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 4

Lack of immune responses to *Mycobacterium tuberculosis* DosR regulon proteins following *Mycobacterium bovis* BCG vaccination.

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Abstract

BCG is widely used as a vaccine against tuberculosis (TB) despite its variable protective efficacy. Relatively little is known about the immune response profiles following BCG vaccination in relation to protection against TB. Here we tested whether BCG vaccination results in immune responses to DosR (Rv3133c) regulon encoded proteins. These so-called TB latency antigens are targeted by the immune system during persistent *Mycobacterium tuberculosis* infection and have been associated with immunity against latent *M. tuberculosis* infection.

In silico analysis of the DosR regulon in BCG and *M. tuberculosis* showed at least 97% homology in amino acid sequence, with 41 out of 48 genes being identical. Transcriptional profiling of 14 different BCG strains, under hypoxia and nitric oxide exposure *in vitro,* revealed a functional DosR regulon similar to that observed in *M. tuberculosis*.

Next, we assessed human immune responses to a series of immuno-dominant TB latency antigens and found that BCG vaccination fails to induce significant responses to latency antigens. Similar results were found in BCG vaccinated BALB/c mice. In contrast, responses to latency antigens were observed in individuals with suspected exposure to TB (as indicated by positive IFNγ responses to TB specific antigens ESAT-6 and CFP-10), and in mice vaccinated with plasmid DNA encoding selected latency antigens.

Since immune responses to TB latency antigens have been associated with control of latent *M. tuberculosis* infection, our findings support the development of vaccination strategies incorporating DosR regulon antigens to complement and improve the current BCG vaccine.

Introduction

Tuberculosis (TB) remains a major global health threat. Each year about eight million new TB cases occur and two million people die from TB. It is estimated that one third of the world population is latently infected with *Mycobacterium tuberculosis*. From this vast latent reservoir about 10% of infected people are expected to develop overt TB disease during their lifetime. However, with the expanding HIV-1/AIDS pandemic this number is expected to soar in the next few decades (11,42).

The current TB vaccine is the live attenuated bacterium *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG). BCG is known to protect against severe forms of TB in young children and against leprosy. However, it does not efficiently and consistently protect against pulmonary TB in adults, the most prevalent and contagious form of TB; neither does BCG offer protection from reactivation of latent TB infection. This partly explains why BCG has little impact on the global TB epidemic despite its widespread use as a prophylactic TB vaccine (43). Over the years many hypotheses have been put forward to explain the apparent variability in protective efficacy of BCG, which varies from 0 to 80% (16). Explanations for this inconsistency include differences in trial methodology, population host genetics, use of different BCG vaccine strains (2) and heterogeneous immunity to a variety of environmental mycobacteria that may interfere with, or mask the protection provided by BCG (7,26).

Immune response profiles following BCG vaccination comprise myriad effector mechanisms, multiple T-cell subsets and many targeted antigens. BCG is capable of inducing Th1 responses (38), which are critical in mycobacterial infections (17). In addition, BCG is also capable of inducing both $CD4^+$ and $CD8^+$ T cell responses to antigens shared with *M. tuberculosis*, such as secreted antigens of the mycolyl transferase family (Ag85) (19,20,33) and non-deleted members of the ESAT-6 family (*e.g.* TB10.4) (32), but also heat shock proteins like Hsp65 and Hsp70 (15). However, it is still not completely known how these and other antigen-specific immune responses contribute to protection against TB.

Recently we studied human T-cell responses to DosR (Rv3133c) regulon encoded antigens (referred to as TB latency antigens) of *M. tuberculosis* (24). We observed preferential recognition of latency antigens by Mantoux skin test positive individuals with latent TB compared to patients with TB disease, suggesting that these immune responses are associated with latent TB disease (14,24). The DosR regulon is expressed by tubercle bacilli under *in vitro* conditions of hypoxia and low dose nitric oxide exposure (40), and in IFNγ-activated macrophages (30). These conditions are thought to mimic the environment encountered by tubercle bacilli *in vivo* when persisting in immuno-competent hosts (31).

One of the most abundantly produced proteins during hypoxia is the 16-kDa α-crystallin homolog HspX (Rv2031c) (12). HspX is part of the DosR regulated genes and is targeted by both $CD4^+$ T cells (9,41) and $CD8^+$ T cells (10). Interestingly, infants vaccinated with BCG do not mount immune responses to HspX, whereas they are capable of generating immune responses to other TB antigens (39). The absence of immune responses to this particular latency antigen led us to the formulation of our hypothesis. In this study we tested the hypothesis that BCG vaccination in humans and

in a mouse model fails to induce immune responses to not only HspX but generally to TB latency antigens that are targeted by the immune system during latent *M. tuberculosis* infection.

