Patterns and Implications of Naturally Acquired Immune Responses to Environmental and Tuberculous Mycobacterial Antigens in Northern Malawi

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Interferon (IFN)-γ responsiveness to 12 purified protein derivative (PPD) and new tuberculin antigens from 9 species of mycobacteria was assessed, using a whole blood assay, in 616 young adults living in northern Malawi, where Mycobacterium bovis bacille Calmette-Guérin (BCG) vaccination provides no protection against pulmonary tuberculosis. The prevalence of IFN-γ responsiveness was highest for PPDs of M. avium, M. intracellulare, and M. scrofulaceum (the MAIS complex). Correlations between responsiveness paralleled genetic relatedness of the mycobacterial species. A randomized, controlled trial was carried out, to assess the increase in IFN-γ responsiveness to M. tuberculosis PPD that can be attributed to M. bovis BCG vaccination. The BCG-attributable increase in IFN-γ response to M. tuberculosis PPD was greater for individuals with low initial responsiveness to MAIS antigens than for those with high initial responsiveness. Although not statistically significant, the trend is consistent with the hypothesis that prior exposure to environmental mycobacteria interferes with immune responses to BCG vaccination.

Geographical variation in the efficacy of Mycobacterium bovis bacille Calmette-Guérin (BCG) vaccination against tuberculosis is well recognized but poorly understood [1]. This has been explored in case-control and cohort studies, and in a vaccination trial, in the Karonga District of northern Malawi. In this population, BCG vaccination (Glaxo 1077 vaccine; Evans Medical) provides >50% protection against leprosy but no protection against pulmonary tuberculosis [2]. Studies in the United Kingdom have consistently shown that this vaccine provides >50% protection against tuberculosis in the British population [3–5].

Several lines of evidence suggest that differences in exposure to environmental mycobacteria may be an important determinant of this variation. Classic studies by Palmer and Long demonstrated that prior exposure of guinea pigs to M. fortuitum, M. avium, or M. kansasi imparted, respectively, 15%, 50%, or 85% as much protection against M. tuberculosis as did BCG, yet the combination of BCG plus an environmental mycobacterium provided no more protection than did BCG alone [6]. Several subsequent studies have demonstrated similar findings in both guinea pigs and mice [7–10]. In humans, prior skin test sensitivity to purified protein derivative (PPD) of M. intracellulare (PPD-B) was found to be associated with reduced risks of tuberculosis [11], and there is much evidence of latitude trends in skin test sensitivity to environmental mycobacteria [12] and in the efficacy of BCG against tuberculosis [1, 13]. Geographic gradients of leprosy prevalence are reported from many areas, and in Karonga District the disease has been more common in the north than in the south, a pattern that has been related to protective mycobacterial exposure in the low-incidence area [14–16]. Results such as these suggest that different environmental mycobacteria are common in different localities, that the cross-reactive immune responses they induce can confer protection against tuberculosis and leprosy, and that this may be related to observed variations in protection by BCG [1].

Environmental mycobacterial exposure may influence BCG protection by providing a degree of partial protection on which BCG cannot improve or by “immunizing” the vaccinee against the vaccine bacilli, which may need to multiply to induce effective immunity [1, 17]. Alternatively, it is possible that natural
exposure to certain environmental mycobacteria might induce an “inappropriate” immune response, which is boosted by the BCG vaccine [9]. Studies of immune responses induced by environmental mycobacterial exposure in humans thus far have been based on delayed type hypersensitivity (DTH) skin testing [16, 18, 19]. However, it is known that DTH does not provide a simple correlate of protective immunity to mycobacterial disease in humans [14, 20]. There has been much interest in IFN-γ production recently as a correlate of protective immunity. This Th1 type cytokine is produced by sensitized T cells in response to antigenic stimulation and leads to macrophage activation [21]; it is also associated with protection against M. tuberculosis in mice [22, 23]. The observation that individuals with mutations in the IFN-γ receptor gene are highly susceptible to environmental mycobacteria and to systemic infection with BCG supports the role of this cytokine in protecting humans against tuberculosis [24].

