CHAPTER 5

The newly identified *Mycobacterium tuberculosis* antigen Rv2034 induces $CD4^+$ T cells that protect against pulmonary infection in HLA-DR transgenic mice

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Submitted

Abstract

Tuberculosis (TB) remains one of the most serious infectious diseases worldwide. *Mycobacterium bovis* BCG, the current vaccine against TB, provides insufficient protection against adult pulmonary TB, indicating the need for improved TB vaccines. Besides being immunogenic, *Mycobacterium tuberculosis* (*Mtb*) antigens that represent potential vaccine candidates should also be expressed *in vivo* during *Mtb* infection. We have analyzed the *in vivo* immunogenicity and protective efficacy of Rv2034, a novel potential vaccine candidate that is expressed in lungs of *Mtb*-infected susceptible mice and that was recognized by T cells from tuberculin skin test-positive individuals. The Rv2034 protein was highly immunogenic in HLA-DR3-immunized tg mice, but not in HLA-DR3-negative littermates. Rv2034 protein immunization induced IFN-γ^+/TNF$^+$ and IFN-γ^+ CD4$^+$ T cells specific for an HLA-DR3-restricted epitope, Rv2034 peptide 31-50. CD4$^+$ T-cell responses were optimally induced when using TLR9 and TLR3 ligand-adjuvants, whereas Rv2034-specific antibodies were observed using ligands for TLR2, TLR3, TLR4, TLR5, TLR7 and TLR9. Finally, Rv2034/CpG immunization reduced the number of bacilli in the lungs after *Mtb* challenge specifically in HLA-DR3 mice indicating the potential of Rv2034 in TB vaccines.

Introduction

8.7 million new tuberculosis (TB) cases and 1.4 million deaths due to TB were recorded for 2011, illustrating the enormous global burden of TB on human health [1]. BCG, the only registered TB vaccine, does protect against severe forms of childhood TB but not sufficiently against pulmonary TB in adults, nor does protect against reactivation from latent TB [2;3]. There is wide consensus that better TB vaccines are urgently needed.

In search of new TB vaccine candidate antigens, we have recently used a genome-wide unbiased antigen discovery approach in which we investigated the expression of 2170 *Mycobacterium tuberculosis* (*Mtb*) genes during infection in the lungs of four genetically related but distinct mouse strains, which ranged in TB susceptibility and TB disease manifestations. We identified a series of *in vivo* expressed *Mtb* (IVE-TB) genes and showed that several of these IVE-TB gene encoded proteins were efficiently recognized by human T cells and thus qualify as candidate TB vaccine antigens [4]. One of the IVE-TB genes identified, *Rv2034*, encodes for an arsenic repressor (ArsR) protein that belongs to the *Synechococcus* metallothionein regulator (SmtB)/ArsR family of metallo-regulatory transcriptional repressors [5]. Rv2034 is suggested to be involved in the regulation of lipid metabolism and the hypoxic stress response of *Mtb* [6-8]. This is further supported by studies showing *Rv2034* gene expression by *Mtb* cultures grown under starvation-, [9] hypoxic- [10], and multi-stress conditions [11], as well as expression in *Mtb* persister populations [12].

SmtB/ArsR transcriptional repressors control resistance to metal ions and bind to DNA elements in genes involved in metal-metabolism and -detoxification. Upon metal binding the SmtB/ArsR family repressor protein dissociates from the ligand DNA, thus activating transcription. The SmtB/ArsR family regulator is widely present and at least one SmtB/ArsR member is encoded by most (sequenced) bacterial genomes. Notably, the *Mtb* genome encodes 12 SmtB/ArsR-like repressors, which is highly unusual compared to bacteria other than Actinobacteria [13]. Recent work identified several DNA binding sites for Rv2034, including promoter regions of major stress response genes such as the DosR gene *Rv2031c* (α-crystallin/ hspX), *Rv0350-Rv0353* (DnaK, GrpE), *Rv0440* (GroEL2), *Rv1907c-Rv1912c* (katG, FurA) and *Rv3197A* (WhiB7), as well as genes involved in fatty acid metabolism; *Rv3543c-Rv3545c* (FadE28, FadE29), *Rv3504-Rv350*5 (FadE26, FadE27), *Rv1094* (DesA2) and *Rv0244* (FadE5) [7]. Interestingly, besides displaying self-regulation, the Rv2034 protein, in contrast to its predicted function as a repressor, positively regulates the expression of g*roEL2* and *phoP*, an important virulence gene of *Mtb* [6;8]. Thus, *Rv2034* gene expression and protein function support a role in adaptation to stress environment and regulation of lipid metabolism, both essential in *in vivo Mtb* infection. In view of its potential mechanistic function in *Mtb*'s strategy to survive inside the human host, the induction of immune responses against Rv2034 would be an attractive option to direct immunity towards *Mtb* proteins that are essential to *Mtb* and that are expressed in the primary target organ, the lung.

In this study, we have investigated the vaccine potency of the Rv2034 protein in the context of HLA using the HLA-DR3 transgenic mouse model [14] and describe the *in vivo* immunogenicity of the Rv2034 protein and synthetic peptides thereof, as well as their efficacy to reduce the number of *Mtb* bacteria after intranasal challenge with live *Mtb*.

Materials and methods

Mouse strains. HLA-DRB1*03:01/DRA, murine class II-deficient (designated HLA-DR3) mice were generated as described [14], backcrossed for 10 generations with C57Bl/10 and eventually intercrossed. During breeding, PBMCs of each mouse were typed by flow cytometry for HLA-DR (FITC-conjugated mouse IgG2 κ anti-HLA-DR; BD Biosciences, Franklin Lakes, NJ), murine-CD4 (PE-Cy5-conjugated Rat IgG2a, κ anti-mouse CD4; BD Biosciences) and PE-conjugated mouse (BALB/c) IgG2a κ anti-mouse I-A^b; BD Biosciences). Littermates lacking HLA-DR expression (designated HLA-$DR3^{neg}$) were used as negative controls. HLA-A*02:01/H2-D Enge/J (designated HLA-A2) mice were purchased from Jackson Laboratory (Bar Harbor, ME) [15]. Surface expression of HLA-A*02:01 was confirmed by flow cytometry analysis. HLA-A2. DR3 transgenic mice were generated by mating the HLA-DR3 with HLA-A2 mice. All mice were bred under specific pathogen-free conditions.

