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# Immunogenicity of Novel DosR Regulon-Encoded Candidate Antigens of *Mycobacterium tuberculosis* in Three High-Burden Populations in Africa▽†

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**Increasing knowledge about DosR regulon-encoded proteins has led us to produce novel *Mycobacterium tuberculosis* antigens for immunogenicity testing in human populations in three countries in Africa to which tuberculosis (TB) is endemic. A total of 131 tuberculin skin test-positive and/or ESAT-6/CFP10-positive, human immunodeficiency virus-negative adult household contacts of active pulmonary TB cases from South Africa ($n$ = 56), The Gambia ($n$ = 26), and Uganda ($n$ = 49) were tested for gamma interferon responses to 7 classical and 51 DosR regulon-encoded *M. tuberculosis* recombinant protein antigens. ESAT-6/CFP10 fusion protein evoked responses in >75% of study participants in all three countries. Of the DosR regulon-encoded antigens tested, Rv1733c was the most commonly recognized by participants from both South Africa and Uganda and the third most commonly recognized antigen in The Gambia. The four most frequently recognized DosR regulon-encoded antigens in Uganda (Rv1733c, Rv0081, Rv1735c, and Rv1737c) included the three most immunogenic antigens in South Africa. In contrast, Rv3131 induced the highest percentage of responders in Gambian contacts (38%), compared to only 3.4% of Ugandan contacts and no South African contacts. Appreciable percentages of TB contacts with a high likelihood of latent *M. tuberculosis* infection responded to several novel DosR regulon-encoded *M. tuberculosis* proteins. In addition to significant similarities in antigen recognition profiles between the three African population groups, there were also disparities, which may stem from genetic differences between both pathogen and host populations. Our findings have implications for the selection of potential TB vaccine candidates and for determining biosignatures of latent *M. tuberculosis* infection, active TB disease, and protective immunity.**

Tuberculosis (TB) remains an ongoing health crisis of global dimensions. The African Region has the highest incidence rate per capita (363 per 100,000 population) and includes 10 of the 22 most high-burden countries in the world (38). It has been estimated that one-third of the world's population is latently infected with *Mycobacterium tuberculosis*. Human immunodeficiency virus type 1 (HIV-1)-infected individuals have a risk of about 5 to 10% per year of progression from latent infection to active TB (4), compared to 2 to 23% in a lifetime for HIV-1-seronegative individuals (24). The only currently licensed vaccine against TB is *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), which has highly variable efficacy against adult pulmonary TB (6). The use of BCG in HIV-1-infected or -exposed infants may be contraindicated (11). The investigation of safe and effective TB vaccines is thus highly prioritized.

The discovery of the precise mechanisms underlying protective anti-TB immunity calls for the identification of new biomarkers (17). A clearer understanding of which *M. tuberculosis* antigens evoke effective immune responses and how they are associated with protection or disease is required. Promising

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antigens that have been identified as immunodominant include the alpha-crystallin homologue (also known as the *M. tuberculosis* 16-kDa protein; Rv2031c, HspX) (9), alpha-cystallin 2 (Acr2; Rv0251) (36), Ag85A (Rv3804) (33), Hsp65 (Rv0440) (23), ESAT-6 (Rv3875) (5), and CFP10 (Rv3874) (32), some of which are currently being tested as potential TB vaccine candidates (28). The search for novel protective antigen(s) has been facilitated by expression profiling of *M. tuberculosis* laboratory strains cultured under conditions of hypoxia and nitric oxide stress, which are thought to resemble conditions that mycobacteria encounter in situ during latent infection (31). Voskuil et al. (35) showed that hypoxia and low concentrations of nitric oxide induced expression of a 48-gene dormancy survival regulon (DosR) believed to be associated with latency. A selection of these proteins has been tested for immunogenicity in relevant mouse models and has established the importance of the regulon in latent infection (26, 29, 34). In addition, human studies of latently *M. tuberculosis*-infected healthy adults living in areas where tuberculosis is not endemic have shown T-cell responses to selected DosR regulon-encoded antigens, suggesting a role in maintenance of the asymptomatic phase of latent infection (18, 30). In order to gain better insight into protection against TB and to expand our current understanding about proteins encoded by the DosR regulon that are targeted by the human immune system, we have tested 51 antigens, spanning the entire 48 genes of the DosR regulon (35), in geographically diverse human populations from three countries in Africa to which TB is endemic. Twenty-five of the antigens studied here have been tested previously (18); however, we report results with an additional 26 new antigens. The immunogenicity of the entire set of DosR regulon-encoded protein antigens in a high-TB-burden African context is described for the first time.

## MATERIALS AND METHODS

**Ethical clearance.** Blood samples were collected at all three African sites only after written informed consent was given. Study protocols were approved by the institutional review boards of Stellenbosch University, Case Western Reserve University, the Uganda National Council for Science and Technology, and the Joint Gambian Government/MRC Ethics Committee.

**Study population.** The study population included 131 individuals: 49 participants from Uganda (Makerere University), 26 participants from The Gambia (Medical Research Council), and 56 participants from South Africa (Stellenbosch University). All Ugandan and Gambian participants were of African descent. All South African participants came from the ethnic group known as South African colored. BCG vaccination status was assigned according to the presence or absence of a typical scar over the deltoid region. All 131 study participants had recorded household exposure to a smear-positive adult pulmonary TB index case diagnosed up to 2 months prior to phlebotomy.

The Mantoux skin test was done using 2 tuberculin units of *M. tuberculosis* purified protein derivative (PPD) RT23 for in vivo use (Statens Serum Institute, Denmark) administered intradermally immediately after venous blood collection. At all sites, indurations were read between 48 and 72 h following test administration. In The Gambia and Uganda, sputum examination is not routinely practiced in nonsymptomatic household contacts. In South Africa, physiotherapy-trained nursing assistants used percussion to assist sputum production by all study participants; sputum was cultured, and all cultures were found to be negative for acid-fast bacilli. Chest X-rays were done on recruitment of all contacts at each of the sites. No abnormalities suggestive of TB disease were found.

