Mycobacterium tuberculosis Defective in Phthiocerol Dimycocerosate Translocation Provides Greater Protective Immunity against Tuberculosis than the Existing Bacille Calmette-Guérin Vaccine

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We demonstrate that Mycobacterium tuberculosis that is unable to export the complex lipid phthiocerol dimycocerosate has a decreased capacity to replicate in mice and affords sustained protective immunity against M. tuberculosis infection. Protection was significantly better than that provided by the existing vaccine, Mycobacterium bovis bacille Calmette-Guérin (BCG), and this improved protective efficacy was maintained for at least 24 weeks after vaccination. Protection afforded by this attenuated strain coincided with a number of factors that were not associated with BCG vaccination: long-term persistence of the strain within the host, sustained and potent induction of antimycobacterial interferon-γ-secreting cells equal to that induced by virulent M. tuberculosis, and elicitation of T cells recognizing dominant M. tuberculosis antigens absent from BCG. These results suggest that the BCG vaccine may be too attenuated to afford effective protective immunity against tuberculosis, and vaccine strains that can provide sustained delivery of mycobacterial antigens are promising antituberculosis vaccine candidates.

Tuberculosis is a major health emergency not only in developing nations but also in industrialized countries where a resurgence has been noted, particularly in association with multidrug resistance and human immunodeficiency virus infection [1, 2]. The current vaccine against tuberculosis, bacille Calmette-Guérin (BCG), has been used for >70 years and has been administered to more people than any other vaccine. Its adverse effects are minimal, and it can prevent miliary and meningeal tuberculosis in young children to an appreciable degree. In contrast, the protection that BCG confers against the most prevalent disease form, pulmonary tuberculosis, is variable (range, 0%–80%) [3]. Thus, vaccination with BCG is not satisfactory, and there is a clear need for a more effective vaccine.

Recent advances in mycobacterial genetics have accelerated the identification of virulence factors and permitted the generation of Mycobacterium tuberculosis strains of attenuated virulence [4]. Live, replicating, attenuated strains of M. tuberculosis may be more potent than nonviable vaccines at stimulating long-lasting immunity. Furthermore, the host immune response may more closely mimic natural infection, because attenuated vaccine strains should contain the majority of antigens produced by the wild-type bacilli in vivo. This
is of particular importance because BCG lacks a number of protective \textit{M. tuberculosis} antigens, such as early secreted antigen target (ESAT)–6 protein [5]. The majority of the \textit{M. tuberculosis} attenuated strains that have been tested as vaccines against tuberculosis have been severely compromised for growth within the host and displayed poor protective efficacy [6, 7]. In some cases, mutants with a highly attenuated phenotype have displayed protection equivalent to BCG [8, 9], and, although such vaccine candidates may be of use in immunodeficient individuals, the nature of the protective immune response induced and their ability to sustain long-term protective immunity is unknown. Thus, the challenge remains to identify attenuated \textit{M. tuberculosis} strains that will stimulate long-term protective immunity and display greater protective efficacy than BCG.

One possible reason for the variable efficacy of BCG vaccination is the inability of the vaccine to induce sustained protective immunity. Studies of BCG efficacy in South India have demonstrated that, although BCG affords some level of protection in younger age groups, protection is lost in individuals aged >15 years [10]. This suggests that the protective immunity induced by BCG is not lifelong. BCG lacks >100 genes that are present in \textit{M. tuberculosis}, and it appears that the deletion of a number of virulence factors led to the highly attenuated vaccine strain currently in use [11–14]. Therefore, it is possible that BCG may be too attenuated and may not be able to sufficiently persist within the host to generate long-lasting protective immunity, a hypothesis that has been previously postulated [15].

We and others [16–18] have recently characterized strains of \textit{M. tuberculosis} that are unable to translocate the capsular lipid phosphatidyl-myo-inositol mannoside (DIM). These mutant strains exhibited heightened cell-wall permeability and display an attenuated phenotype in the lungs of infected mice. In the present article, we demonstrate that an \textit{M. tuberculosis} strain that was defective in DIM secretion was more able to persist in the organs of mice than BCG and provided improved and sustained protective immunity, compared with vaccination with BCG. Improved protection was associated with the induction of prolonged and potent antmycobacterial immunity, which suggests that an important prerequisite for effective vaccination against tuberculosis is the long-term production of protective antigens by candidate vaccine strains.

