MAJOR ARTICLE

A Human Challenge Model for *Mycobacterium tuberculosis* Using *Mycobacterium bovis* Bacille Calmette-Guérin

Angela M. Minassian, Iman Satti, Ian D. Poulton, Joel Meyer, Adrian V. S. Hill, and Helen McShane

The Jenner Institute, University of Oxford, United Kingdom

(See the editorial commentary by Dockrell, on pages 1029–31.)

Background. There is currently no safe human challenge model of *Mycobacterium tuberculosis* infection to enable proof-of-concept efficacy evaluation of candidate vaccines against tuberculosis. In vivo antimycobacterial immunity could be assessed using intradermal *Mycobacterium bovis* bacille Calmette-Guérin (BCG) vaccination as a surrogate for *M. tuberculosis* infection.

Methods. Healthy BCG-naive and BCG-vaccinated volunteers were challenged with intradermal BCG. BCG load was quantified from skin biopsy specimens by polymerase chain reaction (PCR) and culture colony-forming units. Cellular infiltrate was isolated by suction blisters and examined by flow cytometry. Prechallenge immune readouts were correlated with BCG load after challenge.

Results. In BCG-naive volunteers, live BCG was detected at the challenge site for up to 4 weeks and peaked at 2 weeks. Infiltration of mainly $CD15^{+}$ neutrophils was observed in blister fluid. In previously BCG-vaccinated individuals, PCR analysis of skin biopsy specimens reflected a degree of mycobacterial immunity. There was no significant correlation between BCG load after challenge and mycobacterial-specific memory T cells measured before challenge by cultured enzyme-linked immunospot assay.

Conclusions. This novel experimental human challenge model provides a platform for the identification of correlates of antimycobacterial immunity and will greatly facilitate the rational down-selection of candidate tuberculosis vaccines. Further evaluation of this model with BCG and new vaccine candidates is warranted.

The tuberculosis vaccine field has had to rely on preclinical animal challenge models of *Mycobacterium tuberculosis* infection or on the development of in vitro models of *M. tuberculosis* killing as surrogate measures of vaccine efficacy [1]. However, it remains unknown how predictive these are of human in vivo protection, and the development of a relevant in vivo human challenge model would be a significant advancement for the field. The existence of human challenge models for pathogens, such as malaria, influenza, dengue, and typhoid, has greatly facilitated vaccine development [2–5]. However, the ethical barriers to challenging humans with virulent replicating mycobacteria have thus far limited the development of a human *M. tuberculosis* challenge model. Here, we introduce a novel in vivo bacille Calmette-Guérin (BCG) challenge model using *Mycobacterium bovis* BCG vaccination as a surrogate for *M. tuberculosis* infection, based on the hypothesis that an effective vaccine against *M. tuberculosis* should also reduce the replication of BCG. Published preclinical studies support the hypothesis that vaccine-induced suppression of a BCG challenge in small animals is comparable to that of an *M. tuberculosis* challenge, and the vaccine most commonly assessed in such challenge studies is BCG [6–8]. BCG is a feasible challenge agent for human use: it is a safe replicating mycobacterium (with 99.95% sequence homology relative to

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Correspondence: Angela M. Minassian, DPhil, Jenner Institute, University of Oxford, Old Road Campus Research Bldg, Roosevelt Dr, Headington, Oxford, Oxfordshire, OX3 7DQ United Kingdom (minassian.angela@gmail.com).

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live *M. bovis*) [9], it causes a self-contained limited infection in immunocompetent animals and humans, and it is licensed for human use.

We have recently demonstrated that live BCG persists in murine skin for ≥4 weeks and that intradermal BCG vaccination consistently protects against an intradermal BCG challenge—an effect that is independent of vaccination dose, route, or vaccination-challenge interval. We have also shown in the mouse model that efficacy of BCG vaccination against subsequent intradermal BCG challenge is comparable to known vaccine efficacy against aerosol *M. tuberculosis* challenge, supporting the relevance of a mycobacterial skin challenge to an aerosol *M. tuberculosis* challenge [10].