**HIV testing.** All participants were tested for antibodies to HIV-1 and -2 after pre- and posttest counseling: rapid test (Determine HIV-1/2; Abbott/Inverness) in Uganda, enzyme-linked immunosorbent assay (ELISA) (Murex 1.2.0; Abbott-Murex Biotec, Dartford, Kent, United Kingdom), and rapid test (Hexagon HIV; Human Diagnostics GmbH, Wiesbaden, Germany) in The Gambia, and rapid test (First Response HIV Card 1-2.0; PMC Medical India Pvt. Ltd., Daman, India) in South Africa.

**Antigens.** All antigens were produced, quality controlled, and distributed by Leiden University Medical Center as described previously (18, 19). Briefly, genes were amplified by PCR and cloned by Gateway Technology (Invitrogen, San Diego, CA) in a bacterial expression vector containing an N-terminal histidine tag. The proteins were overexpressed in *Escherichia coli* BL21(DE3) and purified, as described previously (8). Purity and size were checked by gel electrophoresis and Western blotting with anti-His antibodies and anti-*E. coli* antibodies. Residual endotoxin levels were determined with a Limulus amebocyte lysate assay (Cambrex) and were found to be below 50 IU/mg recombinant protein. Due to size constraints, Rv0570, Rv1736c, and Rv1997 were expressed in two parts (C-terminal and N-terminal), giving a total of 51 test antigens. In Table 2, the C- and N-terminal fragments of Rv0570 are denoted as Rv0570-C and Rv0570-N, respectively. The same nomenclature is used for the C-terminal and N-terminal parts of Rv1736c and Rv1997.

Recombinant antigens were freeze-dried and shipped at ambient temperature to the African research sites. Each site obtained aliquots of the same batches of the classical TB control antigens and DosR regulon-encoded proteins (for batch numbers, see Table 2), which were reconstituted (following a detailed protocol provided to all sites by Leiden University Medical Center) in dimethyl sulfoxide and 1× phosphate-buffered saline (10 μl and 1 ml per mg of antigen, respectively) and stored at −80°C until testing in whole-blood assays was done.

**Antigen screening.** For the seven classical antigens (Rv0288 [TB10.4], Rv0440 [GroEL2/HSP65], Rv1886c [Ag85-B], Rv3019c [TB10.3], Rv3804c [Ag85-A], Rv3875 [ESAT-6], and a fusion protein of ESAT-6 and CFP-10), 51 *M. tuberculosis* DosR regulon-encoded antigens, and the number of participants tested for each antigen at each site, see Table 2. In The Gambia, 26 study participants were tested with all 51 DosR regulon-encoded antigens. In Uganda, three subgroups were tested: 24 subjects with 16 antigens, 15 subjects with the remaining 35 antigens, and 11 subjects with all 51 antigens. Three subgroups were also tested in South Africa: 18 subjects with 16 antigens, 19 subjects (subgroup A; see Table 2) with a further 16 antigens, and 19 subjects (subgroup B; see Table 2) with the remaining 19 antigens.

**Whole-blood assay.** The whole-blood assay and gamma interferon (IFN-γ) ELISA procedures followed were those in use at each of the sites at the time of antigen testing. In South Africa and The Gambia, the whole-blood assay (WBA) was done as described previously (2). The same WBA protocol was followed in Uganda except that diluted blood was added to single wells of 48-well tissue culture plates (as opposed to triplicate wells of 96-well plates) and the final blood dilution after addition to antigen was 1 in 5 (compared to 1 in 10 in South Africa and The Gambia) (25). All recombinant antigens were used at a final concentration of 10 μg/ml. Ag85A and Ag85B protein antigens were combined for screening to create a single stimulatory condition, with each protein being tested at a final concentration of 10 μg/ml. At all sites the negative control was diluted blood cultured without antigen, and the positive control was phytohemagglutinin (PHA) (lot numbers 22K8935 [MAK], 115K8916 [MRC], and 015K8913 [SUN]; Sigma), used at a final concentration of 5 μg/ml. Cultures were incubated at 37°C with 5% $CO_2$. At all sites, supernatants from each well were harvested on day seven and stored at −80°C before testing by ELISA. In The Gambia and South Africa, the supernatants from triplicate wells were pooled prior to storage.

**IFN-γ ELISA.** South Africa and The Gambia followed the method described previously (2) with the following differences: the IFN-γ standard (554616, lot 33306; Pharmingen) curve ranged from 2,000 to 15 pg/ml; the substrate was Fast *o*-phenylenediamine dihydrochloride (Sigma). ELISA plates were read at 450 nm, and linear curve fitting was used. The protocol followed in Uganda was similar, with variations as follows: the coating antibody was Endogen M-700A monoclonal antibody, the blocking agent (10 to 15 min) was Pierce Superblock (Pierce 37515), the IFN-γ standard (lot no. DH58587, catalog no. Pierce Endogen RIFNG50; Endogen) curve ranged from 1,000 to 25 pg/ml, the secondary biotin-labeled antibody was Endogen M-701b, the enzyme was alkaline-phosphatase-conjugated streptavidin (016-050-084; Jackson Immuno Research), and the substrate was alkaline phosphatase (N-9389; Sigma) in diethylanolamine buffer (Sigma) at 1:10. The reaction was stopped with 5% EDTA in phosphate-buffered saline (no. E-1644; Sigma), plates were read at 405 nm, and the four-parameter curve fit was used.

**Data analysis.** The general distribution of IFN-γ responses in each sample population was highly skewed to the right (indicating positive skewness). Therefore, a log–10 transformation was used whenever a summary measure required that the distribution be normalized.

