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Variable BCG efficacy in rhesus populations: Pulmonary BCG provides protection where standard intra-dermal vaccination fails

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ABSTRACT

M.bovis BCG vaccination against tuberculosis (TB) notoriously displays variable protective efficacy in different human populations. In non-human primate studies using rhesus macaques, despite efforts to standardise the model, we have also observed variable efficacy of BCG upon subsequent experimental *M. tuberculosis* challenge. In the present head-to-head study, we establish that the protective efficacy of standard parenteral BCG immunisation varies among different rhesus cohorts. This provides different dynamic ranges for evaluation of investigational vaccines, opportunities for identifying possible correlates of protective immunity and for determining why parenteral BCG immunisation sometimes fails. We also show that pulmonary mucosal BCG vaccination confers reduced local pathology and improves haematological and immunological parameters post-infection in animals that are not responsive to induction of protection by standard intra-dermal BCG. These results have important implications for pulmonary TB vaccination strategies in the future.

1. Introduction

Tuberculosis (TB) remains a major global health issue in spite of widespread *M. bovis* BCG vaccination campaigns for many years [1]. Protection conferred by live *M. bovis* BCG, which is administered routinely by intra-dermal injection, is variable, depending on the target population [2,3]. Both intrinsic and environmental factors such as age, genetic predisposition and mycobacterial pre-exposure, as well as possible BCG strain variation and virulence of prevalent *M. tuberculosis* (*M.tb*) strain(s) have all been suggested to influence the efficacy of BCG [4–6]. However, the mechanisms ultimately determining the success (or failure) of BCG to protect from TB infection or disease remain poorly understood.

To improve TB prevention better immunisation strategies are required [7]. However, without conclusive mechanistic insights into protective immunity or correlates of vaccine induced protection, the development of a better TB vaccination strategy than BCG remains largely empirical. Animal models therefore play a pivotal role in vacccine development, providing proof-of-concept for protective efficacy and enabling head-to-head evaluation of candidates to identify the most effective vaccines to progress to clinical trials [8]. Amongst these, non-human primate (NHP) macaques are considered to be of primary importance. Like humans, macaques

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are naturally susceptible to *M.tb* infection and pathophysiologically exhibit most of the typical characteristics of human TB disease [9]. The (partial) protective efficacy of BCG vaccination in rhesus monkeys was reported four decades ago [10–13] and more recently a number of vaccine candidates have been tested in rhesus and cynomolgus macaques [14–22].

Macaques are geographically widespread, genetically distinct populations are well recognised and they are outbred [23]. Our laboratory was the first to suggest that the protective efficacy of BCG may vary in different macaques [24]. In that study BCG showed little protective efficacy in rhesus macaques while in cynomolgus macaques there was reduced TB disease after prior BCG vaccination, although the numbers of animal were too small for the difference to reach statistical significance. These findings were unexpected in the light of the early studies, but later we demonstrated protective effects of BCG (as well as investigational candidate vaccines) in rhesus macaques [17], although occasional experiments during this period failed to show any reduction of disease burden following parenteral BCG vaccination.

Recently there has been increased interest in using the NHP model as an important step in vaccine development [9], but as BCG is frequently used as a "gold standard" positive control in the evaluation of novel vaccines, variability in the response to BCG is a potential confounding factor. Here we summarise the findings of our earlier studies and in order to rule-out formally inter-experimental variation we describe a simultaneous head-to-head comparison of two rhesus cohorts of different breeding background in a BCG vaccination-*M.tb* challenge experiment. We demonstrate differential efficacy of BCG in NHP cohorts, reminiscent of the clinical performance of BCG and providing the opportunity to identify factors that affect BCG mediated protection and to determine correlates of protection.

In the light of the failure of the first parenteral TB booster vaccine in an efficacy trial [25], alternative immunisation strategies are being evaluated. Pulmonary mucosal immunisation has the advantage of targetting the portal of entry of *M.tb* and may induce responses that can inhibit early growth of the organism as well as inducing tissue resident memory cells [26–29]. Therefore, as a second objective, we investigated the efficacy of local pulmonary BCG vaccination in this rhesus model. Furthermore, prompted by observations in mice that simultaneous systemic and local BCG administration, a strategy termed SIM-BCG [26], significantly improves the outcome of experimental TB infection, we sought proof for this beneficial synergistic effect in NHP rhesus macaques. Immune monitoring confirmed BCG vaccine take by intra-dermal and pulmonary immunisation, while cutaneous recall testing supported the notion that separate immune memory compartments are targeted by the two routes of immunisation. We show that pulmonary vaccination reduces local pathology in rhesus monkeys that are not protected by standard intra-dermal BCG vaccination, a result with important implications for the development of future vaccine strategies.

2. Materials and methods

2.1. Experimental design

Rhesus macaques (*Macaca mulatta*) used in these studies were all captive bred for research purposes. For each, experiment animals were from a single homogeneous breeding origin, unless specified otherwise (in the case of direct cohort comparison). Rhesus genotype was confirmed by mitochondrial DNA typing for maternal descent, which validated the identification of animals as Chinese or Indian type rhesus monkeys [30]. Healthy animals were selected and shown to be immunologically naive to mycobacterial exposure prior to the start of the study by standard tuberculin skin testing (TST) by palpebral injection and DTH readout within 72 h using *Mycobacterium tuberculosis* (*M.tb*) tuberculin (Old Tuberculin) made up according to the manufacturer's instructions (Synbiotics Corporation, San Diego, CA). Additionally, an interferon-gamma release assay was shown to be negative prior to starting the experiment using whole blood or freshly isolated PBMC and purified protein derivative of *M. tuberculosis* (PPD, Statens Serum Institute (SSI), Copenhagen), *M. bovis* and *M. avium* (both from Prionics AG, Schlieren-Zürich) for specific *in vitro* recall stimulation.

Animals were stratified into treatment groups of N = 6 animals each. Random distribution was used for the following stratification parameters whenever relevant and applicable: gender, age, body weight and indicators to warrant social pair-wise housing. Specific treatment was assigned to the groups randomly.

Prior to starting, NHP study protocols were approved by the institutional animal use and care committee as a prerequisite of Dutch law on the use of animals in scientific research. All housing and animal care procedures were in compliance with European directive 86/609/EEC and later 2010/63/EU, as well as the "Standard for Humane Care and Use of Laboratory Animals by Foreign Institutions" provided by the Department of Health and Human Services of the US National Institutes of Health (NIH, identification number A5539-01). Experiments were limited in time by study protocol and pre-defined humane endpoints were in place to avoid severe animal discomfort. BPRC acquired AAALAC accreditation in 2012.

Animals were housed throughout in appropriately classified experimental facilities at BPRC with treatment groups randomly distributed over the animal rooms. All animal handling and biosample collection was executed under sedation by intramuscular injection of ketamine (10 mg/kg). Heparinised blood for immune monitoring, EDTA blood for standard haematology, and serum for C-reactive protein measurement were collected by venipuncture at specific time points defined by protocol. All clinical and pathological readings were done while blinded for treatment.