Materials and Methods

In vitro expression of the BCG DosR regulon

Expression of the DosR regulon by *M. tuberculosis* strain H37Rv and 14 different BCG strains (Connaught (JPG), Pasteur 140, Sweden, Connaught, Japan, Canada, Vietnam, Danish SSI 1331, Russian, Brazil, Tice, Moscow, Pasteur 133A and Pasteur 1173, (kindly provided by Dr. Angelo Izzo, Colorado State University, USA) was determined using standard methods of microarray RNA expression analysis as previously described (40). Briefly, mycobacteria were grown in 7H9 medium (supplemented with bovine serum albumin, NaCl, glucose, and glycerol) in 250 ml vented tissue culture flasks and shaking 90 rpm. At a culture OD 0.15, 30 ml cultures were placed in an anaerobic GasPak chamber (Becton, Dickinson and Company) and continued to shake. Reference cultures were treated in the same manner but not placed in the anaerobic chamber. After four hours RNA was isolated and microarray analysis was conducted as previously reported (40) with TB oligonucleotide microarrays provided by Colorado State University through the Tuberculosis Vaccine Testing and Research Materials contract.

Study subjects

Cross sectional study (The Netherlands) comprising 43 healthy adult persons (10 male, 33 female) who were vaccinated with BCG between 1 and 37 years before this study (median time after vaccination 25 years). The age at the time of blood sampling ranged from 20-60 years. Thirty-eight individuals were of Dutch origin; the remaining 5 individuals were of German, British, Netherlands Antillean, Philippine and South Korean descent. Blood samples were obtained from all study subjects after written informed consent was obtained, using standard venous puncture and blood collection in heparinized tubes. Subsequently, peripheral blood mononuclear cells (PBMC) were isolated using a Ficoll density gradient and stored in liquid nitrogen as described previously (24). The study protocol (P207/99) was approved by the ethical review board of the Leiden University Medical Centre.

Longitudinal BCG vaccination study (United Kingdom) comprising adolescents who were recruited through the UK schools' BCG vaccination Programme (4). Year 8 pupils (age 12-13 years) were informed verbally and by an information sheet of the study and recruitment proceeded following written informed consent from parent or guardian and verbal consent from the child. Exclusion criteria were evidence of previous BCG vaccination (BCG scar or vaccination records) or serious illness. Ethnical backgrounds of participants were as follows: White British (30%), White Irish (3%), Black Caribbean (22%), Black African (13%), Black other (7%), Asian Pakistan (12%), Asian other (5%) and mixed/other (8%).

Ethical approval for this study was given by the Local Research Ethics Committee of Redbridge and Waltham Forest Health Authority (R&WFHA) and by the Ethics Committee of the London School of Hygiene & Tropical Medicine.
Baseline blood samples were collected from participants at the time of tuberculin skin testing. Skin testing was carried out on the volar surface of the forearm using the Heaf technique with tuberculin PPD. A baseline of 10 ml of intravenous blood was taken and transferred into tubes containing 100 U of preservative-free sodium heparin (Monoparin, CP Pharmaceuticals Ltd. Wrexham, UK). Blood samples were set up in the whole blood assay as soon as possible on the same day as venapuncture. The Heaf test induration was inspected after 7 days and graded appropriately by experienced nurses. Those due to receive BCG following a negative Heaf test received BCG immediately (n=22). Two to four weeks following vaccination, depending upon school availability, recruits were revisited for a follow-up, 10 ml blood sample.

M. tuberculosis antigens

Recombinant proteins were produced as previously described (18). Briefly, nucleotide sequences of selected *M. tuberculosis* H37Rv genes were obtained from http://genolist.pasteur.fr/TubercuList. Genes were amplified by PCR from genomic DNA of *M. tuberculosis* H37Rv and cloned by Gateway Technology (Invitrogen, San Diego, CA) 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 (18). Sequencing was performed to confirm the identity of the cloned DNA fragments. Size and purity were checked by gel electrophoresis and Western blotting with anti-His antibodies (Invitrogen). Residual endotoxin levels were determined with a Limulus Amebocyte Lysate assay (Cambrex) and were found to be below 50 IU/mg recombinant protein. Protein batches were 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.
Mycobacterium tuberculosis lysate, grown under low oxygen conditions (MTB), was obtained from growing *M. tuberculosis* H37Rv for 24 hours in tubes with tightly screwed caps as previously described (29). The low oxygen derived *M. tuberculosis* lysate was kindly provided by Dr Karen Weldingh and Dr Peter Andersen (Statens Serum Institute (SSI), Copenhagen, Denmark).

In vitro proliferation assays

Lymphocyte stimulation assays (cross-sectional BCG vaccination study) were performed using isolated PBMC as previously described (24). Briefly, PBMC (1.5×10^5/well) were cultured in Iscove's modified DMEM (Gibco, Paisley, UK) supplemented with 10% pooled human serum and 40 U/ml penicillin and 40 μg/ml streptomycin, in 96-well round-bottom microtiter plates (Nunc, Roskilde, Denmark) at 37°C, 5% CO_2, in the absence or presence of stimulant. Antigens were tested in the following concentrations: latency antigens, ESAT-6, CFP-10 and Ag85B all at 0.33 μM, *M. tuberculosis* hypoxic lysate and purified protein derivative (PPD) of *M. tuberculosis* (batch RT49 SSI, Denmark) at 5 μg/ml and positive control phytohaemagglutinin

