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Discovery of dormancy associated antigens of Mycobacterium tuberculosis : novel targets for the development of post-exposure or therapeutic tuberculosis vaccines

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Chapter 7

Pulmonary delivery of DNA encoding *Mycobacterium tuberculosis* latency antigen Rv1733c associated to PLGA-PEI nanoparticles enhances T cell responses in a DNA prime/protein boost vaccination regimen in mice

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Abstract

During persistent infection and hypoxic-stress, *Mycobacterium tuberculosis* (*M. tuberculosis*) expresses a series of *M. tuberculosis* latency antigens. The aim of this study was to evaluate the immunogenicity of a DNA vaccine encoding the *M. tuberculosis* latency antigen Rv1733c and to explore the effect of pulmonary delivery and co-formulation with poly (D,L-lactide-*co*-glycolide) (PLGA) - polyethyleneimine (PEI) nanoparticles (np) on host immunity. Characterization studies indicated that PLGA-PEI np kept their nanometer size after concentration and were positively charged. The np were able to mature human dendritic cells and stimulated them to secrete IL-12 and TNF-α comparable to levels observed after lipopolysaccharide (LPS) stimulation. *M. tuberculosis* latency antigen Rv1733c DNA prime combined with Rv1733c protein boost enhanced T cell proliferation and IFN-γ secretion in mice in response to Rv1733c and *M. tuberculosis* hypoxic lysate. Rv1733c-DNA adsorbed to PLGA-PEI np and applied to the lungs increased T cell proliferation and IFN-γ production more potently compared to the same vaccinations given intramuscularly. The strongest immunogenicity was obtained by pulmonary priming with np-adsorbed Rv1733c DNA followed by boosting with Rv1733c protein. These results confirm that PLGA-PEI np is an efficient DNA vaccine delivery system to enhance T cell responses through pulmonary delivery in a DNA prime/protein boost vaccine regimen.

Introduction

Tuberculosis (TB) is the second leading cause of death from an infectious disease, following HIV-1 infection. Two million people die from TB each year while one-third of the world's population is latently infected with the tubercle bacillus. Although the majority (90-95%) of these people does not become ill, they present an immense reservoir of latently infected individuals who may progress to active TB disease when their immune system becomes affected (e.g. upon HIV-1 infection) (1, 2). Therefore, it is highly important to explore the development of post-exposure or therapeutic TB vaccines that can protect already infected individuals against endogenous reactivation of TB disease (3-5).
Persisting tubercle bacilli are ordinarily contained within granulomatous lesions in infected tissues and shift to a dormancy state with reduced metabolic activity that enables them to survive in conditions of nutrient and oxygen deprivation and nitric oxide stimulation (6-8). In vitro expression profiling studies of tubercle bacilli have shown that in this state, a regulon is modulated of about 48 genes that are under the control of its putative regulatory factor DosR (Rv3133c) (8, 9). Recently, we observed preferential recognition of these so-called TB latency antigens in individuals with latent *Mycobacterium tuberculosis* (*M. tuberculosis*) infection compared to TB-patients. In particular the DosR regulon encoded antigens Rv1733c, Rv2029c, Rv2627c and Rv2628 induced strong IFN-γ responses in latently infected individuals. These results suggested that these antigens are targets of the immune system during persistent *M. tuberculosis* infection and may thus be of interest as potential vaccine candidates to help protecting individuals already infected with *M. tuberculosis* (10, 11). DosR antigen Rv1733c was shown to be the most immunodominant amongst the *M. tuberculosis* infected individuals tested and responses to this single antigen were significantly higher in individuals with latent *M. tuberculosis* infection compared to those in TB patients (10). Recent vaccination studies of plasmid DNA encoding latency antigens confirmed their immunogenicity also in mice models (12). Based on the human data and the preliminary mice studies, Rv1733c was selected as a DNA vaccine antigen for detailed studies here.
DNA vaccines against tuberculosis are widely explored by virtue of their ability to induce strong cellular immunity, which is needed for TB control (13, 14). Although there are some conflicting reports regarding the efficiency of DNA vaccines for the immunotherapy of tuberculosis in animal infection models, it is believed that the combination of DNA vaccines with drug treatment can help prevent re-infection and reactivation of the disease in already infected populations (15).
Despite the fact that DNA vaccines have shown promising results in animal models (13, 16, 17), low immunogenicity has been observed in human clinical trials so far (18, 19). Thus, strategies are needed to enhance the immunogenicity of DNA vaccines. These include: improving transfection of host cells and antigen expression; augmenting antigen presentation; enhancing co-stimulation and increasing T lymphocyte expansion (reviewed in (20)). Several novel delivery systems and application methods are being developed to potentiate the performance of DNA vaccines, aiming at enhanced transfection of host cells (21). One of the best-studied