Synthetic peptides. Peptides were purchased from Peptide 2.0 Inc. (Chantilly, VA, USA). Homogeneity and purity were confirmed by analytical HPLC and by mass spectrometry. Purity of all peptides was ≥ 80%. All impurities consist of shorter versions of the peptides caused by < 100% coupling efficiency in each round of synthesis.

Recombinant proteins. *Mtb* genes were amplified by PCR from genomic *Mtb* DNA and cloned using Gateway technology (Invitrogen, Carlsbad, CA) [16]. Purified recombinant proteins were produced and analyzed as described [16;17] and contained endotoxin levels below 50 IU per mg recombinant protein as tested using a Limulus Amebocyte Lysate (LAL) assay (Cambrex, East Rutherford, NJ). Recombinant proteins were tested to exclude protein non-specific T-cell stimulation and cellular toxicity in IFN-γ release assays using PBMC of *in vitro* purified protein derivative (PPD)-negative, healthy Dutch donors recruited at the Blood Bank Sanquin, Leiden, The Netherlands. None of these controls had experienced any known prior contact with TB patients.

Immunizations. For protein immunization, mice (4 - 5 animals per group) were injected subcutaneously (s.c.) three times with 25 μg protein generally in combination with 50 μg CpG (ODN1826 5'-TCC ATG ACG TTC CTG ACG TT -3'; InvivoGen, San Diego, CA; TLR9 ligand) in 200 μl PBS in the right flank with two weeks intervals. Control mice were injected s.c. with 10^6 colony forming units (CFU) BCG 1331 (*M. bovis* Bacillus Calmette Guérin; Statens Serum

Institute, Copenhagen, Denmark) from frozen ampoules. Splenocytes were harvested 21 days after final injections. Since ODNs containing unmethylated CpG motifs can activate immune cells to produce cytokines [18], we routinely immunize with CpG alone as a (negative) control to assess the antigen specificity of immunization. For adjuvants comparison experiments identical amounts of Rv2034 protein were administered s.c. with either: 50 µg PAM3CysK4 (N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]- lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine; Invivogen; TLR2 ligand), 50 µg Poly(I:C) HMW (polyinosinic-polycytidylic acid high molecular weight; Invivogen; TLR3 ligand), 10 µg MPL/TDM (monophosphoryl lipid A/ trehalose dicorynomycolate; Sigma-Aldrich, St. Louis, MO; TLR4 ligand), 10 µg Flagellin (Invivogen; TLR5 ligand) or 50 µg R848 (Imidazoquinoline compound, Invivogen; human TLR7/8 and mouse TLR7 ligand).

Ethics statement. The handling of mice was conducted in accordance with the regulations set forward by the animal care committee of the LUMC and in compliance with European Community Directive 86/609 for the care and use of laboratory animals.

Welfare monitoring. All animals included in the experiments were weighed once a week and observed daily in order to ensure ethics requirements and to monitor any adverse effects possibly related to the vaccination.

In vitro *cultures*. Splenocytes were isolated from individual animals by homogenizing spleens through a plastic cellstrainer (BD Biosciences) and splenocytes (3 x 10^6 cells/ml) were resuspended in IMDM (Invitrogen) supplemented with 2 mM L-glutamine (Invitrogen) penicillin (100 U/ml), streptomycin (100 µg/ml) (Invitrogen), 8% heat-inactivated FCS (Greiner Bio-One) and $5x10^{-5}$ M β-mercaptoethanol (Sigma). Cell suspensions (100 µl) were added to 96-well round-bottomed microtiter plates (Costar, Corning Incorporated). Cells were incubated in quadruplicates with 100 µl of medium, recombinant protein (1 or 10 µg/ml), or synthetic peptide (1 or 10 µg/ml). The mitogen concanavalin A (conA; 2 µg/ml; Sigma) was used in all experiments as a positive control for cell viability. After 6 days supernatants were taken from each well, quadruplicates pooled and frozen at -20 ºC until performing IFN-γ ELISA assay.

IFN-γ ELISA. Before ELISAs were performed on supernatants from *Mtb* infected murine material, supernatants or sera were transferred into 0.2 µM filter plates (Corning, NY, USA) and centrifuged for 3 minutes at 1300 rpm. The filtrated material was collected in clean 96-wells plates and transferred out of the MLIII lab for further analyses. Detection of IFN-γ in culture supernatants of *in vitro* cultured splenocytes was performed by ELISA (BD Biosciences) according to the manufacturer's instructions. OD values were converted into concentrations using Microplate Manager software, version 5.2.1 (Bio-Rad Laboratories, Veenendaal, The Netherlands). The cut-

off value to define positive responses was set beforehand at 100 pg/ml. The assay sensitivity level was 20 pg/ml. Values for unstimulated whole blood cultures were typically < 30 pg/ml.

Intracellular cytokine staining. For polychromatic flow cytometry, splenocytes (3 x10^6cells/ml) were cultured *in vitro* with peptide (5 μg/ml). After 7 days, cells were incubated with medium or fresh peptide (5 μg/ml). After 4 hours brefeldin A (Sigma; 2.5 μg/ml) was added for overnight (20 hour) incubation after which cells were permeabilized and fixed using Cytofix/Cytoperm (BD Biosciences) and Perm/Wash (BD Biosciences) according to the manufacturer's instructions and stained using phycoerythrin (PE)-conjugated anti-CD8β_2 (BD Pharmingen), PECy5-conjugated anti-CD4 (BD Pharmingen), eFluor450-conjugated anti-CD19 (eBioscience, San Diego, CA, USA), Vivid (Invitrogen), Allophycocyanin (APC)-conjugated anti-IL-2 (BD Pharmingen), Alexafluor700-conjugated anti-IFN-γ (BD Pharmingen) and PeCy7-conjugated anti–TNF (BD Pharmingen). As positive controls for detection of IL-2, IFN-γ or TNF, MiCK-1 cytokine positive control cells (BD Pharmingen) were used.

Determination of anti-Rv2034 antibodies (Ab). Levels of antibody directed against Rv2034 in serum from immunized mice were determined by ELISA. Briefly, plates were coated overnight at 4 °C with recombinant Rv2034 protein or PBS (0.4% BSA) as a negative control. Plates were blocked for 2 hours using PBS containing 1% BSA and 1% Tween-20. Different sample dilutions (100 μl/well) were added to wells and incubated at 37°C for 2 hours. Plates were washed three times using PBS containing 0.05% Tween-20 and 100 μl/well horse radish peroxide (HRP)-conjugated, rabbit-anti mouse total IgG, IgA and IgM (P0260 Dako, Glostrup, Denmark). After 2 hours at 37°C, plates were washed three times using PBS containing 0.05% Tween-20 and 100 μl/well tetramethylbenzidine substrate (TMB; Sigma) was added for 15 min at RT. The reaction was stopped by addition of H_2SO_4 (1M; 100 μl/well). OD values at 450 nm were determined using BioRad Microplate reader 680 (BioRad Laboratories, Veenendaal, The Netherlands). Mean Ab concentrations were calculated from the linear part of the titration curve.

