Published in final edited form as:

Vaccine. 2009 March 13; 27(12): 1816–1824. doi:10.1016/j.vaccine.2009.01.119.

Vaccination Strategies to Enhance Local Immunity and Protection Against *Mycobacterium tuberculosis*

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Abstract

To determine the immunogenicity and protective efficacy of the *Mycobacterium tuberculosis* 10 kD culture filtrate protein (CFP10), and to evaluate strategies that enhance local immunity, we used C57Bl/6 DR4 mice that were transgenic for human HLA DRB1*0401, because CFP10 contains epitopes for DRB1*0401 but not for C57Bl/6 mice. Intramuscular immunization with a DNA vaccine encoding CFP10 elicited production of IFN- γ by systemic CD4⁺ T cells, and one intravenous dose of the CFP10-based DNA vaccine coated with polyethylenimine (PEI) stimulated IFN- γ production by lung CD4⁺ cells and reduced the pulmonary bacillary burden. We conclude that CFP10 is a potential vaccine candidate and that coating vaccines with PEI enhances local protective immunity to tuberculosis.

Keywords

CFP10; Tuberculosis; Local immunity

Introduction

Development of an effective vaccine against *Mycobacterium tuberculosis* is a critical priority to reduce the worldwide morbidity and mortality from tuberculosis. Most evidence indicates that secreted *M. tuberculosis* antigens stimulate protective immunity [1]. Two important secreted proteins are the 6 kD early stage antigen for T cells (ESAT-6) and the 10 kD culture filtrate protein (CFP10), which stimulate T-cells to produce IFN- γ and exhibit CTL activity, both in animal models and in humans infected with *M. tuberculosis* [2;3;4;5]. ESAT-6 and CFP10 are encoded by the RD1 region of the *M. tuberculosis* genome, which is deleted from *M. bovis* BCG, and restoration of RD1 enhanced the capacity of BCG vaccination to protect against subsequent infection with *M. tuberculosis* [6]. Thus, evaluating the immunogenicity

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and protective efficacy of secreted proteins encoded by RD1 is important for development of subunit and DNA antituberculosis vaccines.

Protective immunity against *M. tuberculosis* hinges on adaptive immune responses that are mediated primarily by CD4⁺ T cells, although CD8⁺ T cells are also likely to contribute to immunity [4;7–15]. In HIV infection, the degree of reduction in CD4⁺ cell counts correlates strongly with the extent of susceptibility to mycobacterial infections, including tuberculosis. Moreover, compared to other pathogens, the local immune response by CD4⁺ T cells to *M. tuberculosis* in the lungs is significantly delayed, allowing establishment of infection and progression to disease [16].

CFP10 is considered a major antigen that is recognized by T cells from people infected with *M. tuberculosis* [17;18]. We recently identified several immunogenic regions in CFP10 for humans and characterized CFP10 peptides that elicit IFN- γ production and CTL activity by CD4⁺ T-cells. These peptides are recognized in the context of multiple HLA molecules, and more than one epitope bound to DRB1*0401 HLA allele with high affinity [19]. In contrast, Kamath et al showed that CFP10 does not contain epitopes for CD4⁺ or CD8⁺ T cells from C57BL/6 mice [20]. To test the capacity of CFP10 to elicit immunogenicity and protection against tuberculosis *in vivo* through activating CD4⁺ T cells, we used C57Bl/6 mice that lacked the antigen recognition domains of murine MHC class II but were transgenic for human DRB1*0401 (DR4 mice). We also compared the immunogenicity and protective efficacy of different strategies to enhance local immunity, to direct CFP10 peptides to the class II processing and presentation pathway, and to deliver vaccine constructs to the lung.

MATERIAL AND METHODS

Mice

We used 6–8 week-old specific pathogen-free wild type C57Bl/6 or the previously described C57BL/6NTac-[KO]*Abb*-[Tg]DR-4 (DR4) [21]. Mice were either purchased from Taconic Farms or bred at the University of Texas Health Center at Tyler, where all experiments were carried out according to protocols approved by the Institutional Animal Care and Use Committee.