We now describe the application of these preclinical findings to a human BCG challenge model, in which the kinetics of BCG were assessed in the skin of healthy BCG-naive volunteers. Few studies have attempted to detect BCG at the vaccination site, other than in the context of a suppurative lesion complicating vaccination, and none have actually quantified the level of live BCG at these sites [11]. Here, we show that live BCG persists in human skin for up to 1 month and that there is a spectrum of mycobacterial growth or protection within a group with prior BCG vaccination, which may reflect the spectrum of protection conferred by BCG against tuberculosis in humans [12]. This BCG challenge model has the potential to enable proof-of-concept vaccine efficacy screening for the first time in humans and to allow the identification of an immunological profile associated with reduced bacterial load in the skin.

METHODS

Recruitment and Enrollment

This study was approved by Oxfordshire Research Ethics Committee A (REC reference 07/Q1604/3). All volunteers gave written informed consent before participation. Twenty-eight healthy, BCG-naive volunteers were recruited, followed by an additional 12 participants previously vaccinated with BCG. For this previously vaccinated group, volunteers were excluded if they had received the BCG vaccine within the past 2 months; however, the minimum period (from prior vaccination to recruitment) of those enrolled was 8 months. The full inclusion and exclusion criteria are described in Supplemental Methods 1.

All enrolled volunteers had normal baseline hematology and biochemistry findings and negative results of hepatitis B and C and HIV antibody testing. Latent *M. tuberculosis* infection was excluded by ex vivo enzyme-linked immunospot (ELISPOT) responses to ESAT6 and CFP10, as described elsewhere [13].

Challenge and Follow-up

The first 28 participants were challenged intradermally with BCG (SSI; 0.05 mL; diluted in saline to 0.1 mL) from a vial containing 2–8 $\times$ 10^6 colony-forming units (CFU)/mL, giving a final dose of approximately 1–4 $\times$ 10^5 CFU into the upper arm (deltoid insertion). The dose administered was confirmed by plating the BCG onto 7H11 Middlebrook agar. A punch biopsy was performed at the challenge site 1, 2, or 4 weeks after challenge. The 12 BCG-vaccinated volunteers were challenged with BCG and underwent biopsy 2 weeks after challenge. After vaccination, all 40 volunteers were followed up at weeks 1, 2, 4, 8, 12, and 24. Vaccination sites were assessed for local reactions and vital signs recorded; 60 mL of blood was taken at each time, and peripheral blood mononuclear cells and serum were isolated and cryopreserved.

Skin Biopsies

The punch biopsy was performed using a sterile technique with a standard 4-mm punch biopsy (Stiefel); 0.5–2 mL of 1% lignocaine with 1:200 000 adrenaline was infiltrated subcutaneously. The punch biopsy specimen was taken from the center of the BCG vaccination site and frozen in liquid nitrogen. Biopsy specimens were later thawed, weighed, and homogenized in 1 mL of sterile phosphate-buffered saline in a Dispomix machine (Thistle Scientific) before plating and DNA extraction.

Culture, DNA Extraction, and Quantitative Polymerase Chain Reaction

Culture of BCG, BCG DNA extraction from skin biopsy specimens, and quantitative polymerase chain reaction (PCR) were performed as described elsewhere [10]. Estimated CFU counts were corrected for the total amount of DNA extracted per biopsy specimen.

Creation of Suction Blisters

Suction blisters were created using an Eschmann suction unit device (Reed et al [14]). Blisters were dressed and left overnight, and the fluid was harvested using a needle and syringe. Leukocytes were isolated and stained for surface cellular markers (Supplemental Methods 2).

Cultured ELISPOT Assays

Cultured ELISPOT assays were performed using frozen peripheral blood mononuclear cells as described elsewhere [15], with some minor modifications, as follows: ~1 $\times$ 10^6 cells/mL/well were cultured in a 12-well plate in fetal calf serum–free medium (AIM-V; Gibco). Cells were stimulated with 2 μg/mL Ag85A (7 peptide pools), 1 μg/mL TB10.3 (1 peptide pool), and 10 μL/mL recombinant human interleukin 7 and incubated for 3 days. A total of 20 U/mL recombinant human interleukin 2 and 10 μL/mL interleukin 7 were added to each well on day 3, and 20 U/mL interleukin 2 was added on days 7 and 10 in 0.5 mL of fresh medium. On day 12, the cells were washed in fresh medium, rested overnight, washed, counted, and plated at 0.3 $\times$ 10^6 or 0.1 $\times$ 10^6 cells/well in an ELISPOT assay. Results are expressed as spot forming cells per 1 million cells at day 0.