(PHA) (Remel, UK) at 2 μg/ml. Total volume was 200 μl/well. All stimulations were performed in triplicate. At day 6, supernatants were harvested (75 μl/well, pooled per triplicate) and stored at maximally −20°C until use in IFNγ detection assay.
Diluted whole blood assay (longitudinal BCG vaccination study), was used as previously described (3). In summary, whole blood was diluted 1 in 5 with serum-free medium (RPMI 1640 supplemented with 2 mM L-Glutamine, Invitrogen) and 100 μl was plated in 96-well, round-bottomed tissue culture plates (Nunc, Roskilde, Denmark). Antigen was also added in 100 μl to give a final whole blood dilution of 1:10 and culture volume of 200 μl. Medium and PHA (5 μg/ml; Remel, UK) containing wells were included as negative and positive controls respectively, in parallel with *M. tuberculosis* antigens at 5 μg/ml: purified protein derivative (PPD) of *M. tuberculosis* (batch RT49, lot 210; SSI, Denmark), Rv1733c, Rv2029c, Rv2623, Rv2627c, Rv2628, Ag85A and ESAT6/CFP10 fusion protein. Cell cultures were incubated on the day of blood collection at 37˚C with 5% CO_2. Supernatants were harvested on day 6 and stored at maximally -20˚C prior to ELISA.

Detection of IFNγ by ELISA
Cross-sectional study: IFNγ concentrations in the supernatants were measured by ELISA (U-CyTech, Utrecht, The Netherlands). The detection limit of the assay was 20 pg/ml. ELISA samples were tested in duplicate. The mean value of unstimulated cultures was subtracted from the mean value of the stimulated cultures. A positive IFNγ response was predefined as ≥100pg/m (24) in the cross-sectional study (The Netherlands). Positive *in vitro* responses of the study subjects to the TB specific antigens ESAT-6 and/or CFP-10 were used as a marker for previous TB exposure (21,37).
Longitudinal study: quantitative IFNγ ELISAs were done in single wells (100 μl) using commercially available antibody pairs (BD Pharmingen) as previously described (3). Recombinant IFNγ BD Pharmingen) was used for the standard curve with lowest detection limit of 31 pg/ml. Negative control (medium) values were subtracted from all results. A positive IFNγ response was predefined as >62 pg/ml (3) in this longitudinal study (United Kingdom). A positive control supernatant was included in duplicate on each ELISA plate to control for interplate and intraplate variation.

In vivo studies
BALB/c (H-2^d) mice were bred at the Animal Facilities of the WIV-Pasteur Institute of Brussels, from breeding couples originally obtained from Bantin & Kingman (United Kingdom). All animals were 8-10 weeks old at the start of the experiments. Experiments were performed in agreement with the Ethical Committee of CODA-PIB-WIV regulations (permit no. 060202-02).
Male BALB/c mice were vaccinated with 0.2 mg (10^6 CFU) of freshly prepared solution of *M. bovis* BCG vaccine (strain GL2) grown as a surface pellicle on synthetic Sauton medium for 14 days and homogenized by ball mill (22). Mice were immunized subcutaneously with 0.2 ml. Female BALB/c mice were anesthetized with ketamine/xylazine and injected intramuscularly in both quadriceps muscles with 2 times 50 μg of V1J.ns-tPA vector encoding one of the latency antigens Rv1733c, Rv1738, Rv2029c, HspX (Rv2031c), Rv2032, Rv2626c, Rv2627c, Rv2628 or ESAT-

6/CFP10 fusion protein (21) or Ag85A (13,28). Mice were vaccinated three times at three-week intervals and immune responses were analyzed four weeks after the third immunization.
BCG vaccinated mice were sacrificed at 1 or 3 months after vaccination and spleens were removed aseptically and homogenized using a loosely fitting Dounce homogenizer. Plasmid DNA vaccinated mice were sacrificed four weeks after the third DNA immunization. Splenocytes (4×10^6 cells/ml) from three mice per group were tested as a pool (month 1 after BCG) or individually (3-4 mice, month 3 after BCG or after last DNA vaccination) for cytokine response to purified recombinant his-tagged latency antigens, Ag85A or ESAT-6-CFP-10 (all 5 μg/ml). Pooled supernatants from at least three wells were harvested after 72h when peak IFNγ values can be measured and stored at -20°C until assayed. IFNγ activity was quantified by sandwich ELISA using coating antibody R4-6A2 and biotinylated detection antibody XMG1.2 (both Pharmingen). Assay sensitivity was 5 pg/ml. Samples were tested undiluted, and at 1:10 or 1:100 dilution. Cytokine content was calculated for the dilution with OD value in the linear part of the standard curve.

Statistical analysis
For comparison of proportion of responders between test groups in the cross-sectional study the Chi Square Test (χ^2 Test) for independent samples was used. However, Fisher's exact test for independent samples was applied if expected values were lower or equal to 5 ($E \leq 5$). Comparison of proportion of responders in the longitudinal study was performed by McNemar's test for paired samples. For all assays $P < 0.05$ was considered statistically significant. For statistical analysis SPSS 11.0 for Windows was used.