Infection with live* Mtb *and determination of bacterial burden. Naive and immunized mice (5 animals per group) were infected with live *Mtb* strain H37Rv 3 weeks after the third protein immunization or 12 weeks after BCG immunization. The mice were anesthetized with isofluran (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane; Pharmachemie BV, Haarlem, The Netherlands) and intranasal (i.n.) infected with 10^5 CFU *Mtb* from frozen ampoules. Mice were sacrificed 6 weeks after *Mtb* challenge and lungs were aseptically removed. The organs were homogenized in sterile PBS and the number of bacteria was determined by culturing serial dilutions of the homogenates on 7H11 agar plates (BD Biosciences) supplemented with BD BBL™ Middlebrook OADC enrichment (100 ml per bottle; BD Biosciences), PANTA [BD Biosciences; 1 vial per liter containing Polymyxin B (6.000 units), Amphotericin B (600 μg), Nalidixic acid (2.400

μg), Trimethoprim (600 μg), Azlocillin (600 μg)] and ampicillin (3.4 mg/ml; Vepidan, Denmark). Colonies were counted after 3 weeks incubation at 37 ºC. In case of animals that received *Mtb* infection combined with BCG vaccination, 7H11 agar plates containing 2-thiophene carboxylic acid hydrazide (2 μg/ml; Sigma) were used to distinguish BCG colonies from *Mtb* colonies. Protective efficacies are expressed as log10 bacterial counts in immunized mice compared to BCG immunized mice.

Statistical analysis. Graph Pad Prism (version 5) software was used for statistical analysis. Bacterial titers were analyzed by the Mann-Whitney test. *In vitro* cytokine levels were compared using student's t test. P-values ≤ 0.05 were considered significant.

Results

Identification of Rv2034 T-cell epitopes in HLA-DR3 tg mice

In order to evaluate the *in vivo* immunogenicity of Rv2034 different HLA-transgenic (tg) mouse models were used: HLA-DR3 tg mice lacking murine MHC class II (HLA-DR3.Ab^{0}), which has been shown to be suitable for *in vivo* identification of HLA-DR3 restricted T-cell epitopes [14;19], and HLA-A*0201 mice [20;21] which express endogenous MHC class II in addition to the hybrid class I MHC (α1 and α2 domains of HLA-A2*0201; α3 of H-2D^{d}) [15].

Mice were immunized three times with Rv2034 protein in CpG as adjuvant and IFN-γ levels in splenocytes induced by the Rv2034 protein as well as ten 20-mer peptides spanning the entirety of Rv2034, were analyzed (Figure 1). Immunization with Rv2034 in CpG induced significant levels of IFN-γ in HLA-DR3 and HLA-A2.DR3 transgenic mice against both the protein and peptides p31-50, p41-60 and p51-70. These peptides represent 20-mers which each overlap 10 amino acids with the previous and subsequent peptide (Table 1). Rv2034-immunized HLA-A2 mice responded to a 13-fold lower extent to the whole protein, but not to any of the 20-mer peptides, perhaps due to the low IFN-γ production observed in these mice in response to the protein.

HLA-DR3^{neg}/murine MHC class II^{neg} mice did not respond to any of the stimuli except the conA control. Also HLA-DR3 mice that received CpG alone did not show any IFN-γ response except to conA. None of the Rv2034-immunized mice recognized the control protein (HPV16E6) or peptide (HLA-DR3-restricted peptide hsp65 p3-13) [22], indicating the Rv2034-specificity. Thus, these results indicate that IFN-γ secretion after Rv2034 immunization in the models used, is predominantly HLA-DR3-restricted and that presentation of Rv2034 protein most likely does not occur via endogenous murine MHC class II such as present in HLA-A2 mice.

