVA:257–264 or Ag85A:101–120 peptides as a control (C). After washing, BM-DC were cocultured with B3Z cells for 18 h. (D to F) For TB10.4:20–28 presentation, BM-DC from BALB/c mice and the TB10.4:20-28-specific CD8^{+} T-cell hybridoma YB8 were cocultured during 18 h with TB10.4-recombinant CyaA, control CyaA (D and E), or TB10.4- or ESAT-6-purified proteins (E) or with TB10.4:20–28 or Ag85A:101–120 peptides as a control (F). Activation of B3Z and YB8 T-cell hybridomas was determined by measuring the amount of IL-2 in the supernatant by CTLL-2 assay. Results are expressed as cpm of incorporated [³H]thymidine and are representative of four to five experiments.

vivo studies, since this construct exhibited the highest efficacy in delivery of the TB10.4 CD8^{+} T-cell epitope (Fig. 3D). We also selected the recombinant CyaA containing the full-length sequence of the TB10.4 protein (position 233) (CyaA-TB10.4:1-96-OVA) because of its potential as an anti-*M. tuberculosis* vaccine candidate.

Immunization with TB10.4 delivered by CyaA induces a TB10.4:20-28-specific CTL response. To test whether CyaA can induce CTL response against the TB10.4:20–28 epitope, we immunized BALB/c mice by a single intravenous (i.v.) injection of the two selected TB10.4 recombinant CyaAs (50 µg) in PBS. This immunization protocol has been successfully used to induce a CTL response against several CD8^{+} T-cell epitopes delivered by CyaA, such as OVA:257–264 (17), or HPV16-E7 epitopes (33). However, neither CyaA bearing TB10.4:15–33 nor CyaA harboring the full TB10.4 protein was able to induce a detectable CTL response against the TB10.4: 20–28 epitope upon a single i.v. immunization. In contrast, this protocol allowed induction of an efficient OVA:257-264-specific response in C57BL/6 mice immunized with the same CyaA constructs (data not shown).

We have previously observed that two injections of the CyaA vector, preferentially by the i.p. route and in the presence of alum, could be necessary for the induction of response against weak CD8^{+} T-cell epitopes (9, 13). Thus, BALB/c mice were immunized (i.p.) at days 0 and 15 with 50 µg of either CyaA-TB10.4:1-96-OVA or CyaA-TB10.4:15-33-OVA in alum. For comparison, BALB/c mice received a single s.c. injection of BCG (10⁶ CFU/mouse) in PBS or the TB10.4 protein (10 µg) in DMT adjuvant. As a control, mice were immunized with control CyaA (two i.p. injections in alum) or with DMT adjuvant alone (one s.c. injection). As shown in Fig. 4, TB10.4-recombinant CyaA in alum were able to induce a strong and specific CTL response. Both recombinant CyaAs, carrying the entire protein or the TB10.4:15–33 fragment, showed similar efficacies in priming CTL. Interestingly, the free TB10.4 protein was able to induce a low but reproducible CTL response when injected in DMT adjuvant. This response was comparable to the CTL response induced by BCG (Fig. 4). From the three vaccination strategies tested, the protocol based on the immunization with the TB10.4-recombinant CyaA was the most effective at priming TB10.4-specific CTL response. No CTL activity was detected in mice immunized with control CyaA or adjuvant alone, confirming the specificity of the CTL activity detected (Fig. 4).