materials for vaccine delivery is the biocompatible and biodegradable polymer poly-D,L-lactide-*co*-glycolide (PLGA). PLGA microparticles prepared with the cationic surfactant cetyltrimethylammonium bromide (CTAB) enhanced the potency of anti-HIV DNA vaccines in a non-human primate model (22) and increased the potency of a tuberculosis DNA vaccine in mice (23). The immuno-potentiating effect of these particles was caused by increasing DNA persistence and recruiting phagocytes to the injection site, activating antigen-presenting cells (APCs) and directly priming dendritic cells (DCs) (24, 25). In a similar approach, we previously synthesized PLGA nanoparticles (np) with polyethyleneimine (PEI) moieties on their surface that could efficiently adsorb DNA. These np were internalized by human bronchial epithelial cells in culture and resulted in protein expression, indicating their ability to serve as a DNA delivery system for pulmonary administration (26).
In this study PLGA-PEI np were further characterized as to their stability and their effect on human-derived DCs. The immunogenicity of plasmid DNA encoding the *M. tuberculosis* latency antigen Rv1733c was explored in mice in a prime-boost vaccination regimen, by combining pulmonary delivery and the use of PLGA-PEI np formulation.

Materials and Methods

Materials

D, L-Lactide/glycolide copolymer (PLGA, PURASORB®, DL663FL, molar ratio: 53/47, inherent viscosity 0.69dl/g) was a generous gift from PURAC (Gorinchem, The Netherlands). Twenty-five kDa branched water-free polyethyleneimine (PEI) and Tween-80® were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Poloxamer 188 (Lutrol F68) was purchased from BASF (Ludwigshafen, Germany). Acetone of analytical grade was from Fisher chemicals (Leicestershire, UK) and dichloromethane of analytical grade from Biosolve BV (Valkenswaard, The Netherlands). Tris/Boric acid/EDTA buffer (TBE), agarose gel and loading buffer were obtained from Bio-Rad Laboratories (Veenendaal, The Netherlands).

Preparation of DNA loaded PLGA-PEI np

PLGA-PEI np were prepared as previously described (26). A solution of 10% (w/v) PLGA in dichloromethane was stirred for 30 minutes. PEI solution in acetone was prepared to a final concentration of 1% (w/v). PEI was added to the PLGA solution to achieve a PLGA-PEI ratio of 10:1, Tween-80® added to a final concentration of 1% (w/v) and acetone added up to 10ml. This organic phase was mixed and poured into an aqueous phase of 20ml of 0.5% (w/v) Poloxamer-188 in milli-Q®. The suspension was stirred overnight at room temperature to evaporate the organic solvents and filtered through 4-7µm retention size paper filter ($595^{1/2}$ folded filters, Schleicher & Schuell, s'Hertogenbosch, The Netherlands) to remove large polymer particles. The filtered np were loaded with V1Jns encoding *M. tuberculosis* Antigen 85B (Ag85B) by adding np suspension to 25µg/ml DNA plasmid solution (the amount of np was determined according to the requested PEI-DNA ratio). The mixture was vortexed for 30 seconds and left at room temperature for at least 1hr before use. We used Ag85B

plasmid as a *M. tuberculosis* DNA vaccine model for our *in vitro* characterization studies because of previous work performed in our laboratory and reagents available for analysis (27). The size, zeta potential, morphology, loading efficiency and ability to transfect human epithelial cells were extensively studied and previously reported (26).

Particle size and zeta potential measurements of concentrated formulations

Formulations were concentrated by centrifugation at 13,000 rpm for 20 minutes. The np were resuspended in saline to achieve a final concentration of 1µg/µl. To verify the physical stability of the np suspension after concentration, particle size and zeta potential measurements were performed using Zetasizer® 3000 HSA (Malvern Instruments, Bergen op Zoom, The Netherlands). Particle size was measured by photon correlation spectroscopy (PCS) at 25°C and a fixed 90° scattering angle. Zeta potential determinations were based on electrophoretic mobility of the np in the aqueous medium.

Generation of human monocyte derived dendritic cells (mo-DCs)

Immature mo-DCs were prepared by incubating human peripheral blood mononuclear cells (PBMC) for 15 minutes at 4°C with CD14 beads (MACS, Miltenyi Biotec, Germany). CD14 positive cells were separated using positive selection LS+ columns (Miltenyi Biotec, Germany) and cultured for 6 days in the presence of granulocyte macrophage colony-stimulating factor (GM-CSF, 1000 U/ml, Biosource, Etten-Leur, The Netherlands) and IL-4 (500 U/ml, Sanvertech, The Netherlands). Cells were harvested on day 7 and were applied with the DNA formulations.

Analysis of mo-DCs surface marker expression following exposure to PLGA-PEI formulation using fluorescence-activated cell sorter (FACS)

Immature mo-DCs were plated at a density of $5x10^5$ cells/well in 24-well plates and incubated with Ag85B plasmid DNA solution (1µg/well), PLGA-PEI np without DNA and np with Ag85B plasmid DNA (1µg/well) for 48 hours. Cells were incubated with culture medium in the absence of any formulation as negative control whereas cells incubated with lipopolysaccharide (LPS, 100ng/ml, Sigma, St. Louis, MO, USA) were used as a positive control. Surface marker expression was measured using saturation concentrations of PE conjugated antibodies for CD83 and CD80 and of FITC conjugated antibody for CD40 and CD86 (Becton Dickinson, Erembodegem, Belgium). Acquisition and analysis were performed using a FACS Calibur and with CellQuest™ software (Becton Dickinson, Erembodegem, Belgium).