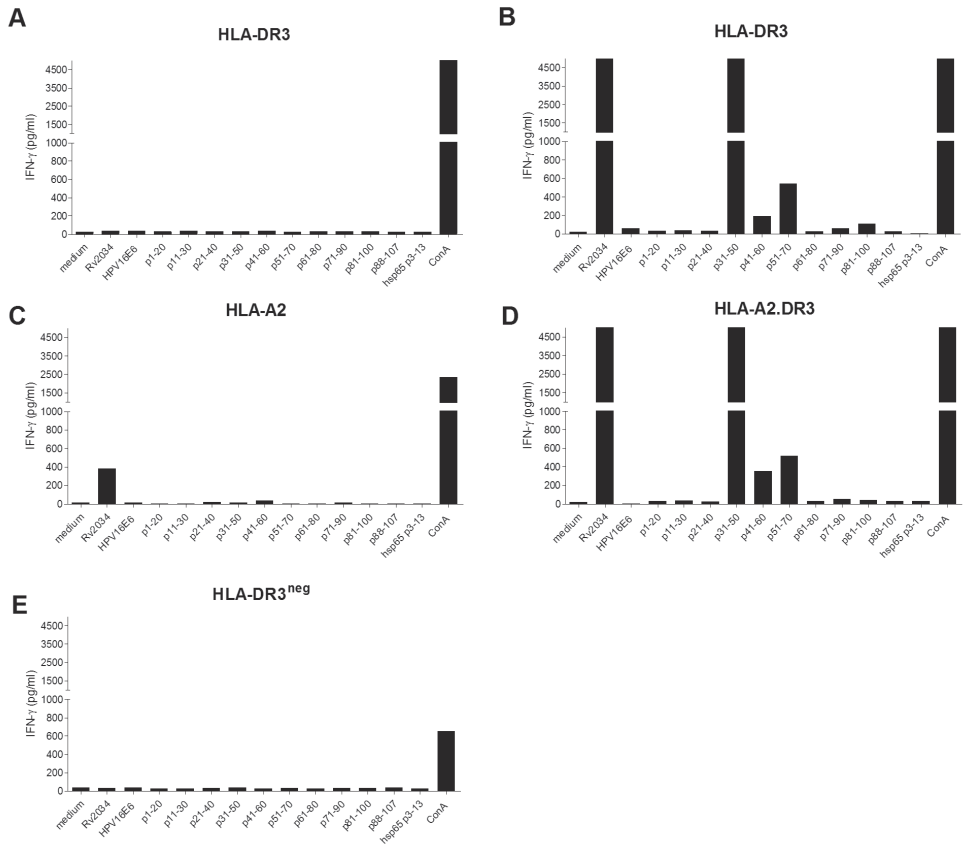

Figure 1. IFN-γ secretion after Rv2034 immunization is HLA-DR3-restricted. HLA-DR3 mice were immunized three times with CpG alone (**A**) or Rv2034 recombinant protein in CpG (**B**). In addition HLA-A2 (**C**), HLA-A2.DR3 (**D**) or HLA-DR3^{neg} (**E**) mice were immunized three times with Rv2034 recombinant protein in CpG. IFN-γ secretion was analyzed by ELISA after 5 days *in vitro* stimulation of splenocytes with Rv2034 protein (10 μg/ml), 10 synthetic Rv2034 peptides (20-mers overlapping 10 amino acids; 10 μg/ml), mitogen (conA; positive control), HPV16E6 recombinant protein (10 μg/ml as negative protein control) and HLA-DR3-restricted epitope hsp65 p3-13 (10 μg/ml as negative peptide control). All groups included five mice. All mice were separately analyzed. Results are shown for one animal, representative for each test group.

Table 1. Rv2034 overlapping 20-mers and 30-mer

peptide	amino acid sequence
p1-20[a]	MSTYRSPDRAWQALADGTRR
p11-30	WQALADGTRRAIVERLAHGP
p21-40	AIVERLAHGPLAVGELARDL
p31-50	LAVGELARDLPVSRPAVSQH
p41-60	PVSRPAVSQHLKVLKTARLV
p51-70	LKVLKTARLVCDRPAGTRRV
p61-80	CDRPAGTRRVYQLDPTGLAA
p71-90	YQLDPTGLAALRTDLDRFWT
p81-100	LRTDLDRFWTRALTGYAQLI
p88-107	FWTRALTGYAQLIDSEGDDT
p31-60	LAVGELARDLPVSRPAVSQHLKVLKTARLV

[a] p: peptide

Rv2034 peptide immunization in HLA-DR3 mice

Synthetic long peptides (SLP) are able to induce $CD4^+$ and $CD8^+$ T cells more efficiently than shorter epitopes [23]. SLP require professional APC such as dendritic cells (DCs) for antigen processing and presentation which increases their vaccine efficacy significantly compared to shorter peptides that are loaded directly onto MHC class II molecules [24]. Therefore, we synthesized the 30-mer sequence Rv2034 p31-60 covering the three immunogenic Rv2034 peptides (p31-50, p41-60, p51-70) and used this to immunize HLA-DR3 mice (Figure 2A). Immunization of HLA-DR3 mice with SLP p31-60 induced efficient responses to Rv2034 p31-50 and p31-60 but also to the Rv2034 protein.

Most current immunization strategies aim at priming immune responses against antigens and epitopes that already are dominant T-cell targets during natural infection. A broader T-cell repertoire including T cells specific for subdominant epitopes not recognized during natural infection, may, when primed sufficiently through immunization, offer additional protection. Peptide epitope-based vaccines can circumvent the propensity of the immune system to focus on immunodominant epitopes by simultaneously targeting the response to dominant as well as subdominant epitopes [25]. Thus, we also used Rv2034 peptide pool composed of ten overlapping peptides with or without the immunodominant p31-50 to immunize HLA-DR3 mice. Rv2034 peptide pool immunization induced responses to the same three core p31-50 related peptides as did the whole Rv2034 protein (Figure 2B). Instead, immunization with a pool of Rv2034 peptides lacking p31-50 induced IFN-γ responses to p41-60, p51-70 and p31-60 but did not result in IFN-γ responses to any of the other peptides, indicating that Rv2034 does not contain any subdominant epitopes in the context of HLA-DR3 (Figure 2C).