TB10.4 induces a strong Th1-polarized response when given in DMT adjuvant or delivered by CyaA. To further explore the immunogenicity of a candidate subunit vaccine based on the TB10.4 protein, BALB/c mice received a single s.c. injection of the TB10.4 protein (10 µg) in DMT adjuvant or adjuvant alone
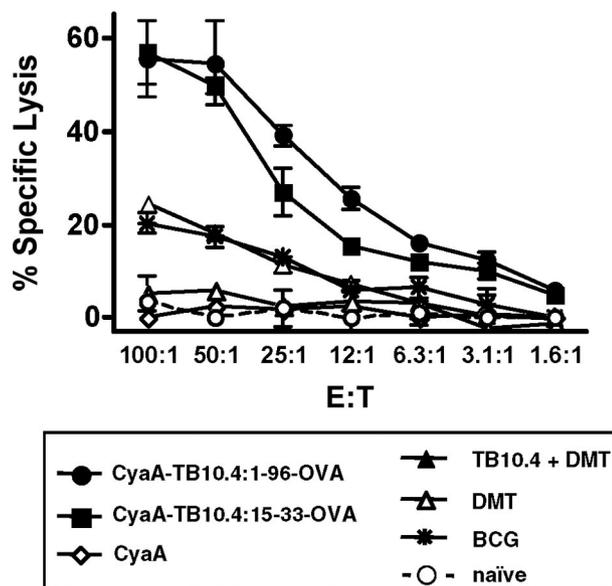
FIG. 4. Comparison of TB10.4:20-28-specific CTL response induced by BCG or by TB10.4 either administered in DMT adjuvant or delivered by CyaA. BALB/c mice received a single s.c. injection of BCG (10^6 CFU) or TB10.4 protein (10 µg) in DMT adjuvant or received DMT adjuvant alone or two i.p. injections (at days 0 and 15) of CyaA-TB10.4:1-96-OVA, CyaA-TB10.4:15-33-OVA, or control CyaA (50 µg) in alum. TB10.4 fragments in the recombinant CyaA were inserted at position 233. Twenty-one days after priming, splenocytes from naïve or immunized mice were stimulated in vitro with the TB10.4:20–28 peptide (10 µg/ml) for 5 days. The CTL activity was measured by ^{51}Cr assay. Data are expressed as the mean ± standard error of the specific percent lysis from duplicate wells. Results are representative of three experiments (three mice per group).

or two i.p. injections (at days 0 and 15) of CyaA-TB10.4:1-96-OVA or control CyaA (50 µg) in alum. T-cell immune responses were analyzed 21 days after immunization using total splenocytes. As depicted in Fig. 5A, TB10.4 was able to induce a strong proliferative response either in adjuvant or when delivered by CyaA. To determine if the immunization with these subunit vaccines induced the same pattern of response as the BCG vaccine (Fig. 1B), splenocytes from mice immunized with the TB10.4 protein in DMT adjuvant or delivered by CyaA were stimulated in vitro with overlapping 15-mer peptides covering the entire TB10.4 protein and IFN-γ levels were determined in culture supernatant by ELISA. The regions containing the TB10.4 T-cell epitopes recognized by splenocytes from mice immunized with the TB10.4 protein in adjuvant (Fig. 5B) were similar to the immunodominant regions identified upon BCG vaccination (Fig. 1B), although some slight differences could be detected. The regions defined by amino acids 16 to 40 and 44 to 65 were immunogenic in both groups. However, the main immunodominant region recognized after BCG vaccination, expanding from residues 63 to 96 (Fig. 1A), was substituted for by a narrower region (amino acids 70 to 91) in mice immunized with TB10.4. The broadness of the immunodominant 63–96 region detected with splenocytes from BCG-immunized mice can be explained by the existence of at least two TB10.4 immunodominant epitopes in this region. However, we cannot exclude that, upon immunization with BCG, there exist