2.2. BCG vaccination

Where appropriate, animals were left untreated as non-vaccinated controls or immunised with BCG Danish 1331 (SSI, Copenhagen) by intra-dermal injection of a standard human dose of 0.1 mL of a stock solution of 2–8x10^6 CFU/mL (unless specified otherwise, see below). BCG was prepared freshly according to manufacturer's instructions immediately prior to immunisation. For each experiment sufficient vials were admixed into a single pool of reconstituted BCG, and all animals were immunised from this pool in random order within less than 2 h from vaccine preparation. For SIM-BCG treatment half a standard human dose in 0.1 mL was injected intra-dermally and half a dose in 10 mL of sterile saline solution (Eurovet Animal Health B.V., Bladel) delivered into the lung using a bronchoscope. For pulmonary mucosal vaccination only, a single human dose of BCG was administered in 10 mL of saline solution endobronchially.

2.3. Mycobacterium tuberculosis infection

For infectious challenge we initially used 1000 CFU of *M.tb* strain Erdman (historical studies A, C and D). This lot of *M.tb* strain Erdman was obtained from a seed vial kindly provided by P. Andersen (SSI, Copenhagen) and which was cultured, aliquoted and stored, courtesy of D. van Soolingen (RIVM, Bilthoven). For historical study B only we used Beijing strain HN878 at 1000 CFU per dose to assess if a highly virulent, clinical isolate of *M.tb* would provide a more stringent model with a greater window of opportunity for finding a

vaccine effect better than BCG. *M.tb* strain Beijing HN878 was kindly provided by M. Reed and C. Barry III (NIAID, NIH, Bethesda, MD). Infectious challenge was achieved by intra-tracheal instillation after intubation of a catheter to the depth of the carina. All animals in any single experiment were infected from a single suspension of *M.tb* in a single session and in random order.

Later, for the prospective head-to-head comparison study in this paper, we infected with 500 CFU of *M.tb* strain Erdman K01 (standard lot S-1), which was prepared as a global harmonisation strain from a seed lot from the Trudeau Institute under a collaborative agreement between WHO and CEBR/FDA with the assistance of Aeras, cultured and filled by Mycos Inc. (CO). *M.tb* strain Erdman K01 challenge was performed by endobronchial instillation using a bronchoscope.

2.4. Immune response analysis

Lymphocyte stimulation tests were used to analyse vaccine induced immunity with readout of specific NHP interferon-gamma (IFNg) secretion by ELISA or ELISPOT (both U-CyTech, Utrecht). For immune readout by IFNg ELISA, freshly isolated PBMC were seeded in triplicate in 0.2 mL of 25 mM HEPES buffered RPMI culture medium, supplemented with penicilin/streptomycin and 10% fetal calf serum, at 200,000 cells per well in a 96-well round-bottom microtiter plate. Cells were either stimulated or not with mycobacterial purified protein derivative (PPD, SSI, Copenhagen) at a final concentration of 5 μg per mL and incubated for 3 days at 37 °C and 5% CO_2. Secreted IFNg levels were measured by ELISA.

A so-called indirect ELISPOT procedure (UCy-Tech, Utrecht) was used to determine the frequency of IFNg producing cells after BCG vaccination and *M.tb* challenge. In brief, 200,000 freshly isolated PBMC were incubated in triplicate in RPMI culture medium in 0.1 mL per well in 96-well flat-bottom microtiter plates for 24 h. Cells were stimulated with *M.tb* PPD or a recombinant fusion protein of ESAT6 and CFP10 (produced at LUMC according to K. Franken et al. [31]), both at a final concentration of 5 μg per mL, or left unstimulated as a negative (medium) control. Subsequently, cells were transferred to specific anti-IFNg coated filter plates (PVDF, Millipore) for an additional overnight (18 h) incubation and spots were developed using biotinylated anti-IFNg detector antibody, streptavidin-horseradish peroxidase conjugate and tetramethylbenzidine substrate (the latter from MAbTech, Stockholm). Spots were quantified using an automated reader (AELVIS, Hannover).

For immunogenicity screening post-BCG by tuberculin skin testing (TST) animals received a Mantoux injection of tuberculin OT (Synbiotics Corporation, San Diego, CA) in 0.1 mL, or 0.1 mL of sterile saline control solution in their upper back. After 72 h injection sites were scored for erythema and induration (adapted Draize score [32]), and biopsies were taken with a circular dermatological knife of 8 mm in diameter. Skin biopsies were treated for 1 h with 1 mg/mL collagenase D and 0.2 mg/mL DNase I (Roche, Mannheim) and homogenised using GentleMACS™ (Miltenyi) to allow for cytometric analysis of the skin resident and recruited lymphocytes. Lymphocytes were stained and characterised as $CD14^{-}CD20^{-}CD3^{+}$ cells on a LSR-II cytometer (Becton Dickinson) and using FlowJo® software.

2.5. Clinical measurements and pathological assessment

Before and after infection and at study endpoint, clinical measures were recorded to assess TB disease progression. Body weights were recorded at all sedation/bleeding time points. Standard haematology was performed using a Sysmex 2000i (Siemens); C-reactive protein (CRP) levels were determined using a Cobas™ Integra400+ (Roche Diagnostics). At endpoint animals were sacrificed in random order and underwent full pathologic evaluation based on gross and histologic examination as previously described [17]. Limited by capacity and logistics these endpoint evaluations took place over a period of 13 days. Gross pathology was recorded and scored according to an algorithm as before [17] or as published by Lin et al. [21] with minor adaptation (large coalescing lesions receiving a maximum score of 4 for prevalence and a score of 3 for size). After collecting small representative samples for histopathology and cryopreservation, whole lungs were minced and homogenised as described previously. In the rhesus cohort comparison study lungs (lung slices) were randomly sampled by the principle of stereology [33]. Serial dilutions of lung homogenates were plated (in duplicate) on 7H10 Middlebrook agar plates containing 100 mg/L cycloheximide to determine the number of colony forming units (CFU) as a measure of bacterial load in the infected lung.

2.6. Statistical analysis

Throughout the manuscript, when depicting results of individual animals, colour coding for each of the N = 6 animals per treatment group is consistent (blue, green, magenta, red, black, brown) to allow for comparison of individual results between assays and readouts at a glance. Results were statistically evaluated using GraphPad Prism™ and non-parametric Mann-Whitney testing for identifying statistically significant differences between group medians.

3. Results

3.1. Historical variation in protection of macaques by intra-dermal BCG immunisation: a retrospective analysis

While developing and refining a rhesus model for TB vaccine research we noticed the variable outcome of BCG vaccination and subsequent *M.tb* challenge. Retrospective analysis suggested that the genetic background of the rhesus macaques was a confounding factor in the results (not shown). From that point we further standardised our experiments and ensured that animals for each experiment were of similar genotype. Thus, using group sizes of N = 6 only, we could demonstrate protective effects of BCG and investigational regimes over non-vaccinated controls, reaching or approaching statistical significance ([17], and unpublished). However, despite standardisation, in two experiments we found no signs of protection by BCG. In Table 1 we summarise retrospectively the animal and treatment group characteristics of four independent, historical studies. Studies A and B exemplify successful prophylactic BCG vaccination in rhesus monkeys, whereas studies C and D show a failure of BCG to protect. (Of note: data from study A have been published previously [17], and are listed here solely for reference purpose. Data from studies B, C and D were hitherto unpublished.)

