A Multicistronic DNA Vaccine Induces Significant Protection against Tuberculosis in Mice and Offers Flexibility in the Expressed Antigen Repertoire\(^\text{7}\)

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Concerns about the safety and efficacy of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) emphasize the need for alternative tuberculosis (TB) vaccines. DNA vaccines are interesting candidates but are limited by the restricted antigen repertoire that they express. Traditional polycistronic vectors are large and have imbalanced expression. Recent advances in molecular genetics and cellular immunology have paved the way toward the rational design of an efficacious vaccine. We exploited self-cleaving peptide 2A from the foot-and-mouth disease virus, because of its small size and high cleavage activity, to generate an efficient TB DNA vaccine (V-2A). V-2A expresses three mycobacterial antigens, Rv3407, Ag85A, and HspX, in a single open reading frame joined by the 2A sequences, which lead to the segmentation of the long translated polypeptide into individual proteins by posttranslational modification. Our in vitro measurements revealed no differences at the transcriptional or translational level between V-2A and the monocistronic expression of the individual antigens. Mice vaccinated with V-2A developed antigen-specific cellular and humoral responses against all three antigens, imparting protection against *Mycobacterium tuberculosis* aerosol challenge equivalent to that imparted by BCG. These results have important implications for the rational design and development of efficacious recombinant subunit vaccines.

Tuberculosis (TB) is a major cause of mortality worldwide. New TB cases in 2006 numbered 9.2 million, and there were 1.7 million deaths. Of these, individuals coinfected with *Mycobacterium tuberculosis* and the human immunodeficiency virus made up 0.7 million cases and more than 0.2 million deaths (2). It has been estimated that 1 billion people will be newly infected, over 150 million people will become ill, and more than 30 million will die of TB in the next decade if control measures are not improved (3). The failure of protection conferred by *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) against pulmonary TB in adults and its negligible impact on the global TB epidemic emphasize the need for a new vaccine (5, 11, 21). The search for an effective vaccination strategy against TB has therefore become a global research priority and has been pursued by the use of a variety of avenues, including recombinant BCG, attenuated *M. tuberculosis*, DNA vaccines, and recombinant protein antigen-based subunit vaccines (8, 16, 34, 37, 39). Due to the complexity of the host immune response against TB and the genetic restrictions imposed by the major histocompatibility complex, it is likely that an effective subunit vaccine comprising multiple epitopes will be required to ensure broad coverage of a genetically heterogeneous population (27, 38). It has previously been demonstrated that vaccines based on a combination of immunodominant antigens can induce levels of protection similar to those induced by BCG in mice (1, 14, 17, 30). Polypeptide subunit vaccines based on the fusion of immunodominant *M. tuberculosis* antigens such as ESAT-6 and antigen 85B (Ag85B) (38) and Mtb72F (33) are currently being tested in clinical trials, following the promising results obtained in preclinical studies. Other reports have described early secreted antigens such as CFP-10, ESAT-6, Ag85B, and Ag85A (1, 27); dormancy antigens such as HspX (32) and Hsp65 and Hsp70 (23, 35), and the Rpf-regulated gene Rv3407 (26) as subunit vaccines against TB.

In this work, we exploited self-splicing 2A sequences of the foot-and-mouth disease virus (FMDV) to generate a DNA vaccine construct expressing multiple full-length antigens from a single open reading frame (ORF). The 2A sequences are oligopeptides located between proteins P1 and P2 in some members of the picornavirus family and can undergo posttranslational self-cleavage to generate mature viral proteins P1 and P2. Among various 2A or 2A-like sequences, FMDV 2A is particularly short (13 amino acids) and is able to cleave at its own C terminus between the last two amino acids through a mechanism believed to be enzyme independent (10). We were able to engineer a DNA vaccine construct (V-2A) that expresses high levels of full-length and functional antigens which are secreted at different stages of disease development and which induce strong immune responses (7, 26, 32). We show that it is possible to coexpress up to four full-length antigens joined by 2A sequences in a single ORF under the control of a single promoter. In the mouse model of TB, the level of protection provided by V-2A outperformed that provided by BCG at day 60 postinfection (p.i.) and leveled off at day 90 p.i.