Detection of IL-12p40 and TNF-α secreted from mo-DCs following exposure to PLGA-PEI formulations

Immature mo-DCs were plated at a density of $5x10^5$ cells/well in 96-well plates and incubated for 24 hours with the formulations described above. In addition, the effect of DNA dose on cytokine secretion was evaluated and 1, 2 or 5 µg DNA was added to the wells associated to PLGA-PEI np or as a solution. LPS (100 ng/mL) and medium

treated wells were used as a positive and negative control, respectively. Each experimental condition was set-up in triplicate and supernatants were collected and stored at -20°C until analysis. The IL-12p40 and TNF-α production were analyzed by Enzyme-Linked Immunosorbent Assay (ELISA; CytoSet,BioSource, Nivelles, Belgium) according to the manufacturer's instructions.

Loading PLGA-PEI np with plasmid DNA for in vivo studies
PLGA-PEI np were loaded with a Gateway® (Invitrogen) adapted pV1J.ns-tPA DNA plasmid encoding *M. tuberculosis* gene Rv1733c (13). Mock DNA plasmid control vector consisted of the same backbone, but with no insert sequence. 200µl PLGA-PEI np suspension was added to 250µl of 200 µg/ml DNA plasmid solution and vortexed for 30 seconds. This mixture gave a final PLGA-PEI ratio of 10:1 and a PEI-DNA ratio of 1:1 (w/w). Formulations were left overnight at room temperature and then centrifuged for 15 minutes at 12,000 rpm. Final DNA concentration adsorbed to np for *in vivo* application was 1 mg/ml.

Immunizations
BALB/c female mice, 6-8 weeks old at the first vaccination, were vaccinated three times at three weeks intervals. Mice were immunized intramuscularly (n=3) or by endotracheal aerosol application (n=5) with 50µg of DNA in solution or adsorbed to 550µg PLGA-PEI np. Non-invasive aerosol application was performed using the technique described by Bivas-Benita et al. (28) where mice were endotracheally intubated with the Penn-Century Microsprayer® (Penn-Century Inc., Philadelphia, Pennsylvania, USA) and the formulation sprayed directly in the airways. Boosting was performed three weeks after the last vaccination by intramuscular injection of 20µg of the Rv1733c protein in incomplete Freund's adjuvant (IFA; Difco Laboratories, Detroit, Michigan, USA) (29). Control mice were immunized subcutaneously with 20µg of the Rv1733c protein in IFA using the same vaccination regimen.

Preparation of antigens
Recombinant Rv1733c was produced as previously described (30). Briefly, the nucleotide sequence of Rv1733c was obtained from http://genolist.pasteur.fr/TubercuList. The gene was amplified by PCR from genomic DNA of *M. tuberculosis* H37Rv and cloned by Gateway Technology (Invitrogen, San Diego, CA, USA) in pDEST™17, a bacterial expression vector containing an N-terminal hexa-histidine tag for rapid purification with nickel-chelating resin. The proteins were over-expressed in *Escherichia coli* BL21(DE3) and purified as previously described (30). Sequencing was performed to confirm the identity of the cloned DNA fragment. Size and purity were checked by gel electrophoresis and Western blotting with anti-His antibodies (Invitrogen, Breda, the Netherlands). The residual endotoxin level was determined with a Limulus Amebocyte Lysate assay (Cambrex, Verviers, Belgium) and was found to be below 50 IU/mg recombinant protein. The protein batch was subsequently tested for non-specific T cell stimulation and for potential cellular toxicity in lymphocyte stimulation assays using PBMC of *M. tuberculosis* unexposed,

BCG unvaccinated, Mantoux skin test negative healthy donors. Recombinant Rv1733c was dissolved in PBS and used in a final concentration of 20 μg/ml.
In vitro, *M. tuberculosis* adapts to low oxygen stress, a condition the bacilli are exposed to during latent infection, by modulating the *dosR* regulon. For this study we used a lysate, which was prepared from *M. tuberculosis* H37Rv cultured under low oxygen conditions as a proxy for the antigenic repertoire that may be presented to the immune system during latent infection. Bacteria were grown for 24 hours in tubes with tightly screwed caps (low-oxygen cultures). Culturing was continued until an OD_{650} of ca. 0.5 was reached. Bacilli were harvested by centrifugation and lysed by using 0.1 mm glass beads as previously described (31-33). This lysate was precipitated with acetone and dialysed against phosphate-buffered saline. The total protein concentration of the resulting preparation was determined by the BCA test (Pierce, Rockford, Illinois, USA). The lysate was used in an end concentration of 1 μg/ml. The *M. tuberculosis* hypoxic lysate was kindly donated by Dr. Karen Weldingh and Dr. Peter Andersen (SSI, Denmark).