Regardless of the variation between individual animals and irrespective of the different pathology levels observed in these experiments, we could detect distinct effects of BCG vaccination on gross (macroscopic) pathology in studies A and B, but not C and D (Fig. S1). Group median total pathology scores (as a percentage of total possible score) of non-vaccinated controls versus BCG vaccinees were 37% versus 14%, 7.0% versus 0%, 9.0% versus 6.5%, and 32% versus 26%, for studies A to D respectively. BCG vaccine take was confirmed in all studies by a specific increase of mycobacterial PPD stimulated IFNg response post-vaccination (Fig. S2). The differential BCG vaccine effect in these studies was reflected both by pulmonary pathology and by extra-thoracic, disseminated TB-associated

Table 1
A retrospective analysis: standard intra-dermal BCG vaccination can be variably efficacious in NHP rhesus monkeys.

	Study A	Study B	Study C	Study D
Protective BCG Effect	Yes	Yes	No	No
Group Size (N = n)	6	6	6	6
Rhesus Spectrotype	Chinese	Chinese	Indian	Chinese
Gender, proportion of males	100%	50%	100%	100%
Age (years ± sd)	7.8 ± 0.6	6.8 ± 1.7	7.2 ± 2.9	8.0 ± 1.0
Body Weight (kg ± sd)	8.9 ± 2.0	5.7 ± 1.8	10.2 ± 2.8	9.3 ± 1.6
BCG Vaccine Strain (2–8 x10^5 CFU)	Danish 1331	Danish 1331	Danish 1331	Danish 1331
M.tb Challenge Strain (1000 CFU)	Erdman	HN878	Erdman	Erdman
Time Interval from Vaccination to Infect	18 weeks	18 weeks	18 weeks	18 weeks
Follow-up Time Post-Infection	17 weeks	17 weeks	17 weeks	24 weeks

Animal and treatment group characteristics and experimental conditions of 4 independent, historical TB vaccination-infection experiments in rhesus macaques (*Macaca mulatta*) are summarised. For studies A to D, naive animals were selected from single populations and confirmed by mitochondrial DNA typing to be of homogeneous spectrotype (Chinese or Indian rhesus). (Data from study A have been published previously [17]).

lesion scores, typically involving spleen, liver and/or kidney (Table S1). Other clinical measures of TB disease and protective BCG vaccine effect, e.g. body weight alteration and changes in serum levels of CRP and haematological erythrocyte-associated values, sometimes reached statistical significance in studies A and B, but never in studies C and D (Table S1).

None of the animal and experimental design characteristics of the historical studies listed in Table 1 - genotype (i.e. Chinese versus Indian type rhesus), gender, age, body weight, BCG vaccine dose/strain, *M.tb* challenge dose/strain, time interval from vaccination to infection and from infection to endpoint - explained the failure of BCG to (partially) protect from TB disease after *M.tb* challenge in studies C and D. Study C was a prospective study anticipating BCG failure on the basis of earlier analyses and selection of animals from a specific cohort. In study D, however, the lack of protective effect of BCG was unexpected. Thus, from these historical studies we learned that the outcome of BCG vaccination seems to be critically determined by unknown factors intrinsically associated with the population of rhesus macaques used in each study.

3.2. Head-to-head comparison of protective response of different macaques to intra-dermal BCG immunisation

Despite our standardisation efforts, however, we could not exclude formally the possibility of accidental variations in experimental conditions compromising BCG efficacy. Therefore, we designed a prospective head-to-head BCG vaccination-*M.tb* infection study of two rhesus cohorts. The animals were obtained from different breeding facilities and represented different - Indian versus Chinese - genotypes. Animals were vaccinated as before with a standard human dose of BCG Danish 1331 intra-dermally and 17 weeks later they were challenged with *M.tb* harmonisation strain Erdman K01 by endobronchial instillation. Non-vaccinated (non-v) control animals were enroled later, at week 12 post-primary BCG, five weeks prior to *M.tb* challenge. The details of the experimental set-up are summarised in Table 2 (see treatment groups T1 to T4 in particular).

Immunogenicity of intra-dermal BCG vaccination was monitored by specific IFNg ELISPOT upon *in vitro* recall stimulation with mycobacterial PPD during the vaccination phase of the study. In both rhesus populations PPD specific IFNg responses could be detected above background and with similar kinetics and similar amplitude (Fig. 1). The IFNg response curves indicate similar immunogenicity of the vaccine in both rhesus cohorts.

To determine BCG vaccine efficacy, vaccinees and non-vaccinated controls from both rhesus populations were infected with *M.tb* strain Erdman in a single randomised session. Two non-vaccinated controls of the Indian rhesus cohort reached the humane endpoint in weeks 8 and 11 post-infection, and pathological examination upon euthanasia confirmed severe pathology in both animals (which are represented in Fig. 2A, B and C in the non-v* designated treatment group by blue and magenta symbols, respectively). The remaining animals were all culled in random order at the endpoint according to protocol. Lung pathology, disseminated disease and bacterial burden in the lung was broadly more prominent in Indian than in Chinese type animals (Fig. 2, see non-v* and non-v designated groups, respectively). BCG vaccination reduced these parameters of disease and infection in the Indian type rhesus, but failed to do so in the animals of Chinese genotype (Fig. 2). BCG vaccination reduced group median lung pathology, extra-thoracic disseminated pathology, and lung colony forming units (CFU) in Indian rhesus (Fig. 2A, B, C, respectively), while no such protective effects were observed in Chinese type animals (Fig. 2D, E, F). This head-to-head study demonstrates that standard intra-dermal BCG vaccination can fail to protect some populations of rhesus macaques. Taken together with the historical

Table 2
Experimental setup and details of study design.

	Treatment	Treatment Abbreviated	Rhesus Genotype	BCG Danish 1331 Dose
T1	non-vaccinated	non-v*	Indian	n.a.
T2	BCG vaccinated, intra-dermally (i.d.)	BCG*	Indian	5×10^5 CFU
T3	non-vaccinated	non-v	Chinese	n.a.
T4	BCG vaccinated, intra-dermally (i.d.)	BCG	Chinese	5×10^5 CFU
T5	Pulmonary BCG vaccinated	MUC	Chinese	5×10^5 CFU
T6	Simultaneously pulmonary & i.d. BCG vaccinated	SIM	Chinese	(2.5 + 2.5) x10^5 CFU

Prospective head-to-head comparison of two cohorts of rhesus macaques comprising N = 6 animals in treatment groups T1 to T4. In each cohort of Indian and Chinese type rhesus macaques, standard (human) intra-dermal BCG Danish 1331 vaccination was compared to non-vaccinated controls (non-v* and BCG* indicate Indian type rhesus in text and figures). Non-vaccinated controls were enroled from week 12 post-BCG onwards. Additionally, sharing standard BCG and non-vaccinated controls (T3 and T4), groups of N = 6 Chinese rhesus macaques were immunised by endobronchial instillation with a standard human dose of BCG (abbreviated as MUC, T5) or by the simultaneous administration of a standard dose of BCG, half intra-dermally and half by endobronchial instillation (SIM-BCG, T6). All animals were challenged 17 weeks post-BCG with 500 CFU of *M.tb* strain Erdman K01 endobronchially and followed for another 13 weeks at max until endpoint.