**MATERIALS AND METHODS**

**V-2A construction.** Rv3407 was cloned into the DNA vaccine vector PCMV.tPA (formerly Chiron-Behring [now Novartis Vaccines], Emeryville, CA), which encodes antigens such as tissue plasminogen activator and leader
peptide fusion proteins, under the control of the cytomegalovirus (CMV) promoter. Ag85A was joined to Rs3407 in the pCMV-Ipa vector by FMDV 2A sequences. The DNA sequence of FMDV was synthesized (Agowa, Berlin, Germany) on the basis of the sequence APVKQTLNFDLLKLAGDVESNPGP (10). We introduced this sequence for the splicing peptide of the FMDV 2A sequence into the forward oligonucleotides during the synthesis of oligonucleotides (Agowa). By using the oligonucleotides listed in Table 1, the corresponding sequences were amplified by PCR with M. tuberculosis H37Rv chromosomal DNA as the template. PCR amplification was carried out in three steps in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany): the first step was an initial denaturation for 10 min at 94°C; the second step was four cycles under conditions of 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min; and then the final step was 30 cycles at 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min. The amplification products were purified by agarose gel electrophoresis and subcloned into the TOPO cloning vector (Invitrogen). The resulting plasmids carrying the subcloned PCR products were identified by restriction endonuclease digestion and confirmed that the sequences were inserted was performed by DNA sequencing. The verified sequences were cleaved from the TOPO backbone by digestion with restriction endonucleases specific for the incorporated 5′ sites of the PCR products and were subsequently ligated to DNA vaccine vector pCMV-Ipa containing Rs3407. Ag85A-2A was first ligated, resulting in the DNA vaccine construct containing Rs3407 and Ag85A joined by FMDV 2A sequences. Verification that the sequences were inserted was determined by DNA sequencing. HspX-2A cleaved from the TOPO backbone by restriction endonuclease digestion specific for the incorporated 5′ sites was then ligated into DNA vaccine vector pCMV-Ipa containing Rs3407-2A and Ag85A, resulting in the DNA vaccine construct containing Rs3407, Ag85A, and HspX joined by FMDV 2A sequences. To further explore the possibility that an additional antigen could be added, we used green fluorescent protein (GFP) as a model protein and cloned it in the same way. (The V-2A construct used for the in vivo experiments did not contain GFP.)

**Cell culture.** Human embryonic kidney 293T (HEK293T) cells were cultured at 37°C in 5% CO₂ and Dulbecco modified Eagle's medium (Invitrogen, San Diego, CA) supplemented with 10% heat-inactivated fetal calf serum (FCS), 3 mM glutamine, penicillin (100 U/ml), and streptomycin (100 U/ml).

**Transfection of HEK293T cells.** HEK293T cells were grown for 24 h until they reached a level of confluence of approximately 70 to 80%, before they were transfected in a 150-cm² flask in antibiotic-free Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% heat-inactivated FCS and 3 mM glutamine. A mixture of DNA and lipofectamine reagent (Invitrogen) was prepared according to the manufacturer's instructions. The transfection was carried out in optimum medium (Invitrogen). The DNA-lipofectamine complexes were plated onto Middlebrook 7H11 plates. The numbers of bacterial colonies were plated onto Middlebrook 7H11 plates. The numbers of bacterial colonies were calculated as the number of CFUs (counts forming units).

**Western blotting.** After 72 h of transfection, the cells were collected; washed with phosphate-buffered saline (PBS); and lysed with lysis buffer containing 50 mM HCl, 150 mM NaCl, 1% Nonidet P-40, 1% Triton X-100, 0.1% sodium dodecyl sulfate, and a cocktail of protease inhibitors (Roche, Basel, Switzerland) on ice for 30 min with intermediate vortexing. After 10 min of centrifugation at 13,000 rpm, the supernatant was collected and analyzed by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane for immunoblotting with anti-HspX monoclonal antibody (MAb; Affinity Bioreagents, Golden, CO) and antisera against Ag85A and Rs3407. Secondary antibodies coupled to horseradish peroxidase were used at a concentration of 1/5,000.