The availability of whole blood assays for cytokine production provides an appropriate means for studying these mechanisms in human populations [25–27]. We have applied the diluted 6-day whole blood method to measure IFN-γ responsiveness to 12 different mycobacterial antigen preparations in BCG scar–negative individuals in the area of northern Malawi where BCG has been demonstrated to provide no protection against pulmonary tuberculosis [2]. We use this approach to reveal patterns of natural exposure or sensitivity to the various antigens by age, sex, and geographic distribution, and we explore whether the magnitude of prior sensitization to mycobacterial species closely related to M. tuberculosis, as reflected by IFN-γ responsiveness to M. avium, M. intracellulare, and M. scrofulaceum (MAIS complex) antigens, influences the magnitude of the BCG-induced IFN-γ response to M. tuberculosis PPD.

Subjects, Materials, and Methods

This work was carried out in the context of the Karonga Prevention Study (KPS), a large vaccine trial and epidemiological study of tuberculosis and leprosy in Karonga District, a rural area in northern Malawi. Basic methods of the KPS project and evidence that BCG vaccination (Glaxo 1077 vaccine; Evans Medical) provides 50% protection against leprosy, but not against tuberculosis, have been published elsewhere [2].

Recruitment of subjects. Recruitment for this study took place between January and November 1998 [28]. Field teams consisted of a paramedic and an interviewer. Candidates with no prior history of BCG vaccination were selected from the project database and were visited in their homes, where the interviewer confirmed identifications, explained the study, and obtained written consent. Candidates were examined for evidence of a BCG scar, generalized rashes, and signs of tuberculosis, leprosy, or other severe illness. Women were tested for pregnancy. Any of these conditions was a criterion for exclusion from the study. All candidates were pre-counseled for human immunodeficiency virus (HIV) antibody testing, which was performed using a particle agglutination test (Edgware modification of Serodia, Mast Diagnostics) and was confirmed by ELISA (Vironostika HIV Uni-form II plus 0; Organon Teknika); any subjects who tested positive were excluded from the study.

Skin testing was carried out using the Mantoux technique, with M. tuberculosis PPD batch RT23 (2 TU; Statens Serum Institut [SSI]). Tests were administered on the volar surface of the forearm and were read after 48–72 h. Indurations were measured across and along the arm, and the mean induration was used for analyses. Individuals with responses >10 mm were excluded from vaccination, referred to a project medical officer, and examined for tuberculosis. For the whole blood assay, a 5-mL intravenous blood sample was transferred into 50 U of preservative-free sodium heparin (Monoparin; CP Pharmaceuticals).

Eligible individuals were randomized to receive BCG vaccine (two thirds of subjects) or placebo (one third). The vaccine was the same strain as that used in the vaccine evaluations in Karonga District (Glaxo 1077), and the placebo consisted of the dextran matrix of the BCG vaccine. Both were kindly donated by Evans Medical. Study subjects were followed up 1 year after their recruitment, at which time the skin tests, whole blood assays, and HIV tests were repeated. An 84% follow-up rate was achieved.

Whole blood assay. The whole blood assays and ELISAs were performed in the project’s laboratory in Chilumba, Malawi [28]. Whole blood (5 mL) containing 50 U of preservative-free sodium heparin (Monoparin; CP Pharmaceuticals) was diluted 1:5 with RPMI 1640 tissue culture medium and 100 µL of diluted blood incubated with or without antigen in 100-µL volumes in quadruplicate wells at 37°C with 5% CO₂. Supernatants were harvested on day 6 (mode, 144 h; range, 140–167 h), pooled, and stored at −20°C or −70°C before ELISA. Previous studies have shown that, in this system, IFN-γ production is not dependent on variations in the number of total white blood cells present in the cultures [29].