The negative control IFN-γ value for each study participant was subtracted from the antigen-induced IFN-γ values so that all response values could be considered over and above the background response. The WBA blood dilution

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TABLE 1. Demographic characteristics of study participants

| Site ($n^a$) | % Male | Mean age in yr (range) | Mean TST size in mm (range) | % BCG scar positive |
|---|---|---|---|---|
| Uganda (49) | 27 | 28 (15–75) | 16 (10–24) | 67 |
| The Gambia (26) | 38 | 30 (15–52) | 18 (0–23) | 36 |
| South Africa (56) | 31 | 32 (12–56) | 24 (8–38) | 68 |

[a] Total number of study participants included in DosR regulon-encoded antigen screening at each site.

and the ELISA protocol used in Uganda varied from that used in both The Gambia and South Africa. Therefore, a direct comparison of the geometric means or medians across each of the sites was not feasible. Instead, ranks and frequencies of positive responders were compared within and between sites. The IFN-γ response to each stimulus was categorized as positive or negative for each participant based on whether the stimulus response was greater than a calculated cutoff value. The cutoff value for determining a positive response was calculated separately for each site as means + 2 standard deviations of log-transformed negative control values. Table S1 in the supplemental material shows calculated cutoff values on the log scale and the original pg/ml scale ($10^{\log X}$, where $X$ is the cutoff value). Fig. S1 in the supplemental material shows a dot plot of the individual negative control responses within each site.

Correlations between antigen responses within each site were assessed with the Pearson correlation coefficient ($r$) after log-10 transformation of the data. The criteria for significance was set to an $r$ value of ≥0.6 and, to account for multiple estimates, a $P$ value of <0.005 ($\alpha = 0.005$). Since this was an exploratory study, it was not necessary to make any further adjustments to the data.

## RESULTS

**Demographics.** Gender, mean age in years, mean tuberculin skin test (TST) size (mm), and BCG scar status of the participants from the three sites are shown in Table 1.

In Uganda, The Gambia, and South Africa, 100%, 84%, and 89% of participants had a TST of >10 mm, respectively. In The Gambia and South Africa, three and two participants, respectively, had no TST data available, but all had a positive whole-blood IFN-γ response to *M. tuberculosis*-specific ESAT-6/CFP10 fusion protein.

Dot plots of IFN-γ responses (pg/ml) to the seven classical TB control antigens and the positive control (PHA) are shown on a log scale in Fig. 1A (Uganda), B (The Gambia), and C (South Africa). The geometric means, medians, interquartile ranges, and percent positive responses to the seven classical *M. tuberculosis* control antigens and PHA are shown in Table S2 in the supplemental material. PHA- and ESAT-6/CFP10 fusion protein-specific responses were of highest magnitude in The Gambia, as were the background responses. Thus, the Gambian response cutoff was higher than those at the other two sites (The Gambia cutoff, 163 pg/ml; Uganda cutoff, 62 pg/ml; South Africa cutoff, 29 pg/ml).

The frequency of responses to PHA in both Uganda (63% responders) and South Africa (78% responders) was less than that observed in The Gambia (96%). The one Gambian participant that did not respond to PHA—at least as assessed by IFN-γ production—also showed no response to ESAT-6/CFP10 or TB10.4 but did respond to two DosR regulon-encoded antigens. In Uganda, of the 18 study participants that did not respond to PHA, 16 (89%) responded to ESAT-6/CFP10 and/or TB10.4 as well as at least one DosR regulon-encoded antigen. The remaining two Ugandan participants did not respond to PHA, ESAT-6/CFP10, or TB10.4 but did respond to 7 and 15 DosR regulon-encoded antigens, respectively. In South Africa, of the 12 nonresponders to PHA, 100% responded to ESAT-6/CFP10 and/or TB10.4, and out of this subgroup only 1 participant did not respond to any DosR regulon-encoded antigens. Thus, despite the reduced percentage of responders to PHA in Uganda and South Africa, none of the participants were anergic.

Responses to the 51 DosR regulon-encoded antigens were ranked by the frequency of responders at each site. The top 10 overall highest-ranked antigens for each site, a total of 19 antigens, are shown in Fig. 2. Antigens are shown in order by Rv code antigen number. Some antigen responses ranked in the top 10 at only a single site, but others ranked in the top 10 at two or three sites, and these are shown by matching box patterns. Rv0081, Rv1733c, Rv1735c, and Rv2006 were among the 10 most frequently recognized antigens in all three population groups. Rv1736c-C, Rv1737c, and Rv1997-C ranked in the top 10 at both Ugandan and South African sites.

For the geometric mean, median, 25th percentile, 75th percen-

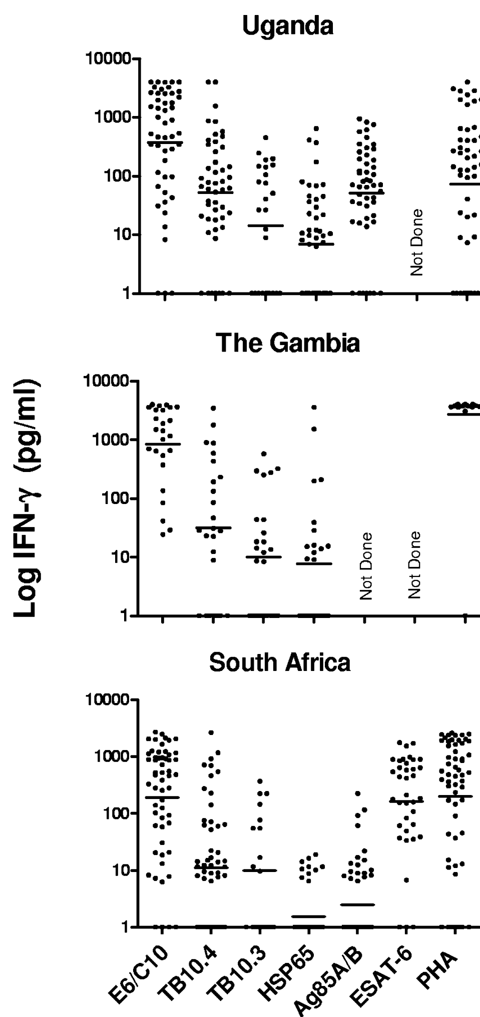


FIG. 1. Dot plots of IFN-γ responses (pg/ml), on a log scale, to the ESAT-6/CFP10 fusion protein (E6/C10), TB10.4, TB10.3, HSP65, Ag85A/B, ESAT-6, and PHA are shown for Uganda (A), The Gambia (B), and South Africa (C). All "0" values were converted to 1 for plotting on the log axis. The horizontal line shows the median response for each condition.