**Fluorescent microscopy.** For microscopy analysis, cells were seeded on top of 12-mm round coverslips in a 24-well cell culture plate. After 24 h, when confluence reached 70 to 80%, the cells were transfected with a mixture of DNA and lipofectamine reagent (Invitrogen), prepared according to the manufacturer's instructions. Transfection was carried out in optimum medium (Invitrogen). After 4 h, the medium was changed and the cells were cultured in antibiotic-free Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% heat-inactivated FCS and 3 mM glutamine for 72 h. The slides were washed with PBS and analyzed with a fluorescence microscope (DM IRB; Leica, Wetzlar, Germany).

**qRT-PCR.** Two days after transfection, the cells were trypsinized, collected, and washed with PBS. Total RNA was isolated by using RNeasy columns (Qiagen, Foster City, CA), according to the manufacturer’s instructions. Total RNA was quantified and analyzed for integrity with a Nanodrop analyzer (model 2100 bioanalyzer; Agilent, Santa Clara, CA). Prior to use in the reverse transcription (RT) reaction, RNA samples were treated with DNase I (Invitrogen). After heat inactivation of DNase I, the RT reaction was performed with a Superscript II kit, according to the manufacturer's instructions (Invitrogen). Each reaction mixture contained 2 μg total RNA. Table 2 lists the oligonucleotides (Agowa) used for the real-time quantitative RT-PCR (qRT-PCR). Real-time RT-PCR was performed with an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) in a 30-μl reaction volume with a SYBR green kit. Each reaction was performed in triplicate. The conditions for the RT-PCR and the data analyses were carried out according to the manufacturer’s instructions (Applied Biosystems). GADPH was used as an internal control for the normalization of mRNA levels.

**Mycobacteria, vaccination, and challenge of mice.** M. bovis BCG strain Pasteur and M. tuberculosis H37Rv were cultured in Dubos broth base (Difco, BD Diagnostics, Sparks, MD) supplemented with 10% Dubos medium albumin (Difco, BD Diagnostics) at 37°C. Medium-logarithmic-phase cultures were aliquoted and stored at −80°C until use. Female C57BL/6 mice were purchased from the Bundesinstitut für Risikobewertung (Berlin, Germany). The experiments were conducted in our animal facility according to German animal protection laws. The mice were vaccinated subcutaneously with 10⁸ CFU of BCG. For DNA vaccination, the mice received 100 μg of DNA (50 μg in each quadriceps) three times at intervals of 21 days. At 90 days postvaccination (p.v.) with BCG and 28 days p.v. with DNA, the animals were aerosol challenged with 200 CFU of M. tuberculosis H37Rv in an aerosol chamber, as described previously (31). At the indicated days p.v. or p.i., mice were killed and serial dilutions of tissue homogenates were plated onto Middlebrook 7H11 ampicillin plates. The numbers of CFU on the plates were counted after 3 to 4 weeks of incubation at 37°C. Counting of the CFU was performed in a double-blind manner.

**IgG subtyping.** Serum samples from individual animals (six per group) were analyzed for antigen-specific immunoglobulin G1 (IgG1) and IgG2a subtypes by enzyme-linked immunoabsorbent assay (ELISA). Recombinant protein (1 μg/well)

### TABLE 1. PCR primers

| Primer | Sequence |
|--------|----------|
| Ag85A_2A_forward | gcgtgcGACCGGTGAAACAGACTTTGAATTTTGACCTTCTCAAGTTGGCGGGAGA |
| Ag85A_reverse | tgatcatctagagccgccctggggcgc |
| HspX2A_forward | tctagaGCACCGGTGAAACAGACTTTGAATTTTGACCTTCTCAAGTTGGCGGGAG |
| GFP_2A_forward | tga3gtcGACCGGTGAAACAGACTTTGAATTTTGACCTTCTCAAGTTGGCGGGAG |
| GFP_reverse | tga3gtcGACCGGTGAAACAGACTTTGAATTTTGACCTTCTCAAGTTGGCGGGAG |

* Lowercase letters that are underlined indicate restriction sites; lowercase letters that are not underlined indicate sequences homologous to the target gene; capital letters indicate the 2A sequence.