T cells specific to other proteins of the ESAT-6 family which may recognize by cross-reactivity epitopes of TB10.4. This could explain why with the same PEPSCAN, the third region seems broader in BCG-immunized mice than the profile obtained with mice immunized with the TB10.4 protein in adjuvant or with CyaA-TB10.4. Moreover, a novel region containing immunodominant peptides (amino acids 56 to 76) was recognized upon immunization with TB10.4 in DMT adjuvant (Fig. 5B). After immunization with CyaA-TB10.4:1-96-OVA (Fig. 5B), the levels of IFN-γ production by peptide-stimulated cells were in general markedly lower, with peptide TB10.4:74–88 being the immunodominant epitope. Splenocytes from mice immunized with DMT adjuvant alone or with control CyaA did not recognize any TB10.4 peptides (data not shown). Figure 5 clearly shows that the context of the epitope is important in determining how it is processed and presented. Indeed, the first region of TB10.4 recognized by T cells after vaccination with the TB10.4 protein (aa 16 to 40) is not the same as the region recognized by T cells elicited by CyaA-TB10.4:1-96-OVA (aa 14 to 36). To further characterize the T helper response induced, splenocytes from mice immunized with TB10.4 in DMT adjuvant or with CyaA-TB10.4:1-96-OVA in alum were stimulated in parallel with various concentrations of the TB10.4:74–88 peptide, using ESAT-6:1–20 peptide as negative control. An intense proliferative response specific for the TB10.4:74–88 epitope was detected in both cases (data not shown). Splenocytes from both groups produced high levels of Th-1 cytokines (IL-2 and IFN-γ) (Fig. 5C), with no detectable IL-4 or IL-5 (data not shown). The highest levels of Th-1 cytokines were, in fact, found in mice immunized with TB10.4 in DMT adjuvant. No production of Th1 and Th2 cytokines against the TB10.4:74–88 peptide was detected after immunization with DMT adjuvant alone or with control CyaA, confirming the specificity of the responses (Fig. 5C). It is noteworthy that immunization with CyaA-TB10.4:1-96-OVA in alum did not generate Th2 responses under our experimental conditions. Finally, as depicted in Fig. 5D, a high frequency of TB10.4-specific IFN-γ-producing cells was detected after immunization with TB10.4 in DMT but not in mice immunized with CyaA-TB10.4:1-96-OVA. It should, however, be noted that the frequency of these cells was fourfold lower than that upon BCG vaccination.