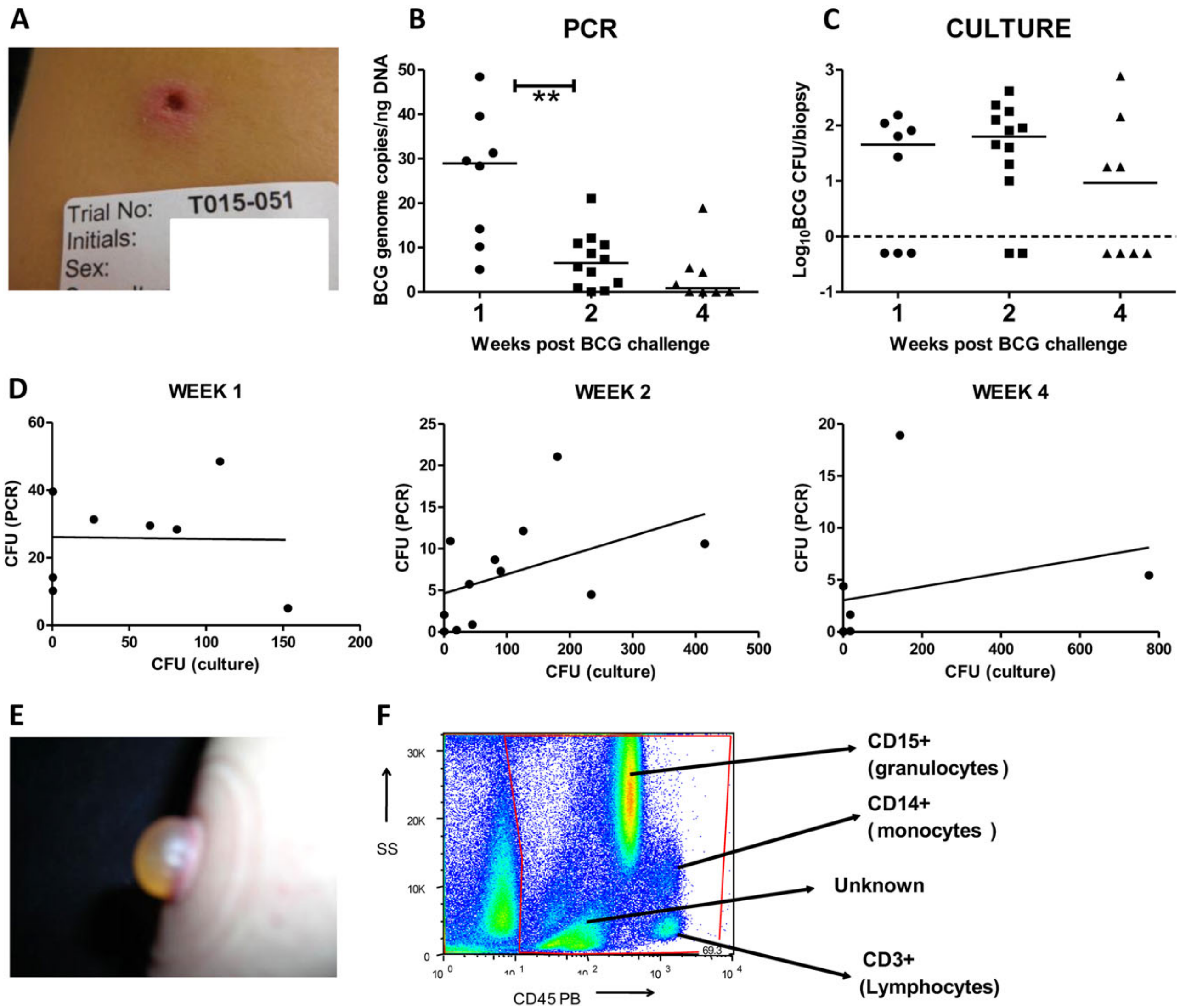

Figure 1. Quantification of bacille Calmette-Guérin (BCG) in skin biopsy specimens from BCG challenge sites. *A*, Appearance of skin 2 weeks after biopsy. *B*, Estimated number of BCG copies (log_{10}) per biopsy specimen (taken at 1, 2, or 4 weeks after challenge) by quantitative polymerase chain reaction (PCR) (corrected for nanograms of DNA extracted). *C*, Colony-forming unit (CFU) counts after 3–4 weeks of incubation on 7H11 Middlebrook agar. Bars represent median per group. *D*, Correlation between CFU counts measured by culture and PCR 1, 2, and 4 weeks after challenge (Spearman rank, week 1, $R = -0.22$, $P = .6$ [left]; week 2, $R = 0.77$, $P = .004$ [middle]; week 4, $R = 0.75$, $P = .03$ [right]). *E*, Appearance of blister at 1 week after challenge. *F*, Application of CD45 marker to blister cells, compared with side scatter (SSC).