Results

In silico sequence analysis and in vitro expression of the DosR regulon of BCG.
The DosR regulon of *M. tuberculosis* is expressed *in vitro* under conditions of hypoxia and low-dose nitric oxide stimulation (40). To evaluate whether BCG is similarly capable of expressing the DosR regulon, we first assessed available genome sequences of *M. tuberculosis*, *M. bovis* and BCG for the level of conservation and then performed *in vitro* transcription profiling of DosR regulon encoded genes.
BLAST searches (http://ncbi.nlm.nih.gov/BLAST/) of DosR encoded sequences showed that all 48 DosR regulon encoded genes were highly conserved between *M. tuberculosis* H37Rv and CDC1551 strains, *M. bovis* AF2122/97 and BCG Pasteur 1173P2. Homology of amino acid sequences was at least 97%, with 85% (41 of the 48) genes being identical (*data not shown*).
Subsequently, we assessed RNA expression profiles of 14 different BCG vaccine strains and *M. tuberculosis* H37Rv under *in vitro* conditions of hypoxia and nitric oxide stimulation. Results showed that all tested BCG strains have a functional DosR regulon with similar expression profiles as compared to *M. tuberculosis* (40). Figure 1 shows the RNA profiles of nine DosR regulated genes of *M. tuberculosis* H37Rv and BCG

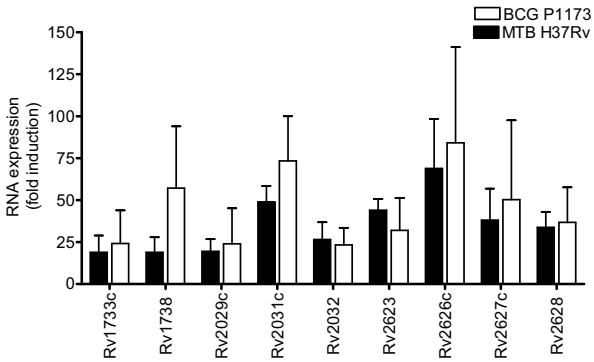

Figure 1. Expression of DosR genes (RNA). *RNA transcript expression profiles (fold induction) of nine DosR encoded genes induced during low oxygen conditions in Mycobacterium tuberculosis H37Rv (n=4) and BCG Pasteur 1173 (n=4). The RNA expression profiles of both strains are highly similar, except for Rv1738. Data shown as mean + SD*

Pasteur strain 1173 when exposed to low oxygen levels. Selection of these nine genes was based on results from our previous study (24) and contains the four most recognized latency antigens in humans. These genes were further analyzed in this study.

Immune responses to DosR regulon encoded antigens in human BCG vaccinees.
Next we addressed the question whether BCG vaccination in human beings leads to the induction of immune responses to DosR regulon encoded latency antigens that are targeted during natural *M. tuberculosis* infection.
IFNγ responses to immuno-dominant latency antigens were assessed in a longitudinal BCG vaccination study in young adolescents in the United Kingdom. Twenty-two tuberculin skin test (TST) negative adolescents were included in the vaccination group; blood was taken before pre- and post (2-4 weeks) BCG vaccination. Results showed that responses to the latency antigens of the participants before BCG vaccination were minimal: median responses to tested latency antigens Rv1733c, Rv2029c, Rv2623, Rv2627c and Rv2628 were below the predefined cutoff level of 62 pg/ml IFNγ as used in this study (Figure 2A). BCG vaccination did not alter the response profiles of the adolescents to the latency antigens: proportion of responders in the pre-vaccination group ranged from 4.5% to 33.3% compared to 0% to 31.8 % post-BCG vaccination. Statistical analysis of the proportion of responders to TB latency antigens showed no significant difference between pre- and post BCG vaccination (McNemar's test, $P = 0.500$ to $P = 1.000$).
Following BCG vaccination there was a significant increase in the IFNγ response to PPD from 46% to 96% responders ($P = 0.001$). There was no significant difference for responses to Ag85A, only a trend towards an increase in response from 64% to 77% responders ($P = 0.375$). Although all eligible subjects were tested negative in the tuberculin skin test (Heaf test) prior to BCG vaccination, *in vitro* stimulation with PPD or Ag85A showed that a considerable proportion of the tested adolescents gave positive whole blood assays (46% and 64% respectively), which is most likely