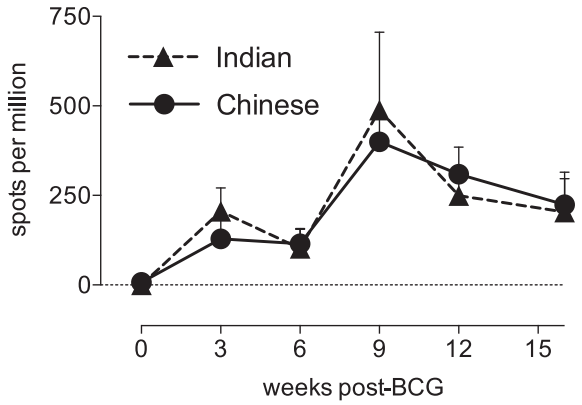

Fig. 1. IFNg response upon BCG vaccination in two distinct rhesus populations. In a prospective head-to-head comparison the immune response to BCG vaccination was monitored in two distinct rhesus cohorts by specific NHP IFNg ELISPOT assay. The frequency of IFNg producing cells, detected upon *in vitro* stimulation with mycobacterial PPD, indicated similar efficacy of BCG vaccine take in Indian and Chinese type rhesus. Group averages of control corrected values (+standard error) are plotted over time.

data as shown in Table 1, it is clear that protection does not strictly correlate with the genotype - Indian versus Chinese.

3.3. Immune responses after pulmonary and parenteral vaccination with BCG

While BCG in man is routinely administered by intra-dermal injection, BCG delivered by aerosol has previously been suggested to improve protection over intracutaneous injection in rhesus monkeys [11] as well as in mice and guinea pigs [26,34–36]. More recently, simultaneous local mucosal and systemic administration of BCG, so-called SIM-BCG, was shown to provide additional protective efficacy in a mouse model and significantly decreased the lung *M.tb* load compared to immunisation by either route alone [26]. To re-assess pulmonary mucosal BCG vaccination in rhesus macaques (abbreviated as MUC) and to evaluate SIM-BCG, two additional groups of N = 6 of the same Chinese rhesus cohort were included in the study (Table 2, T5 and T6 for MUC and SIM-BCG, respectively). Again, vaccination and infection were performed from single BCG vaccine and *M.tb* challenge suspensions, in single sessions and with animals from all treatment groups handled in random order.

The immune response post-vaccination was monitored by specific IFNg ELISPOT using freshly isolated PBMC. As soon as 3 weeks after vaccination frequencies of antigen specific IFNg producing cells were significantly increased over baseline values in all three vaccine groups, standard intra-dermal BCG, MUC and SIM (Fig. 3A). The highest levels of IFNg response were obtained at week 9 post-vaccination in the standard BCG group, significantly higher than in the MUC but not SIM-BCG group. The MUC PBMC IFNg response remained significantly lower compared to standard BCG up to week 16, 1 week before infectious challenge ($p = 0.028$ by Mann-Whitney). Nevertheless, up to that point, all three vaccine groups displayed significantly elevated IFNg responses. At study week 16, the group median IFNg response values were 147.5, 45.0, and 115.0 for BCG, MUC and SIM, respectively, and highly significantly greater than the median of 10 spots per million in non-vaccinated control animals (Fig. 3A).

Twelve weeks after primary vaccination a tuberculin skin test (TST) was performed to assess the antigen specific recall response *in vivo*. Although classical DTH responses of induration or erythema were not informative if recordable at all, skin biopsies from PPD injection versus saline control sites revealed significant mycobacterial antigen associated influxes of $CD3^+$ T cells upon flow cytometric analysis (Fig. 3B). Local T cell recruitment in the skin was most prominent in the two groups immunised with BCG intradermally and significantly above the median specific T cell influx in the MUC group. Yet, despite the pulmonary route of vaccination, MUC treated animals also showed significant antigen-specific recruitment of T cells to the TST site over that in non-vaccinated controls (Fig. 3B).

In summary, immune response monitoring by ELISPOT and TST-DTH site analysis confirmed vaccine take. The decreased PBMC and skin responses of the MUC group are in accordance with the expectation that pulmonary vaccination preferentially induces a lung homing immune response [36–38].

3.4. Protective effect of pulmonary compared to intra-dermal BCG immunisation

The results of *M.tb* challenge of the MUC and SIM groups compared to non-v and standard BCG groups are shown in Fig. 4. Median lung pathology scores are 22.0 and 26.0 for non-v and standard BCG respectively, while the median scores for MUC, 6.5, and SIM, 10.0, show a strong trend toward a decrease ($p = 0.061$ by Mann-Whitney for MUC in comparison to non-v controls) (Fig. 4A). Note, however, that the pulmonary immunisation of the MUC and SIM groups is not identical because the SIM animals received half the lung dose of BCG given to MUC treated monkeys. Disseminated extra-thoracic pathology scores were relatively low and not significantly different between any of the groups, although appearing worse in the standard BCG group compared to non-v, MUC and SIM (Fig. 4B, not significant). Individual CFU counts from the lungs displayed considerable variation as expected, but median values of ^{10}log CFU were 3.16 and 3.18 for MUC and SIM, respectively, and about half a log lower in comparison to non-v and BCG animals with medians of 3.64 and 3.69, respectively (Fig. 4C). These results confirm earlier data indicating the superiority of pulmonary mucosal over parenteral immunisation with BCG in NHPs, but more importantly indicate that pulmonary delivery can show a protective effect under circumstances when standard intradermal BCG fails to induce protection. It is not clear from these data whether SIM can provide superior protection against *M.tb* challenge in NHPs since the systemic component (intra-dermal BCG) of the SIM regime failed, indeed may have been deleterious, in this cohort.

3.5. Clinical and immunological indicators of protective efficacy of pulmonary BCG

As in human TB, NHP macaques can show clinical signs of disease after experimental *M.tb* infection, which can be used as a readout of treatment efficacy. Severe loss of body weight (wasting) has been reported previously in *M.tb* infected Chinese type rhesus macaques [17], but was not prominent in the present cohort for reasons unclear at the moment. Levels of CRP, an acute phase protein in the serum reflecting systemic inflammation, were elevated after *M.tb* challenge in most animals, but highly variable and without significant difference between the treatment groups in this experiment (median change in CRP over the infection period: 21.1, 32.6, 12.0, and 12.2 mg/L for non-v, BCG, MUC and SIM, respectively).