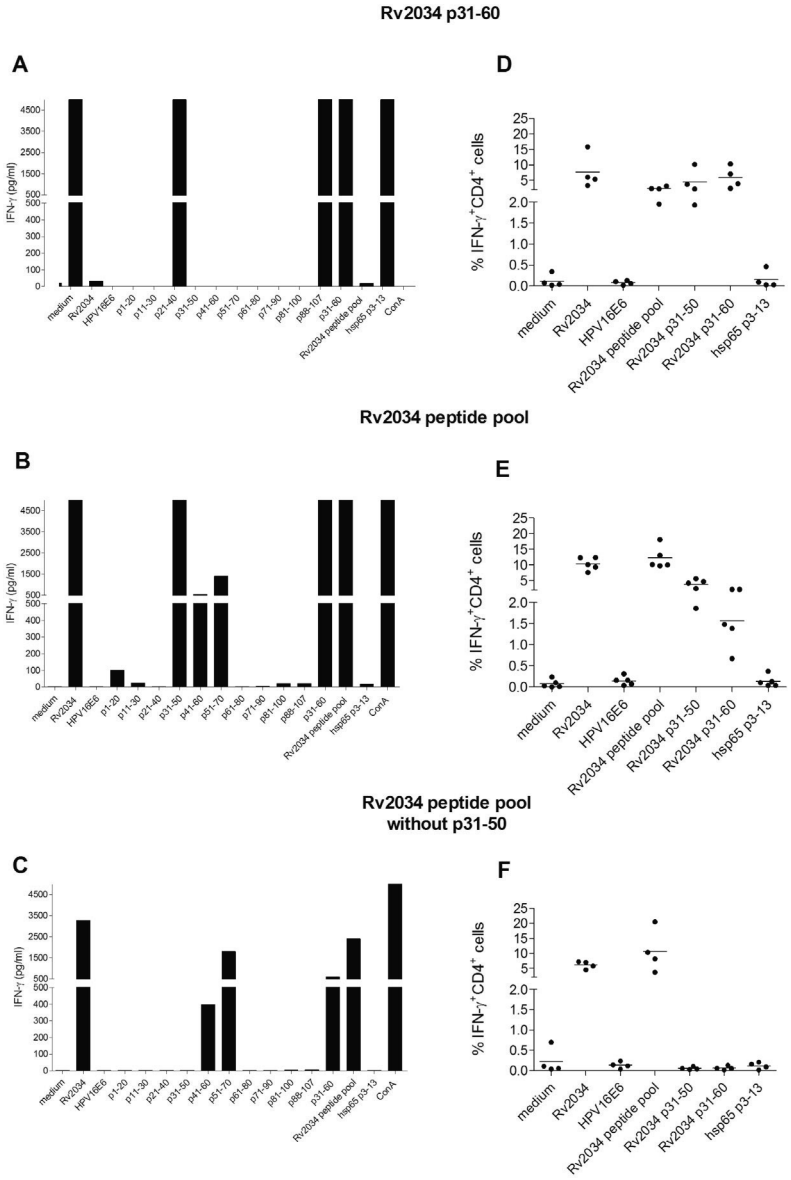

Figure 2. IFN-γ production after Rv2034 peptide immunization. HLA-DR3 mice were immunized three times with Rv2034 30-mer p31-60 in CpG (**A** and **D**), Rv2034 peptide pool composed of ten 20-mers overlapping 10 amino acids (10 μg/ peptide) including p31-50 in CpG (**B** and **E**) or without p31-50 in CpG (**C** and **F**). IFN-γ secretion was analyzed by ELISA (**A**, **B**, **C**) after 5 days *in vitro* stimulation of splenocytes with Rv2034 protein or peptides, control protein HPV16E6, control peptide hsp16 p3-13 (all 10 μg/ml) or control mitogen (1 μg/ml). Intracellular IFN-γ production by $CD4^+$ T cells was measured (**D**, **E**, **F**) after *in vitro* stimulation of splenocytes of immunized mice with the same antigen as used for immunization. After 7 days, cells were incubated with medium or fresh peptides for 1h before addition of brefeldin A and analysis for intracellular IFN-γ production. No IFN-γ was detected in $CD8^+$ T cells (data not shown). All groups included five mice. All mice were separately analyzed.

Since T-cell subtypes differ in their ability to induce protection against *Mtb*, we identified the phenotype of Rv2034-responsive T cells: splenocytes of HLA-DR3 mice immunized with Rv2034 peptide or peptide pools were stimulated *in vitro* for 7 days. Subsequently, intracellular IFN-γ production was assessed by flow cytometry analysis (Figure 2D-F). Rv2034 peptide pool immunization of HLA-DR3 mice induced IFN-γ production by $CD4^+$ T cells after *in vitro* stimulation with Rv2034 protein, Rv2034 peptides and peptide pool, whereas no responses were observed in mice that were injected with CpG alone nor in HLA-DR3-negative littermates and HLA-A2 mice (data not shown). The control protein HPV16E6 and HLA-DR3-restricted peptide hsp65 p3-13 did not induce IFN-γ in any of the immunized mice. As expected, immunization with the peptide pool lacking the dominant peptide Rv2034 p31-50 did not induce any responses to p31-50 or p31-60, whereas immunization with 30-mer p31-60 induced strong responses to the whole protein as well. $CD8^+$ T cells did not produce IFN-γ following *in vitro* peptide re-stimulation (data not shown) confirming $CD4^+$ T cell-specificity for the HLA-DR3-restricted response to Rv2034.

Induction of polyfunctional T cells in response to Rv2034

Antigen-specific IFN-γ production is often used as a proxy biomarker of vaccine-induced and protection-associated Th1 responses. More recently, the induction of polyfunctional $CD4^+$ Th1 cells was found to correlate better with vaccine-induced protection [26] in several model settings, even though the value of polyfunctional T cells as biomarkers of protection in *Mtb* infection remains questionable [27-29]. To estimate the frequency of polyfunctional T cells in the Rv2034-immunized HLA-DR3 mouse vaccine model, intracellular production of TNF, IL-2 and IFN-γ was assessed simultaneously using polychromatic flow cytometry (Figure 3). Splenocytes of immunized HLA-DR3 mice that were *in vitro* restimulated with medium mainly showed TNF^+ (0.5%) single positive and IFN-γ^+ (0.12%) single positive $CD4^+$ T cells. *In vitro* restimulation with Rv2034 protein and Rv2034 p31-60 increased the number of IFN-γ^+ $CD4^+$ T cells to 1.28% and 1.93%, and induced 0.71 % and 0.54 % double (IFN-γ^+/TNF^+) positive $CD4^+$ T cells, respectively. The percentages of triple (IFN-γ^+/IL-2^+/TNF^+) positive $CD4^+$ T cells, however, remained low. These data indicate that immunization of HLA-DR3 mice with Rv2034 or Rv2034 p31-60 induces a significant proportion of single and dual functional T cells specific for Rv2034.

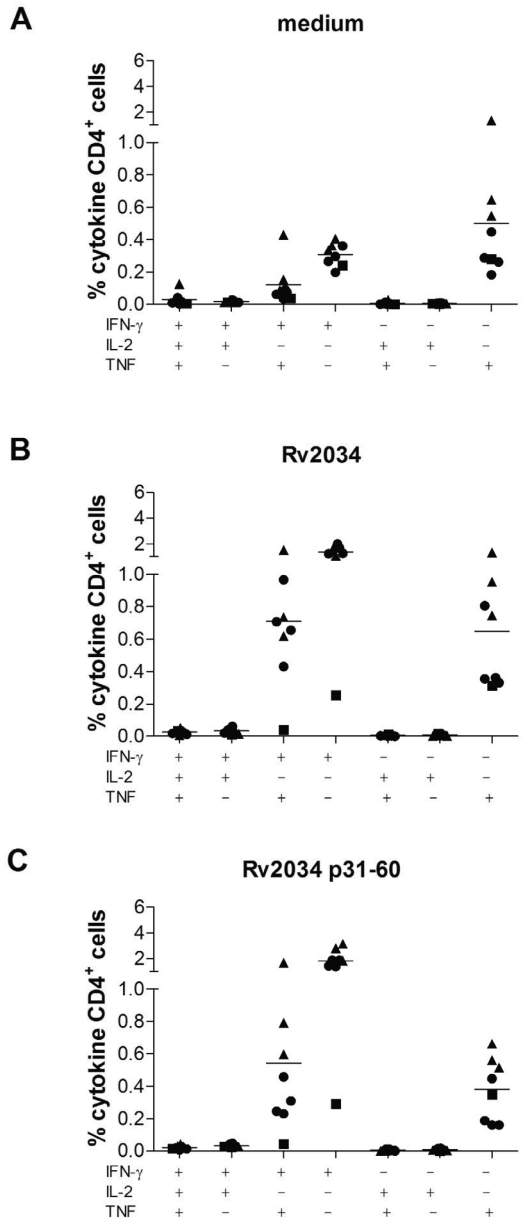

Figure 3. Frequency of polyfunctional CD4⁺ T cells. Percentage of IFN-γ, IL-2 and/or TNF producing CD4⁺ T cells in splenocytes of HLA-DR3 mice immunized with Rv2034 protein or Rv2034 p31-60 in CpG and re-stimulated *in vitro* with medium (**A**), Rv2034 protein (**B**) or Rv2034 p31-60 (**C**). Each symbol represents one mouse immunized with Rv2034 protein (●), Rv2034 p31-60 (▲) or unimmunized (■). The total number of CD4⁺ T cells analyzed in immunized mice was 56,000. Only CD4⁺ populations of > 5 x 10^4 events were analyzed.