Proliferation assay
Spleens were harvested 3 weeks after the last vaccination or 10 days after the protein boost and ground with a 70μm nylon cell strainer (BD biosciences, Erembodegem, Belgium) to obtain a uniform single cell-suspension. Splenocytes were labeled at 10^7 cells/ml with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Leiden, The Netherlands) for 10 minutes at 37°C in PBS with 0.5% BSA (Sigma-Aldrich, Zwijndrecht, The Netherlands). Following labeling, 10% heat-inactivated fetal bovine serum (FBS; Greiner, Alphen a/d Rijn, The Netherlands) was added to the cell suspension and washed in PBS with 0.5% BSA. Splenocytes were resuspended in RPMI-1640 medium (Gibco, Breda, The Netherlands) supplemented with 10% FBS. Cells were added to 96-well U-bottom plates (Corning, Schiphol-Rijk, The Netherlands) at 3×10^5 cells/well and stimulated in triplicate with antigens. Seventy-two hours later, supernatants were taken; triplicates were pooled and stored at -20°C until IFN-γ was assayed. Cells of triplicates were pooled and washed with PBS with 0.1% BSA. Cells were evaluated by flow cytometry where cells gated from a live lymphocyte population were analyzed for CFSE proliferation. The Δ geometric mean was used as a measure of proliferation. The Δ geometric mean was obtained by subtracting the geometric mean CFSE fluorescence of the total population from the geometric mean CFSE fluorescence of the undivided cell population: Relative proliferation (or Δ geometric mean) = geometric mean (unproliferating cells) - geometric mean (total cells).
Relative proliferation was expressed as a percentage of the maximal proliferation measured using phytohaemagglutinin (PHA) stimulation (end concentration 2 μg/ml) and was corrected for spontaneous proliferation:

$$\left(\frac{[\Delta \text{ geometric mean sample - } \Delta \text{ geometric mean medium}]}{[\Delta \text{ geometric mean PHA- } \Delta \text{ geometric mean medium}]} \right) * 100 = \% \text{ of maximal proliferation}$$

IFN-γ analysis

Supernatants were evaluated for their IFN-γ content using IFN-γ CytoSet™ ELISA kit (Biosource, Etten-Leur, The Netherlands). The assay was performed according to the manufacturer's instructions. ELISA samples were tested in duplicate and the mean value of the unstimulated cells was subtracted from the mean value of the samples.

Statistical analysis

Statistical analysis of proliferation and IFN-γ secretion was performed using one-way ANOVA test. Multiple comparisons between the 9 immunized mice groups were performed by a subsequent Tukey's correction test. Statistical significance is represented by * ($P<0.05$), ** ($P<0.01$) and *** ($P<0.001$).

Results

Stability of concentrated formulations loaded with plasmid DNA

Concentration of the formulations is a first, but necessary step to prepare for the in vivo studies in which 50μg of DNA needs to be applied in 50μl volume to the airways. After centrifugation, resuspension of the np was performed using water or saline. Loading and concentration of the np resulted in a small mean size increase from 235nm to 275nm when resuspended in water and 271nm in saline (Table 1). After concentration, np remained positively charged, however, mean zeta potential was reduced from +64.3mV to +38.8mV after resuspension in water and to +40.6mV after resuspension in saline.

Table 1. Size and zeta (ζ) potential of np 10:1 PLGA-PEI ratio and 1:1 PEI-DNA ratio after concentrating formulations for in vivo application

	Size (nm)	ζ Potential (mV)
Not loaded, not concentrated	235	64.3
Loaded, concentrated, resuspended with H2O	27 ± 39	38.8 ± 10.9
Loaded, concentrated, resuspended with Saline	271 ± 20	40.6 ± 4.0

Values are mean averages ± S.D. of 3 separately loaded samples. All size measurements had a polydispersity index <0.2.

Effect of DNA loaded PLGA-PEI np on mo-DC maturation

PLGA-PEI np were evaluated for their ability to stimulate and induce maturation of human DCs in culture. The latter was evaluated by measuring the up-regulation of surface expression of the molecules CD40, CD80, CD83 and CD86 compared to unstimulated cultures. Exposure of mo-DCs to Ag85B DNA in solution or loaded onto PLGA-PEI np resulted in increased surface expression of all four markers to a level comparable to the increase noted in response to the positive control LPS (Figure 1). Incubation with non-loaded PLGA-PEI np did not cause DC maturation as levels of surface CD40, CD80, CD83 and CD86 were similar to the base line expression (Figure 1, 4th row).