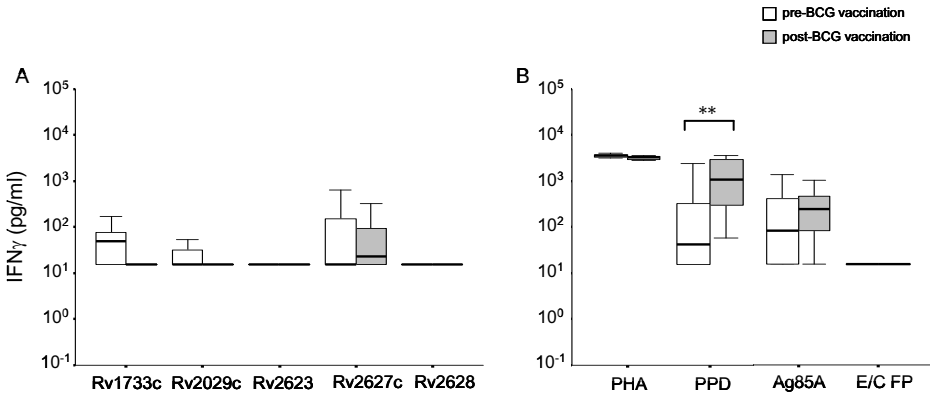

Figure 2. IFNγ responses to M. tuberculosis latency antigens in a longitudinal BCG study. *Box and Whisker plot of IFNγ responses to the latency antigens in schoolchildren pre- and post-BCG vaccination. Horizontal black line in the box represents the median; lower boundary of the box represents the 25th percentile and upper boundary the 75th percentile. Whiskers extend from the box to the highest and lowest value with exclusion of the extreme values and the outlier values. Supernatants were taken after 6 days for measurement of IFNγ production. (A) IFNγ responses of the pre-BCG vaccinated group to tested latency antigens. (B) Responses to PHA and control antigens PPD, Ag85A and ESAT-6/CFP-10 fusion protein. E/C FP, ESAT-6/CFP-10 fusion protein. Vaccinated adolescents, n=22, except for antigen Rv1733c in the pre-vaccinated group where 12 individuals were tested. An IFNγ response ≥ 62.5 pg/ml was considered positive. The McNemar's test for paired samples was used to compare the proportions of responders per group, ** P=0.001.*

as a result of exposure to cross-reactive environmental mycobacteria. As expected, none of the participants responded to the *M. tuberculosis* specific ESAT-6/CFP-10 fusion protein (Figure 2B). Thus, these results show that in adolescents following BCG vaccination virtually no increases in responses to *M. tuberculosis* latency antigens are observed.

Next, we carried out a cross-sectional study (The Netherlands) including BCG vaccinated adults without exposure to TB (n=27) compared to subjects with likely exposure to TB (n=16). For the purpose of this study, individuals with an IFNγ response >100 pg/ml to *M. tuberculosis* specific antigens ESAT-6 and CFP-10 (21,37) were considered previously exposed to TB.

Figure 3 shows the immune recognition profiles of both BCG vaccinated groups to the latency antigens. Overall, BCG vaccinated individuals without evidence of exposure to TB showed significantly lower IFNγ production to the latency antigens compared to the group that had positive responses to ESAT-6 and/or CFP-10. The median IFNγ responses to all tested latency antigens in the unexposed group were all below the cut-off level of 100 pg/ml, whereas this was the opposite in the TB exposed group. In the latter group median IFNγ values to all tested latency antigens, except for protein Rv2628, were above 100 pg/ml; indicating that over half of the individuals strongly recognized the antigens (Figure 3A).