Statistical Analysis

Data were not normally distributed. Consequently, medians with interquartile ranges are presented, and nonparametric tests have been applied. Differences in BCG CFU counts between groups were analyzed using the Kruskal-Wallis test (for comparison of >2 independent groups) and Mann–Whitney test (for comparison of 2 groups). Correlations (within individuals) were analyzed by Spearman rank. The statistical software used was Stata (StataCorp). Differences were considered statistically significant at $P < .05$ (*$P < .05$, **$P < .01$, and ***$P < .001$).

RESULTS

Safety and Tolerability of BCG Challenge Model

Twenty-eight BCG-naive and 12 previously BCG-vaccinated volunteers were challenged with BCG intradermally. Skin biopsy specimens were taken from the center of the challenge site of naive volunteers 1, 2, or 4 weeks after challenge. Biopsy specimens were taken 2 weeks after challenge in the previously BCG-vaccinated group. There were no unexpected local reactions or systemic complications after BCG challenge. Three of 28 volunteers in the BCG-naive group and all 12 in the

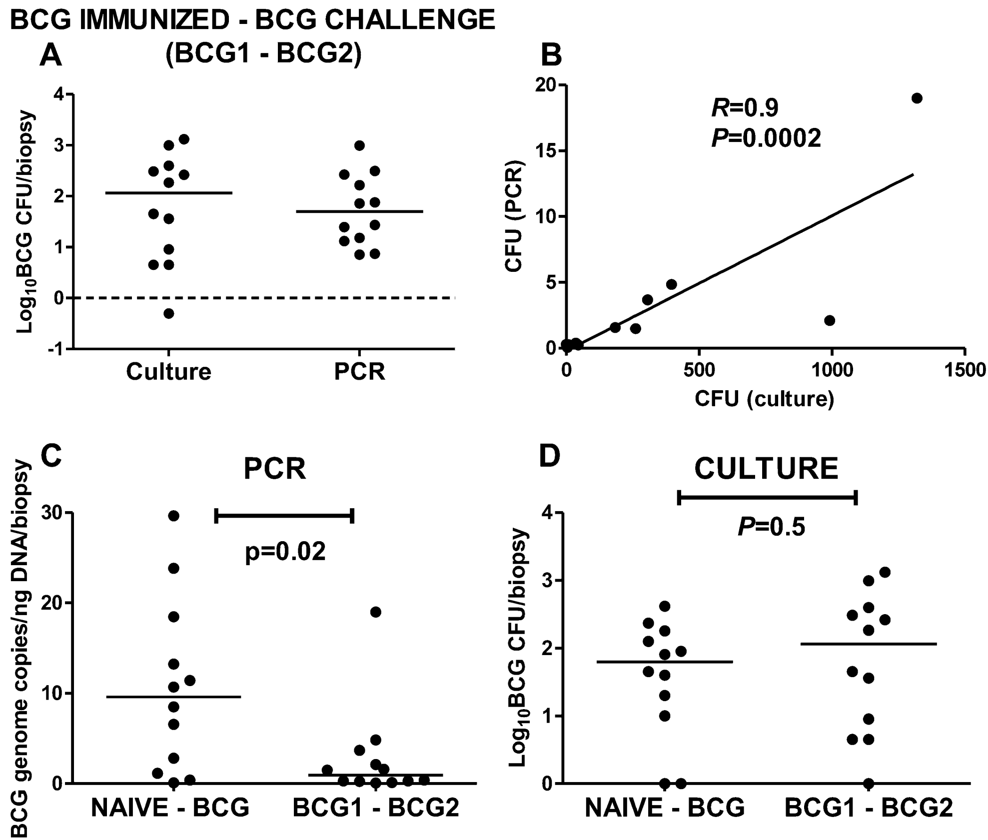

Figure 2. Variability in postchallenge colony-forming unit (CFU) counts in bacille Calmette-Guérin (BCG)–vaccinated humans. *A*, Comparison between culture and polymerase chain reaction (PCR) challenge results in BCG-vaccinated volunteers, log scale. *B*, Correlation between culture (CFU count) and PCR (Spearman rank). Comparison of PCR and culture challenge results in naive (NAIVE–BCG) and BCG-vaccinated (BCG1–BCG2) volunteers. *C*, PCR values (BCG copies [$\log_{10}$] per biopsy specimen, corrected for nanograms of DNA extracted) in BCG-naive and BCG-vaccinated groups. *D*, Corresponding culture values ($\log_{10}$ BCG CFU count per biopsy specimen). Exact *P* values are shown.