Beside body weight and CRP we also recorded haematologic alterations from the start of the infection until endpoint. Red blood cell associated measures of mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH), reflecting infection associated anemia, are decreased in non-v and BCG vaccinated animals in

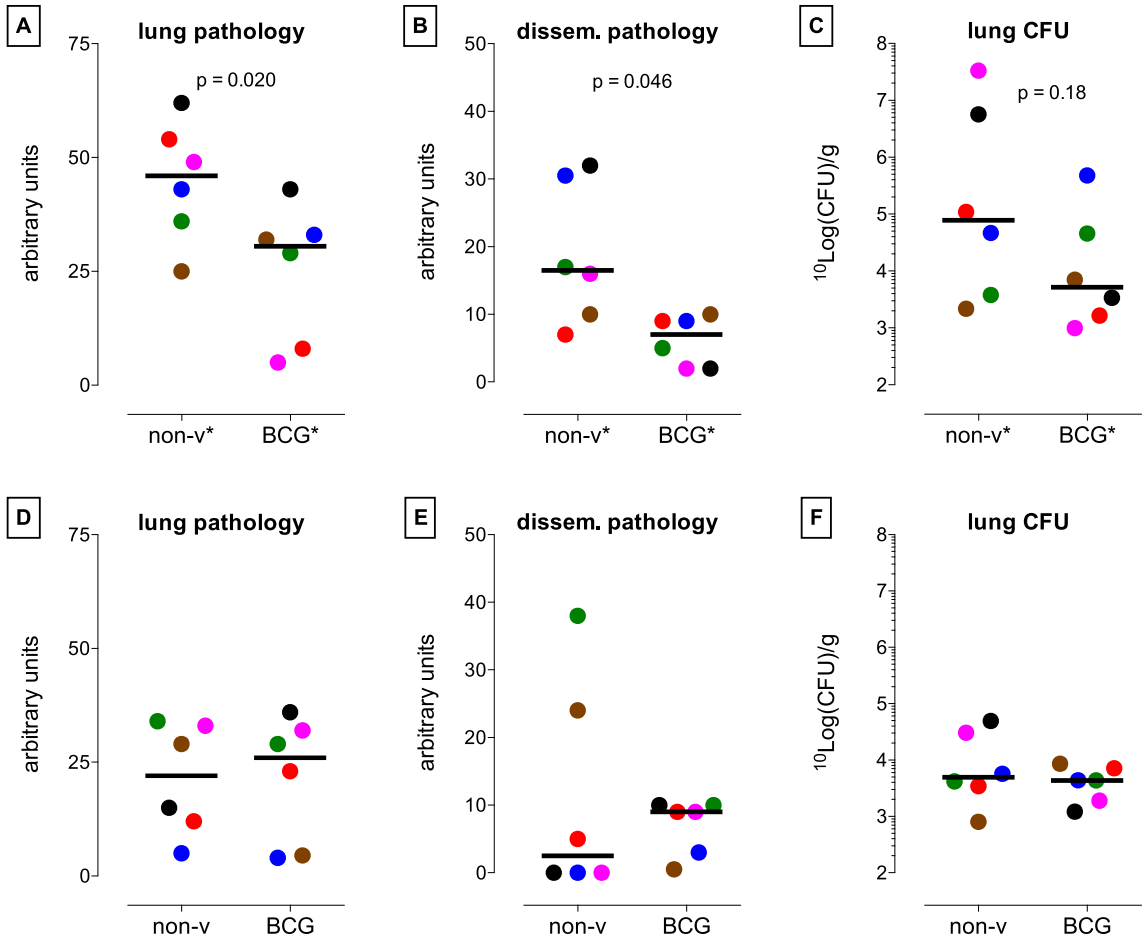

Fig. 2. Head-to-head analysis of differential protective effect of BCG in distinct rhesus populations. Groups of N = 6 rhesus macaques were either vaccinated by standard intra-dermal BCG or left unvaccinated, and 17 weeks later infected by endobronchial *M.tb*. The top panels (**A-C**) present results from the Indian rhesus cohort (non-v* versus BCG*); the bottom panels (**D-F**) from the Chinese rhesus cohort (non-v versus BCG). At endpoint animals were sacrificed for evaluation of TB disease and infection. Lung pathology (**A, D**) and extra-thoracic TB lesions (**B, E**) were scored in arbitrary units. Mycobacterial burden was enumerated from random lung sample homogenates **(C, F)**. Colour coding of individual animals per treatment group is consistently applied throughout the manuscript to allow for correlation at glance. Horizontal lines represent group median values. P-values by Mann-Whitney testing reached significance only for the Indian type rhesus cohort for lung and disseminated pathology scores only (top panels A and B, respectively).

particular (Fig. 5A and B). Prior MUC and SIM treatment, however, reduces the adverse effect of *M.tb* infection on the erythrocyte compartment (Fig. 5A and B).

The ratio of monocytes over lymphocytes (Mo/Lf ratio) has been identified as a risk factor for TB in BCG vaccinated children [39], while we have previously identified a correlation between Mo/Lf ratio post-infection and the severity of TB pathology in rhesus macaques (unpublished). In this study infectious challenge resulted in increased Mo/Lf ratios over the infection period in the non-v control and BCG groups, but not in the MUC or SIM treated animals (Fig. 5C).

Altogether, while systemic inflammation assessed by serum CRP did not reveal a clear vaccine effect, alterations in haematologic measures of the erythrocyte compartment (MCV, MCH) as well as Mo/Lf ratio post-infection provide suggestive evidence of decreased disease severity and protective efficacy of local pulmonary but not standard intra-dermal BCG vaccination.

3.6. Immune responses post-M.tb infection

As well as measuring IFNg responses of PBMC after vaccination to provide evidence of vaccine take, immune responses were monitored by specific ELISPOT after infectious challenge. IFNg responses 1 week before infection are highest in the animals that received intra-dermal BCG (BCG and SIM groups) (see above and Fig. 3A, week 16 post-vaccination). That response pattern is unchanged 1 week after challenge, but by week 3 post-infection the pattern changes completely (Fig. 6A and B). Both MUC and SIM vaccinated animals at week 3 display significantly lower IFNg responses after *in vitro* PPD stimulation in comparison to standard BCG vaccinated animals ($p = 0.004$ and $p = 0.026$, respectively) and non-v controls ($p = 0.009$ and $p = 0.041$, respectively) (Fig. 6A). The greatest differences in median response values between pulmonary mucosal vaccination strategies (MUC and SIM) versus standard intra-dermal BCG and non-v controls are apparent at the peak of the PPD specific IFNg response at week 6 with group medians of 1100, 1147, 5941 and 5688 spots per million respectively, albeit statistically less robust (Fig. 6C).

IFNg release against ESAT6 and CFP10, two antigens that are absent from BCG and therefore in this experiment specifically associated with *M.tb* infection, revealed similar response kinetics (Fig. 6D). While 1 week post-challenge no ESAT6-CFP10 response was detectable (Fig. 6E), at week 6 the MUC and SIM treatment groups show lower median IFNg responses than BCG and non-vaccinated controls (713, 344, 2207 and 2201, respectively; for SIM vs non-v, $p = 0.065$) (Fig. 6F).