Antigens. The 12 antigen preparations described here were obtained from 5 separate suppliers and laboratories. SSI was the supplier for PPDs of M. tuberculosis (batch RT48, lot 191), M. avium (batch RS10/2, lots 37 and 39; avium SSI), M. intracellulare (batch RS23, lot 27; intracellulare SSI), M. scrofulaceum (batch RS95, lots 17 and 18), M. kansasi (batch RS30, lots 18 and 19; kansasi SSI), M. fortuitum (batch RS20, lots 16 and 17), and M. marinum (batch RS170, lots 10 and 11). All of these antigens were prepared according to a standardized protocol [30]. In brief, the mycobacteria were grown as surface cultures on modified Sauton’s medium for 4.5–5.5 weeks, were sterilized by heating, were filtered, were concentrated by ultrafiltration, and were precipitated with trichloroacetic acid prior to drying in ether and subsequent dilution in PBS. A second M. avium antigen (standard avian PPD, produced in 1954; avium CVL [Central Veterinary Laboratory]) was obtained from the Veterinary Laboratories Agency [31]. A second M. intracellulare antigen (“PPD-B”) was obtained from the US Public Health Service. This antigen (batch 55-7005 1 3) was manufactured in 1970 by the Mycobacterial Immunology Section, PHS/Centers for Disease Control and Prevention, according to a standard protocol [32]. A second M. kansasi preparation (batch 14-4-77) and an M. vaccae antigen (batch R877R) were obtained from Dr. J. Stanford (University College London [UCL]). These were prepared as “new tuberculins” by growth on Sauton’s medium solidified with 1.5% agar at 32°C–35°C. The growth was harvested into M/15 borate buffered saline (pH 8.0) and was ultrasonicated, followed
by filter sterilization and dilution in borate buffered saline [33]. M. bovis PPD was obtained from the Central Veterinary Laboratory via the National Institute of Biological Standards and Controls. This International Standard was originally produced by the Central Diergeneeskundig Instituut in The Netherlands. After initial experiments to determine the optimal concentration, all these preparations were used at a final concentration of 5 µg/mL.

Positive controls included the mitogen phytohemagglutinin (PHA; Difco Laboratories/Becton Dickinson), at a final concentration of 5 µg/mL, and a nonmycobacterial antigen, streptokinase-streptodornase (SK/SD [Varidase]; Wyeth Laboratories), at a final concentration of 250 U/mL. Culture medium alone served as the negative control.

**Measurement of cytokines.** Quantitative IFN-γ ELISAs were carried out in single wells testing 100-µL aliquots of the previously pooled supernatants, using commercially available antibody pairs (PharMingen), as described elsewhere [28]. Recombinant cytokine (31–2000 pg/mL; PharMingen) was used for the standard curve; the lower detection limit of the ELISA was 31 pg/mL. Negative control values were subtracted from all results. To control for interplate and intraplate variation, a positive control supernatant was used in duplicate on each ELISA plate. The mean intraplate variation of these duplicate measurements was 3.7%. The coefficient of variation between plates (interplate variation) was 12.3%.

**Statistical analysis.** Associations between pairs of antigens were quantified by using Spearman’s rank correlation coefficient. IFN-γ responses were presented on a log scale, representing doubling concentrations (from 31 to 62, 125, 250, 500, and so forth). Age and sex trends for IFN-γ responses were analyzed by using logistic regression, with thresholds of 62, 250, and 500 pg/mL. The prevalence of responsiveness to the mycobacterial preparations separately for individuals from the north and the south of the district, and by season. No clear geographic or seasonal pattern was found.

**Results**

The distributions of IFN-γ responses to the control stimuli RPMI, PHA, and SK/SD have been described elsewhere [28]. Only 4% of individuals had a “positive” IFN-γ response (>62 pg/mL) in unstimulated cultures, whereas 99% had a positive IFN-γ responses to PHA. A positive IFN-γ response to SK/SD was detected in 83% of the samples.

To ascertain the optimal PPD concentration for cytokine measurement, the mycobacterial antigens were tested over a range of dilutions. Results indicated that optimal IFN-γ responses were detected at 5–10 µg/mL, and a concentration of 5 µg/mL was thus selected for use in subsequent work. Figure 1 shows the frequency distributions of the IFN-γ responses detected to the 12 antigen preparations. The proportion of individuals with a positive IFN-γ response (>62 pg/mL) varied among antigens. For some (e.g., M. avium, M. intracellulare, and M. scrofulaceum; figure 1C, 1D, 1E, 1F, and 1G), the prevalence of responsiveness was higher than that observed for M. tuberculosis (figure 1A). For M. marinum it was very similar (figure 1H), whereas for others (e.g., M. bovis, M. kansasii, M. fortuitum, and M. vaccae; figure 1B, 1I, 1J, 1K, and 1L) it was lower.

Table 1 shows the correlations between IFN-γ responses for all possible pairs of antigens used in this study. All correlation coefficients between pairs of antigens belonging to the closely related MAIS species complex are ≥ .7, with lower values for pairs including the less closely related, slow-growing M. kansasii or the more distantly related, fast-growing species M. vaccae and M. fortuitum. Two pairs of particular interest, because they represent different antigenic preparations for the same species, are M. intracellulare SSI versus M. intracellulare PPD-B and M. avium SSI versus M. avium UCL (r = .77 and .73, respectively). High correlations of response (r > .75) were also observed for M. tuberculosis PPD-RT48 with M. bovis, M. intracellulare (SSI), M. scrofulaceum, and M. avium CVL.