Mycobacterial antigens

Cell culture grade PPD was purchased (Statens Serum Institute, Copenhagen, Denmark). His-tagged CFP10 was produced by propagating the pMRLB46 plasmid expressing Rv3874 in *Escherichia coli*, followed by purification of CFP10 by a standard protocol. The pMRLB46 plasmid and protein purification protocol were provided by Colorado State University through the TB Vaccine Testing and Research Materials contract. CFP10_{71–90} peptide was synthesized commercially (Invitrogen) and reconstituted with DMSO.

Plasmid constructs

The open reading frame of *M. tuberculosis* encoding CFP10 (Rv3874) was cloned into the pcDNA3.1(+) eukaryotic expression vector (Invitrogen) using *Kpn*I and *Xba*I flanking restriction sites (Fig. 1). Based on the genomic sequence of *M. tuberculosis* H37Rv, the gene encoding CFP10 was amplified with sequence-specific primers, the 3' primer containing a TAG stop codon (Fig. 1). To generate fusion constructs, the CFP10 sequence was amplified from genomic DNA of H37Rv and flanked by *Kpn*I and *Eco*RI restriction sites. LAMP-1-C-terminal and LIMP-2-C-terminal sequences were amplified from cDNA of DR4 splenocytes, using sequence-specific primers. LAMP-1 and LIMP-2 sequences were flanked by *Eco*RI (5'-end) and *Xba*I (3'-end) and inserted in frame with the CFP10 sequence (Fig. 1). Plasmid constructs were verified by sequence analysis.

DNA immunization

Plasmid DNA was purified using the EndoFree® Plasmid Giga Kit (Qiagen), and its endotoxin level, measured by the Limulus Amebocyte Lysate assay, was below the limit of detection. Endotoxin-free plasmid was re-suspended in PBS at 1 mg/mL for subcutaneous inoculation, or at 2.5 mg/mL for intranasal immunization. For subcutaneous or intranasal immunization, DR4 mice were immunized twice with 100 or 50 µl of DNA, respectively, 3 weeks apart. In experiments in which linear polyethylenimine (PEI, Bridge Bioscience) was used, the plasmid was diluted in 5% glucose to 1 mg/mL, 100 µg of plasmid DNA was mixed with PEI solution according to manufacturer's instruction, and 200 µl of the mixture containing 100 µg of plasmid DNA was administered once, either intranasally, s.c. or i.v. via the retro-orbital route [22].

Isolation of splenocytes and lung mononuclear cells

Mice were sacrificed, their organs were harvested and passed through a 40 µm strainer (BD Biosciences) followed by red blood cell lysis. Cells were cultured in complete medium, containing RPMI1640 (Invitrogen), 100 mM sodium pyruvate, 100 U/mL penicillin/streptomycin, 100 mM minimal non-essential amino acids, 10 mM HEPES, 50 µM 2-mercaptoethanol, and 10% heat inactivated fetal bovine serum (Atlanta Biologicals).

Measurement of the frequency of IFN-γ-producing cells

Splenocytes were cultured in 24-well plates at 6×10^6 cells/well with either peptide CFP10₇₁₋₉₀ (10 µg/ml), CFP10 (5 µg/ml), PPD (5 µg/ml) or in complete medium only as a negative control. After 48 h incubation at 37°C, CD4⁺ cells were separated by positive selection using magnetic beads conjugated to anti-CD4 (Miltenyi Biotech) and placed on an ELISPOT plate that was precoated with anti-IFN-γ (Clone AN-18, Mabtech). After 48 h incubation, ELISPOT plates were developed according to manufacturer's instruction (Mabtech). The spots were counted using a stereomicroscope. In some experiments, CD4⁺ cells were separated from freshly harvested splenocytes or lung mononuclear cells of *M. tuberculosis*-infected mice and seeded directly onto the ELISPOT plate for 48 h, along with Ag-pulsed and irradiated splenocytes (5×10^4 cells/well) from uninfected naïve mice.