BCG-vaccinated group developed purulent discharge from the challenge site 1–2 weeks after vaccination, but all cases spontaneously subsided within 2–4 weeks. Punch biopsies healed well and left a small scar superimposed on the challenge site.

Detection of Live BCG in Human Skin After Vaccination

BCG was detected in punch biopsy specimens (Figure 1*A*) from all 28 BCG-naive volunteers by PCR (Figure 1*B*) and from 19 of 28 by culture (Figure 1*C*). Live BCG was detected by culture up to 2 weeks in 10 of 12 volunteers and up to 4 weeks in 4 of 8 (Figure 1*C*). There was a decrease in genome copies, as identified by PCR, during weeks 1–2 but not in CFU counts, and there was considerable variability in the culture data, up to 3 logs at week 4 (Figure 1*C*). Although this makes it more difficult to show a statistically significant difference between time points, there remained a trend for a reduction in live BCG in the skin over time. Quantification of BCG by PCR was a mean of 1 log higher than by culture.

Local Cellular Profile at BCG Vaccination Site

Blister cells were isolated 1 week after BCG vaccination (Figure 1*E*), and surface staining revealed distinct populations of leukocytes in all volunteers. The main CD45-staining populations were $CD15^+$, $CD14^+$, and $CD3^+$ cells (Figure 1*F*), with a relative predominance of $CD15^+$ cells. A significant unknown $CD45^+$ population (with a small proportion costaining for CD14 and CD15) remained to be fully characterized. Direct inspection of the cellular types by direct microscopy of formalin sections confirmed the cellular populations identified by flow cytometry and confirmed that there were almost no platelets. There were very small numbers of $CD1a^+$ dendritic cells and $CD56^+$ natural killer cells (data not shown).

Comparison of Challenge Results: PCR vs Culture CFU Counts

The PCR and culture data for the group with prior BCG vaccination are shown in Figure 2*A*. Within this group, there was a strong correlation between the 2 methods of bacterial quantification 2 weeks after challenge (R = 0.87; P = .0002)