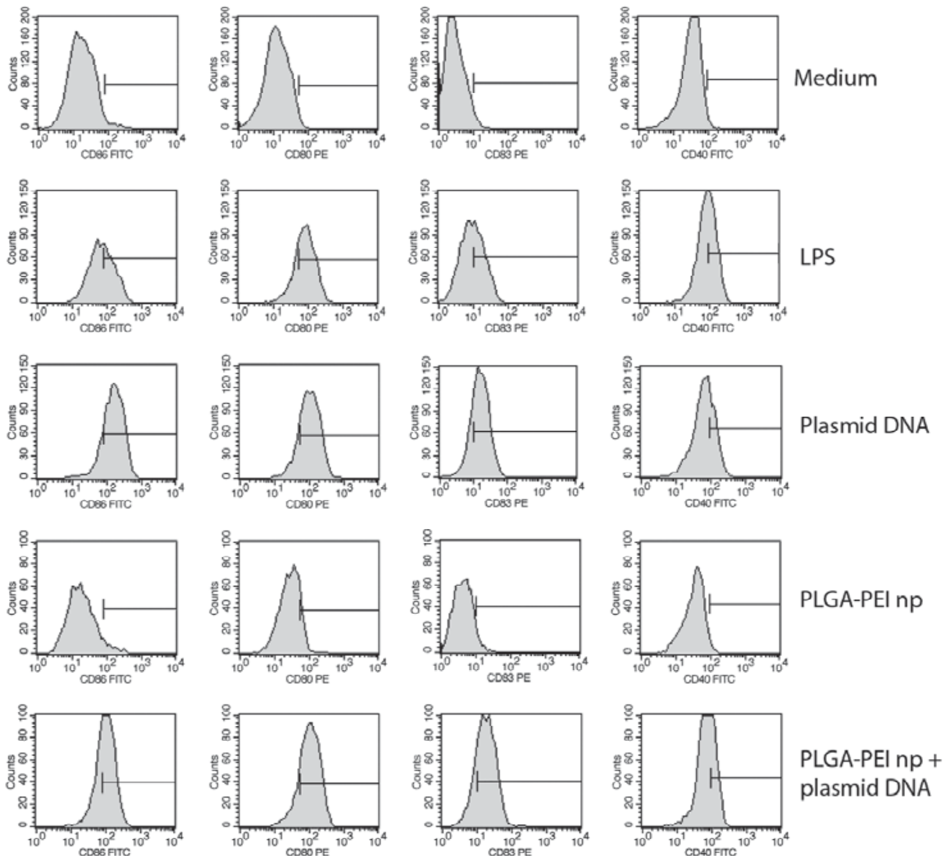

Figure 1. Maturation of mo-DCs following incubation with PLGA-PEI np formulations. *Surface markers on DCs were detected by FACS following incubation with FITC-labeled CD86 and CD40 MAb and PE-labeled CD80 and CD83 MAb.*

IL-12 and TNF-α secretion from mo-DCs following exposure to PLGA-PEI formulations

Activation of DCs can also be evaluated functionally by the secretion of cytokines. The levels of the stimulatory cytokines IL-12 (p40 subunit) and TNF-α were evaluated in 24 hours DCs culture supernatants by ELISA with detection limits of 0.4 and 0.28 ng/ml, respectively. IL-12 secretion was the highest after application of 1µg plasmid DNA adsorbed to PLGA-PEI np with values similar to those obtained after LPS stimulation (Figure 2). Increasing the dose of DNA adsorbed to the np resulted in reduced IL-12 production and after application of the 5µg DNA dose there was no detectable IL-12. Applying DNA in solution resulted in lower IL-12 levels than those obtained by 1µg DNA adsorbed to np but DCs exposed to PLGA-PEI np without DNA did not secrete any IL-12. Similar results were obtained for TNF-α where application of 1µg DNA adsorbed to PLGA-PEI np induced the highest TNF-α levels which were comparable to those obtained by the positive control, LPS (Figure 3).

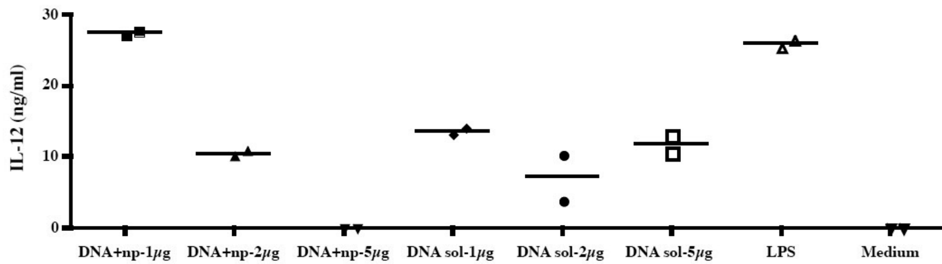

Figure 2. IL-12 secretion from mo-DCs following incubation (24hrs) with different formulations. *IL-12 levels were measured using ELISA and LPS was used as a positive control. The results are the mean of duplicate samples from one representative experiment out of two performed.*

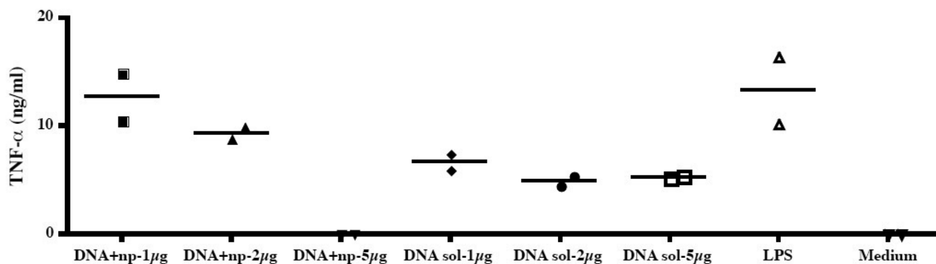

Figure 3. TNF-α secretion from mo-DCs following incubation (24 hrs) with different formulations. *TNF-α levels were measured using ELISA and LPS was used as a positive control. The results are the mean of duplicate samples from one representative experiment out of two performed.*