Measurement of IFN-γ concentrations

Splenocytes (6×10^6 cells/well) were cultured in the presence of 10 µg/mL of peptide CFP10₇₁₋₉₀, CFP10 protein (5 µg/mL) or medium alone in a 24-well plate and incubated at 37°C. Supernatants were collected after 72 h, and stored at -70°C. IFN-γ concentrations were measured by sandwich ELISA, using matched Ab pairs (BD Biosciences).

Infection of mice with *M. tuberculosis*

Mice were infected with 25–100 CFU of *M. tuberculosis* H37Rv by using an aerosol exposure chamber, made to order by the University of Wisconsin. Infected mice were sacrificed at different time points, and several dilutions of homogenates of the lungs, mediastinal lymph nodes and spleens were plated on 7H10 medium. Inoculated 7H10 plates were incubated at 37°C and 5% CO₂, and CFU were counted after 14–21 days.

Statistical analysis

All statistical analysis was done with Prism software (GraphPad Software, Inc. San Diego, CA). Student's t-test was used to evaluate differences between two groups, and ANOVA was used for analysis of variance. A p value <0.05 was considered statistically significant.

RESULTS

CFP10 is recognized by CD4⁺ cells from DR4 mice

It has previously been shown that T-cells from C57Bl/6 mice do not recognize CFP10 [20]. In a pilot study, we infected C57Bl/6 and DR4 mice with aerosolized *M. tuberculosis* H37Rv. The burden of bacteria was similar in both groups at all time points, peaking at 4–5 weeks post infection, and reaching a plateau by 8–9 weeks post infection. The pathologic findings in the lung, as determined by staining with hematoxylin and eosin, were also similar in both groups (data not shown).

DR4 mice express human HLA-DRB1*0401 but not the Ag recognition domains of murine MHC class II molecules. To investigate whether CFP10 can be processed and presented by DR4 mice during natural infection, we infected wild type C57Bl/6 and DR4 mice with *M. tuberculosis* by aerosol. After 5 and 9 weeks, mice were sacrificed, their splenocytes and CD4⁺ T cells were stimulated with mycobacterial Ags, and an ELISPOT assay was used to detect IFN- γ -producing cells (Fig. 2). Infected DR4 mice showed a high precursor frequency of CD4⁺ T cells that produced IFN- γ in response to CFP10 and CFP10_{71–90}, a peptide that contains at least two epitopes recognized in the context of DRB1*0401 [19]. In contrast, the numbers of CFP10-responsive IFN- γ -producing cells were at baseline levels in infected C57Bl/6 mice. This was not due to an overall inability of cells from C57Bl/6 mice to respond to mycobacterial Ags, as splenocytes and CD4⁺ cells from C57Bl/6 and DR4 mice produced IFN- γ in response to PPD.

Immunogenicity of a pcDNA3.1+ expression vector encoding CFP10

DR4 mice were vaccinated with a eukaryotic expression vector, pcDNA3.1(+), encoding CFP10. As seen in Fig. 3A, the frequency of IFN- γ -producing CFP10-responsive CD4⁺ T cells in the spleen was significantly increased after two subcutaneous immunizations with a plasmid encoding CFP10, but not by intranasal immunization with the same construct. The number of IFN- γ -producing cells was much lower than that induced after natural infection (compare Figs. 2 and 3A). Neither subcutaneous nor intranasal immunization produced detectable IFN- γ responses in the lungs (data not shown).

Lysosomal targeting to increase the immunogenicity of DNA immunization

DNA vaccines are delivered to target cells, and the encoded antigens are expressed in the cytosol, favoring processing and presentation through the class I pathway. Because CD4⁺ T cells are pivotal in protecting against *M. tuberculosis*, we used lysosomal targeting to direct the DNA vaccine antigens to the class II processing and presentation pathway [23–25]. DR4 mice were immunized subcutaneously with pcDNA3.1(+) expressing CFP10, alone or fused with either lysosome-associated membrane protein 1 (LAMP-1) or lysosomal integral membrane protein 2 (LIMP-2). Three weeks after the second immunization, immunogenicity was assessed by ELISPOT. As shown in Fig. 3B, fusion of CFP10 to LAMP-1 did not improve the immunogenicity of CFP10, while its fusion to LIMP-2 yielded a robust splenic CD4⁺ T cell response that was three-fold higher than that induced by the CFP10 construct alone. However, subcutaneous immunization with the CFP10 LAMP-1 or LIMP-2 constructs did not elicit detectable CD4⁺ T cell IFN- γ responses in the lungs of immunized mice (data not shown).