Evaluation of the protective capacity of a *M. tuberculosis* subunit vaccine based on the TB10.4 recombinant protein given in adjuvant or delivered by CyaA. Considering the strong immunogenicity of the TB10.4 protein, we next evaluated the capacity of this Ag to protect BALB/c mice against *M. tuberculosis* challenge. To this end, mice were vaccinated s.c. with BCG (1 × 10^6 CFU/mouse) at day 0 or with TB10.4 (10 µg/mouse) adjuvanted in DMT at day 15. At day 30, all mice were infected by low dose of aerosolized *M. tuberculosis* H37Rv. At 1 month postchallenge, bacterial burden was determined in lungs of infected mice and CFU load was determined (Fig. 6). As typically observed in the mouse model, vaccination by BCG conferred protection leading to CFU counts 1 order of magnitude lower than those in unvaccinated mice. TB10.4 adjuvanted in DMT induced a highly statistically significant protection ($P < 0.005$), which, however, did not reach the level of protection induced by BCG ($P < 0.003$). Interestingly, DMT adjuvant alone provided a significant de-
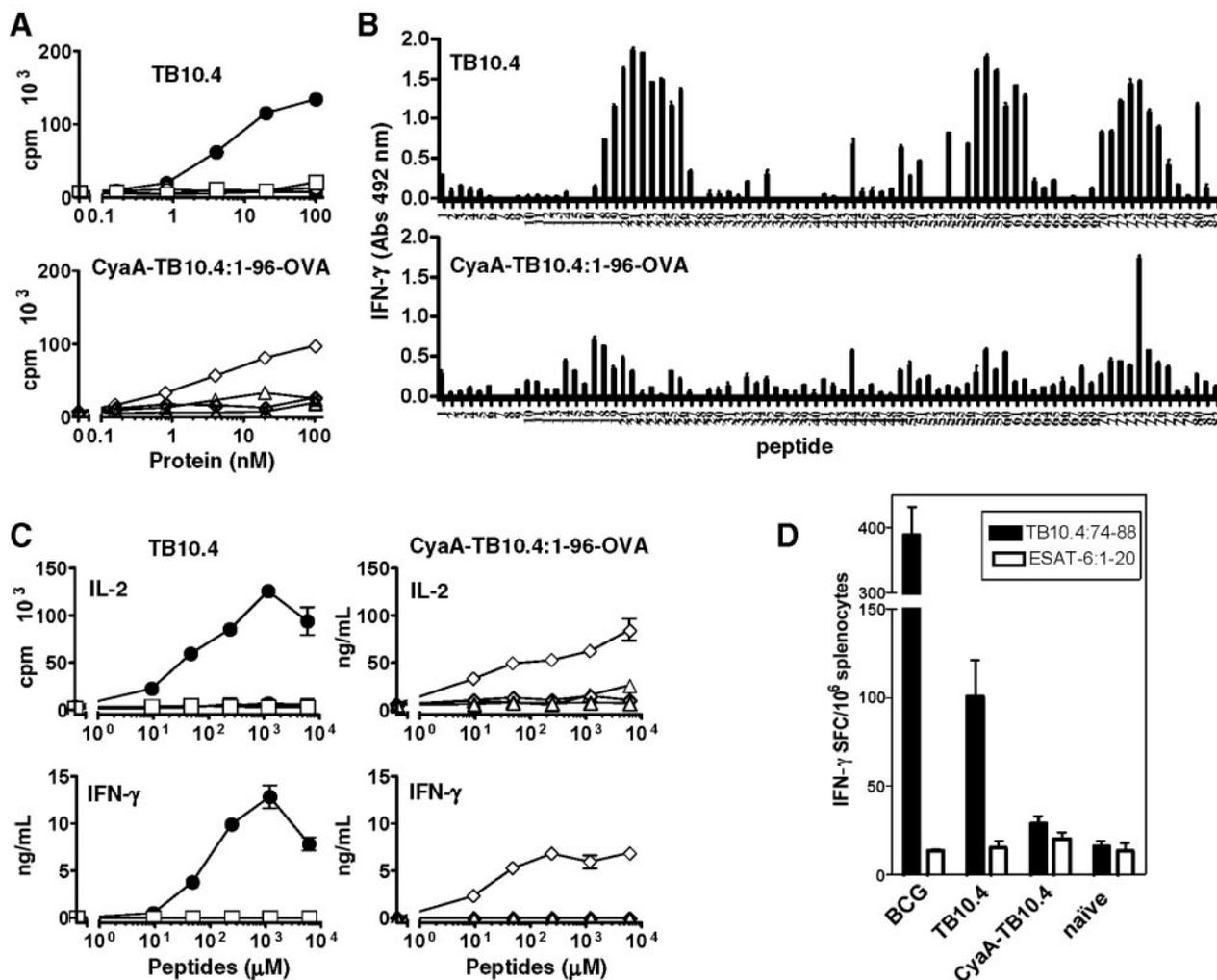
FIG. 5. Comparison of the TB10.4-specific T-helper responses induced by TB10.4 either administered in DMT adjuvant or delivered by CyaA. BALB/c mice received a single s.c. injection of the TB10.4 protein (10 μg) in DMT adjuvant or received adjuvant alone or two i.p. injections (at days 0 and 15) of CyaA-TB10.4:1-96-OVA:257–264 or control CyaA (50 μg) in alum. The TB10.4 protein was inserted at position 233. T-cell responses were analyzed 21 days after priming using total splenocytes. (A) Proliferation against the TB10.4 protein (solid symbols) or the control protein ESAT-6 (open symbols) of splenocytes from mice immunized with TB10.4 in DMT adjuvant (circles) or DMT adjuvant alone (squares) (top panel) or with CyaA-TB10.4:1-96-OVA (diamonds) or control CyaA (triangles) (bottom panel). (B) Mapping of the dominant T-cell determinants of the TB10.4 protein after immunization with TB10.4 in DMT adjuvant or delivered by CyaA. Splenocytes from mice immunized with TB10.4 protein in DMT adjuvant (top panel) or delivered by CyaA (bottom panel) were stimulated in vitro with overlapping 15-mer peptides (1 to 3 μg/ml) covering the entire TB10.4 protein. Peptides are referred to by the NH$_2$-terminal amino acid of each peptide. IFN-γ levels were determined in culture supernatant by ELISA. The absorbance values (Abs) depicted correspond to the difference between the levels of absorbance of supernatants from naïve and primed mice. Absorbance values corresponding to supernatants from naïve mice never exceeded an optical density of 0.1. (C) TB10.4-specific T-helper responses induced after immunization with TB10.4 in DMT adjuvant or delivered by CyaA. Splenocytes from mice immunized with TB10.4 in DMT adjuvant (circles) or DMT adjuvant alone (squares) (left panels) or with CyaA-TB10.4:1-96-OVA (diamonds) or control CyaA (triangles) (right panels) were in vitro stimulated with the TB10.4:74–88 peptide (solid symbols) or with a negative control peptide (Ag85A:101–120) (open symbols). Cytokine levels were determined in culture supernatant by CTLL-2 assay (IL-2) or by ELISA (IFN-γ). (D) Comparison of the frequency of TB10.4-specific IFN-γ-producing cells after immunization with BCG (10^6 CFU) or TB10.4 in DMT adjuvant or delivered by CyaA. Splenocytes from naïve or primed mice were stimulated with TB10.4:74–88 or ESAT-6:1–20 peptide (10 μg/ml), and IFN-γ-producing cells were measured by ELISPOT assay. Results are representative of two to four experiments (three mice per group).