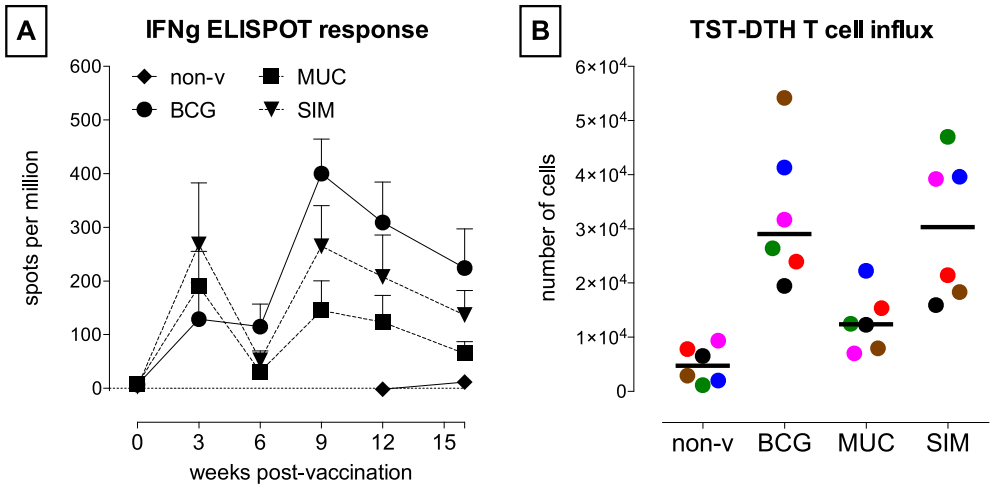

Fig. 3. Immunogenicity of pulmonary and SIM BCG vaccination. **A)** The frequency of antigen specific IFNg secreting cells in freshly isolated Chinese rhesus PBMC was determined by specific ELISPOT. Group averages of (medium control corrected) PPD specific responses (+standard error) are plotted for standard intra-dermal BCG (BCG), pulmonary BCG (MUC) and SIM-BCG treatment groups over time up to 16 weeks post-vaccination. Non-vaccinated control animals were included in the analysis from week 12 onward. **B)** Twelve weeks post-BCG vaccination a standard tuberculin skin test (TST) was performed by the intra-dermal injection of tuberculin or saline control. Three days later the skin injection sites were sampled and processed for flow cytometric evaluation. The individual specific influx of $CD3^+$ T lymphocytes upon tuberculin over saline *in vivo* recall stimulation is plotted with horizontal lines indicating group medians. Colour coding of individual animals in each treatment group is consistently applied throughout to allow for correlation at a glance (for animals in non-v and BCG treatment groups as in Fig. 2). All differences between MUC and BCG, MUC and SIM, MUC and non-v are significant with p = 0.0043, 0.0152 and 0.0152 respectively (by Mann-Whitney).

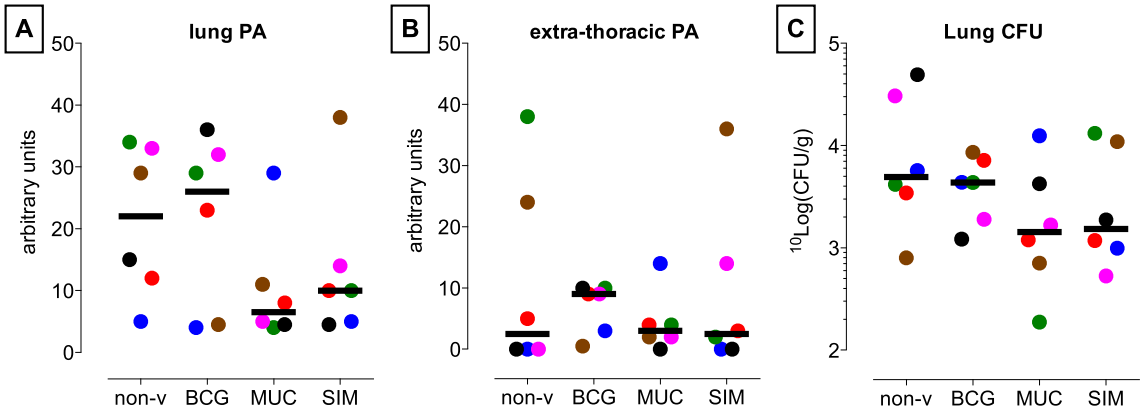

Fig. 4. Gross pathology and lung bacterial load. Intradermal BCG, pulmonary BCG and SIM-BCG treated Chinese rhesus monkeys (BCG, MUC, and SIM, respectively) were challenged by endobronchial infection with *M.tb* strain Erdman and 13 weeks later sacrificed for pathological and bacteriological evaluation of protective efficacy. **A)** Lung and **B)** extra-thoracic TB pathology were determined by pre-defined arbitrary scoring algorithm. **C)** Mycobacterial burden was enumerated from random lung sample homogenates by plating serial dilutions. Horizontal lines represent group medians. Colour coding of individual animals as in Fig. 3. The p value of the difference in median lung pathology between MUC versus non-v by Mann-Whitney is 0.061; for all other comparators: p > 0.01.

The lower antigen specific IFNg levels after MUC and SIM vaccination and *M.tb* challenge could be due to more efficient homing of IFNg producing effector cells to the local sites of infection, to a better control of bacterial replication, or to both. In any case, since the magnitude of PBMC TB antigen specific immune responses post-*M.tb* challenge has repeatedly been shown to correlate with disease severity [17,40,41], the immune response patterns observed are compatible with improved prognosis after pulmonary MUC or SIM immunisation with BCG.

4. Discussion

To our knowledge this is the first study to show in a head-to-head experiment that Chinese and Indian rhesus macaques may either develop some protective immunity or fail to do so in response to parenteral BCG immunisation. Parenteral BCG vaccination in rhesus macaques has previously been reported to provide partial protection against intratracheal, endobronchial or aerosol infection using *M.tb* strain Erdman or H37Rv and a challenge dose ranging from 14 to 3000 CFU [10,12,16,17,20]. However, failure of standard intra-dermal BCG to protect in rhesus macaques, despite immunological evidence of vaccine take, has been demonstrated previously in a small study comparing the response to BCG of cynomolgus and Indian type rhesus macaques; a finding ascribed to genetic factors [24]. The hitherto unpublished historical data from Chinese and Indian macaque vaccination-infection experiments described here, together with the head-to-head experiment, illustrate variability in protective efficacy of parenteral BCG in both Indian and Chinese type rhesus macaques, but taken together show that this difference is not due to the genotype of the animals. The data reported here and the head-to-head evaluation in particular, exclude the possibility that variability in BCG efficacy