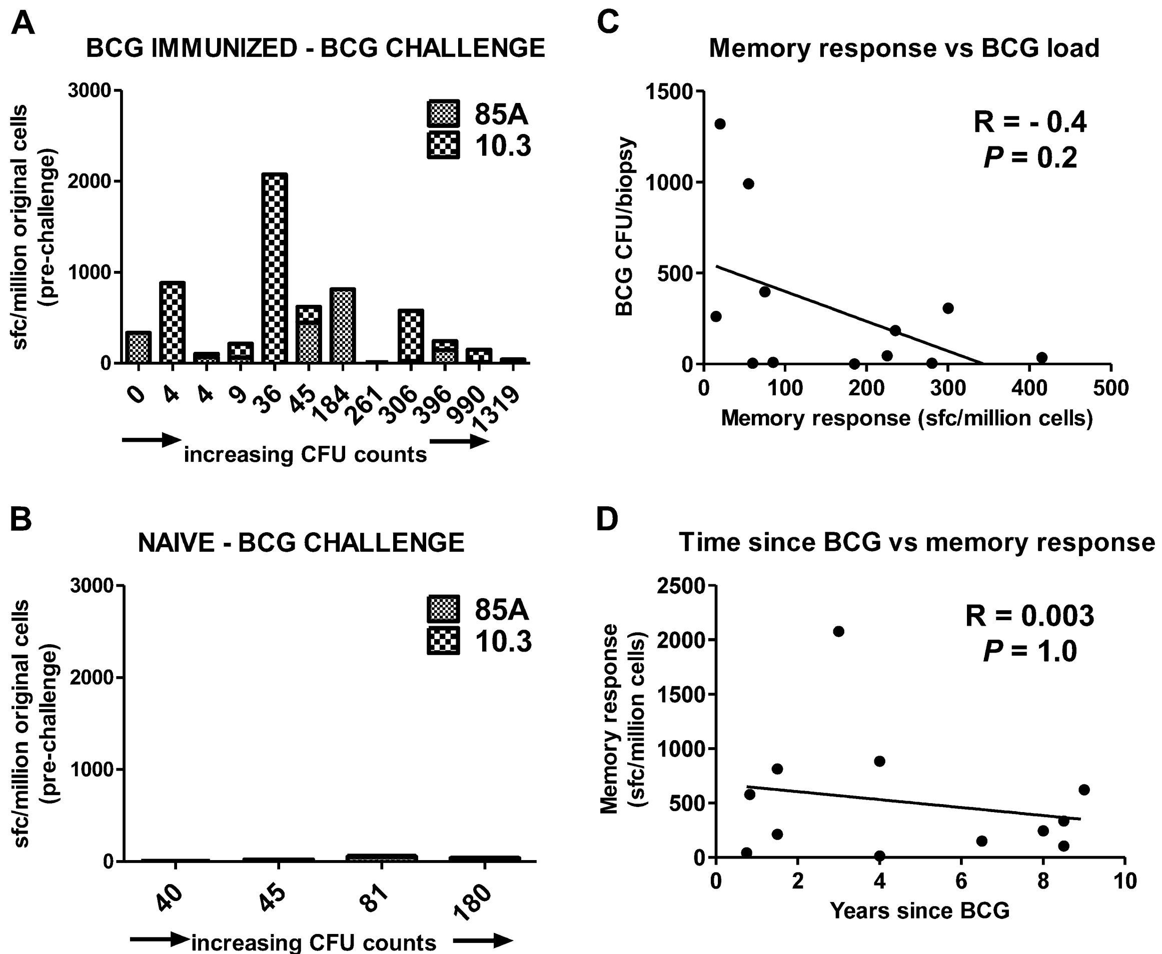

Figure 3. Prechallenge cultured enzyme-linked immunospot (ELISPOT) responses in bacille Calmette-Guérin (BCG)–vaccinated volunteers. Graphs show the responses per million original cultured cells, measured at day 0 (total culture period, 13 days). Combined cultured interferon γ T-cell responses to TB10.3 and 85A peptide pools are shown for each volunteer. Volunteers in order of increasing challenge colony-forming unit (CFU) count are shown along the x-axis, labeled by exact counts per biopsy specimen. Prechallenge responses are shown for BCG-vaccinated volunteers (n = 12) (*A*) and BCG-naive volunteers (n = 4, *B*); there were only enough frozen peripheral blood mononuclear cells for 4 of the naive volunteers to allow processing of cultured ELISPOT assays. *C*, Correlation between magnitude of memory T-cell response and bacterial load of challenge (BCG CFU count per biopsy specimen; $R = -0.4$, $P = .2$). *D,* Time since prior BCG vaccination versus magnitude of prechallenge cultured ELISPOT responses.

(Figure 2*B*). This correlation was stronger than for the corresponding BCG-naive group ($R = 0.77$; $P = .004$).

A comparison of the 2-week postchallenge CFU counts between the BCG-vaccinated and BCG- naive groups is shown in Figure 2*C* and 2*D*. The PCR results (Figure 2*C*) suggest that the immunity conferred by prior BCG vaccination can protect against a challenge dose by ~1 log CFU ($P = .02$ with correction for the total DNA extracted). However, the culture data were not supportive of the PCR findings in detecting a difference between naive and vaccinated groups (Figure 2*D*).

Associations Between Prechallenge Immune Parameters and Challenge CFU Counts

The T-helper 1 cytokine interferon (IFN) γ is essential but not sufficient for protective immunity against *M. tuberculosis* [1]. Ex vivo IFN-γ ELISPOT assays to measure effector T-cell responses against BCG-immunodominant antigens, TB10.3, and antigen 85A were thus evaluated before BCG challenge. However, these responses did not correlate with the CFU data (BCG naive, $R = -0.1$ and $P = .8$; BCG vaccinated, $R = -0.25$ and $P = .5$; data not shown). There was a trend for a negative correlation between prechallenge cultured ELISPOT responses against the same antigens (which measures central memory T-cell responses [16]) and CFU count after challenge, although this did not reach statistical significance ($R = -0.4$; $P = .2$) (Figure 3*C*). The cultured ELISPOT responses in BCG-naive volunteers were uniformly low (Figure 3*B*). There was no correlation between time since BCG vaccination and cultured ELISPOT responses (Figure 3*D*).