Rv2034 immunization induces antibodies directed against *Mtb*

Since we recently showed that even mycobacterial peptides can induce specific antibody responses *in vivo* in HLA-A2 mice [20] and HLA-DR3 [19], we also analyzed the humoral response induced by the Rv2034 protein. A high antibody titer against Rv2034 protein was observed after Rv2034/CpG immunization of HLA-DR3 mice (Figure 4). Mock-immunized mice did not show any antibody reactivity, nor were antibodies detected in Rv2034-immunized mice against an unrelated recombinant protein HPV16 E6. Finally, no antibodies were generated in Rv2034-immunized HLA-A2 mice. These data indicate that Rv2034 protein induces strong cellular as well as humoral immune responses *in vivo*.

Effect of the use of different TLR ligands as adjuvants for Rv2034 immunization

Besides the *Mtb* antigen, the adjuvant used also plays a major role in the induction of an effective Th1/Th2 balance and subsequent protection *in vivo* [30]. In order to estimate the adjuvant-effect on protection-associated Th1 immunity, HLA-DR3 mice were immunized three times with identical amounts of Rv2034 protein adjuvanted with different TLR ligands: PAM3CysK4 (TLR2), Poly(I:C) (TLR3), MPL/TDM (TLR4), Flagellin (TLR5) and R848 (Imidazoquinoline; TLR7). The TLR9 ligand (CpG) as well as the TLR3 ligand, Poly(I:C), induced strong Th1 responses as measured by IFN-γ^+CD4$^+$ T cells, whereas the TLR4 ligand, MPL/TDM, induced detectable but significantly reduced numbers of IFN-γ^+ CD4$^+$ T cells in response to Rv2034 (Figure 5A). TLR2, 5 and 7 ligands did not induce IFN-γ^+ CD4$^+$ T cells at all. In none of the conditions CD8$^+$ IFN-γ^+ T cells were detected (data not shown). In contrast, analyses of humoral responses induced by immunization with various adjuvants showed that all different TLR ligands induced strong, Rv2034-specific antibody responses (Figure 5B), indicating a clear separation of these TLR specific adjuvants on the induction of humoral vs. cellular immune responses at least to *Mtb* antigens.

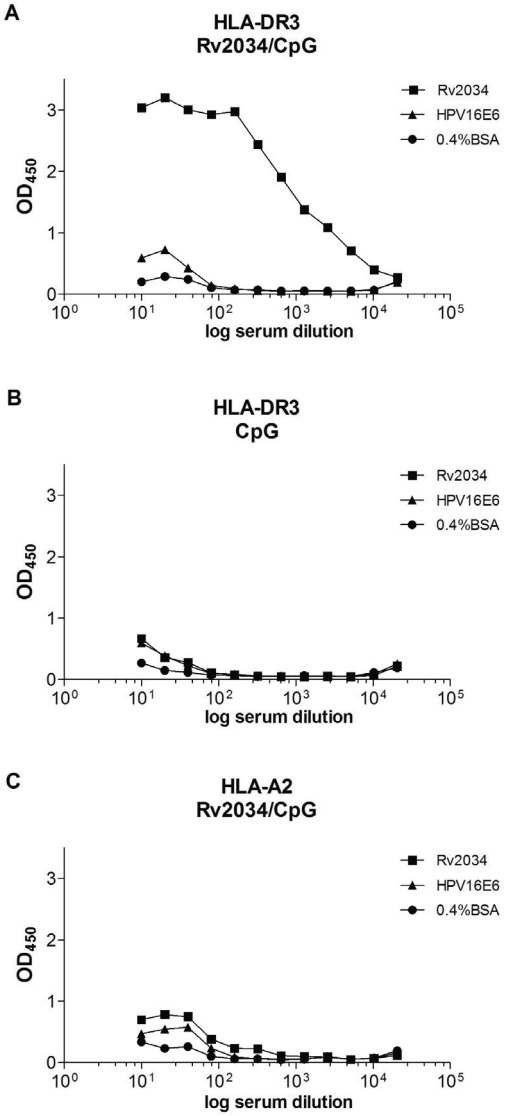

Figure 4. Quantification of serum antibodies to Rv2034. Following immunization of HLA-DR3 mice with Rv2034 in CpG (**A**) or with CpG alone (**B**) antibody titer (OD_{450}) against Rv2034 (■) was determined by ELISA. As a specificity control HPV16E6 (▲) or 0.4% BSA in PBS alone (●) were tested. Rv2034/CpG immunization of HLA-A2 mice were included as negative control (**C**). Serum dilutions are shown on the *x*-axis. All groups included five mice. All mice were separately analyzed.