Immunogenicity of *M. tuberculosis* Rv1733c administered in different delivery regimens and formulations: proliferative responses

Proliferative responses of the splenocytes from the different groups of immunized animals were evaluated using CFSE based cell proliferation measured by flow cytometry. In vitro restimulation with recombinant Rv1733c antigen was used to evaluate antigen specific responses. In addition, *M. tuberculosis* hypoxic lysate was used as a proxy for the antigenic repertoire that may be presented to the immune system during latent infection. The responsiveness of the splenocytes to this stimulation indicates in vivo expression of the Rv1733c in bacilli grown under low oxygen conditions. The total proliferation of splenocyte cultures restimulated with Rv1733c was increased in all the groups that received the recombinant Rv1733c protein boost after the homologous DNA immunization, compared to DNA vaccination without protein boost (Figure 4a). Of interest was that the group that received Rv1733c plasmid DNA adsorbed to PLGA-PEI np endotracheally followed by a subsequent protein boost had significantly higher proliferative responses than when the same vaccination regimen was administered intramuscularly ($P<0.05$). In addition, this pulmonary vaccination regimen induced the strongest Rv1733c total

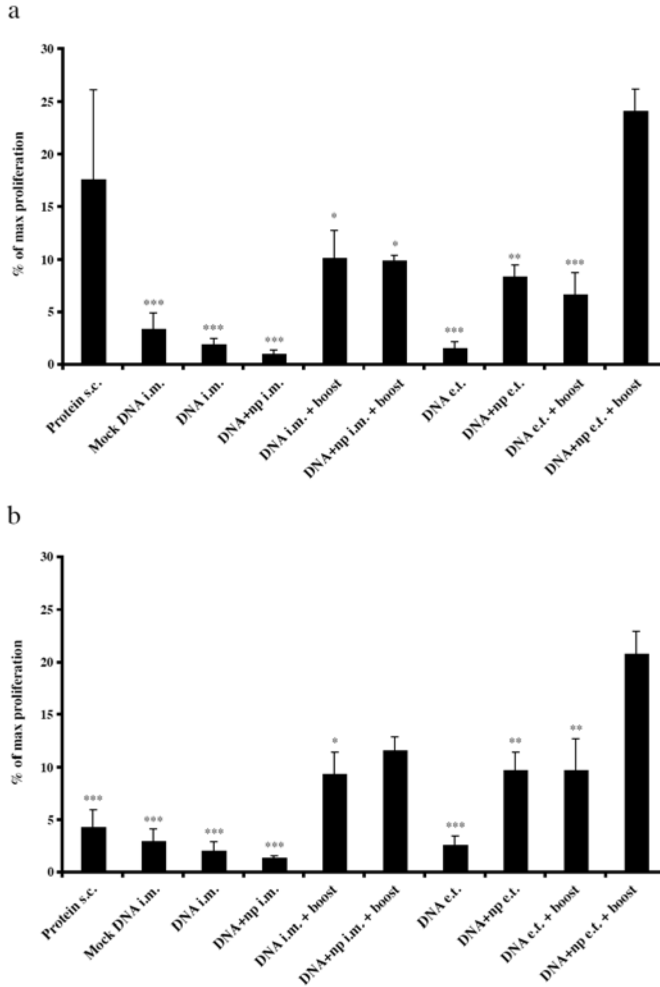

Figure 4. T cell proliferation of immunized mice in response to Rv1733c protein (a) and M. tuberculosis hypoxic lysate (b) restimulation. *Relative proliferation is represented as the mean percentage of the measured relative proliferation per group ± SEM (n=3-5). Statistical significant differences as compared to the DNA+np e.t. + boost vaccinated group are represented by * (P < 0.05), ** (P < 0.01) and *** (P < 0.001).*

proliferative T cell responses among all groups studied here. In order to exclude that inherent adjuvant activity through TLR-9 triggering by CpG motifs carried by the plasmid vector might have accounted for the observed Rv1733c responses, we also used empty non-coding vector DNA as a control. This did not induce significant proliferative (Figure 4a) responses, which indicates that responses are induced by the specific antigen encoding sequence in the context of the delivery platform.
Similar results for total proliferation were obtained after restimulation of splenocytes from the same mice with *M. tuberculosis* hypoxic lysate (Figure 4b). T cell proliferation

was again significantly increased after endotracheal immunization with Rv1733c DNA adsorbed to PLGA-PEI np followed by protein boost, in comparison to all the other groups studied.

Immunogenicity of Rv1733c administered in different vaccine regimens and formulations: IFN-γ responses

To further evaluate the cellular immune responses initiated in the vaccinated groups, splenocytes from immunized mice were restimulated with Rv1733c or *M. tuberculosis* hypoxic lysate for 72 hours, followed by IFN-γ analysis using ELISA.