DISCUSSION

Here, we have presented a novel BCG challenge model for the assessment of mycobacterial immunity and candidate tuberculosis vaccine efficacy testing and down-selection.

To our knowledge, this is the first reported clinical study undertaken to evaluate the feasibility of such a challenge model, although another study used similar methodology for a different purpose [11]. In that study, serial punch biopsy specimens were taken immediately adjacent to (not from the center of) the BCG vaccination site to detect nonquantitative mycobacterial shedding. At 1 month, swab cultures from drainage vaccine ulcerations in 5 volunteers detected viable BCG; however, PCR analyses of biopsy specimens did not detect BCG in 4 of 7 volunteers [11]. In comparison, in the volunteers participating in the study reported here, a larger, 4-mm biopsy specimen was obtained once, at the center of the challenge site, to detect and quantify the majority of the total BCG present, and BCG was detected by PCR in all 28 volunteers. Obtaining standard-size central biopsy specimens also allowed comparison of the levels of BCG detected between groups, giving an indication of the peak time for BCG detection.

The data from the pilot study in BCG-naive individuals suggested that week 2 after challenge was the most suitable time to enable an observable difference in BCG replication between vaccinated and control groups; at this time, the highest median CFU count was observed with the least variability. The variability probably reflects, in part, the genetic diversity and differential environmental mycobacterial exposure of volunteers and the limited sample size. The level of BCG in the skin at 4 weeks was both too low and too variable to enable a significant difference between a control and a vaccinated group to be observed, and the week 1 time-point may have only allowed assessment of innate immune processes in control of BCG replication, rather than true vaccine-induced adaptive immunity. Similar results have been observed in preclinical studies: at 14 days after challenge, a clear difference in *M. tuberculosis* CFU count was seen in the lungs of *M. tuberculosis*–infected naive mice, compared with BCG-vaccinated mice [17], and a significant difference in BCG CFU count was seen in the lungs and ears of BCG-challenged naive mice, compared with BCG-vaccinated mice [10]. In light of these data, the week 2 time-point was chosen for the punch biopsy in the second part of the study, which involved volunteers who had been previously vaccinated with BCG. The 1-log discrepancy between CFU counts by PCR and culture may reflect a failure of PCR to distinguish between live and dead mycobacteria and would explain the weak PCR and culture correlation at the early week 1 time-point (and the discrepancy in values), compared with the significant positive correlation at later time-points (Figure 1*D*).

The PCR results (Figure 2*C*) suggest that the immunity conferred by prior BCG vaccination can protect against a challenge dose by ~1 log CFU ($P = .02$ when corrected for the total DNA extracted). This level of protection is comparable to that seen in the murine model [6–8]. However, the culture data were not supportive of the PCR findings in detecting a difference between the naive and vaccinated groups (Figure 2*D*). It is possible that the PCR assay overestimated the protective effect of BCG, by detecting dead and live BCG in the naive group (as discussed above). Alternatively, the difference seen by PCR may be real, because agar plating of the biopsy specimens from the 2 groups was done separately in real time at the end of the study period for each group. Unlike this CFU count analysis, which could only be done once because of sample availability, PCR of extracted DNA was performed for both groups in parallel in the same assay at the end of the study period for the second group. Therefore, it is likely that the CFU data are less directly and less reliably comparable between the groups than the PCR data.

In addition, because volunteers were recruited sequentially over a 2-year period for the naive groups of the study, it was not logistically possible to vaccinate all volunteers with the same batch of BCG, and there was a 1-log CFU count variability between some of the batches. However, there was no correlation between the number of CFUs administered and the CFU count at the biopsy site 2 weeks later ($R = 0.15$; $P = .65$, by Spearman rank). This is reassuring, because the outcome measure was independent of the amount of BCG administered.

A repeat controlled study, with parallel administration of the same batch of BCG, is required to confirm and validate the findings of this pilot proof-of-concept study. Nevertheless, the PCR data suggest a degree of antimycobacterial immunity in the previously BCG-vaccinated group. Similarly, analysis of both groups by PCR and culture showed a spread of challenge BCG load. This is likely to reflect a spread of human immunity because of (1) variable effects of prior BCG vaccination, (2) differential exposure to nontuberculous mycobacteria, and (3) genetic differences in innate and adaptive immunity.