**MATERIALS AND METHODS**

**Bacterial strains and media.** \textit{M. tuberculosis} Mt103, the wild-type strain used in the present study, was isolated from an immunocompetent patient with tuberculosis [6]. The \textit{M. tuberculosis} strain that lacks the \textit{drrC} gene (\textit{drrC}'; MYC2261) was obtained by random mutagenesis and harbors an insertion sequence 1096\textit{Km} insertion within the \textit{drrC} gene [16]. The construction of the \textit{M. tuberculosis} lacking the \textit{purC} gene (\textit{purC}'; MYC1552) has been described elsewhere [6]. The BCG strain used was \textit{M. bovis} BCG Pasteur 1173P2. Strains were grown in Middlebrook 7H9 medium (Difco Laboratories) supplemented with 0.5% glycerol, 0.05% Tween 80, and 10% albumin-dextrose-catalase (ADC) or on solid Middlebrook 7H11 medium (Difco Laboratories) supplemented with oleic acid–ADC. When required, the antibiotic kanamycin (Km) was added at a concentration of 20 \textmu g/mL. Hypoxanthine (20 \textmu g/mL) was used to supplement 7H9 and 7H11 for the growth of \textit{M. tuberculosis} \textit{purC}’.

**Animal infections.** Six- to eight-week-old female C57BL/6 mice were obtained from Animal Resources Centre and maintained in specific pathogen–free conditions. For the analysis of bacterial persistence, C57BL/6 mice (4/group) were infected intravenously (iv) via the lateral tail vein with \textasciitilde10\textsuperscript{9} parental \textit{M. tuberculosis}, \textit{M. bovis} BCG, or the \textit{M. tuberculosis} mutant strains. Bacterial load was determined by killing mice at 1 day and at 4, 8, and 24 weeks after infection and plating serial dilutions of homogenates of liver, lung, and spleen onto 7H11 plates. Plates were incubated at 37°C for 3–4 weeks. Lung samples were also fixed in 10% buffered formalin, embedded in paraffin, and stained with hematoxylin-eosin for histological analysis. To calculate the proportion of the lung that contained granulomas or lesions present after aerosol \textit{M. tuberculosis} infection (percentage involvement), whole lung sections were scanned using a coolSNAP-Pro/ digital camera and Image-Pro Plus software (Media Cybernetics). The percentage of involvement was calculated by color differentiating involved and normal lung using Photoshop 5 (Adobe Systems). For the assessment of protective efficacy, C57BL/6 mice (5/group) were immunized subcutaneously (sc) with \textasciitilde10\textsuperscript{4} BCG or mutant \textit{M. tuberculosis} strains. Eight or 24 weeks after vaccination, mice were challenged with aerosol \textit{M. tuberculosis} H37Rv using an inhalation exposure apparatus (Glas-Col) with an infective dose of \textasciitilde100 viable bacilli/lung. Four weeks after the challenge, the number of bacteria within the lung and spleen was enumerated on 7H11 agar with or without Km. No residual bacteria were detected in the spleens or lungs of vaccinated mice at either 8 or 24 weeks after vaccination. Approval for all animal experiments was obtained from the University of Sydney Animal Ethics Committee, and all experiments were done using their guidelines.

**Cytokine measurements.** Single-cell suspensions were prepared from the spleen or mediastinal lymph node (MLN) of immunized mice in RPMI medium supplemented with 10% fetal calf serum and 2 mmol/L L-glutamine and tested individually. Interferon (IFN)–\gamma–producing cells were detected by ELISPOT, as described elsewhere [19]. Culture filtrate protein (CFP) was used in IFN-\gamma assays at a concentration of 3 \textmu g/mL. ESAT-6 antigen was used at a concentration of 6 \textmu g/mL.
and was a gift from Peter Andersen (Statens Serum Institut, Copenhagen, Denmark).

**Statistical analysis.** Statistical analysis of the results from immunological assays and log-transformed bacterial counts were conducted using analysis of variance (ANOVA). Fisher’s protected least significant difference ANOVA post hoc test was used for pairwise comparison of multigrouped data sets. Differences with \( P < .05 \) were considered to be significant.