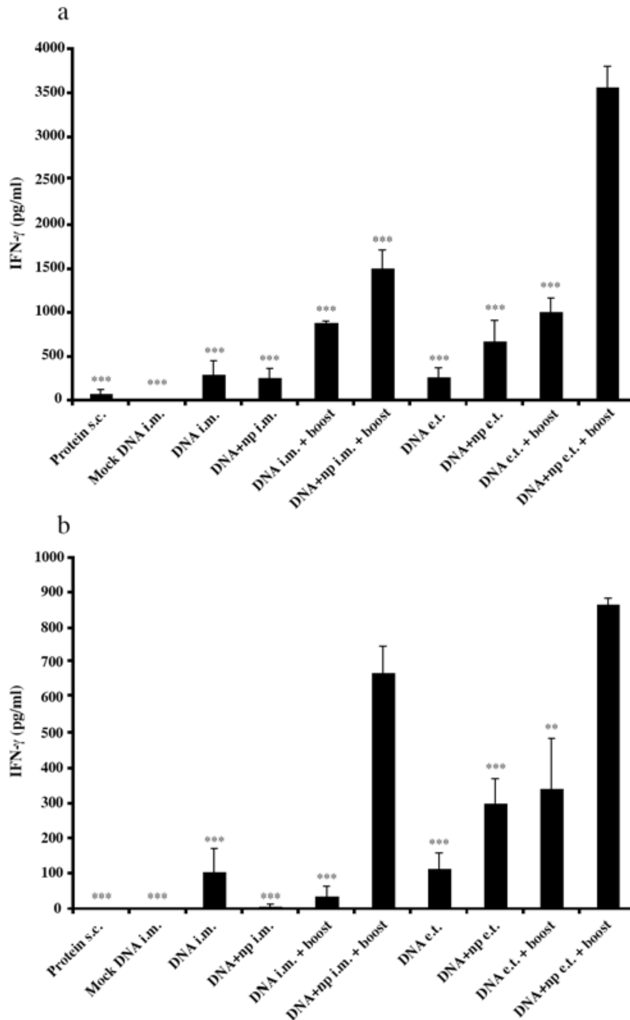

Figure 5. IFN-γ production from spleen cell cultures of immunized mice in response to Rv1733c protein (a) and M. tuberculosis hypoxic lysate (b)*. IFN-γ levels from splenocytes culture supernatants were measured by ELISA and represented as mean concentration values ± SEM (n=3-5). Statistical significant differences as compared to the DNA+np e.t. + boost vaccinated group are represented by * (P < 0.05), ** (P < 0.01) and *** (P < 0.001).*

IFN-γ production in response to restimulation with recombinant Rv1733c protein was elevated in the groups that received DNA followed by protein boost in both intramuscular and pulmonary applications, when compared to the corresponding groups without the protein boost (Figure 5a).
Moreover, PLGA-PEI np increased IFN-γ production in the protein-boosted groups, in comparison to the same vaccination regimen without the particles. The vaccination regimen of pulmonary priming with Rv1733c DNA vaccine adsorbed to PLGA-PEI np followed by a protein boost gave the highest levels of IFN-γ and was significantly different from all other immunized groups ($P<0.001$). Splenocytes from mice immunized with only recombinant Rv1733c protein produced low levels of IFN-γ, although they proliferated strongly (Figure 4a), suggesting the involvement of a non IFN-γ associated proliferative T cell component in the response to the protein only immunization.
The secretion of IFN-γ in response to *M. tuberculosis* hypoxic lysate restimulation was lower in magnitude but indicated a comparable trend (Figure 5b). In the vaccine regimens that included the protein boost, PLGA-PEI np showed higher production of IFN-γ in comparison to the groups receiving the DNA vaccine as a solution. Also here, pulmonary delivery of Rv1733c plasmid DNA induced higher levels of IFN-γ than intramuscular delivery after priming with DNA associated to np and boosting with protein. Like the results obtained after Rv1733c restimulation, splenocytes of protein-immunized animals responded poorly to stimulation with *M. tuberculosis* hypoxic lysate. Additionally, intramuscular administration of a mock DNA plasmid resulted in no IFN-γ secretion from splenocytes in response to either Rv1733c or *M. tuberculosis* hypoxic lysate.

Discussion

The 2 billion latently *M. tuberculosis* infected individuals worldwide represent an enormous reservoir of potential TB cases, since TB reactivation occurs in about 5-10% of latently infected people. Targeting mycobacterial antigens expressed by persisting *M. tuberculosis* bacilli represents a new strategy to help control latent infection and prevent TB disease reactivation. The *M. tuberculosis* DosR regulon encoded genes are such late stage specific genes. We have previously shown that responses to *M. tuberculosis* DosR antigen Rv1733c were significantly higher in individuals with latent *M. tuberculosis* infection compared to TB patients (10). These results suggest that responses to this antigen are associated with control of latent *M. tuberculosis* infection.
The present study therefore evaluates the immunogenicity of a DNA vaccine encoding *M. tuberculosis* latency antigen Rv1733c in mice in a prime-boost setting, employing mucosal application of a novel DNA formulation (26, 28).
We have previously developed PLGA-PEI np as a novel delivery platform for pulmonary DNA vaccination (26). We have now further improved this system by concentrating np to reach the desired DNA dose for mice. Particles kept their nanometer size and positive surface charge, which will enable adherence to the pulmonary mucosal membranes and subsequent cellular uptake. The effect of PLGA-