The range of challenge CFU counts in the BCG-vaccinated group suggests there is potential within this model for the identification of immunological correlates of protection. The level of detectable CFU counts in the BCG-vaccinated group also allows for the evaluation of BCG booster vaccines. This is essential for application of this model to tuberculosis vaccine testing, in which showing improvement over BCG is necessary and has often been difficult in preclinical models, in particular, the guinea pig model [18]. If BCG is shown in subsequent human studies to protect against a BCG skin challenge, a similar study in a country with higher endemic mycobacterial exposure would be important to investigate whether the protective effect of BCG on a skin challenge is reduced in persons who have been environmentally primed. If this was observed, it would further support the relevance of an intradermal challenge model. The evaluation of BCG replication in a no-vaccine control group

would also be important to evaluate whether such a model has use in areas where tuberculosis is endemic.

BCG survival in a skin lesion may not necessarily reflect pathogenic survival in the lungs, because the immune environments of skin and lung are different and this could have a differential impact on bacterial survival. Of importance, we previously compared the effect of BCG with intradermal (skin) and intranasal (lung) challenge in parallel and found highly comparable results [10]. This supports the relevance of a myocbacterial intradermal (skin) challenge to an aerosol *M. tuberculosis* challenge. Nevertheless, the ear represents a comparatively different immune compartment, with relatively fewer lymphoid cells, and there is likely to be a balance in the level of bacterial replication and clearance in the ear, with the clearance attributable to either bacterial death and phagocytic clearance or phagocytic transport or draining of live bacteria to the local draining lymph nodes. Indeed, the abundant $CD15^+$ neutrophil population observed in blister fluid (Figure 1*F*) is consistent with data from animal models of intradermal BCG infection in which neutrophils were shown to be induced by BCG in the skin and engineer the induction of T cells through their interactions with dendritic cells [19, 20].

Although there is no guarantee that protection against an attenuated BCG strain in humans will predict protection against a virulent *M. tuberculosis* strain, the evidence from preclinical models is, however, supportive of this prediction. As shown in our murine BCG challenge model [10], the protective effect of intradermal BCG on both intradermal and intranasal BCG challenges mirrors the effect of parenteral BCG on *M. tuberculosis* aerosol challenge in multiple previous studies published by many different research groups during the past 30 years. These data suggest that, if a BCG-based vaccine regimen is protective against aerosol *M. tuberculosis*, its effect against a BCG skin challenge would predict its effect on an aerosol *M. tuberculosis* challenge. Further experimental validation of the relevance of this BCG challenge model to *M. tuberculosis* is planned in the more relevant cattle and nonhuman primate preclinical animal models, in parallel with the establishment of this model in humans.

Ex vivo IFN-γ ELISPOT responses to BCG-immunodominant antigens did not correlate with the challenge CFU count. This assay measures circulating effector and effector memory T-cell responses, and these results are in agreement with recent studies of viral and parasitic infections in mice and humans that have shown central memory responses to correlate with pathogen clearance and protection [16, 21, 22]. Tuberculosis vaccine studies in cattle have also demonstrated the importance of the central memory T-cell response, as measured by cultured ELISPOT assay, in correlating with protective efficacy against *M. bovis* challenge [23, 24]. Data from this clinical study showed a trend for prechallenge cultured ELISPOT responses to negatively correlate with CFU count after challenge, although this did not reach statistical significance. Larger controlled studies are underway to validate these preliminary findings.

This study has demonstrated the feasibility of developing a challenge model to assess mycobacterial vaccines in humans. Validation of an intradermal BCG challenge as a surrogate for aerosol *M. tuberculosis* infection is planned in large preclinical animal models, and results from this human challenge will need validating against field efficacy trials. However, the standardization of microbial challenge models has been essential to the development of vaccines for other diseases [25], and thus, this approach now promises to also allow such cost-effective, small-scale, phase IIa vaccine efficacy trials to be undertaken for tuberculosis.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://www.oxfordjournals.org/our_journals/jid/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. H. M. is a Wellcome Trust Senior Clinical Research Fellow and Jenner Investigator. A. V. S. H. is a Wellcome Trust Principal Research Fellow and Jenner Investigator. All other authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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