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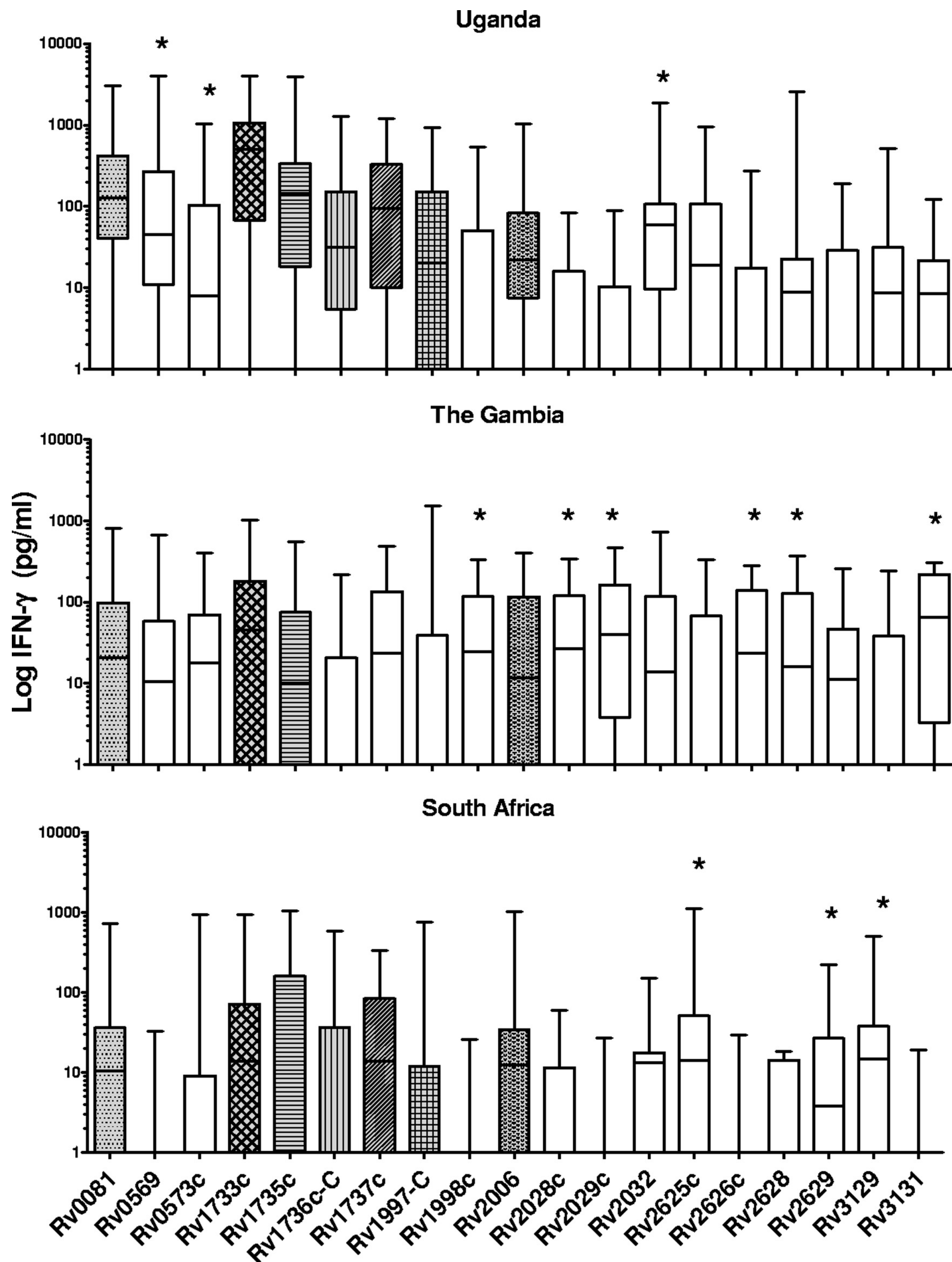


FIG. 2. Box-and-whisker plots showing on a log-10 scale minimum and maximum IFN-γ levels (pg/ml) in supernatants of *M. tuberculosis* DosR regulon-encoded antigen-stimulated 7-day whole-blood cultures. The top 10 ranking antigens in Uganda (A), The Gambia (B), or South Africa (C) are shown, with a total of 19 antigens. Patterned boxes represent antigens that were among the 10 most frequently recognized antigens at two or three sites. Antigens that ranked in the top 10 at only a single site are marked by an asterisk above the top whisker. The line within the box shows the median.

tile, and percent positive responses for the 10 most immunogenic antigens at each site, listed in antigen number order, see Table 3.

In order to investigate associations between the IFN-γ response and the TST size, the data were analyzed using the Pearson correlation coefficient (*r*). TST induration was significantly correlated with the cytokine response to TB10.3 in Uganda ($P = 0.01$) and with the response to the ESAT6/CFP10 fusion protein in South Africa ($P = 0.003$). None of the

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responses to the classical TB antigens correlated with TST size in The Gambia; the same observation was made for *M. tuberculosis* PPD ($r = 0.173$), which was tested as an additional culture condition in The Gambia (data not shown) but was not included as an antigen in Uganda or South Africa.

Next, data were analyzed for associations between the magnitude of the responses to the classical TB antigens and the 51 DosR regulon-encoded antigens. Significant associations were observed at each of the sites for a small number of comparisons. Responses to TB10.4 were significantly correlated with responses to Rv2628 in Uganda ($P < 0.0001$) and with responses to Rv0574c in South Africa ($P = 0.0015$). No correlations between TB10.4 and any DosR regulon-encoded antigens were observed in The Gambia. Responses to the ESAT-6/CFP10 fusion protein were significantly associated with responses to Rv2623 in Uganda ($P = 0.0017$); however, no significant correlations between the ESAT-6/CFP10 fusion protein and any DosR regulon-encoded antigens were observed in The Gambia or South Africa. There were no significant associations between IFN-γ responses to any of the DosR regulon-encoded antigens and TST size at any of the sites.