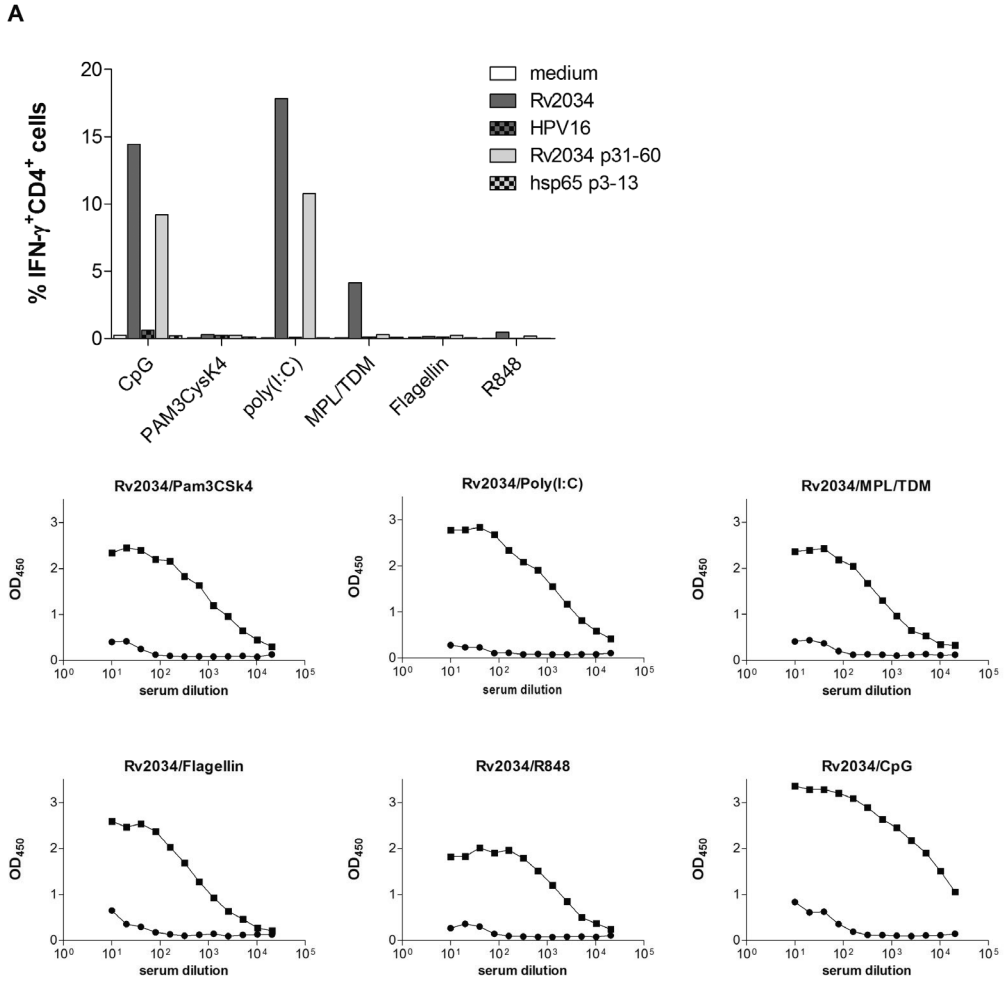

Figure 5. Rv2034 immunization of HLA-DR3 mice using different adjuvants. (**A**) HLA-DR3 mice were immunized three times with Rv2034 protein in either PAM3CysK4, Poly(I:C), MPL/TDM, Flagellin, R848 or CpG. Splenocytes were stimulated *in vitro* with Rv2034 protein, HPV16E6 (control recombinant protein), Rv2034 p31-60 or hsp65 p3-13 (control HLA-DR3-restricted peptide). After 7 days, cells were incubated with medium or fresh antigen for 1h before addition of brefeldin A and analyzed for intracellular IFN-γ production. Adjuvants are indicated on the *x*-axis.
(**B**): Sera of HLA-DR3 mice immunized as above or with CpG only were used to determine antibody titers (OD_{450}) by ELISA against Rv2034 (■) or BSA (0.4% in PBS) alone (●). Adjuvants are indicated above each figure. Serum dilutions are shown on the *x*-axis. Groups included 4-5 mice. Results are shown for one animal, representative for each test group.

Protective efficacy of Rv2034 immunization against live *Mtb* challenge

To assess the vaccine potential of the Rv2034 protein adjuvanted by CpG, its prophylactic protective effect was evaluated in a live *Mtb* challenge model, by enumerating the colony forming units (CFU) in the lungs. Interestingly, Rv2034 immunization reduced the number of CFU in the lungs by almost one log (from $6x10^6$ to $9x10^5$) whereas BCG caused a reduction of over a log to $1.8x10^5$ CFU (Figure 6). As a reference, recombinant hybrid protein Ag85-ESAT6 (H1) [31] adjuvanted in CpG was used as well: immunization with H1/CPG decreased the number of *Mtb* bacteria in the lung to a similar extent as Rv2034 (to $5x10^5$) being slightly less effective than BCG. The combination of BCG followed by Rv2034/CpG immunization 6 weeks afterwards also resulted in a decreased CFU load, but did not lead to an improvement compared to BCG alone. Importantly, in HLA-DR3^{neg} mice Rv2034 vaccination did not reduce the number of CFU, confirming the HLA-DR3-restriction of the T-cell mediated protection.

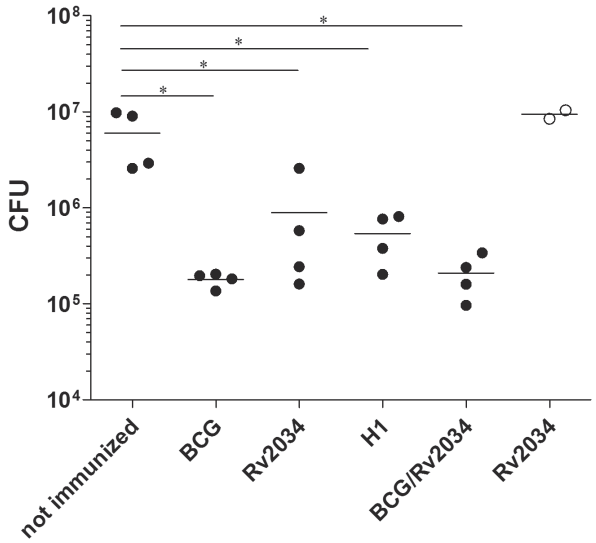

Figure 6. Determination of colony forming units (CFU) in the lungs of *Mtb*- infected mice. CFU were determined in lung homogenates derived from *Mtb* infected HLA-DR3 mice (●) or HLA-DR3^{neg} (○) mice that were left unimmunized or had been immunized with BCG, Rv2034 in CpG, H1 in CpG or immunized with BCG followed by a boost with Rv2034 in CpG as indicated on the *x*-axis. Protective efficacies are expressed as log10 bacterial counts. All groups included 4 mice.

Discussion

New vaccines against TB need to induce immune responses that target *Mtb* at the primary site of infection, which is the lung. Thus, *Mtb* antigens *in vivo* expressed during pulmonary *Mtb* infection (IVE-TB) might represent interesting vaccine candidates. In this study we have analyzed one of our newly identified IVE-TB proteins, the ArsR protein Rv2034, and show that it is strongly immunogenic *in vivo* in HLA-DR transgenic mice. Immunization of HLA-DR3 mice with Rv2034 induced specific cellular as well as humoral immunity against the protein and its dominant HLA-DR3-restricted epitope p31-50. Importantly, prophylactic immunization of HLA-DR3 mice with Rv2034 in CpG induced a significant protective response to *Mtb* which was similar to BCG, as judged by decreased bacterial numbers upon *Mtb* challenge. Importantly, previous work showed that Rv2034-specific T cells were induced by *Mtb* infection in mice and humans [4], supporting a biological role for Rv2034 in *in vivo* infection and rendering it an interesting vaccine target antigen.