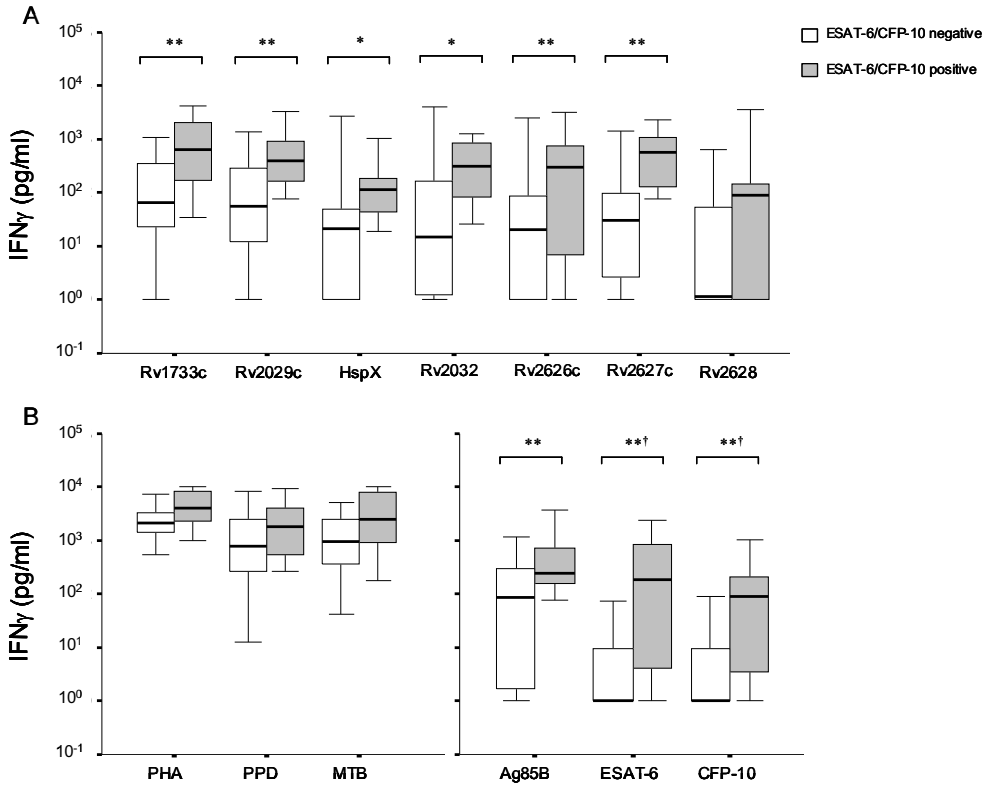

Figure 3. IFNγ responses to M. tuberculosis latency antigens in a cross-sectional BCG study. *Box and Whisker plot of IFNγ responses to the latency antigens by BCG vaccinated individuals. Horizontal black line in the box represents the median; lower boundary of the box represents the 25th percentile and upper boundary the 75th percentile. Whiskers extend from the box to the highest and lowest value with exclusion of the extreme values and the outlier values. Supernatants were taken after 6 days for measurement of IFNγ production (A) IFNγ responses to the latency antigens of the group that is BCG vaccinated without TB exposure (no IFNγ production to ESAT-6 or CFP-10) and with exposure to TB (positive IFNγ production to ESAT-6 or CFP-10). (B) IFNγ responses to control stimuli PHA, PPD, MTB, Ag85B, ESAT-6 and CFP-10.*
MTB, lysate of M. tuberculosis cultured under low oxygen conditions. BCG vaccinated group without TB exposure, n=23, BCG vaccinated group with TB exposure, n=16. An IFNγ response ≥100 pg/ml was considered positive. χ^2-test, P<0.05, ** P<0.01, † Fishers exact test.*

The proportion of responders (i.e. >100 pg/ml IFNγ) per antigen was as follows (BCG vaccinated without TB exposure vs. BCG vaccinated with TB exposure): Rv1733c 44.4 % vs. 93.8 % (P = 0.001), Rv2029c 37.0 % vs. 81.3 % (P = 0.005), HspX 22.2 % vs. 56.3 % (P = 0.024), Rv2032 29.6 % vs. 68.8 % (P = 0.013), Rv2626c 20.0 % vs. 68.8 % (P = 0.003), Rv2627c 25.9 % vs.75 % (P = 0.002) and Rv2628 25.9 % vs. 43.8 % (P = 0.228). Furthermore, the levels of IFNγ production in the BCG vaccinated group with suspected TB exposure were similar to those we previously found in Mantoux positive individuals (24). In addition to the responses to latency antigens, exposure to TB was also associated with higher IFNγ production to Ag85B (P = 0.005) which is shared between BCG and *M. tuberculosis.* The data also suggest that the responses to mycobacterial complex antigens like PPD (P = 0.139) and *M. tuberculosis* lysate (from

bacteria cultured under hypoxic conditions) (P = 0.279) are higher but no significant difference was detected (Figure 3B).
Time after BCG vaccination and moment of sampling varied considerably in our study population. We therefore attempted to seek a correlation between the level of IFNγ production and time since vaccination. However, no time dependent association was observed (data not shown).
Taken together, BCG vaccination in the skin in general appears to induce poor responses to not only HspX but also to other latency antigens of *M. tuberculosis*. In contrast, following exposure to TB, significant responses to latency antigens are seen.

Immune responses to DosR regulon antigens in mice.
In order to determine whether BCG vaccination also fails to induce adequate T-cell responses to *M. tuberculosis* latency antigens in mice, BALB/c mice were vaccinated subcutaneously with BCG (n=3). The mice were sacrificed 4 weeks and 3 months after BCG vaccination in order to test splenocytes for IFNγ production in response to latency antigens, the homologous or shared mycobacterial antigen Ag85A and the fusion protein of ESAT-6 and CFP-10 of *M. tuberculosis* (21).

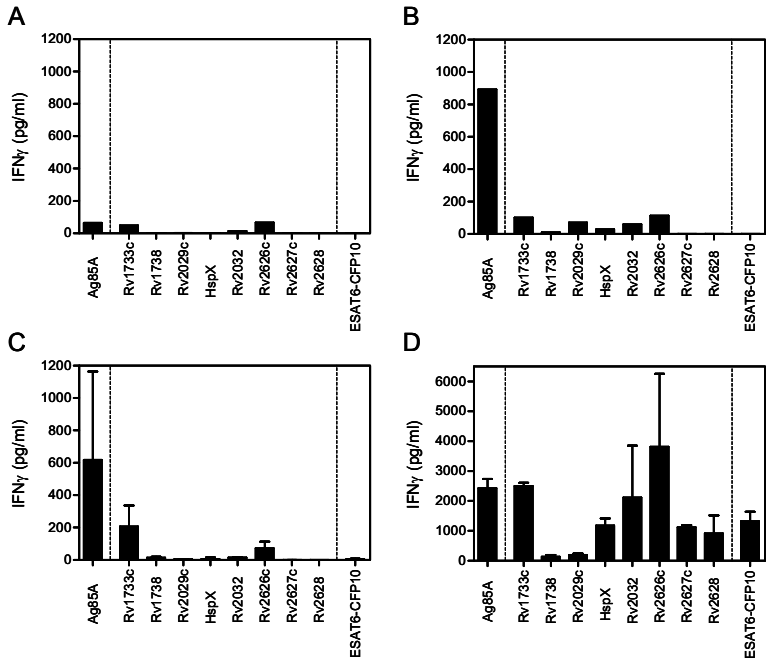

Figure 4. IFNγ responses to M. tuberculosis latency antigens in BALB/c mice following BCG vaccination (s.c.) or vaccination (i.m.) with plasmid DNA*. Supernatants were taken after 3 days (72 hours) for measurement of IFNγ production. (A) Naïve, non-BCG vaccinated, tested as a pool, n=3. (B) Animals 4-weeks post BCG vaccination, mice tested as a pool, n=3. (C) Animals 3-months post BCG vaccination, n=3. (D) Mice were immunized 3x with plasmid DNA encoding for one the eight latency antigens, Ag85A or ESAT-6/CFP-10. Splenocytes were in vitro restimulated with their respective antigens, n=4.*