Finally, an exploratory analysis was done to investigate associations between the DosR regulon-encoded antigens. In Uganda and South Africa, subgroups of study participants were tested with subsets of the DosR regulon-encoded antigens. Therefore in order for pairwise associations between antigen responses to be evaluated across all three study populations, it was necessary to do the Pearson analysis within antigen clusters. Antigens were clustered together if they had pairwise data available at each African site and were also included in the top 19 most frequently recognized antigens shown in Tables 2 and 3. Clusters 1 (Rv0081, Rv0569, Rv1733c, Rv2029c, Rv2626c, and Rv2628) and 2 (Rv0573c, Rv1735c, Rv1736c-C, Rv1737c, Rv1997-C, and Rv1998) each contain six antigens, and cluster 3 (Rv2006, Rv2028c, Rv2032, Rv2625c, Rv2629, Rv3129, and Rv3131) contains seven antigens (see Tables S3A, B, and C in the supplemental material). Due to the number of comparisons made, *P* values could not be used to judge whether the adjusted probability of type I error is $<0.05$.

For antigen cluster 1, IFN-γ responses to Rv0081 and Rv1733c were positively associated ($r > 0.6$) at all three sites (see Table S3A, M/M/S, in the supplemental material). In both South Africa and Uganda, a strong correlation was observed between Rv0081 and Rv0569. Other significant pairwise correlations between antigens in cluster 1 were observed at a single site only. Overall, antigen cluster 1 had the smallest number of positive pairwise associations across all sites.

For antigen cluster 2, all three sites showed strong correlations between Rv1997-C and each of Rv1735c, Rv1736c-C, and Rv1737c. In both South Africa and Uganda, positive associations were observed for four pairwise comparisons (Rv1736c-C versus both Rv0573 and Rv1735c, Rv1737 versus Rv1735c, and Rv1997-C versus Rv0573). Other positive pairwise correlations between antigens in cluster 2 were observed in either Uganda or South Africa only.

For antigen cluster 3, all three sites showed positive associations when responses to Rv2006 versus those to Rv2625c were measured. In Uganda and The Gambia, correlation coefficients of $>0.6$ were observed for pairwise evaluations between the antigens Rv2028c and Rv3129 and between Rv2625c and Rv2032. The Ugandan and South African groups showed associations between Rv2006 and Rv3129. Positive correlations were observed in The Gambian and South African groups between Rv2028c and each of Rv2006, Rv2625c, and Rv2629 and between Rv2629 and both Rv2032 and Rv2625c. Other pairwise correlation coefficients of $>0.6$ between antigens in cluster 3 were observed at single sites only.

## DISCUSSION

Effective vaccines against TB are urgently required, but progress is hampered by our lack of knowledge about which antigens of *M. tuberculosis* are immunogenic in relevant human populations and should therefore be included in new vaccines (15, 22). Additionally, there are no reliable predictive biomarkers of latent *M. tuberculosis* infection, active TB disease, or vaccine-induced protection against TB. In a large-scale attempt to begin to address these issues, we have screened 7 classical and 51 candidate antigens, spanning the entire DosR regulon, for their ability to induce IFN-γ responses in whole-blood cultures from *M. tuberculosis*-exposed contacts of smear-positive TB patients in three TB-endemic settings in Africa. None of the study participants at any site had evidence of active TB disease. Although the duration of infection may have influenced the extent of reactivity to the DosR regulon-encoded antigens, the point in time at which the TB index cases became infectious, which is likely to be prior to the date of diagnosis, remains unknown, such that it is not possible to be certain about the true duration of infection in the contacts. However, the range and mean time of exposure to the index cases were comparable for the three population groups due to the application of a 2-month limit between index case diagnosis and contact recruitment.

The WBA was selected since this has been widely used as a tool for measuring cytokine production in response to antigenic stimulation. Although the source of IFN-γ in the long-term (6- to 7-day) WBA has not been fully characterized, a recent infant BCG study indicates that this assay detects an antigen-specific T-cell-mediated immune response rather than nonspecific cytokine production (16).

Although the percentage with positive BCG scar status was different in The Gambia (36%) from those in Uganda (67%) and South Africa (68%), it has been shown (19) that BCG vaccination in adults fails to induce significant responses to many of the latency proteins tested here and is therefore unlikely to affect the antigen recognition preferences of the study participants.

From this study, Rv1733c came out as one of the most frequently recognized DosR regulon-encoded antigens in all three African sites. Of interest, it has also been shown to induce IFN-γ responses in both T-cell lines and peripheral blood mononuclear cells from a majority of TST-positive individuals in a Dutch study (18). Thus, there is a shared ability for T cells from different populations to respond to this antigen. In this descriptive study, we have investigated response patterns across population groups but have made no attempt to directly compare assay performances at the different sites. The striking similarity in recognition profiles of a select number of antigens is clear, regardless of different blood dilutions and possible

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TABLE 2. Seven classical and 51 *M. tuberculosis*-derived DosR regulon-encoded antigens screened for immunogenicity by IFN-γ release in Uganda, The Gambia, and South Africa