The data were examined to determine whether individuals with strong responses to one of the MAIS complex PPDs also showed high responses to the other two. Figure 2 indicates a high degree of concordance among responses to the SSI PPDs of the 3 closely related mycobacterial species, since few individuals responded to only one or two of the antigens at each response threshold, and a large majority tended to respond either to none or to all three, which is consistent with considerable cross-reaction among these 3 closely related antigens.

Because environmental mycobacteria are prevalent in the soil and water and might be expected to vary with season, and because of our evidence for geographic trends in leprosy and in M. leprae skin test sensitivity, we examined the IFN-γ responses to the mycobacterial preparations separately for individuals from the north and the south of the district, and by season. No clear geographic or seasonal pattern was found. For each area/season combination, the proportions of individuals responding to >62 pg/mL to the 4 closely related antigens of the MAIS complex were similar and were higher than those for other antigens. The prevalence of responsiveness to the M. tuberculosis PPD was consistently higher than that to M. bovis (data not shown).

Skin test responsiveness to mycobacterial antigens increases with age in Karonga District, as in most populations, reflecting cumulative exposure, and is typically higher among male than among female individuals >15 years old. Therefore, we tested whether there was any evidence of an age–sex interaction in the IFN-γ responses to the 12 PPD antigens. For most antigens, evidence of such interaction did not reach statistical significance. However, the median IFN-γ responses and percentages...
Figure 1. Frequency distributions of the interferon (IFN–γ) responses to mycobacterial antigen preparations of *Mycobacterium tuberculosis* and of environmental mycobacteria, as measured in the whole blood assay. Proportions of subjects making IFN–γ responses of 0–31 pg/mL, 32–62 pg/mL, and so forth are shown. The numbers of subjects tested with each antigen were as follows: *M. tuberculosis* (Statens Serum Institut [SSI]), n = 614; *M. bovis*, n = 465; *M. avium* (SSI), n = 564; *M. avium* (Central Veterinary Laboratory [CVL]), n = 614; *M. intracellulare* (purified protein derivative [PPD]–B), n = 284; *M. intracellulare* (SSI), n = 615; *M. scrofulaceum* (SSI), n = 616; *M. marinum*, n = 609; *M. kansasii* (SSI), n = 99; *M. kansasii* (University College London [UCL]), n = 391; *M. fortuitum*, n = 607; and *M. vaccae*, n = 448.
Table 1. Correlations between interferon (IFN)-γ responses to the different Mycobacterium antigen preparations.

<table>
<thead>
<tr>
<th>Mycobacterium</th>
<th>M. avium (SSI)</th>
<th>M. avium (CVL)</th>
<th>M. intracellulare (SSI)</th>
<th>M. scrofulaceum (SSI)</th>
<th>M. marinum (SSI)</th>
<th>M. kansasii (SSI)</th>
<th>M. fortuitum (UCL)</th>
<th>M. vaccae (SSI and UCL)</th>
<th>SK/SD</th>
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<tr>
<td>M. tuberculosis</td>
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<td>.71</td>
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<td>.72</td>
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<td>M. bovis</td>
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<td>M. avium (SSI)</td>
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<td>M. avium (CVL)</td>
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<td>.79</td>
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<td>M. intracellular (PPD-B)</td>
<td>.77</td>
<td>.80</td>
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<td>.67</td>
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<td>M. intracellular (SSI)</td>
<td>.83</td>
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<td>M. scrofulaceum</td>
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<td>M. marinum</td>
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<td>.54</td>
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<tr>
<td>M. kansasii (UCL)</td>
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<td>M. kansasii (SSI)</td>
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<td>M. vaccae</td>
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NOTE. IFN-γ responses to the 12 mycobacterial antigen preparations, tested at final concentrations of 5 μg/mL, and to an irrelevant antigen, streptokinase/streptodornase (SK/SD), tested at a final concentration of 250 U/mL, were measured by ELISA in day 6 supernatants of diluted whole blood cultures, as described in Subjects, Materials, and Methods. Spearman’s rank correlation coefficients of IFN-γ responses between the antigens are shown. In blank cells, duplicated information has been omitted for clarity. CVL, Central Veterinary Laboratory; M. intracellular, M. intracellulare; MAIS, Mycobacterium avium, M. intracellulare, M. intracellulare; has been omitted for clarity. CVL, Central Veterinary Laboratory; SSI, Statens Serum Institut; UCL, University College London.

of responders (>62 pg/mL) were higher for male than for female individuals among those ≥15 years old for all antigens except M. vaccae (SSI and UCL) and M. kansasii (data not shown).