### TABLE 2. Quantitative real time RT-PCR primers

| Primer | Sequence |
|--------|----------|
| RV3407_forward | ggtgcGACCGGTGAAACAGACTTTGAATTTTGACCTTCTCAAGTTGGCGGGAGA |
| RV3407_reverse | cggtgcGACCGGTGAAACAGACTTTGAATTTTGACCTTCTCAAGTTGGCGGGAG |
| GFP_forward | tga3gtcGACCGGTGAAACAGACTTTGAATTTTGACCTTCTCAAGTTGGCGGGAG |
| GFP_reverse | tga3gtcGACCGGTGAAACAGACTTTGAATTTTGACCTTCTCAAGTTGGCGGGAG |
in 100 μl of 0.1 M sodium carbonate-bicarbonate buffer was coated overnight at 4°C. The plates were then blocked with 1% bovine serum albumin (BSA) in PBS for 2 h at 37°C, followed by three washings with PBS-Tween 20 (0.02%). This step was followed by incubation of the serum samples (1:100 dilution) for 2 h at room temperature and washing with PBS-Tween 20. Secondary antibodies (antimouse IgG1 and IgG2a conjugated with horseradish peroxidase) at 1:1,000 were incubated for 2 h at 37°C. Color was developed by adding o-phenylenediamine and H₂O₂ in citrate buffer (pH 5.0), and the absorbance was measured at 490 nm.

Isolation of cells. Cells from the lymph nodes and spleens were prepared by homogenizing the lymph nodes and spleens with a nylon mesh. The cells were then washed and the red blood cells were lysed (22). The lungs were perfused with PBS via the right vena cava and homogenized by using an iron mesh sieve. The cells were washed, and the remaining red blood cells were lysed. The cells were purified by use of a 40/70% Percoll gradient.

In vitro restimulation of cells and flow cytometric determination of cytokine expression. Cells (1 × 10⁶ to 3 × 10⁶) were cultured in a 1-ml volume of RPMI 1640 medium supplemented with glutamine, sodium pyruvate, 2-mercaptoethanol, penicillin, streptomycin, and 10% heat-inactivated FCS and buffer with rat serum and anti-CD16/CD32 MAb. After 5 min, fluorescein isothiocyanate-conjugated anti-tumor necrosis factor alpha (anti-TNF-α) MAb (BD), allophycocyanin-conjugated interleukin-2 (IL-2) MAb (eBioscience, San Diego, CA), and PE-conjugated CD40L MAb (eBioscience) were added. After 20 min, the cells were washed and fixed for 15 min at room temperature with PBS–2% paraformaldehyde. The cells were washed with PBS–0.1% BSA, permeabilized with PBS–0.1% BSA–0.5% saponin, and incubated in this buffer with rat serum and anti-CD16/CD32 MAb (BD). After 5 min, fluorescein isothiocyanate-conjugated anti-tumor necrosis factor alpha (anti-TNF-α) MAb (BD), and PE-conjugated CD40L MAb (eBioscience, San Diego, CA), and PE-conjugated CD40L MAb (eBioscience) were added. After a further 15 min at room temperature, the cells were washed with PBS and fixed with PBS–1% paraformaldehyde. The cells were analyzed with a FACS Canto flow cytometer and FACS Diva software (BD).