| Antigen name | aa[a] size (gene) | Name/description[a,b] | Batch no. | No. tested[c] | | | | Previously published |
|---|---|---|---|---|---|---|---|---|
| | | | | MAK | MRC | SUN[f] | Total | |
| Classical TB antigens | | | | | | | | |
| Rv0288 | 96 (*esxH*) | Low-molecular-wt protein antigen 7 (ESXH; TB10.4) | 041111 | 49 | 26 | 56 | 132 | Yes |
| Rv0440 | 540 (*groEL2*) | 60 kDa chaperonin 2 GROEL2—heat shock protein 65 | 051016 | 49 | 26 | 56 | 132 | Yes |
| Rv1886c | 325 (*fbpB*) | Secreted antigen 85-B (FBPB)[d] | 050409 | 49 | 0 | 56 | 107 | Yes |
| Rv3019c | 96 (*esxR*) | Secreted ESAT-6 like protein (ESXR; TB10.3) | 030411 | 26 | 26 | 19 | 70 | Yes |
| Rv3804c | 338 (*fbpA*) | Secreted antigen 85-A (FBPA)[d] | 041007 | 49 | 0 | 56 | 107 | Yes |
| Rv3875 | 95 (*esxA*) | 6-kDa early secretory antigenic target (ESXA; ESAT-6) | 051202 | 0 | 0 | 38 | 38 | Yes |
| | 95 (*esxA*) | ESAT-6 (N-terminal) and CFP10 (C-terminal) fusion protein | 040101 | 49 | 26 | 56 | 132 | Yes |
| Rv3874 | 98 (*esxB*) | | | | | | | |
| DosR regulon-encoded antigens[e] | | | | | | | | |
| Rv0079 | 273 | Hypothetical protein | 030515 | 35 | 26 | 19A | 80 | Yes[g] |
| Rv0080 | 152 | Conserved hypothetical protein | 050209 | 35 | 26 | 19A | 80 | No |
| Rv0081 | 114 | Probable transcriptional regulatory protein | 050212 | 35 | 26 | 19A | 80 | No |
| Rv0569 | 88 | Conserved hypothetical protein | 051104 | 35 | 26 | 19A | 80 | Yes[g] |
| **Rv0570** | 692 (*nrdZ*) | Probable ribonucleoside-diphosphate reductase | | | | | | |
| Rv0570-C | 354 | Rv0570 C-term part (aa 1–354) | 050611 | 26 | 26 | 19B | 71 | No |
| Rv0570-N | 360 | Rv0570 N-term part (aa 333–692) | 050604 | 26 | 26 | 19B | 71 | No |
| Rv0571c | 443 | Conserved hypothetical protein | 050601 | 35 | 26 | 19A | 80 | No |
| Rv0572c | 113 | Hypothetical protein | 030403 | 26 | 26 | 19B | 71 | Yes[g] |
| Rv0573c | 463 | Conserved hypothetical protein | 050307 | 26 | 26 | 19B | 71 | No |
| Rv0574c | 380 | Conserved hypothetical protein | 050509 | 26 | 26 | 19B | 71 | No |
| Rv1733c | 210 | Probable conserved transmembrane protein | 051105 | 35 | 26 | 19A | 80 | Yes[g] |
| Rv1734c | 80 | Conserved hypothetical protein | 050306 | 26 | 26 | 19B | 71 | No |
| Rv1735c | 165 | Hypothetical membrane protein | 051012 | 26 | 26 | 19B | 71 | No |
| **Rv1736c** | 652 (*narX*) | Probable nitrate reductase | | | | | | |
| Rv1736c-C | 380 | Rv1736c C-term part (aa 1–380) | 050605 | 26 | 26 | 19B | 71 | No |
| Rv1736c-N | 308 | Rv1736c N-term part (aa 345–652) | 050702 | 26 | 26 | 19B | 71 | No |
| Rv1737c | 395 (*narK2*) | Possible nitrate/nitrite transporter | 051201 | 26 | 26 | 19B | 71 | No |
| Rv1738 | 94 | Conserved hypothetical protein | 030210 | 35 | 26 | 19A | 80 | Yes[g] |
| Rv1812c | 400 | Probable dehydrogenase | 050415 | 26 | 26 | 19B | 71 | No |
| Rv1813c | 143 | Conserved hypothetical protein | 031205 | 26 | 26 | 19B | 71 | Yes[g] |
| Rv1996 | 317 | Conserved hypothetical protein | 030311 | 26 | 26 | 19B | 71 | Yes[g] |
| **Rv1997** | 905 (*ctpF*) | Probable metal cation transporter P-type ATPase A | | | | | | |
| Rv1997-C | 430 | Rv1997 C-term part (aa 1–430) | 050703 | 26 | 26 | 19B | 71 | No |
| Rv1997-N | 504 | Rv1997 N-term part (aa 402–905) | 050603 | 26 | 26 | 19B | 71 | No |
| Rv1998 | 258 | Conserved hypothetical protein | 050501 | 26 | 26 | 19B | 71 | No |
| Rv2003c | 285 | Conserved hypothetical protein | 050411 | 26 | 26 | 19B | 71 | No |
| Rv2004c | 498 | Conserved hypothetical protein | 050416 | 26 | 26 | 19B | 71 | No |
| Rv2005c | 295 | Conserved hypothetical protein | 050410 | 26 | 26 | 19B | 71 | No |
| Rv2006 | 1327 (*otsB1*) | Probable trehalose-6-phosphate phosphatase | 050506 | 26 | 26 | 18 | 71 | No |
| Rv2007c | 114 (*fdxA*) | Probable ferredoxin | 041206 | 26 | 26 | 18 | 71 | Yes[g] |
| Rv2028c | 279 | Conserved hypothetical protein | 050412 | 26 | 26 | 18 | 71 | No |
| Rv2029c | 339 (*pfkB*) | Probable phosphohexokinase | 050714 | 35 | 26 | 19A | 80 | Yes[g] |
| Rv2030c | 681 | Conserved hypothetical protein | 030128 | 26 | 26 | 18 | 71 | Yes[g] |
| Rv2031c | 144 (*acr*) | Heat shock protein X (Hspx; alpha-crystallin homolog) | 050706 | 35 | 26 | 19A | 80 | Yes[g] |
| Rv2032 | 331 (*acg*) | Conserved hypothetical protein | 020919 | 26 | 26 | 18 | 71 | Yes[g] |
| Rv2623 | 297 (*TB31.7*) | Conserved hypothetical protein | 030312 | 35 | 26 | 19A | 80 | Yes[g] |
| Rv2624c | 272 | Conserved hypothetical protein | 030308 | 26 | 26 | 18 | 71 | Yes[g] |
| Rv2625c | 393 | Probable conserved transmembrane protein | 050610 | 26 | 26 | 18 | 71 | No |
| Rv2626c | 143 | Conserved hypothetical protein | 030229 | 35 | 26 | 19A | 80 | Yes[g] |
| Rv2627c | 413 | Conserved hypothetical protein | 050705 | 35 | 26 | 19A | 80 | Yes[g] |
| Rv2628 | 120 | Hypothetical protein | 050713 | 35 | 26 | 19A | 80 | Yes[g] |