PEI greatly enhances the pulmonary immune response to DNA vaccination

Delivery of plasmid DNA to relevant anatomic sites and survival of plasmid DNA in host cells significantly influence the outcome of DNA vaccination. For efficient antigen expression, it is critical for the plasmid to survive lysosomal degradation and to enter the nucleus. Recent reports suggest that PEI-coated DNA exhibits 400-fold greater expression of Ag than naked

DNA [22;26]. In addition, intravenous immunization of mice with PEI-coated DNA significantly enhanced Ag expression in the lung [22]. Hence, we evaluated the efficacy of coating our DNA vaccine candidate expressing CFP10 with PEI. When DR4 mice were immunized subcutaneously once with the CFP10-LIMP-2 construct, either in the presence or absence of PEI, there was no significant IFN- γ response (Fig. 4A). However, when the PEI-coated CFP10-LIMP-2 construct was administered once intravenously, the number of IFN- γ -producing splenic CD4⁺ cells was comparable to that obtained with two subcutaneous immunizations with the CFP10-LIMP-2 construct without PEI (compare Figs. 4A and 3B). Furthermore, when splenic CD4⁺ T cells of mice immunized with the PEI-coated construct were stimulated *in vitro* with CFP10 or CFP10₇₁₋₉₀, high levels of IFN- γ were produced (data not shown).

The most striking effect of intravenous administration of the PEI-coated vaccine was the generation of an extremely high frequency of pulmonary CFP10-specific IFN- γ -producing cells, constituting approximately 1% of all CD4⁺ cells in the lungs (Fig. 4B). Subcutaneous administration of the PEI-coated vaccine and intravenous immunization with the CFP10-LIMP-2 construct that was not coated with PEI did not elicit IFN- γ -producing cells in the lungs. When the CFP10-LIMP-2 construct coated with PEI was given intranasally, the number of CFP10-responsive IFN- γ -producing CD4⁺ cells in the spleen and lung were low (data not shown), compared to values obtained by intravenous immunization with the same construct (Fig. 4).

Protective efficacy of the PEI-coated DNA vaccine

Because the PEI-coated DNA vaccine elicited a strong response by CD4⁺ T-cells in the lung, we wished to determine if this vaccine would protect against challenge with *M. tuberculosis*. We immunized DR4 mice with different DNA vaccine constructs, and then infected them by aerosol with virulent *M. tuberculosis* H37Rv.

A single intravenous immunization with the PEI-coated DNA vaccine expressing CFP10 protein fused to LIMP-2 was the only intervention that significantly reduced CFU in the lungs by approximately 90% (Fig. 5A). In contrast, two subcutaneous immunizations with the CFP10-LIMP-2 construct did not affect the mycobacterial burden. The PEI-coated DNA vaccine also significantly reduced CFU in the mediastinal lymph nodes (Fig. 5B) but not in the spleen (Fig. 5C).

To determine the potential mechanisms that underlie the protective efficacy of the PEI-coated DNA vaccine, we isolated CD4⁺ cells from lungs, lymph nodes and spleens of vaccinated DR4 mice that had been challenged with *M. tuberculosis*. CD4⁺ cells were stimulated with naïve irradiated splenocytes as APCs, pulsed with either PPD or CFP10 *in vitro*, and the number of IFN- γ -producing cells was measured by ELISPOT. After 4 weeks of infection, mice vaccinated with the PEI-coated CFP10-LIMP-2 construct had significantly higher numbers of IFN- γ -producing CFP10-responsive CD4⁺ T cells per lung than those immunized with the PEI-coated empty plasmid (Fig. 6). However, there were no significant differences in the number of CFP-10-responsive IFN- γ -producing CD4⁺ T cells in the mediastinal lymph nodes or spleens of mice immunized with the vaccine or control constructs. In addition, PPD-induced IFN- γ responses were similar in both groups of mice.