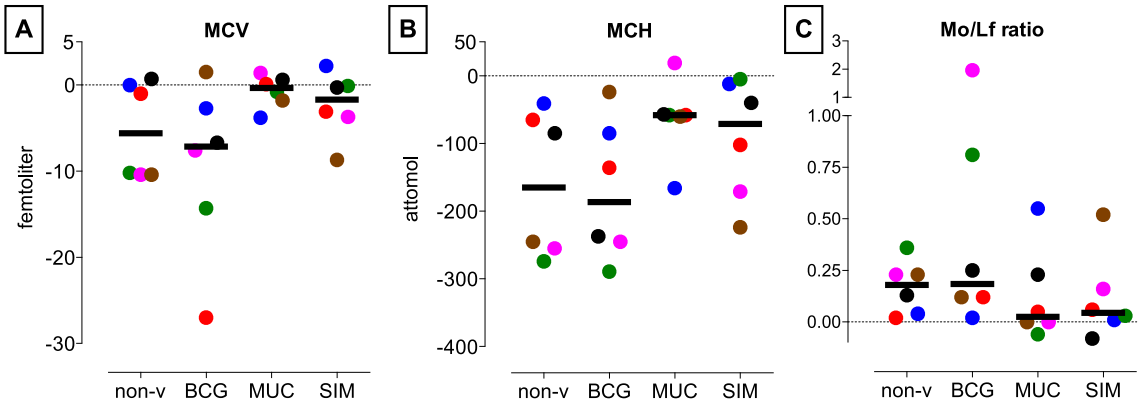

Fig. 5. Changes in haematological values following infectious challenge. Changes in clinical values over the infection period are depicted for **A)** mean corpuscular volume (MCV), **B)** mean corpuscular hemoglobin (MCH) and **C)** ratio of monocyte to lymphocyte counts (Mo/Lf ratio). Colour coding of individual animals as in Fig. 3, and horizontal lines indicating group median values. The p value of the difference in median change in MCH of MUC versus non-v by Mann-Whitney is 0.084; for all other comparators: $p > 0.1$.

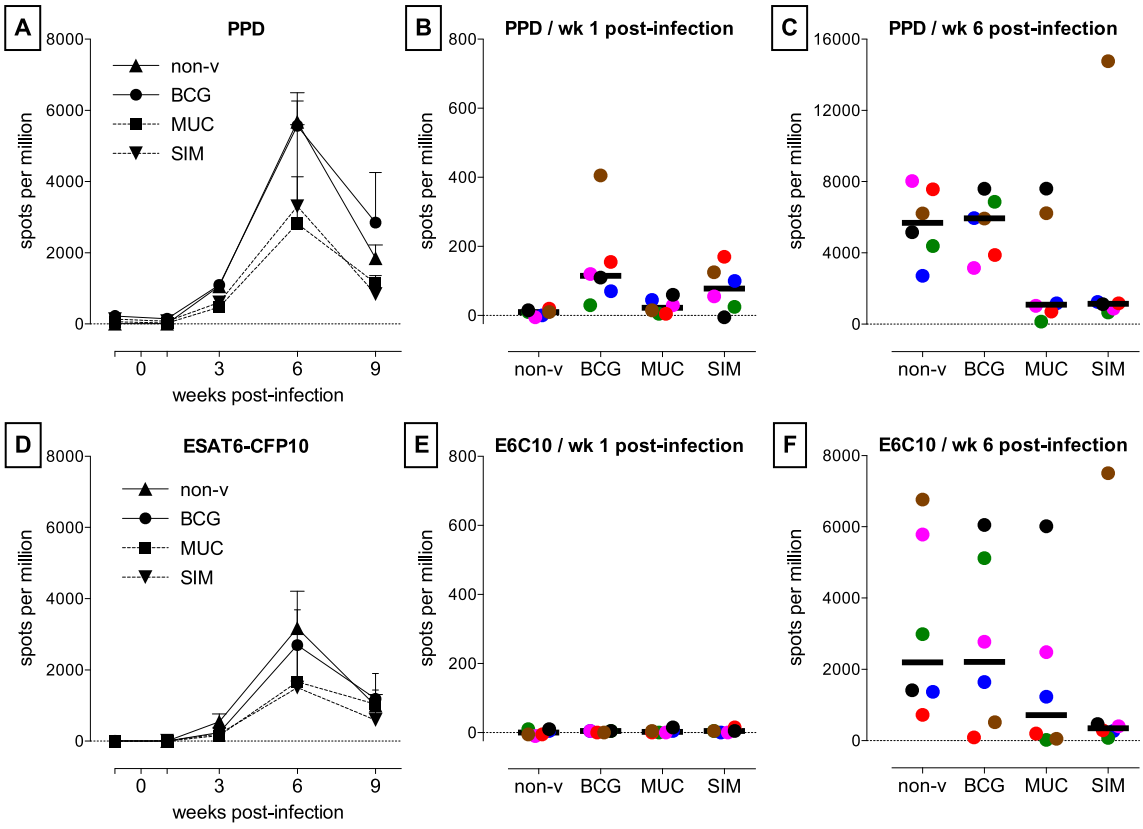

Fig. 6. IFNg responses post-*M.tb* infection. Immune responses post-infection were monitored using freshly isolated PBMC and a specific IFNg ELISPOT assay. PBMC were stimulated *in vitro* with PPD (**A-C**) or a recombinant ESAT6-CFP10 (E6C10) fusion protein (**D-F**). **A, D)** Group averages of saline control corrected frequencies of IFNg secreting cells (+standard error) are plotted over time. Individual results with horizontal lines indicating group medians, are plotted at 1 week **B, E)** and the peak of response at week 6 **C, F)**. Colour coding of individual animals as in Fig. 3. At the peak, $p = 0.24$ and $p = 0.18$ for MUC versus BCG and non-v respectively, and $p = 0.065$ for both SIM versus BCG and SIM versus non-v (Mann-Whitney).

relates to the magnitude of the vaccine induced response in the blood or at the DTH skin site. Nor can variation in the *M.tb* challenge be the cause of BCG failure in the Chinese rhesus cohort. Although recently lack of protection after immunisation of Indian rhesus was ascribed to use of a high challenge dose of 275 CFU of *M.tb* Erdman K01 [22], the present data using the same challenge strain from the same source, indicate that BCG efficacy can be demonstrated irrespective of challenge dose, but is affected by other factors (see below).

It remains possible that genetic differences between rhesus macaque populations explain the variability of parenteral BCG efficacy, for example, in genes controlling immune functions not measured by our assays for T cell immunity. However, as animals in our experiment were protected by endobronchially administered BCG, this does not seem likely. It is therefore tempting to speculate that environmental parameters may alter the (innate) immune

status of macaques and the efficacy of BCG, as has been suggested for man (see below).

BCG has long been known to vary geographically in efficacy [2], an effect often ascribed to exposure to environmental mycobacteria (EM), and a growing body of evidence shows that alterations to the gut microbiota can have profound effects on subsequent immune responses. Experimentally, oral or parenteral immunisation of mice with EM can block subsequent induction of protective immunity by parenteral BCG or subunit vaccines [42,43]. Similarly, the presence or absence of a single species of microorganism can profoundly alter the balance between T_{H17} cells, shown to be important in protection of cynomolgus macaques after BCG immunisation [44], and IL-10 producing T_{reg} in mice [45–47]. Intestinal helminths in man and *Helicobacter pylori* in NHPs and man, are implicated in modulating immunity to tuberculosis [48,49] and interestingly, different macaque populations have been found to harbour distinct intestinal bacterial communities [50]. We speculate that differences in the microbiota might account for the variability of protective immunity induced by parenteral BCG in rhesus macaques.