*Continued on following page*

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TABLE 2—*Continued*

| Antigen name | aa[a] size (gene) | Name/description[a,b] | Batch no. | No. tested[c] | | | | Previously published |
|---|---|---|---|---|---|---|---|---|
| | | | | MAK | MRC | SUN[f] | Total | |
| Rv2629 | 374 | Conserved hypothetical protein | 050417 | 26 | 26 | 18 | 71 | No |
| Rv2630 | 179 | Hypothetical protein | 050701 | 26 | 26 | 18 | 71 | No |
| Rv2631 | 432 | Conserved hypothetical protein | 050510 | 26 | 26 | 18 | 71 | No |
| Rv3126c | 104 | Hypothetical protein | 030304 | 26 | 26 | 18 | 71 | Yes[g] |
| Rv3127 | 344 | Conserved hypothetical protein | 030231 | 26 | 26 | 18 | 71 | Yes[g] |
| Rv3128c | 337 | Conserved hypothetical protein | 050502 | 26 | 26 | 18 | 71 | No |
| Rv3129 | 110 | Conserved hypothetical protein | 030129 | 26 | 26 | 18 | 71 | Yes[g] |
| Rv3130c | 463 | Conserved hypothetical protein | 030706 | 26 | 26 | 18 | 71 | Yes[g] |
| Rv3131 | 332 | Conserved hypothetical protein | 021003 | 26 | 26 | 18 | 71 | Yes[g] |
| Rv3132c | 578 (*devS*) | Two-component sensor histidine kinase | 030612 | 35 | 26 | 19A | 80 | Yes[g] |
| Rv3133c | 217 (*dosR*) | Two-component transcriptional regulatory protein | 030404 | 35 | 26 | 19A | 80 | Yes[g] |
| Rv3134c | 268 | Conserved hypothetical protein | 041208 | 35 | 26 | 19A | 80 | Yes[g] |

[a] aa, amino acid.
[b] C-term, C-terminal; N-term, N-terminal.
[c] MAK, Uganda; MRC, The Gambia; SUN, South Africa.
[d] The Ag85A and Ag85B protein antigens were combined for screening to create a single stimulatory condition, with each protein being tested at a final concentration of 10 μg/ml.
[e] For DosR regulon-encoded antigens, bold type indicates where proteins were expressed in two parts due to size constraints.
[f] "A" indicates the first group and "B" the second group of 19 subjects (each) tested at SUN.
[g] See reference 18.

differences in ELISA sensitivity. These observations give hope for the inclusion of antigens such as these in future vaccines and immunologic biomarker assays. Moreover, they also provide a rational basis for identifying relevant epitopes targeted by the immune system that may achieve protection against TB (10, 21). Rv1733c, Rv1735c, and Rv1737c were ranked in the top 10 most frequently recognized antigens across all three African countries. IFN-γ responses to Rv1735c and Rv1737c were highly correlated in Uganda and South Africa and positively correlated in The Gambia. A strong association was also observed between Rv1733c and Rv1735c and between Rv1733c and Rv1737c in The Gambia. However, due to the study design, these pairwise comparisons were not available for Uganda or South Africa. The genes encoding these three proteins share close chromosomal proximity (35), and our findings indicate that they might constitute an "immunogenicity island." In this study, correlations were not strong enough to conclude that one antigen could provide as much information as a pair or a cluster of antigens. If further larger studies indicate strong associations in responsiveness to Rv1733c, Rv1735c, and Rv1737c or the same pattern is found for other potential "immunogenicity islands," then the information obtained from one protein could be representative of multiple antigens. Such subselection of proteins may eliminate redundancy and maximize efficiency in future vaccine trials, although the impact of excluding potentially useful antigens would need careful investigation.

Rv1736c (C-terminal) (*narx*) and Rv1737c (*narK2*) ranked among the top 10 most frequently recognized antigens in Uganda and South Africa. It has been reported (13) that these two genes are not expressed by BCG vaccine strains, although they are present in BCG's genome(s), suggesting that responses induced by these two antigens may be *M. tuberculosis*-specific and that they could be of potential interest as immunodiagnostic reagents. However, in our study we did not observe significant correlations between either Rv1736c C-term or Rv1737c and any of the classical TB-specific antigens tested.

In contrast, prominent differences in antigen recognition, such as the case of Rv3131, highlight the importance of including a well-defined set of antigens when investigating anti-TB immunity in populations from geographically distinct locations. The observed differences may be human population related (host genetic, including HLA) (12, 20) and/or *M. tuberculosis* lineage related (pathogen genetic) (7). During hypoxia, *M. tuberculosis* upregulates the expression of the 16-kDa protein (α-crystallin, Rv2031c, HspX) (39), which has been shown in other studies to induce both $CD4^+$ and $CD8^+$ T-cell responses in latently infected individuals (3, 9, 37). The lack of recognition of this antigen in any of the three TB-endemic populations studied here might be related to the dormant state of the bacilli in its resting form. The conditions that *M. tuberculosis* is exposed to while residing in human hosts may not fully reflect those encountered under hypoxia in vitro. Of note, an earlier study also showed that Rv2031c was less potently recognized than other DosR regulon-encoded antigens, although it was highly antigenic in *M. tuberculosis*-stimulated short-term T-cell lines (18). Alternatively, the Rv2031c antigen might activate non-IFN-γ-producing cells, such as Th17 or Treg cells, which would not be detected in our current study design (14). This issue will be addressed in future studies, in which we will test multiple cytokines in response to DosR regulon-encoded antigens.