Phenotype of CD4⁺ T cells after vaccination with the PEI construct

To determine the phenotype of CD4⁺ T cells in mice immunized with the PEI-coated vaccine after challenge with *M. tuberculosis*, we measured the percentages of central memory cells (CD62L^{hi}CD44^{hi}), effector memory cells (CD62L^{lo}CD44^{hi}) and naïve cells (CD62L^{hi}CD44^{lo}). Two weeks after infection, most cells in the lungs and spleens were naïve

cells, with relatively few effector memory and central memory cells (Fig. 7). However, four weeks after infection, the majority of CD4⁺ T cells in the lungs showed the effector memory phenotype, and this percentage was significantly higher in mice immunized with the PEI-coated vaccine than in those immunized with PEI-coated control empty pcDNA. The percentage of effector memory cells was also significantly higher in the lymph nodes but not in the spleens of animals that received the PEI-coated vaccine. The percentages of central memory cells in all organs increased from two to four weeks after infection, but there were no significant differences between mice immunized with the CFP10-expressing PEI-coated vaccine and those immunized with PEI-coated control pcDNA.

Discussion

CFP10 is a small mycobacterial protein that is recognized by most persons who are infected with *M. tuberculosis* [4;5;18;19], in part because it contains promiscuous epitopes that bind with high affinity to multiple HLA alleles [19;27]. However, CFP10 has no epitopes for C57Bl/6 mice [20], the most commonly used strain for the study of the immune response to *M. tuberculosis*. By evaluating the response to CFP10 in DR4 C57Bl/6 mice, the current report demonstrated several important findings. First, we found that the course of infection with aerosolized *M. tuberculosis* was similar in DR4 and C57Bl/6 mice and that CD4⁺ cells from DR4 mice produce IFN- γ in response to CFP10 during natural *M. tuberculosis* infection, indicating that they recognize epitopes in the context of human DRB1*0401. Second, immunization with a DNA vaccine encoding CFP10 elicited production of IFN- γ by systemic CD4⁺ cells which was enhanced by lysosomal targeting using a vaccine construct encoding a fusion protein of CFP10 and LIMP-2. Finally, a single intravenous dose of the CFP10-based DNA vaccine coated with PEI was the only strategy that yielded a high frequency of IFN- γ -producing CD4⁺ cells in the lungs and a significant reduction in the pulmonary bacillary burden. These findings indicate that CFP10 elicits protective immunity by CD4⁺ T cells and is a potential vaccine candidate. In addition, we conclude that coating vaccines with PEI can enhance the local pulmonary immune response to mycobacterial antigens.

CD4⁺ T cells play a pivotal role in the human immune response to tuberculosis, and optimal vaccination strategies hinge on a comprehensive understanding of the capacity of specific mycobacterial Ags and epitopes to elicit protective T cell responses. To address this issue, we took advantage of a transgenic mouse that expresses human HLA-DRB1*0401, which has previously been used to study human metabolic diseases. DR4 mice express chimeric MHC class II molecules on their APCs, with the Ag-binding domains of DRB1*0401, and the remaining domains from murine IE^d- α and - β chains [21], allowing recognition of Ag in the context of DRB1*0401, but signaling and interactions with murine CD4 through murine portions of the MHC molecule. To our knowledge, this is the first report in which human HLA-transgenic animals have been used to evaluate the potency of a vaccine candidate and to evaluate the effects of different vaccine delivery systems on the systemic and local immune response to a pulmonary pathogen. T cells from C57Bl/6 mice have previously been reported not to recognize CFP10 or its peptides [20], and we confirmed that CD4⁺ cells from *M. tuberculosis*-infected C57Bl/6 mice did not produce IFN- γ in response to CFP10 (Fig. 2). In contrast, approximately 0.3–0.5% of all CD4⁺ splenic T-cells from infected DR4 mice produced IFN- γ in response to CFP10 or its CFP10₇₁₋₉₀ peptide (Fig. 2), indicating that these animals recognized Ag in the context of DRB1*0401.