In addition to demonstrating variability in the protective efficacy of BCG in different NHPs, we show in this study that pulmonary mucosal administration of BCG can overcome the failure of parenteral BCG. Although a very recent study in (Indian type) rhesus macaques has failed to demonstrate a significant improvement of pulmonary BCG delivered 11 weeks after parenteral BCG [51], abundant evidence indicates that delivery of TB vaccines to the respiratory tract mucosa is highly effective. Direct comparisons in mice indicate that both BCG and subunit vaccines are more [26,36,38,52] or at least equivalent in efficacy when delivered mucosally [26,36]. Also, in accordance with what we report here in NHP, in DBA/2JRj mice that are not protected by parenteral (subcutaneous) BCG vaccination, intranasal mucosal administration of BCG overcomes susceptibility and confers protection by an IL17-dependent mechanism [53]. In guinea pigs very small numbers of BCG organisms delivered by aerosol are as potent as much larger numbers delivered parenterally [54,55]. In cattle endobronchial is at least as protective as parenteral BCG delivery [56], while endobronchial or aerosol administered BCG induced a stronger lung immune response [57,58] and had superior protective efficacy to parenteral BCG in NHPs [12]. Skin test conversion has been reported in humans exposed to a BCG aerosol [59].

Although mucosal BCG is effective in animal models, currently BCG is administered parenterally to humans. However, parenteral boosting with subunit vaccines has so far not proved dramatically effective in improving parenteral BCG efficacy over BCG alone, either in animal models [52] or humans [25]. In contrast mucosal boosting after parenteral BCG can provide markedly improved efficacy in mice [38,52] and guinea pigs [60]. In the light of these data, and because pulmonary mucosal and parenteral immunisation induce very different immune responses (see below), the alternative immunisation strategy of simultaneous parenteral and mucosal immunisation (SIM) was investigated and shown to be effective in mice [26,27]. SIM with parenteral BCG and endobronchial administration of a subunit vaccine has also been shown to be highly effective in cattle [56]. However in the present experiments, although a SIM BCG group was included, the protective effect in this group is difficult to interpret, since parenteral BCG alone failed to induce protective immunity.

Nevertheless there are good reasons to continue to explore the efficacy of (pulmonary) mucosal delivery and SIM (reviewed in Ref. [61]). Immune responses to *M.tb* infection in the lungs are delayed [62] so that *M.tb* grows unchecked for the first two weeks, even in parenterally immunized animals [27,63,64]. In contrast, in mice immunized by the respiratory route *M.tb* does not grow for the first week [26,27]. This may be due to the nature of immune cells induced by different routes of immunisation. In mice, after parenteral immunisation antigen specific cells are found in lung tissue, but after mucosal immunisation or respiratory infection, they are also found in bronchoalveolar lavage (BAL) [65,66]. These tissue resident T cells (T_{RM}) play an important role in protection against other respiratory infections (for example influenza, Sendai or respiratory syncytial viruses) in mice and have distinct phenotypic properties [28,67,68]. In humans lung T cells differ in many respects from those in blood and are enriched for cells that can respond to respiratory pathogens [29], while PPD responsive T cells are found in BAL of skin test positive donors [69].

However, although in mice parenteral and pulmonary immunisation result in distinct patterns of localisation of antigen specific cells in the lungs, when NHPs or humans were immunized parenterally or by aerosol with the vaccine candidate MVA85A, neither systemic nor BAL cellular responses differed dramatically [70,71]. This might be a technical issue attributable to the nature of the aerosol, as particles with a maximal size of 4 μm, which reach the lower respiratory tract (LRT), induce strong BAL responses and powerful systemic immunity in NHPs. In contrast immunisation of the upper respiratory tract (URT) induces much weaker lung and systemic responses [72], a result reminiscent of experiments in mice showing that URT immunisation induces poor responses in the LRT [37]. However, as is the case for parenteral vaccines including BCG, powerful LRT immune responses do not necessarily equate to protection. When the Aeras-402 vaccine (recombinant adenovirus type-35 (rAd35) expressing Ag85A, Ag85B and TB10.4) was administered as an aerosol to NHPs, it induced strong and prolonged BAL CD4 and CD8 T cell responses and transient responses in the blood [73], but there was no protection against *M.tb* challenge [22].

This result was attributed to the high dose (275 CFU) of *M.tb* used for challenge, but it is also possible that the vaccine activates inappropriately the innate immune system, although inducing a T_{H1} like immune response. In a mouse experiment, parenteral priming with a recombinant Ad5 expressing Ag85A was followed by a mucosal boost with the same construct or a VSV recombinant also expressing Ag85A. Both regimes induced equally strong LRT T cell immune responses, but only the mice primed and boosted with the adenoviral construct were protected against *M.tb* challenge [74]. The Ad boost stimulated, whereas VSV blunted (through increased IFNβ production), IL12 responses and anti-mycobacterial host defence. In other experiments activation of NK cells via IL-21 contributed to early non-specific protection against *M.tb* challenge, so that there may be several pathways to protective immunity against tuberculosis [61]. Much other evidence indicates the importance of innate immunity in protection against *M.tb* [62,75], while BCG induces a state of “trained immunity” in macrophages [76]. The innate signals activated by Ad35 (used in Aeras-402, above) are not known, but different adenoviruses vary greatly in efficacy as vaccine vectors [77], although only a minority have been tested by the (pulmonary) mucosal route.

5. Conclusions

Both parenteral and pulmonary mucosal immunisation regimes may fail for multiple reasons, but the failure of parenteral BCG to protect in some NHP experiments has important implications for development of TB vaccines. Clearly the use of parenteral BCG as a “gold standard” positive control vaccine is fraught with difficulties. Assessment of parenteral/parenteral, parenteral/pulmonary mucosal prime boosts, or in our case a SIM BCG regime, is impossible when parenteral BCG immunisation fails. On the other hand, investigation of the reasons for failure of parenteral BCG in NHPs might throw light on the mechanism of the variation in BCG

efficacy in humans [78]. It is intriguing that endobronchial BCG is effective in NHPs when parenteral immunisation fails and that after oral adminstration of EM or BCG, mucosal administration overcomes the failure of a parenterally administered subunit vaccine to protect against *M.tb* challenge in mice [43]. We suggest that one reason to pursue further work on pulmonary mucosal immunisation with both subunit vaccines and BCG, may be because this route might circumvent at least some of the geographical variation in protection after immunisation against tuberculosis.

Author contributions

F.A.W.V. and A.W.T. conceived and designed studies, and the pulmonary vaccination experiments together with E.Z.T. and P.C.L.B.; F.A.W.V., E.Z.T. and P.C.L.B. wrote the paper; R.A.W.V., C.C.S., O.A.E., V.S., and N.M.v.d.W. processed samples and performed assays and data analyses; I.K. was responsible for pathology assessments, E.V. for animal typing, G.B. and J.B. for veterinary care, T.H.M.O. for immunological consultancy, critical feedback and reagents, and K.W.v.K. for clinical consultancy and radiology; J.A.M.L. and P.J.H. bore overall responsibility as chairs of the Animal Science Dpt. and A.W.T. and C.H.M.K. as chairs of the Dpt. of Parasitology of BPRC.

Conflict of interest

The authors declare no conflict of interest.

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Competing interests

None declared.

Ethical approval

The studies were approved by the institutional animal use and care committee as a prerequisite of Dutch law on the use of animals in scientific research.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tube.2017.02.003.

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