gree of protection ($P < 0.018$). The difference between the group of mice immunized with DMT and that of mice immunized with TB10.4 adjuvanted in DMT was statistically significant ($P < 0.0075$) and was due to TB10.4 protein antigen.

We further investigated the potential of CyaA-TB10.4:1-96-OVA or CyaA-TB10.4:15-33-OVA constructs to protect BALB/c mice against *M. tuberculosis* challenge. Negative or

positive control mice were, respectively, left unvaccinated or vaccinated s.c. with BCG at day 0. At days 0 and 15, CyaA-TB10.4:1-96-OVA or CyaA-TB10.4:15-33-OVA was injected i.p. with alum. At day 30, all mice were challenged with a low dose of aerosolized *M. tuberculosis* H37Rv. At 1 month postchallenge, bacterial burden was determined in lungs of infected mice (Fig. 7). Neither CyaA-TB10.4:15-33-OVA nor CyaA-
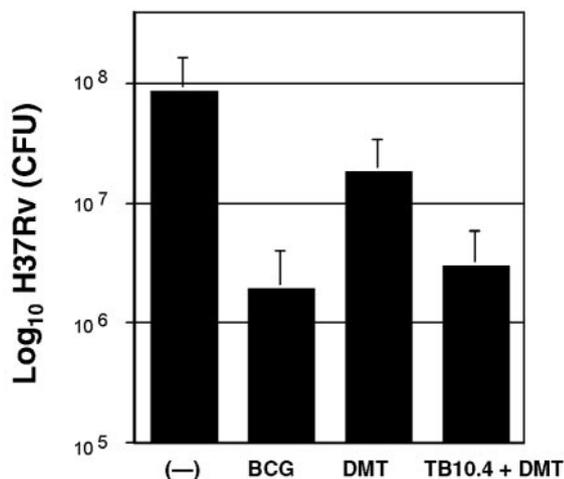
FIG. 6. Evaluation of the protective capacity of TB10.4 against infection with *M. tuberculosis*. BALB/c mice were left unvaccinated or were immunized with BCG or with TB10.4 in DMT or with DMT alone. BCG was injected s.c. (10^6 CFU) at day 0, whereas TB10.4 (10 µg) was injected s.c. at day 15. At day 30, mice were infected with aerosolized *M. tuberculosis* H37Rv ($\approx$100 CFU/mouse) and bacterial burden in lung was determined by CFU counting at day 30 postinfection. Mean lung counts were analyzed statistically using analysis of variance. Bacterial counts in the lungs from mice immunized with BCG or with TB10.4 in DMT were statistically different from the nonvaccinated ones ($P < 0.0032$ and $P < 0.0050$, respectively). CFU in the lungs from mice immunized with TB10.4 in DMT were statistically different from those immunized only with DMT ($P < 0.0075$). Results are means ± standard deviation and are representative of two independent experiments.
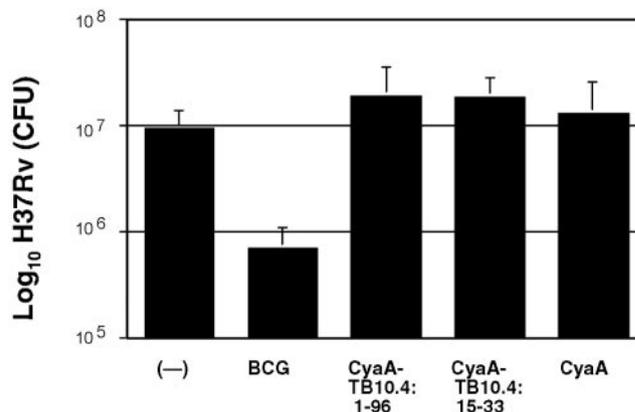