Local immunity is critical for protection against infection by gastrointestinal and pulmonary pathogens [28]. Vaccination with live attenuated oral polio virus, influenza A virus, and *Salmonella* results in effective mucosal immunity, as well as systemic humoral and cellular immune responses [29]. Also, establishment of *M. tuberculosis* infection in the lungs has been attributed to delayed initiation of adaptive immunity, especially by CD4⁺ T cells [16].

Therefore, local immunity in the lung is likely to play a critical role in protecting against tuberculosis. Previous studies have demonstrated that intranasal DNA vaccination efficiently deposits DNA plasmid in lungs [30] and can generate potent cell mediated immune responses in murine models [31]. However, in our hands immunization with naked DNA intranasally induced no response.

As an alternative means to enhance the local pulmonary immune response, we coated our DNA vaccine constructs with PEI, a cationic polymer that compacts DNA by electrostatic interactions, protecting it from enzymatic degradation and providing an overall positive charge that facilitates uptake by cells and enhances transfection efficiency [32]. PEI is particularly effective at delivering complexed DNA to pulmonary tissue [33]. In the current report, PEI induced a high frequency of IFN- γ -producing CFP10-specific splenic CD4+ T-cell after a single intravenous immunization, comparable to results obtained with two subcutaneous immunizations with the non-PEI-coated LIMP-2 construct (Figs. 4A and 3B). Furthermore, intravenous administration of PEI-coated DNA was the only method that elicited a vigorous CFP10-specific pulmonary response, with approximately 1% of lung CD4+ cells producing IFN- γ , compared to 0.2% of splenic CD4+ cells (Fig. 4). This potent local response was only induced by intravenous, but not by subcutaneous, administration of the PEI-coated CFP10-based DNA vaccine (Fig. 4B), as subcutaneous administration would not be expected to deliver CFP10 to the lung. It was notable that intranasal immunization with the PEI-coated vaccine yielded a minimal pulmonary and systemic CD4+ IFN- γ response (data not shown) perhaps because through the intranasal route vaccine did not reach the lungs and/or it was taken up by nasal associated lymphoid tissues, rather than being deposited in the lower airways.

For several antituberculosis vaccines, the magnitude of the systemic IFN- γ response does not correlate with the capacity to lower the burden of organisms after challenge with *M. tuberculosis* [34;35]. Similarly, subcutaneous administration of the LIMP-2 CFP10 fusion construct did not elicit protective immunity (Fig. 5), despite a substantial splenic CD4+ T-cell IFN- γ response (Fig. 3B). The PEI-coated DNA vaccine, the only preparation to induce a strong pulmonary immune response, significantly reduced the lung and lymph node bacillary burdens (Fig. 5). This vaccine also increased the percentage of CD4+ effector memory cells in the lungs (Fig. 7), suggesting that these cells contribute to inhibition of bacterial growth in the early stages of infection. The PEI-coated vaccine did not reduce the splenic bacillary burden, indicating that a mucosal response alone does not prevent bacterial dissemination and replication in other organs. We speculate that combining a PEI-coated vaccine with other strategies to foster strong systemic immune responses could reduce extrapulmonary bacillary growth. Alternatively, or in addition, coating vaccines that encode several immunogenic *M. tuberculosis* Ags, such as ESAT-6, Ag 85 and CFP10 with PEI, may elicit a more potent mucosal immune response that could drastically lower the pulmonary and systemic burden of organisms. Additional studies are needed to evaluate these strategies. Although PEI-coated DNA vaccines in their current form cannot readily be used in humans, our data provide proof of principle that mechanisms that enhance pulmonary delivery of DNA vaccines can protect against pulmonary tuberculosis.

In summary, we demonstrated that DR4 mice transgenic for human HLA DRB1*0401 could be used to demonstrate the capacity of vaccination with CFP10 to elicit production of IFN- γ by Ag-reactive CD4+ cells. Lysosomal targeting by fusion of CFP10 to LIMP-2, combined with PEI coating to deliver the vaccine to the lungs, yielded a potent pulmonary immune response and reduced the bacillary burden after challenge with *M. tuberculosis*. These findings indicate that CFP10 is a potential vaccine candidate, and that coating vaccines with PEI is an effective means to enhance the local mucosal immune response to mycobacterial infection.