Four weeks post-BCG vaccination, splenocytes showed very low to no production of IFNγ when stimulated with latency antigens (Figure 4B). The levels of IFNγ were similarly low as those of naïve control mice (Figure 4A). At this same time point cells produced significant amounts of IFNγ to Ag85A (Figure 4B) and PPD (data not shown). As expected, responses to the fusion protein ESAT6-CFP-10 were absent. When responses were assessed at 3 months post-immunization, similar results were obtained: IFNγ responses of splenocytes of vaccinated mice to latency antigens remained at levels comparable to those in naïve mice (Figure 4A), and only to Ag85A significant IFNγ production was found (Figure 4C). To rule out that absence of IFNγ responses to latency antigens was due to an inherent inability of BALB/c mice to respond to these latency antigens, mice were vaccinated with DNA. BALB/c mice (n=4) were vaccinated three times with three weeks intervals with naked DNA plasmid encoding one of eight different latency antigens, Ag85A or ESAT-6/CFP-10. Four weeks after the last immunization spleen cell IFNγ production was analyzed. Vaccination with six out of eight DNA plasmids induced significant antigen specific IFNγ production (Figure 4D). Robust levels of IFNγ were produced after *in vitro* re-stimulation with Rv1733c, HspX, Rv2032c, Rv2626c, Rv2627c and Rv2628. Antigens Rv1738 and Rv2029c appeared to be not very immunogenic for T cells in BALB/c mice, albeit significant antibody responses to the latter protein could be induced (29a). Vaccination with DNA plasmids encoding Ag85A or ESAT6-CFP-10 fusion protein was also very effective in inducing strong IFNγ responses. The results thus demonstrate that mice are able to generate strong Th1 type immune responses to latency antigens after immunization with DNA plasmid but that subcutaneous BCG administration fails to induce immune responses to *M. tuberculosis* latency antigens in BALB/c mice.

Discussion

Relatively little is known about the antigen specificity of immune response profiles following BCG vaccination in relation to protection against TB. In this study we addressed the question whether BCG vaccination induces immune responses to DosR regulon encoded antigens, in particular to those TB latency antigens that are targeted during persistent *M. tuberculosis* infection (24).
Comparison of available genome sequences of *M. tuberculosis, M. bovis* and BCG showed that the entire set of DosR regulon coding sequences is conserved in BCG; except for a few minor point mutations, which are not expected to have a major impact on expression or immunologic recognition of these antigens. Subsequently, transcriptional analysis of 14 different BCG vaccine strains under *in vitro* conditions of low oxygen and nitric oxide exposure were studied. Our results show that RNA expression profiles of the DosR regulon latency antigens reported in this study, were highly similar to those previously reported for *M. tuberculosis* (40). In addition, BCG is reported to be capable of adapting to anaerobiosis *in vitro* by shifting down to a non-replicating persistent state similar to *M. tuberculosis* (25). When tested in the so-called Wayne model or in standing (*i.e.* hypoxic) cultures it was previously shown that BCG is

capable of producing at least four DosR regulon encoded proteins: Rv2623, Rv2626c, HspX (Rv2031c, *acr*) and DosR (Rv3133c) (5,6). We conclude from these *in vitro* observations that BCG has a functional DosR regulon, although no complete proteomics data is available to demonstrate that every single protein encoded by the regulon is indeed expressed.

Next we monitored immune responses to a series of immuno-dominant TB latency antigens following BCG vaccination, both in a longitudinal and a cross-sectional study in two different human cohorts, and corroborated our findings in a BALB/c mouse model.

As mentioned before, neonates vaccinated with BCG at birth do not develop immune responses to the latency antigen HspX, whereas they are capable of mounting responses to other non-dormancy associated TB antigens (including PPD) (39). BCG vaccinated adolescents (longitudinal study) and BCG vaccinated adults who have not been exposed to TB (*i.e. in vitro* negative for production of IFNγ to ESAT-6 and/or CFP-10) (cross-sectional study), mounted immune responses to secreted antigens of the Ag85 complex and PPD, but did not develop responses to HspX and other tested latency antigens (Figure 2, 3).

In contrast to the above, BCG vaccinated adults with previous exposure to TB (*i.e. in vitro* positive production of IFNγ to ESAT-6 and/or CFP-10) in the cross-sectional study showed significantly higher IFNγ responses to selected TB latency antigens next to increased and significant (only Ag85B) responses to common TB antigens. These results agree with our recent findings that T cells from individuals with latent TB infection preferentially recognize a set of TB latency antigens (*i.e.* Rv1733c, Rv2029c and Rv2627c) while patients with (past or active) TB disease preferentially recognized secreted antigens such as CFP-10 and ESAT-6 (14,24).