Statistical analysis. All results are representative of those from at least two independent experiments with similar results. Bacterial titers and antibody titers were analyzed by the Mann-Whitney test with two-tailed P values for nonparametric data by using GraphPad Prism (version 4.0) software. The frequencies and percentages of T cells were compared by analysis of variance and Bonferroni’s multiple-comparison test.

RESULTS

V-2A construct coexpressing three M. tuberculosis antigens. Three M. tuberculosis antigens, Rv3407, Ag85A, and HspX, which are expressed at three different stages of disease development, i.e., at the metabolically active (Ag85A), dormant (HspX), and resuscitation (Rv3407) stages and which have been well characterized as antigens of cell-mediated immunity (7, 26, 32) against M. tuberculosis were chosen as model antigens to evaluate their expression by using FMDV 2A self-processing peptides. A cassette was generated in which the expression of the three antigens, total protein extracts from HEK293 cells transiently transfected with V-2A were analyzed by using Western blots. Antisera against Ag85A and Rv3407 and anti-HspX MAb were used to detect bands of 11 kDa, 30 kDa, and 14 kDa, which corresponded to Rv3407, Ag85A, and HspX, respectively, in the lysates of HEK cells transfected with V-2A but not in those transfected with the control vector pCMV.tPA (Fig. 1B). Bands that corresponded to the homologous recombinant proteins were used as positive controls in the Western blot analysis. Thus, FMDV 2A efficiently facilitated the coexpression of the three antigens from a single ORF.

Coexpression of four proteins. We next explored the possibility of expressing additional antigens by use of the FMDV 2A strategy. We linked GFP to V-2A at the fourth position downstream of HspX, after deleting its stop codon to generate V-2A–GFP. HEK293 cells were transiently transfected with V-2A–GFP and monitored by fluorescence microscopy after 48 h of transfection for GFP expression. Microscopic analysis confirmed the expression of active GFP (Fig. 2A). GFP expression was detectable only in cells transfected with V-2A–GFP and not in cells transfected with pCMV.tPA as a control. Furthermore, we compared the levels of expression of the individually expressed GFPs under the control of the CMV promoter (pCMV.tPA-GFP) to the level of expression of the GFP expressed from V-2A–GFP under the control of the same promoter in HEK293 cells using fluorescence microscopy. We observed no significant differences in the levels of expression of GFP between cells transfected with pCMV.tPA-GFP and those transfected with V-2A–GFP (Fig. 2A and B). These results confirm the possibility that additional antigens may be expressed by the use of FMDV 2A sequences.

Equal expression levels. To assess the levels of expression of the genes in V-2A and to compare the levels of expression of those of the genes expressed in the pCMV.tPA-Rv3407, pCMV.tPA-GFP, V-2A, and V-2A–GFP vectors, qRT-PCR analysis was performed. HEK293 cells were transiently transfected with V-2A. RNA was isolated after 24 h of transfection and analyzed by qRT-PCR. The RNA was quantified and analyzed for its integrity with a Nanodrop analyzer (model 2100 bioanalyzer; Agilent). Prior to use, the RNA was reverse transcribed and used in the qRT-PCR. The expression results demonstrated the proper and equal expression of GFP and Rv3407 from V-2A–GFP compared to that from pCMV.tPA-GFP and pCMV.tPA-Rv3407 when GADPH was used as the internal control. Furthermore, the levels of expression of Rv3407 and GFP in the V-2A, V-2A–GFP, and monocistronic vectors pCMV.tPA-Rv3407 and pCMV.tPA-GFP were compared. The expression results verified that Rv3407 and GFP were equally expressed by both vectors (Fig. 2B). This supports the findings of Western blot and microscopy that all genes in V-2A are coexpressed at equal strengths and that their levels of expression are comparable to the level of expression of a monocistronic vector.