FIG. 7. Evaluation of the protective capacity of CyaA harboring the complete sequence of TB10.4 or the subfragment TB10.4:15–33 against infection with *M. tuberculosis*. BALB/c mice were left unvaccinated or were immunized with BCG or with CyaA-TB10.4:1-96-OVA, CyaA-TB10.4:15-33-OVA, or control CyaA in alum. BCG was injected s.c. (10^6 CFU) at day 0, whereas recombinant or control CyaA (50 µg/mouse) was injected i.p. at days 0 and 15. At day 30, mice were infected with aerosolized *M. tuberculosis* H37Rv ($\approx$100 CFU/mouse) and bacterial burden in lungs was determined at day 30 postinfection. Results are means ± standard deviation and are representative of two independent experiments.

TB10.4:1-96-OVA was able to confer any significant protection as compared to that in the unvaccinated controls.

DISCUSSION

The TB10.4 protein has been previously shown to induce strong CD4$^+$ T-cell responses following TB infection either in humans (41, 42) or in animal models (11, 26). Recently, we have also shown that CD8$^+$ T cells from mice infected with BCG or *M. tuberculosis* specifically lysed target cells pulsed with the TB10.4:20–28 epitope (*H-2^d*)-containing peptide, showing that this protein is naturally processed and presented by MHC class I molecules in both BCG-immunized and *M. tuberculosis*-infected mice (26). Moreover, as shown in the present study, upon vaccination with BCG, a strong TB10.4-specific Th1 T-cell response is induced in BALB/c mice but not in C57BL/6 mice. CD4$^+$ T cells from BALB/c mice were able to strongly recognize several T-cell epitopes along the sequence of the TB10.4 protein. This fact could explain the high immunogenicity of TB10.4 in the context of this haplotype. The low response of BCG-vaccinated C57BL/6 mice was clearly due to lack of recognition of strong immunodominant epitopes. Absence of response in C57BL/6 mice to TB10.4 can, however, be overcome by hyperimmunization with 10 µg of TB10.4 emulsified in DMT adjuvant (data not shown). Following this protocol, we have observed that splenocytes from TB10.4-primed C57BL/6 mice recognize, although weakly, peptides belonging to the N-terminus part of the protein (aa 1 to 19). In humans, such genetic control of the T-cell response should be taken in consideration, especially when using small proteins as subunit vaccines. In fact, genetic control would explain the lack of TB10.4 recognition by around 30% of TB patients (41). Thus, in order to generate T-cell responses with broader specificities, larger subunit vaccines or multivalent subunit vaccines should be more appropriate for vaccination of a genetically outbred human population.