The absence of *in vitro* detectable responses to *M. tuberculosis* latency antigens in BCG vaccinated adults may in part be explained by the common belief that BCG-induced protection to TB wanes over a period of 10-15 years (34). Since time after BCG vaccination in our study group varied between 1 and 37 years we attempted to correlate the level of responses to latency antigens to time elapsed since vaccination, but found no significant associations.

Somewhat unexpectedly, 46% of the adolescents who tested negative in the Heaf skin test prior to BCG vaccination, showed *in vitro* responses to PPD. Our own observations (unpublished observations) and those of others (4) show that *in vitro* detectable immune responses to PPD can be observed in a significant group of Mantoux negative, non-BCG vaccinated and TB unexposed, healthy individuals. This phenomenon is likely caused by previous exposure to cross-reactive environmental mycobacteria (4, 7, 16, 26). In addition, previous exposure to environmental mycobacteria might also have induced low level of responses (4.5%-33% responders) in the pre-vaccinated BCG group in the longitudinal study (Figure 2) since DosR encoded antigens have not been reported to be solely expressed in *M. tuberculosis.*

Our findings in humans were corroborated in BCG vaccinated BALB/c mice. Upon subcutaneous vaccination with BCG, the mice generated immune responses to the common *M. tuberculosis* antigens Ag85A and PPD. In contrast, poor responses were detectable against *M. tuberculosis* latency antigens both one and three months post-vaccination. Yet, vaccination with recombinant DNA plasmids induced a strong T cell

response to several latency antigens including Rv1733c, HspX, Rv2032, Rv2626c, Rv2627c and Rv2628, as well as Ag85A and ESAT6-CFP-10 fusion protein. DNA plasmids encoding Rv1738 and Rv2029c were not immunogenic in mice. This demonstrated that the mice were able to generate strong Th1 type immune responses to *M. tuberculosis* latency antigens when immunized with DNA plasmids, but that subcutaneous BCG administration failed to induce immune responses to such antigens in BALB/c mice. Using HLA-transgenic mice (19, 20) a similar observation concerning HspX was recently made in our laboratory: mice vaccinated with BCG did not develop immune responses to HspX whereas immunization with HspX did induce responses to HspX and its predefined T cell epitopes (10, 20a).

Of interest is our observation that the recognition pattern of latency antigens seems to be different between humans and BALB/c mice. It appears that humans responded highest to antigens Rv1733c, Rv2029c and Rv2627c whereas mice responded most to Rv1733c, Rv2032 and Rv2626c (although both not statistically significant). However this may be related to different mode of antigen exposure being natural infection in humans versus DNA vaccination in mice. This observation might have implications for proof of concept studies which involve TB latency antigens in mouse models and their extrapolation to latent TB in humans.

Finally, two other animal models confirmed the lack of T cell responses to *M. tuberculosis* latency antigens following BCG vaccination. First, adult male rhesus monkeys were vaccinated with BCG (23) and *in vitro* immune responses to *M. tuberculosis* latency antigens were monitored at nine weeks post-vaccination. Similar to observations in humans and mice, significant immune responses to PPD were seen following BCG vaccination, but no responses to *M. tuberculosis* latency antigens (Dr F Verreck, personal communication). Second, cattle were vaccinated with BCG or infected with *M. bovis* (8) and immune responses were measured against a series of latency antigens, (bovine) PPD as well as ESAT6-CFP-10 fusion protein. Significant responses to PPD were observed and animals infected with virulent *M. bovis* also responded significantly to ESAT6 and CFP-10, but no significant responses to any of the tested latency antigens were observed (Dr M Denis and Dr B Buddle, personal communication). Although it is difficult to establish positive controls for responses to TB latency antigens in these two animal models, the results are likely accounted for by the same mechanisms underlying the observed effects in humans and mice following BCG vaccination.

The studies summarized above have been performed with different strains of BCG and this may potentially confound interpretation and comparison of results. However micro-array expression profiling of 14 different BCG vaccine strains showed highly similar regulation of the DosR regulon. This rather suggests that lack of immune responses to TB latency antigens following BCG vaccination is not a strain-specific phenomenon, but an intrinsic property of dermal vaccination with BCG.

Upon administration in the skin, BCG is ordinarily not expected to persist in immuno-competent humans, although rare cases have been reported of persistence and reactivation of BCG in individuals who developed immune deficiencies many years after vaccination (1, 27, 35, 36). It therefore seems unlikely that BCG in the skin will encounter the necessary environmental triggers needed to express the latency antigens and subsequently to trigger immune responses Moreover, absence of T cell

responses are not due to restrictions in T cell repertoire since mice respond to these antigens when immunized with DNA plasmids and BCG vaccinated individuals exposed to TB also respond to latency antigens by IFNγ production. Further studies will be aimed at identification of exact mechanisms that are responsible for regulation and expression of the DosR regulon and consecutively immunity to latency antigens following BCG vaccination.
Since the start of its first use in the 1920s, 3 billion doses of BCG have been delivered throughout the world, unfortunately with relatively little impact on the global TB epidemic, underlining the need for a better vaccine regimen. Our findings suggest future studies evaluating the protective efficacy of latency antigens against (latent) TB and potential use of latency antigens as vaccine candidates, either alone, with BCG or alternatively, as post-exposure TB vaccines.

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