Induction of specific T-cell response after V-2A vaccination. Mice were vaccinated three times intramuscularly (i.m.) with 100 μg DNA per mouse (50 μg per quadriceps) at intervals of 21 days. Fourteen days after the last DNA injection, lymphocytes were isolated from the spleens and lymph nodes and incubated for 5 h with combinations of peptides Ag85A99-118(FPVLSTELPGWLOANKHVKPT) and Ag85A145-152(VVAGASMGL) or HspX1-20(MATTLPVQRHPRSLFPEFSE) or Rv340764-73(IPARRPQNL), and HspX1-20(MATTLPVQRHPRSLFPEFSE) or Rv340768-77(RPQNLLDVT) (7, 26, 32). During the final 4 h of culture, 10 μg/ml brefeldin A was added. The cultured cells were washed and incubated for 5 min with rat serum and anti-CD16/CD32 MAb to block FcR-mediated antibody binding. Subsequently, the cells were stained with phycoerythrin (PE)– and peridinin chlorophyll protein-conjugated anti-CD8 MAb and allophycocyanin-conjugated anti-CDM Mab (all from BD) (Franklin Lakes, NJ) and, after 15 min on ice, the cells were washed with PBS and fixed for 15 min at room temperature with PBS–2% paraformaldehyde. The cells were washed with PBS–0.1% BSA, permeabilized with PBS–0.1% BSA–0.5% saponin, and incubated in this buffer with rat serum and anti-CD16/CD32 MAb (BD). After 5 min, fluorescein isothiocyanate-conjugated anti-tumor necrosis factor alpha (anti-TNF-α) MAb (MPIB, Berlin, Germany) and PE-Cy7-conjugated anti-immunofluorescence (anti-IFN-γ) MAb (BD), alkaliphycocyanin-conjugated interleukin-2 (IL-2) MAb (eBioscience, San Diego, CA), and PE-conjugated CD40L MAb (eBioscience) were added. After a further 15 min at room temperature, the cells were washed with PBS and fixed with PBS–1% paraformaldehyde. The cells were analyzed with a FACS Canto flow cytometer and FACS Diva software (BD).
The T-cell responses were determined by intracellular staining for the cytokines IFN-γ, TNF-α, and IL-2. BALB/c mice vaccinated with V-2A showed strong antigen-specific T-cell responses in their spleens and lymph nodes (Fig. 3). High frequencies of CD4+ and CD8+ T cells producing IFN-γ, TNF-α, and IL-2 were detected in the V-2A-vaccinated group of mice but not in mice vaccinated with the naked vector or naïve mice. We detected a small proportion of polyfunctional T cells (secreting multiple cytokines) in the V-2A-vaccinated group but not in the mice vaccinated with the naked vector or naïve mice (data not shown). These results demonstrate that all V-2A-encoded antigens are coexpressed in vivo and elicit strong T-cell responses in the lymph nodes and spleen.

Humoral response induced by V-2A. Sera from six individual mice vaccinated three times i.m. with 100 μg DNA per mouse (50 μg per quadriceps) at intervals of 21 days were collected at 14, 21, and 35 days p.v. Their sera were analyzed by ELISA for IgG1 and IgG2a responses. On days 21 and 35, IgG2a responses against HspX were also detected. We did not detect Ag85A-specific IgG1 or IgG2 in the V-2A-vaccinated mice (Fig. 4).

V-2A vaccination-induced protection against M. tuberculosis in mice. In the case of DNA vaccination, the mice were challenged with M. tuberculosis by low-dose aerosol infection 4 weeks p.v. The mice were vaccinated three times i.m. at intervals of 21 days. BCG-vaccinated mice received 10^6 CFU subcutaneously 90 days prior to M. tuberculosis challenge. The M. tuberculosis titers in the lungs were determined 30, 60, and 90 days p.i. The mycobacterial loads were significantly reduced in the lungs of mice vaccinated with BCG and V-2A compared with those in the nonvaccinated controls. The numbers of CFU in the lungs of V-2A-vaccinated mice were reduced by 1.0 to 1.5 log units compared to those in the naive mice at 30, 60, and 90 days p.i. This protection was equivalent to the protection induced by BCG vaccination at days 30 and 90 p.i. and was better than that induced by BCG vaccination at day 60 p.i. (Fig. 5). These data serve as a proof of concept for the protective efficacy of the V-2A vaccination strategy. Further explorations by use of a prime-boost regimen should be considered.