Based on the strong immunogenicity of the TB10.4 protein, we have compared the protective efficacies of this Ag given in DMT adjuvant (containing DDA, MPL, and TDM) or delivered to DC by fusion to the *Bordetella pertussis* CyaA vector that binds CD11b/CD18 integrin (18). To examine the potential of CyaA as a vaccine vector for TB10.4, we have constructed different detoxified CyaA harboring the entire TB10.4 protein or short sequences containing the TB10.4:20–28 CD8$^+$ T-cell epitope with or without its natural flanking regions. These sequences have been genetically inserted at two different sites of CyaA (position 233 or 336). Both insertion sites have been previously shown to be permissive for delivery of short exogenous sequences (25). Six out of eight CyaA constructs were able to deliver the TB10.4:20–28 epitope to the MHC class I pathway, with an in vitro efficiency of presentation comparable to or even higher than that of the TB10.4:20–28 peptide. Except for CyaA-TB10.4:20-28-OVA, insertion of Ag at position 233 allowed better efficiency of Ag delivery and presentation than insertion at position 336. It is interesting to note that CyaA harboring the entire TB10.4 protein delivered the TB10.4:20–28 CD8$^+$ T-cell epitope as efficiently as its counterparts bearing the minimal epitope alone or with natural flanking regions. In contrast, the purified TB10.4 protein was unable to gain access to the MHC class I pathway. The ability of CyaA to deliver in vitro the TB10.4:20–28 epitope to the MHC class I pathway was also confirmed in vivo, as immunization of mice with recombinant CyaA bearing the entire pro-

tein or the TB10.4:15–33 sequence led to the induction of TB10.4-specific CTL response even though two injections of recombinant CyaA in the presence of alum were necessary. It should be mentioned that since the TB10.4:20–28 CD8$^+$ T-cell epitope does not induce IFN-γ responses (data not shown), it was not possible to analyze the frequency of TB10.4:20-28-specific CD8$^+$ T-cell responses by ELISPOT assay.

In this study, we have also compared the CD4$^+$ T-cell response induced by the TB10.4 protein when given in DMT adjuvant or delivered by CyaA. Using the TB10.4:74–88 peptide containing the H-2^d CD4$^+$ T-cell epitope, both vaccination strategies were shown to stimulate a specific Th1 CD4$^+$ T-cell response. However, as compared to immunization with CyaA-TB10.4:1-96-OVA in alum, the frequency of TB10.4:74-88-specific T cells induced after injection of TB10.4 in DMT adjuvant was much higher, while remaining fourfold lower than that after vaccination with BCG. Moreover, immunization with TB10.4 in DMT adjuvant induced a highly polyclonal T-cell response, whereas the CD4$^+$ T-cell response induced by the TB10.4 protein by CyaA was mainly focused to the TB10.4: 74–88 epitope. Interestingly, the fine specificity of the T-cell response induced by TB10.4 in DMT adjuvant was comparable to the T-cell response induced after BCG vaccination. Finally, it is worth noting that this strong and polyclonal CD4$^+$ T-cell response induced by TB10.4 in the context of the DMT adjuvant was achieved upon a single injection.

When both strategies of vaccination (immunization with TB10.4 formulated in DMT adjuvant or CyaA harboring a TB10.4 CD8$^+$ T-cell epitope or the entire protein) were tested for their capacity to protect against $M.$ $tuberculosis$ infection in the mouse model, only immunization with TB10.4 in DMT was able to induce protection. Thus, our data confirm the recent results of Dietrich et al. (11), showing that vaccination of mice with TB10.4 formulated in a cocktail of adjuvants containing DDA and MPL induced significant protection against $M.$ tu-$berculosis$. These two studies identified the TB10.4 protein as an attractive Ag candidate for designing a TB subunit vaccine. So far, only a few Ag have been shown to induce a protective immunity against $M.$ $tuberculosis$ (reviewed in references 12 and 45). BCG does not confer adequate protection against pulmonary disease in adults, although it does confer consistent protection against disseminated forms of TB in childhood. Since TB10.4 is expressed by BCG, boosters with the TB10.4 protein could potentially enhance the rate of immune protection of BCG.