**RESULTS**

Inability to translocate DIM limits the in vivo growth of *M. tuberculosis*. Using a signature-tagged mutagenesis approach, we previously isolated a mutant strain of *M. tuberculosis, drrC* (MYC2261), which had markedly reduced capacity to export DIM, a complex, cell-wall–associated lipid [18]. Complementation of the mutant strain with the *drrC* gene restored DIM secretion [18]. In preliminary experiments, the *drrC* mutant displayed attenuated growth in the mouse lung 3 weeks after infection [16]. We undertook a detailed analysis of the long-term persistence of this strain within C57BL/6 mice. In addition, we examined the persistence of the parental *M. tuberculosis* strain Mt103, the existing BCG vaccine, and *purC* (MYC1552). The latter strain was chosen as an example of a highly attenuated mutant [6], to allow us to examine the relationship between vaccine strain persistence and protective immunity. Mice were infected iv, and bacterial loads were assessed after 1 day and 4, 8, and 24 weeks in the lungs, liver, and spleen. *M. tuberculosis* Mt103 grew rapidly in the lungs during the first 4 weeks of infection, and bacterial loads remained high up to 24 weeks after infection (figure 1A). Mt103 continued to replicate over time in the spleen and, to a lesser extent, in the liver (figure 1B and 1C). The *drrC* mutant persisted in the spleen and lungs over the 24-week period but displayed a reduced ability to replicate at these sites, compared with the parental *M. tuberculosis* strain. The strain was completely cleared from the liver by 24 weeks. The BCG load was progressively reduced in all organs examined, especially at the later time points of 8 and 24 weeks. *M. tuberculosis* *purC* was the most attenuated of the strains tested, with numbers markedly reduced at the 8-week time point and no bacteria recovered from the 3 organs 24 weeks after infection. Therefore, this *M. tuberculosis* strain, which is unable to translocate DIM to the cell wall, displays attenuated growth in vivo but shows greater persistence within the host than BCG.

*M. tuberculosis* *drrC* mimics the immunity induced after natural *M. tuberculosis* infection. We next assessed whether the *drrC* strain was capable of generating a strong immune response, despite its attenuated phenotype. We chose to examine the generation of splenic IFN-\( \gamma \)–secreting cells after immunization, because this cytokine, which is produced predominantly by activated T cells, is a critical component of protective immunity against *M. tuberculosis* [20, 21]. Immunization with *drrC* resulted in the strong induction of splenic IFN-\( \gamma \)–secreting cells in response to CFP of *M. tuberculosis*, which was maintained at 24 weeks after immunization (figure 2A and 2C). A similar pattern of changes in antigen-specific IFN-\( \gamma \)–secreting cells was observed in cells of the MLNs draining the lung (data not shown). The magnitude of the response was identical to
that induced by *M. tuberculosis* Mt103, even at later time points. BCG elicited markedly reduced numbers of CFP-reactive IFN-γ-secreting cells at all time points analyzed (figure 2A and 2C). The purC− mutant did not generate any appreciable IFN-γ response, which was most likely a consequence of its poor ability to persist in vivo.

BCG lacks a number of important antigens that are present in *M. tuberculosis*, including the immunodominant secreted ESAT-6 antigen [11]. This may be, in part, the reason for the reduced antimycobacterial immunity induced by BCG, because ESAT-6 is a major component of *M. tuberculosis* CFP [5]. To address this, we determined the magnitude of IFN-γ-secreting cells recognizing the ESAT-6 antigen that were generated after immunization with all strains. Neither BCG nor purC− elicited an IFN-γ response on recall with ESAT-6 (figure 2D and 2F). By contrast, drrC− was a potent inducer of IFN-γ-secreting cells that recognized ESAT-6, and this strain produced a similar response to that induced by natural infection with the parental *M. tuberculosis* strain, even at extended time points after immunization (figure 2D and 2F). These results suggest the drrC− strain is able to present to the immune system a broader repertoire of important *M. tuberculosis* antigens than the existing BCG vaccine.

**M. tuberculosis drrC− mutant provides sustained protection against aerosol *M. tuberculosis* challenge.** We next determined whether the potent antimycobacterial immunity induced by the drrC− mutant would translate to effective protection against *M. tuberculosis* infection. Mice were immunized sc with 5 × 10^4 cfu of drrC−, purC−, or BCG, and the ability to protect against aerosol challenge with *M. tuberculosis* was determined 8 weeks after vaccination. Mice vaccinated with the purC− mutant were not protected against infection, which demonstrates the crucial link between vaccine persistence and effective protective immunity (figure 3A and 3B). BCG vaccination reduced the bacterial load by ∼1.1 log in the lungs, compared with unvaccinated mice. Immunization with the *M. tuberculosis* drrC− strain further reduced the bacterial burden by 0.5 log (figure 3A). This effect was also observed in the spleen, with drrC− affording significantly better protection than vaccination with BCG (figure 3B).

An important prerequisite for any vaccine is the ability to provide long-term protective immunity. We compared the protection induced by the vaccine strains at 24 weeks after vaccination. After challenge with *M. tuberculosis*, unvaccinated animals and purC−-immunized mice displayed equivalent and elevated bacterial loads in both the lungs and spleen (figure 3C and 3D). BCG vaccination was able to significantly reduce the bacterial burden at both sites. Of importance, vaccination with drrC− maintained its superiority over BCG—*M. tuberculosis* numbers were significantly reduced in both the lung and spleen compared with BCG vaccination (figure 3C and 3D). Therefore, vaccination with drrC− is superior to BCG at controlling infection at the primary site of exposure and in limiting the dissemination of *M. tuberculosis* to distant sites, and the protective immunity generated is long lasting.