FIG. 1. V-2A–GFP/V-2A-coexpressing Mycobacterium tuberculosis antigens and GFP. (A) Map and principle of V-2A–GFP/V-2A. After the removal of the stop codons of Rv3407, Ag85A, and HspX, these genes were linked to GFP with the FMDV 2A sequence. Expression is under the control of the CMV promoter. Transcription leads to the formation of one long mRNA transcript. Posttranslational cleavage of the 2A sequence causes segmentation of the long translated polypeptide into individual proteins. (B) Protein coexpression by V-2A. HEK293 cells were transiently transfected with V-2A and a control vector. Protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions; transferred to a polyvinylidene difluoride membrane; and probed with biotinylated polyclonal antibodies to Rv3407 (Biogenes, Berlin, Germany), Ag85A, (MPIIB, Berlin, Germany), and HspX (Affinity Bioreagents), followed by addition of streptavidin-conjugated horseradish peroxidase (MPIIB), and then developed with an ECL Western blotting analysis system (Amersham Biosciences). Bands of 11 kDa, 30 kDa, and 14 kDa corresponding to Rv3407, Ag85A, and HspX, respectively, were detected in the lysates of cells transfected with V-2A but not in the lysates of cells transfected with the control vectors.
DISCUSSION

Two major avenues of vaccine design against TB are currently being pursued (20): first, viable vaccines either as gene deletion mutants of \( M. \) \( \text{tuberculosis} \) or as recombinant BCG expressing additional antigens or immunomodulators and, second, subunit vaccines as protein adjuvant formulations, naked DNA vaccines, or viral carriers, such as modified vaccinia virus Ankara (24) and nonreplicating adenoviral vectors (29). The various efficacies and failure of protection against pulmonary TB and the fatal dissemination of diseases in immunocompromised patients, notably, AIDS patients (15), have raised the question of vaccine safety in areas of endemicity such as Africa, where AIDS and TB coexist. For these reasons, efforts to develop a subunit vaccine have increased during the last decade (1, 4, 27, 38). The advantages of subunit vaccines delivered as protein, DNA, or nonreplicating viral vectors over BCG include their safety profiles as well as the lack of tissue damage or scarring. This permits their repeated use as booster doses to maintain effective T-cell memory. Important factors include the extent of the T-cell repertoire required to protect individuals from diverse human populations against TB and a universally effective delivery vehicle which will stimulate the necessary broad antigen spectrum of CD4+ and CD8+ T cells.

Thus far, subunit vaccines based on fusion proteins, such as ESAT-6–Ag85B and Mtb72F, have entered clinical trials (33, 38).

In previous studies, subunit vaccines were shown to induce lower levels of protection and protection rarely equal to that induced by BCG. This has been attributed to the limited antigen repertoire of these vaccines, which is restricted to a single antigen or to a maximum of two antigens in the case of fusion proteins. More recently, it has been shown that vaccines based on a mixture of immunodominant antigens can induce levels of protection similar to those induced by BCG (1, 14, 17, 38). Grover et al. (14) compared the protective efficacies of Ag85B, CFP10, and CFP21, all of which are considered immunodominant antigens of \( M. \) \( \text{tuberculosis} \). That group tested these antigens both as single formulations and as a combination of all three antigens. They observed that the multivalent vaccine had a higher degree of efficacy than the single antigens. We have compared Rv3405 as a single antigen to V-2A and found that V-2A had a higher degree of protective efficacy in mice (data not shown).