PEI np formulations on human DC maturation was evaluated next, by measuring the expression of the surface molecules CD40, CD80, CD83 and CD86. We showed that PLGA-PEI np by themselves were inert and did not cause an increase in surface markers while DNA-loaded np increased the expression of these markers to levels comparable to those seen after LPS activation. Similar stimulation was induced by empty control plasmid DNA, suggesting that plasmid DNA itself caused the activation, probably through Toll-like receptor 9 (TLR9) recognition and TANK-binding kinase-1 (TBK1)-dependent signalling (34). We observed equivalent DC maturation using different plasmid DNAs (encoding *M. tuberculosis* Ag85B, a mock plasmid vector and a comparable polyepitope plasmid DNA vector (35)), demonstrating that this stimulation is intrinsic to the bacterial DNA vector, independent of the insert characteristics. Franco et al. reported similar observations with *Mycobacterium leprae* HSP65 (36). These results thus bridge our human DC studies, using plasmid encoding *M. tuberculosis* Ag85B, with our mouse immunization studies using DNA plasmids encoding *M. tuberculosis* latency antigen Rv1733c.
The stimulatory function of DCs also depends on cytokine secretion. IL-12 and TNF-α are two essential cytokines for efficient microbial resistance (37-41). Our results show that PLGA-PEI np adsorbed with 1µg of plasmid DNA are able to induce similar levels of IL-12 and TNF-α secretion as LPS. Application of higher DNA doses adsorbed to PLGA-PEI np abolished the cytokine secretion, suggesting PEI induced toxicity. Since the PEI-DNA ratio was constant, an increased particles/DNA dose resulted in a higher PEI dose, which could lead to cell toxicity and decreased cell viability (26). This was strengthened by the observation that when DNA alone was given in the same amounts, it induced moderate cytokine secretion in each of the three DNA doses. These results imply that the enhanced cytokine secretion seen for PLGA-PEI np adsorbed with 1µg DNA is likely due to better cellular uptake (42).
Our strategies to improve DNA vaccine immunogenicity included DNA prime-protein boost vaccine regimens, previously shown to enhance immunogenicity for mycobacterial antigens such as Ag85A (29), HSP65 (43, 44), ESAT-6 (45) and MPB 70 (46). Using our new delivery platform, we observed enhanced cellular proliferation and IFN-γ secretion in mice that received the DNA prime followed by Rv1733c protein boost, in response to either Rv1733c protein or *M. tuberculosis* hypoxic lysate. These results emphasize the importance of protein boosting in DNA based vaccination regimens in order to optimize stimulation of cellular immunity. In future studies we plan to validate this strategy by evaluating its protective efficacy in (latent) *M. tuberculosis* infection models in mice.
In case of mucosal infectious diseases such as TB, immunization via the mucosal linings of the lung seems a highly relevant approach, since this can induce both systemic and local immune responses at the site of mycobacterial entry (47). Recent studies suggested that airway antigen delivery could also restore protective mucosal immunity following intramuscular DNA vaccine delivery (48). In the case of latent *M. tuberculosis* infection, boosting DCs and resident T cells in the lungs with relevant *M. tuberculosis* latency antigens could increase local immunity, and help control local dormant bacteria, thus preventing reactivation of the infection. We show here for the first time that Rv1733c DNA adsorbed to PLGA-PEI np applied to the lungs increased

proliferation and IFN-γ secretion in comparison to the same vaccination regimen given intramuscularly. By contrast, the immunogenic capacity of DNA alone was not affected by the application method. This strongly suggests that np are taken up more efficiently by the lung mucosa compared to muscle tissue, thus enhancing immunity to *M. tuberculosis* Rv1733c. Since PLGA-PEI np are positively charged they adhere better to negatively charged cellular membranes, thus promoting intracellular uptake and subsequent protein expression in human airway epithelial cells as we have observed previously (26). Our in vivo results support the in vitro observations, suggesting that DNA adsorbed to the delivery system could promote its survival in the mucosal environment against degradative enzymes, and thus result in protein expression within the pulmonary tissue.
The immunogenic potential of the DNA/PLGA-PEI np applied to the lungs followed by a protein boost could be a result of different T cell subsets stimulation, like $CD4^+$ T cells, which are stimulated more vigorously following a protein boost (29) and could synergize to achieve higher antigen specific responses. Another population that could contribute to the increased cell proliferation and IFN-γ secretion is the antigen specific memory T cell population (49). The effect of pulmonary application of PLGA-PEI np carrying DNA vaccine on DCs in vivo and on specific T cell populations will be an important aspect of future studies.
Taken together, these data demonstrate that the immunogenicity of DNA vaccines can be strongly enhanced in case of pulmonary delivery by formulating the DNA with PLGA-PEI np, followed by protein boosting. Further studies will need to elucidate the exact mechanisms responsible for the increased immunogenicity following pulmonary vaccination via PLGA-PEI np. This work may help to design better vaccines against mucosal infectious diseases, such as TB.

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