Acknowledgments

The authors thank Dr. Joanne Flynn for helpful discussions. The pMRLB46 plasmid encoding CFP10 was provided by Colorado State University, under the TB Vaccine Testing and Research Materials contract.

This work was supported by grants from the American Lung Association (RG-846-N to H. Shams), the National Institute of Health (AI064898 to H. Shams and A1063514 to P.F. Barnes), Flight Attendant Medical Research Institute (052338-Clinical Innovator Award to H. Shams), and the Margaret E. Byers Cain Chair for Tuberculosis Research and the James Byers Cain Research Endowment (both to P.F. Barnes).

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CFP10

ATG GCA GAG ATG AAG ACC GAT GCC GCT ACC CTC GGG CAG GAG GCA GGT AAT TTC GAG CGG ATC TCC
M A E M K T D A A T L G Q E A G N F E R I S

GGC GAC CTG AAA ACC CAG ATC GAC CAG GTG GAG TCG ACG GCA GGT TCG TTG CAG GGC CAG
G D L K T Q I D Q V E S T A G S L Q G Q

TGG CGC GGC GCG GCG GGG ACG GCC GCC CAG GCC GCG GTG GTG CGC TTC CAA GAA GCA GCC
W R G A A G T A A Q A V V R F Q E A A

AAT AAG CAG AAG CAG GAA CTC GAC GAG ATC TCG ACG AAT ATT CGT CAG GCC GGC GTC CAA
N K Q K Q E L D E I S T N I R Q A G V Q

TAC TCG AGG GCC GAC GAG GAG CAG CAG CAG GCG CTG TCC TCG CAA ATG GGC TTC TAG
Y S R A D E E Q Q A L S Q M G F STOP

LAMP I - Tail

ATG TTG ATC CCC ATT GCT GTG GGC GGT GCC CTG GCA GGG CTG ATC CTC ATC GTC CTC ATT GCC TAC
M L I P I A V G G A L A G L I L I V L I A Y

CTC ATT GGC AGG AAG AGG AGT CAC GCC GGC TAT CAG ACC ATC TAG
L I G R K R S H A G Y Q T I STOP

LIMP II - Tail

CGA GGA CAG GGG TCT ATG GAT GAG GGA ACT GCA GAT GAA AGA GCA CCC CTC ATA CGA ACC TAA
R G Q G S M D E G T A D E R A P L I R T STOP

Figure 1. Construction of DNA vaccines that express CFP10

The open reading frame encoding *M. tuberculosis* CFP10 (Rv3874) was cloned into the insertion site of the pcDNA3.1(+) eukaryotic expression vector. To generate fusion constructs, the CFP10 sequence was amplified and flanked by *KpnI* and *EcoRI* restriction sites. LAMP-1 and LIMP-2 C-terminal sequences were amplified from cDNA of DR4 mouse splenocytes, and inserted into pcDNA3.1(+), in frame with Rv3874, flanked by *EcoRI* and *XbaI*.

Figure 2. Evaluating the capacity of CD4⁺ splenocytes from wild type C57Bl/6 and DR4 mice to recognize CFP10 during *M. tuberculosis* infection

DR4 and wild type C57Bl/6 mice were each infected with 50–100 *M. tuberculosis* bacteria by aerosol. After 5 and 9 weeks, mice were sacrificed, and their splenocytes, or immunomagnetically selected CD4⁺ T cells and APCs from uninfected mice, were cultured in the presence or absence of mycobacterial Ags. The frequency of IFN-γ-producing cells was determined by ELISPOT. Mean values and SE for 5–6 mice per group are shown. Experiments for each mouse were done in triplicate. Depicted data are representative of four independent experiments.