To overcome the limitation of a restricted immune response expressed by DNA vaccines expressing a single antigen, we have utilized the self-splicing features of the 2A sequences of FMDV (10, 28) to generate a DNA vaccine construct containing multiple antigens. By harnessing the special sequences from FMDV, we were able to express four full-length proteins (three \( M. \) \( \text{tuberculosis} \) antigens and one GFP) from a single ORF under a single promoter (Fig. 1A). Our results demonstrate that all four genes from V-2A–GFP were coexpressed equally (Fig. 1 and 2). By conventional techniques, it is technically difficult to fuse more than two proteins, since this frequently leads to the unstable expression of the fusion proteins, and for larger proteins, incorrect folding can become a critical issue. The use of the 2A sequences allowed us to successfully express four antigens without the compromise of either expression stability or protein folding. We assume that the use of bidirectional promoters to further increase the number of antigens that can be expressed can be explored.

So far we have generated V-2A containing three \( M. \) \( \text{tuberculosis} \) antigens: Rv3407, Ag85A, and HspX. Our results provide a proof of concept for the coexpression of three genes in a single vector. To explore the possibility of adding more genes, we fused GFP with the 2A sequence downstream of HspX at position four of V-2A to generate V-2A–GFP. The use of GFP allowed the direct in vitro verification of expression by fluorescence microscopy. The successful expression of GFP at this position (Fig. 2A) demonstrated the possibility of expressing at least four genes by use of this vector system. The level of expression of GFP was shown to be comparable irre-
ated by interactions between antigen-specific T cells and macrophages (12) and that different cytokines promote these interactions. Although a broad spectrum of cytokines contributes to protection, the type 1 response, which is dominated by IFN-γ, TNF-α, and IL-2 secretion, is considered critical for protective immunity against TB (9, 12).

We detected IFN-γ, TNF-α, and IL-2 secretion by antigen-specific CD4⁺ and CD8⁺ T cells after vaccination with V-2A.
A small proportion of these cells secreted multiple cytokines. Immunity against all V-2A-encoded antigens demonstrated the proper expression of these antigens in vivo. The immune response to V-2A vaccination should establish long-lasting immunological memory and should give rise to a strong and robust secondary response upon challenge with *M. tuberculosis*. This notion is supported by our findings of multifunctional T cells coexpressing IFN-γ, IL-2, and TNF-α. Studies with other systems indicate the importance of such T cells for protective immunity (6). Accordingly, the lungs of mice vaccinated with V-2A showed significant reductions in pulmonary CFU levels at 30, 60, and 90 days p.i. The protection achieved by the V-2A construct was equal to the protection offered by BCG at days 30 and 90 p.i. and better than that offered by BCG at day 60 p.i. We therefore conclude that in the V-2A combination vaccine, the antigens did not interfere with each other and, hence, did not affect each others’ protective potential.

The high degree of protective efficacy of V-2A can be attributed to the rapid expansion of antigen-specific IFN-γ, TNF-α, and IL-2-secreting T cells early in the course of infection (19, 25). We believe that a combination of immunodominant antigens, when they are presented to the immune system by mimicking natural infection, can activate relevant immune components, in addition to cytokine secretion, and therefore prove more efficacious than a single-antigen vaccine or a mixture of single-antigen-based vaccines. In this regard, the selection of appropriate antigens for the next generation of multicomponent vaccines against TB is an important goal. The subunit vaccines currently under development are almost exclusively based on the early antigens secreted by the replicating bacteria and recognized in the first stage of infection. For a successful subunit vaccine, late-stage antigens induced during the dormant stage (metabolically inactive *M. tuberculosis* during latent infection) or the resuscitation stage (i.e., the transition from low to high bacterial metabolic activity prior to disease reactivation) need to be incorporated to achieve the maximum impact on all stages of *M. tuberculosis* infection.

The novel strategy of coexpressing multiple proteins under the control of a single promoter constitutes a new perspective for DNA vaccination against TB. In addition to being cost-
effective and less time-consuming, the delivery of multiple proteins in a single vaccine construct has the potential advantage of inducing a more profound response than the use of the same proteins separately. Furthermore, different immunodominant antigen combinations can be tested and the best possible combinations can be determined by the Mann-Whitney test.

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