The inflammatory response in the lungs of mice vaccinated for 24 weeks and then infected via an aerosol method with *M. tuberculosis* Mt103 was investigated. Large, diffuse lesions were present in lungs of unvaccinated mice (figure 4A) that were composed of large foamy macrophages with lymphocytes interspersed throughout the lesion (figure 4B). Occasional neutrophils were also seen. Areas of mild-to-moderate interstitial pneumonia, characterized by cellular influx and thickening of the alveolar wall, were evident throughout the lung. In comparison, the lesions seen in BCG-vaccinated mice were signif-
As the mean bacterial load was found to be significantly smaller and more compact than in the unvaccinated group (figure 4C). Lesions were located around blood vessels and were predominantly lymphocytic (figure 4D), with some macrophages and occasional neutrophils also evident. Most of the lung was normal alveolar space and airways, although mild interstitial pneumonia was evident in some areas. The lesions in the purC- vaccinated mice were generally smaller yet more numerous than those present in unvaccinated animals (figure 4E). These lesions were composed of interspersed macrophages and lymphocytes, with some neutrophils evident in most lesions (figure 4F). Lungs from mice vaccinated with the drrC- strain contained small, compact lesions localized around blood vessels (figure 4G) that had a strong lymphocytic appearance, with some foamy macrophages and occasional neutrophils (figure 4H). Some mild interstitial pneumonia was evident, but large areas of the lung retained a normal structure. The area of the lung displaying inflammatory involvement was significantly reduced in drrC- vaccinated mice, compared with unvaccinated mice, which further demonstrates the ability of vaccination with the drrC- strain to protect against destructive lung pathology (figure 5).

**DISCUSSION**

Despite its widespread use, the current BCG vaccine has had little effect on the control of tuberculosis in countries where it is highly endemic. The vaccine protects against childhood tuberculosis but has generally been less effective against pulmonary tuberculosis in adults [3]. An analysis of BCG trials concluded that the vaccine does not afford adequate protection >10 years after vaccination [22]. Therefore, one limitation of BCG is that the induced protective immunity does not appear to be sufficiently long lasting. Furthermore, BCG lacks a large number of proteins that are present in *M. tuberculosis*, including the ESAT-6 and major protein tuberculosis–64 antigens, which can induce partial protection in animal models [23]. Therefore, a suitable replacement should persist long enough to afford sustained protective immunity and deliver most, if not all, of the important antigens encountered during natural infection with *M. tuberculosis*. This has been the rationale for the use of *M. tuberculosis* strains of attenuated virulence, although, to date, no attenuated vaccine strain has proved more effective than BCG [6–9]. Our results demonstrate that immunization with *M. tuberculosis* drrC-, an attenuated vaccine strain that persists for a long time within the host, provides significantly greater protective efficacy than does BCG. This superiority was manifested both in the lung and spleen of immunized mice, which implies that the immunity induced by drrC- is able to initially limit the replication of *M. tuberculosis* at the primary site after natural aerosol infection and to reduce the bacterial load at secondary sites of dissemination.

The product of the drrC gene is directly involved in the proper localization of DIM in the cell envelope. The attenuated phenotype described in the present article and in previous studies [16, 17] directly implicates a role for DIM in the virulence of *M. tuberculosis*. Because mycobacterial lipids have been implicated in the avoidance of host immunity by pathogenic mycobacteria [24], it is tempting to speculate that the disruption of DIM transport may “relax” an immune inhibitory mechanism and thus contribute to the strong immunogenicity of the drrC- strain. Alternatively, a lack of DIM may lead to an increased release of antigenic components, thereby improving the antigenicity of the mutant strains. We have observed that, even when low numbers of drrC- are present in the lungs of mice after intranasal infection, a potent anti-CFP response is still generated.
Figure 4. Vaccination with *Mycobacterium tuberculosis* drrC reduces inflammatory responses within the lung after *M. tuberculosis* infection. Mice were vaccinated as described in figure 3 and were challenged 24 weeks after immunization by the aerosol route with *M. tuberculosis* H37Rv. Twenty-eight days after infection, lung tissue was fixed in buffered formalin, sectioned, and stained with hematoxylin-eosin (A, C, E, and G, magnification, ×25; B, D, F, and H, magnification, ×400). Unvaccinated mice showed large granulomatous lesion composed of large foamy macrophages (crosses) interspersed with lymphocytes and some neutrophils (arrowhead) (A and B). Bacille Calmette-Guérin–vaccinated mice showed small compact lesions associated with blood vessels, although most of the lung was normal (C). Lesions were highly lymphocytic (arrow), with some macrophages and occasional neutrophils (D). *M. tuberculosis* purC–vaccinated mice displayed multiple lesions, greater in no. but of reduced size than those in unvaccinated mice (E), with lesions showing interspersed lymphocytes and macrophages with occasional neutrophils (F). *M. tuberculosis* drrC–vaccinated mice developed small compact vessel-associated lesions (G). Most of the lung still appeared normal. Lesions were highly lymphocytic, with some macrophages present (H). The section is representative of 1 of 5 lung sections per group from 1 of 2 representative experiments.
The area of the lung displaying inflammatory involvement was calculated as described in Materials and Methods. The significance of differences between the percentage of lung involved in vaccinated compared with unvaccinated (Unvacc) mice was determined by analysis of variance (*P < .005). Data are representative of 2 separate experiments.