Upon immunization with CyaA-TB10.4:15-33-OVA, able to induce CD8$^+$ CTL responses, and more surprisingly, upon immunization with CyaA-TB10.4:1-96-OVA, able to induce both CD8$^+$ and Th1 CD4$^+$ responses, no protection has been observed against infection with $M.$ $tuberculosis$. This fact can be explained by the weak frequencies of TB10.4-specific Th1 cells induced by these immunogens. Indeed, in our model, the frequency and polyclonality of IFN-γ-producing TB10.4-specific T cells correlate with protection. Although a quantitative analysis of the CTL response induced could not be provided, the lack of protection observed despite the induction of TB10.4-specific CD8$^+$ T cells addresses the role of these cells in the host defense against $M.$ $tuberculosis$ infection. Whereas it is generally accepted that CD4$^+$ T cells are of primary importance in the protection against $M.$ $tuberculosis$ (8, 30, 36, 38,

43), there is some controversy about the role played by CD8$^+$ T cells, with some studies claiming the importance of CD8$^+$ T cells (3, 15) but with others suggesting that these cells are not required (3, 8, 15, 29, 30, 32, 36, 38, 43).

Our results also highlight the importance of adequate triggering of the innate immune system to ensure the induction of a fully protective immune response against $M.$ $tuberculosis$. Subunit-based vaccines need effective adjuvants and/or vectors to elicit appropriate immune responses, since highly purified Ag are usually poorly immunogenic. Another important limitation of vaccines based on soluble Ag is that they rarely have access to the MHC class I presentation pathway and hence are not capable of activating CD8$^+$ T cells. Moreover, the only adjuvants currently approved for use in humans (alum and MF59) induce a Th2-polarized immune response (40) and are unable to promote a protective response against $M.$ $tuberculosis$ (1, 2, 24). The improved understanding of the activation of the immune system in response to conserved molecules on pathogens has led to a flood of new adjuvants. DDA is a cationic micelle-forming surfactant with potent adjuvant activity. Despite the fact that DDA is a weak Th1 immunomodulator, it has been shown to potentiate the immunogenicity of several $M.$ $tuberculosis$ Ag (4, 19, 24), probably by promoting the long-lasting delivery and/or uptake of the Ag (19). In order to achieve higher levels of protection, several adjuvants have been tested in combination with DDA. From a long list of immunomodulators, which include cytokines like IFN-γ, IL-2, and IL-12 and molecules such as saponin, n-hexadecane, β-glucan, and muramyl dipeptide (19, 24, 39), only MPL and a derivative of TDM (trehalose 6,6-dibehenate) were able to synergize with DDA and to induce a strong protective immune response against ESAT-6 (19). The combination of DDA plus MPL, DDA plus TDM, or DDA plus MPL plus TDM has also been effective at boosting the immunogenicity of other $M.$ $tuberculosis$ Ag such as Ag85A and TB10.4 protein (11, 19; this paper).

Besides the DDA- and MPL-TDM combinations, other mixtures composed of oligodeoxynucleotides and polycationic amino acids and several live vectors have proven to be effective for the delivery of mycobacterial Ag and for the adequate triggering of the innate system, allowing induction of a protective response against $M.$ $tuberculosis$ (12). We have extensively shown that CyaA can induce in vivo both a Th1 response and a CTL response, in the absence of adjuvant or even when injected with a Th2 adjuvant such as alum. CTL responses are still induced following injection of CyaA in MHC class II$^{-/-}$ mice, excluding the possibility that indirect activation of DC by CD4$^+$ T cells is responsible for the high immunogenicity of CyaA. This suggests that, in addition to allowing the delivery of epitopes into DC, CyaA may also trigger the innate system to promote the induction of an adaptive immune response. However, these immunomodulatory signals are not sufficient to induce a protective response against $M.$ $tuberculosis$, at least in the mouse model and in combination with Ag such as TB10.4 (this paper), ESAT-6, or Ag85A proteins (27) However, despite this limitation, the capacity of CyaA to directly deliver entire Ag into DC, activating both the CD4$^+$ and CD8$^+$ arms of immunity, could be a major advantage in designing novel TB vaccines, as compared to live vectors or adjuvanted formulations of $M.$ $tuberculosis$ Ag. In order to further improve the

immunological properties of CyaA, Th1 immunomodulators might be coadministered with CyaA-based vaccines to achieve better protection. Studies are currently under way in our laboratory to identify immunomodulators that would synergize with the CyaA vector.

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