Figure 3. Immunogenicity of different pcDNA3.1+ constructs expressing CFP10

DR4 mice were immunized twice, 3 weeks apart, with 100 μ g of pcDNA3.1(+), encoding different CFP10 constructs. Three weeks after the second immunization, mice were sacrificed and splenocytes were cultured in medium alone (No Ag) or with CFP10 (5 μ g/ml). After 48 h, CD4⁺ cells were isolated by positive immunomagnetic selection and the frequency of IFN- γ -producing cells was measured by ELISPOT. A) Mice were immunized intranasally (i.n.) or s.c. with either empty plasmid or with pcDNA3.1(+) expressing CFP10. B) Mice were immunized with either empty pcDNA3.1(+) or pcDNA3.1(+) encoding the proteins shown. Experiments for each group were done in triplicate and mean values and SE are shown. Depicted data are representative of two independent experiments and five mice were used per group.

Figure 4. Effect of coating DNA vaccine constructs with PEI on splenic (A) and lung (B) CD4 $^{+}$ cell production of IFN- γ in response to CFP10

DR4 mice were immunized once, either s.c. or i.v., with either empty pcDNA3.1(+) plasmid coated with PEI, or pcDNA3.1(+) expressing CFP10-LIMP-2, with or without coating with PEI. Two weeks after immunization, mice were sacrificed, and their lung and spleen CD4 $^{+}$ T cells were isolated by positive immunomagnetic selection. Purified CD4 $^{+}$ cells were co-cultured with irradiated splenocytes from naïve mice as APCs, in medium alone, or with CFP10₇₁₋₉₀ (10 μ g/ml) or CFP10 (5 μ g/ml). The frequency of IFN- γ producing cells was measured by ELISPOT. Mean values and SE for 3–5 mice per group are shown. The experiments were performed three times with comparable results.

Figure 5. Effect of coating DNA vaccine constructs with PEI on the bacillary burden after challenge with *M. tuberculosis*

DR4 mice were immunized s.c. twice, 3 weeks apart, with the pcDNA3.1(+) constructs shown. Another group of mice were immunized i.v. only once with pcDNA3.1(+) expressing CFP10-LIMP-2, coated with PEI. Two weeks after the last immunization, all groups were infected via aerosol with 25–50 bacteria per mouse. Four weeks after infection, mice were sacrificed and their organs were removed, homogenized and plated to enumerate CFU. Each dot represents the mean value for an individual mouse. The horizontal line shows the mean value for each group. Depicted data are representative of two independent experiments with comparable results (* $p = 0.03$, ** $p = 0.01$).

Figure 6. Effect of immunization with PEI-coated DNA vaccine constructs on production of IFN- γ after challenge with *M. tuberculosis*

DR4 mice were immunized once i.v., with either empty pcDNA3.1(+) plasmid, or pcDNA3.1(+) expressing CFP10-LIMP-2, both coated with PEI. Two weeks after immunization, mice were each infected via aerosol with 25–50 bacteria. Four weeks after infection, mice were sacrificed, and their lung, mediastinal lymph node and splenic CD4⁺ T cells were isolated by positive immunomagnetic selection, and cultured with irradiated splenocytes from naïve mice as APCs, pulsed with PPD or CFP10, both at 5 μ g/ml. The frequency of IFN- γ producing cells was measured by ELISPOT. Each spot shows the result for an individual mouse and the

horizontal line shows the mean value for each group. Experiments for each mouse were done in triplicate. * $p = 0.01$. A representative result of two independent experiments is shown.

Figure 7. Phenotype of CD4⁺ T cells after vaccination with the PEI-coated DNA vaccine constructs and infection with *M. tuberculosis*

DR4 mice were immunized once i.v., with either empty pcDNA3.1(+) plasmid, or pcDNA3.1(+) expressing CFP10-LIMP-2, both coated with PEI. Two weeks after immunization, mice were each infected via aerosol with 25–50 bacteria. Two and four weeks after infection, mice were sacrificed, and their lungs, mediastinal lymph nodes and spleens were removed. The phenotype of CD4⁺ T cells was defined by staining with Abs to CD44 and CD62L, followed by cytofluorometric analysis. Each spot represents an individual mouse. The horizontal line shows the mean value.