Figure 5. Quantitation of lung inflammation after vaccination with Mycobacterium tuberculosis drrC and challenge with aerosol M. tuberculosis. The area of the lung displaying inflammatory involvement was calculated as described in Materials and Methods. The significance of differences between the percentage of lung involved in vaccinated compared with unvaccinated (Unvacc) mice was determined by analysis of variance (*P < .005). Data are representative of 2 separate experiments.

The superior protective efficacy of BCG/H11002 cutaneous vaccination of mice with the drrC/ H11002 strain was related to its ability to replicate and persist to a greater extent than BCG within the mouse yet display an attenuated phenotype in vivo. We have observed that strains that are less attenuated than drrC/ H11002 afford poorer protective efficacy (data not shown), which indicates that there is a delicate balance between vaccine virulence and protective immunity; a vaccine strain that is too virulent may induce a destructive pathological process that will limit the capacity of the host to adequately deal with subsequent infection. Conversely, strains that are unable to persist sufficiently to deliver protective antigen are poor vaccines, as has been demonstrated by the purC/ strain used in the present study. Intrinsically linked to persistence of the vaccine is the immunity generated after vaccination. The drrC/ strain was able to induce anti-M. tuberculosis IFN-γ–secreting T cells to a level equal to that induced by M. tuberculosis infection, and this effect was sustained long-term. This was in contrast to purC/, whose complete lack of protective efficacy correlated with an inability to generate IFN-γ–secreting T cells in response to mycobacterial antigens. BCG displayed a markedly reduced response to M. tuberculosis culture filtrate antigens. One possible reason for this observation is the absence from BCG of important, secreted antigens that are present in CFP, thus limiting the pool of activated T cells recognizing M. tuberculosis generated after immunization. Accordingly, we observed that drrC/ was able to generate high numbers of IFN-γ–secreting cells that recognize the M. tuberculosis ESAT-6 antigen. BCG was unable to do this, because it does not express the antigen [25], whereas purC/ lacked this ability because of insufficient presentation of the antigen to the immune system. ESAT-6 is a major secreted antigen of M. tuberculosis and is protective in the form of both protein and DNA vaccines [23, 26]. Therefore, it is assumed that the presence in drrC/ of this and other M. tuberculosis–specific proteins increases the array of protective antigen encountered by the immune system. It is unclear what the relative contribution is of the expanded antigen repertoire of drrC/ and its increased persistence; do these 2 factors synergize to result in the potent protective efficacy of the drrC/ mutant, or is one property more dominant? Addressing this fundamental question is crucial to our understanding of antituberculosis protective immunity and the development of effective vaccines.

The persistent phenotype of drrC/, although no doubt contributing to the strong level of protection afforded by the strain and its superiority over conventional BCG vaccination, may limit use of the vaccine in its present form. However, after the subcutaneous vaccination of mice with the drrC/ strain for protection studies, we could not detect any residual bacteria in the lungs, spleen, or liver either 8 or 24 weeks after immunization (data not shown). Therefore, the drrC/ strain can elicit effective protective immunity when it is used at an immunizing dose that does not result in a progressive infection. This is reflected in the limited pulmonary pathological abnormalities after drrC/ vaccination and subsequent M. tuberculosis challenge, similar to that induced by BCG vaccination, which suggests little if no deleterious affect after immunization with the drrC/ strain. Using the parameters of protective immunity defined in the present study, it will now be possible to assess other attenuated vaccine candidates, which possibly incorporate the drrC/ mutation, that afford potent and sustained protection while exhibiting a suitable safety profile for use in human populations.

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References