Recently, we have shown that immunization of HLA-DR3 mice with an *Mtb* polyepitope in CpG induced strong cellular as well as humoral (antigen-specific Ig) responses [19]. Similar to Rv2034 immunization, this *Mtb* polyepitope immunization caused a significant reduction of bacterial load upon *Mtb* challenge. In contrast to the role of T cells, the role of *Mtb* antigen-specific antibodies in the control of *Mtb* remains largely unknown and has long been debated [32;33]. Antibodies can induce multiple immune-modulatory effects which can play a role in reducing the bacterial load such as mediating effector functions via binding to Fc receptors on immune cells, including activation of T-cell immunity and antibody-dependent cell-mediated cytotoxicity (ADCC) [34]. Indeed, several studies showed that immunoglobulins mediate protection against TB [35-37], whereas the organs of mice that are deficient for humoral immunity [μ-chain knock out (B cell$^{-/-}$ or Ig^{-}) and γ-chain subunit knock out (Fcγ-chain$^{-/-}$)] displayed 3- to 8-fold elevated numbers of viable bacilli compared with normal littermates, while splenic IFN-γ responses to whole antigen were unimpaired [38-40]. Moreover, in *Mtb* infected non-human primates active B cells were present in clusters within granulomas. Furthermore, high levels of *Mtb* antigen specific antibodies were detected within *Mtb* infected tissues, indicating that B cells play a role in *Mtb* infection [41]. Thus, Rv2034 antibody responses may substantially contribute to protection against *Mtb* infection.

Studies on animal models have shown that polyfunctional T cells which secrete IFN-γ, TNF-α and IL-2 simultaneously, are functionally superior in vaccine-induced protection and also often induced in *Mtb* vaccine studies [31;42-44]. In our current study, Rv2034 immunization of HLA-DR3 mice induced strong IFN-γ^{+}/TNF^{+} and IFN-γ^{+} CD4^{+} T cells specific for Rv2034, but no IFN-γ^{+}/TNF^{+}/IL-2^{+} CD4^{+} T cells. Importantly, control cytokine producing cells (MiCK-1) displayed IL-2 production demonstrating that IL-2 was detectable using this assay. Thus, despite the lack of substantial numbers of triple positive (IFN-γ^{+}/TNF^{+}/IL-2^{+}) CD4^{+} T cells, Rv2034 immunization reduced the number of bacteria in HLA-DR3 mice, but not in HLA-DR3^{neg} littermates or HLA-A2 mice. Therefore, it remains uncertain whether the presence of IFN-γ^{+}/TNF^{+}/IL-2^{+} CD4^{+} T cells

is an absolute requirement for vaccine-induced protection against *Mtb*. In view of our data, it is of note that the described *Mtb* vaccine studies also identified double and single cytokine producing $CD4^+$ T cells [31;42-44]. Interestingly, it is uncertain whether polyfunctional T cells correlate with TB protection [27-29]. Multiple factors are suggested to be involved in development or detection of polyfunctional T cells such as antigen dose and time point of T-cell analysis [28;45;46]. Also, previous work showed that the frequency of IFN-γ producing $CD4^+$ T cells does not directly associate to vaccine-induced protection [47] or might even be irrelevant in killing *Mtb* [48;49]. This further indicates that other (yet unknown) mechanisms are involved in controlling *Mtb* infection [50;51].

The use of TLR9 ligand as adjuvant in mouse models has shown efficient induction of both humoral and cellular immunity [19;20;52]. Of note is that the distribution of TLR9 in mice differs from that in humans being either broadly expressed on all types of DC in C57BL/6 mice or restricted to plasmacytoid DC in humans [53]. Out of the TLRs tested in this study that are broadly expressed on several DC types of both species (TLR2, 3, 4, 5 and 7) [54], only poly(I:C) (TLR3) and to a lesser extent MPL/TDM (TLR 4) induced Th1 responses. In contrast, regardless of the TLR ligand used as adjuvant for Rv2034 immunization in this study (TLR2, 3, 4, 5, 7 or 9), strong Ig responses were observed specific for Rv2034. Thus, these findings clearly show the separation of the effect on humoral and cellular responses by these adjuvants. Similar data have been reported in a single long peptide vaccination model in which all different adjuvants activated DC *in vitro* but only MPL and CpG (TLR4 and TLR9) induced a strong functional T-cell response *in vivo* [30]. This effect depended mainly on the capacity of ligands to mature pro-inflammatory DC and the duration of their *in vivo* stimulatory effect.

Besides the choice of adjuvant, the route of administration might play an important role in the development of robust Th1 immunity upon immunization [30;55]. In this study, adjuvanted *Mtb* antigens were administered subcutaneously representing a custom vaccination route in human vaccination programs.

Immunization with Rv2034/CpG resulted in decreased bacterial load in the lung after *Mtb* challenge, which was dependent on induced HLA-DR3-restricted T-cell immunity. Although Rv2034 immunization resulted in reduction of CFU, boosting BCG with Rv2034 did not further improve the protective effect of BCG alone. In addition, immunization with Ag85B/ESAT6 (H1) in CpG decreased the number of *Mtb* bacteria in the lung to a similar extent as Rv2034, slightly less than the BCG induced reduction. Interestingly, fusion protein H1, Ag85B/ESAT6/Rv2660 (H56), Ag85B/TB10.4 (HyVac4) and Mtb10.4/HspX (MH) showed an improved CFU reduction when used as BCG booster [44;56;57]. However, when administered as a subunit vaccine alone, the fusion proteins showed less protection compared to BCG, in line with previous findings [44;57] and this study. Thus, since Rv2034 is highly expressed in the lungs during *Mtb* infection, incorporating Rv2034 in a fusion protein may induce efficient protection when used as BCG booster. Rv2034 may thus have potential prophylactic and therapeutic vaccine properties when combined with early stage and TB latency proteins into a multistage vaccine [44], which should be capable of targeting relevant different stages

of the *Mtb* infection cycle. Additionally, the immunogenic peptide may also be integrated in a poly-epitope vaccine, containing multiple immunogenic epitopes from different *Mtb* proteins [19].

In summary, we have shown that the IVE-TB protein Rv2034 is immunogenic *in vivo* and has protective efficacy as evidenced by its ability to reduce the bacterial load *in vivo* after a live challenge with *Mtb*. Thus Rv2034 represents an interesting new antigen for subunit TB vaccination.

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Conflict of interest. The authors declare to have no financial/ commercial conflicts of interests. TO is co-inventor of an *Mtb* latency antigen patent, which is owned by LUMC.

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