It is possible that immune recognition of the antigens tested here is partially primed by exposure to or infection with microorganisms or mycobacteria other than *M. tuberculosis* (1, 6). It has been shown that Rv1733c also induces responses in T cells from *M. tuberculosis*-unexposed but PPD-responsive persons (18; M. Y. Lin and T. H. M. Ottenhoff, submitted). Further studies will be needed to elucidate the impact of antigenic cross-reactivity in the immune recognition of the DosR regulon-encoded antigens. A recent study by Rustad et al. (27)

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TABLE 3. IFN-γ responses to the 10 most immunogenic antigens in Uganda, The Gambia, and South Africa[a]

| Antigen name | IFN-γ response (pg/ml) | | | | | | | | | | | | % Responders[d] | | | Rank[e] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Geometric mean | | | Median | | | P25[b] | | | P75[c] | | | | | | | | |
| | MAK | MRC | SUN | MAK | MRC | SUN | MAK | MRC | SUN | MAK | MRC | SUN | MAK | MRC | SUN | MAK | MRC | SUN |
| Rv0081 | 136 | 27 | 15 | 128 | 21 | 10 | 43 | 0 | 0 | 369 | 92 | 36 | 70 | 16 | 26 | 2 | 5 | 5 |
| Rv0569 | 54 | —[f] | — | 45 | — | — | 12 | — | — | 267 | — | — | 41 | — | | 6 | — | — |
| Rv0573 | 23 | — | — | 8 | — | — | 0 | — | — | 97 | — | — | 38 | — | — | 7 | — | — |
| Rv1733c | 236 | 41 | 24 | 501 | 46 | 14 | 69 | 0 | 0 | 1045 | 159 | 71 | 79 | 24 | 47 | 1 | 2 | 1 |
| Rv1735c | 93 | 18 | 22 | 145 | 10 | 0 | 21 | 0 | 0 | 297 | 72 | 159 | 61 | 16 | 42 | 3 | 5 | 2 |
| Rv1736c-C | 38 | — | 16 | 32 | — | 0 | 7 | — | 0 | 123 | — | 37 | 38 | — | 26 | 7 | — | 5 |
| Rv1737c | 66 | — | 22 | 96 | — | 14 | 11 | — | 0 | 329 | — | 84 | 50 | — | 37 | 4 | — | 3 |
| Rv1997-C | 32 | — | 12 | 20 | — | 0 | 0 | — | 0 | 135 | — | 12 | 35 | — | 21 | 8 | — | 7 |
| Rv1998 | — | 26 | — | — | 25 | — | — | 0 | — | — | 101 | — | — | 17 | — | — | 4 | — |
| Rv2006 | 30 | 22 | 17 | 22 | 12 | 12 | 10 | 0 | 0 | 78 | 116 | 29 | 31 | 13 | 22 | 9 | 6 | 6 |
| Rv2028c | — | 27 | — | — | 26 | — | — | 0 | — | — | 120 | — | — | 17 | — | — | 4 | — |
| Rv2029c | — | 43 | — | — | 40 | — | — | 4 | — | — | 162 | — | — | 21 | — | — | 3 | — |
| Rv2032 | 44 | — | — | 59 | — | — | 10 | — | — | 101 | — | — | 46 | — | — | 5 | — | — |
| Rv2625c | — | — | 20 | — | — | 14 | — | — | 0 | — | — | 36 | — | — | 33 | — | — | 4 |
| Rv2626c | — | 27 | — | — | 24 | — | — | 0 | — | — | 138 | — | — | 17 | — | — | 4 | — |
| Rv2628 | — | 23 | — | — | 16 | — | — | 0 | — | — | 107 | — | — | 17 | — | — | — | — |
| Rv2629 | — | — | 11 | — | — | 4 | — | — | 0 | — | — | 26 | — | — | 17 | — | v | 8 |
| Rv3129 | — | — | 17 | — | — | 15 | — | — | 0 | — | — | 36 | — | — | 26 | — | — | 5 |
| Rv3131 | — | 48 | — | — | 65 | — | — | 6 | — | — | 207 | — | — | 38 | — | — | 1 | |

[a] Data are shown for the 19 DosR regulon-encoded antigens that represent the 10 most immunogenic antigens at each African site. The order of antigens is the same as that shown in Fig. 2. MAK, Uganda; MRC, The Gambia; SUN, South Africa.
[b] P25, 25th percentile.
[c] P75, 75th percentile.
[d] Percent responders was calculated based on the site-specific cutoffs for a positive response as shown in Table S1 in the supplemental material.
[e] Antigens were ranked for immunogenicity at each site based on percent responders.
[f] For each site, data are not shown (—) for antigens that did not rank in the top 10.

suggested that the extended in vitro response of *M. tuberculosis* to hypoxia presumably involves additional genes next to the DosR regulon, and this enduring hypoxic response regulon was shown to involve more than 200 genes. These findings suggest that so-called latency antigens additional to those described here may become available for immunogenicity screening in human populations. The procedures used for the whole-blood and ELISA assays have undergone further harmonization, which will add strength to future data emerging from this work.

We show data from a relatively small but multicenter cross-sectional study at a single time point. Our most significant finding is the observed immune recognition of a large set of new *M. tuberculosis* antigens. While there was an apparent lack of recognition of other antigens, we cannot exclude that the observed *M. tuberculosis* antigen recognition profiles may change throughout the course of *M. tuberculosis* infection, with or without active TB disease. Of fundamental importance is how testing of DosR regulon-encoded antigens and other novel antigens can best contribute to our understanding of whether responses to these proteins are associated with the prevention of progression from latent *M. tuberculosis* infection to active TB disease. We also need to ascertain which antigens provide information that can be included in immunologic biomarker signatures that predict the outcome of infection with *M. tuberculosis*. We hope to be able to address these issues and gather further information about the antigens described over the duration of an ongoing longitudinal study within the Grand Challenges in Global Health (http://www.gcgh.org) Biomarkers for TB Consortium (http://www.biomarkers-for-tb.net). These future studies may provide more-detailed information about which antigens may be important.

By studying genetically and geographically diverse human populations and different *M. tuberculosis* lineages, it will be possible to capture potential correlates of protection in the context of various genetic backgrounds of host and *M. tuberculosis* populations. Such insights will allow us to define immune correlates and host markers of disease that can predict whether or not new TB vaccines will be effective and facilitate the iterative process of optimization during clinical vaccine trials (15).

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