The distribution of observed Mantoux skin test responses to M. tuberculosis PPD-RT23 in this population has been described elsewhere [28]. A total of 281 individuals (49%) had no measurable induration (0 mm). Of the remaining subjects, 214 (38%), 54 (9%), and 24 (4%) had indurations >0.5, >10, or >15 mm respectively. Figure 3 shows the median IFN-γ response for each antigen as a function of the M. tuberculosis PPD RT23 skin test sensitivity. The strongest association is seen for the M. tuberculosis PPD. We also see evidence of strong positive associations with the closely related MAIS antigens and with M. marinum, but there is little evidence of such a relationship with M. kansasii, M. fortuitum, or M. vaccae.

Among individuals with prevaccination IFN-γ responsiveness ≤250 pg/mL to the M. tuberculosis RT48 antigen, there were 44 individuals with low (≤62 pg/mL) prevaccination IFN-γ responses to all MAIS (SSI) complex antigens and 30 individuals with high (>250 pg/mL) IFN-γ responses before vaccination. The vaccine-associated changes in IFN-γ responses to M. tuberculosis PPD-RT48 are illustrated in figure 4. For those with low responses to MAIS before vaccination, the ratio of the (postvaccination + 1)/(prevaccination + 1) IFN-γ response to M. tuberculosis PPD RT48 was, on average, 6.5 times higher in those who received BCG vaccination than in those who received placebo (95% confidence interval [CI], 0.8–54.8; P = .083). For those with high prevaccination responses to MAIS, the ratio of (postvaccination + 1)/(prevaccination + 1) IFN-γ response to M. tuberculosis PPD-RT48 was, on average, only 1.7 times higher among those who received BCG vaccination than among those who received placebo (95% CI, 0.2–16.1; P = .638). The difference between the effects (6.5 vs. 1.7) was not statistically significant (P = .38).

Discussion

This study provides evidence that individuals living in a rural area of northern Malawi, with no history or scar evidence of prior BCG vaccination, have considerable exposure to mycobacteria, as reflected in IFN-γ responsiveness to a variety of mycobacterial antigens. The fact that M. avium, M. intracellulare, and M. scrofulaceum are recognized more strongly than M. tuberculosis suggests that many individuals meet these or closely related agents in the environment and that sensitivity is not a reflection of M. tuberculosis exposure alone. This is supported by a relatively low prevalence of responses detected to individual recombinant antigens known to be restricted to the M. tuberculosis complex (G. F. Black, P. E. M. Fine, S. D. Chaguluka, A. C. Crampin, L. Mwaungulu, L. Sichali, S. Floyd, R. E. Weir, L. Bliss, E. Jarman, L. Donovan, P. Andersen, W. Britton, G. Hewinson, K. Huugen, J. Paulsen, M. Singh, R. Prestidge, and
H. M. Dockrell, unpublished data). We have shown elsewhere that there is a relatively high prevalence of skin test sensitivity to antigens of *M. intracellulare* and *M. scrofulaceum*, moderate prevalence of sensitivity to *M. marinum* and *M. kansasii*, and moderate or low prevalence of sensitivity to *M. fortuitum* in this population [16], which is in accord with the relative IFN-γ responses observed here. The lower prevalence of sensitivity to *M. bovis* relative to *M. tuberculosis* reflects the specificity of these antigens, since *M. bovis* is known to be rare in Karonga District (it has been identified only twice in >2000 positive cultures from tuberculosis suspects), and since the subjects in this study had no evidence of having received BCG vaccine.

Mycobacterial preparations such as those employed in this study contain a large number of antigenic components, many of which are small peptides. Our data indicate that differences in the methods used to prepare the *M. avium* and *M. intracellulare* antigens did not greatly influence their ability to induce T cell responses. In contrast, there were quite large differences between the 2 *M. kansasii* preparations, one of which is a “true” PPD (*M. kansasii* SSI) and the other a “new tuberculin” (*M. kansasii* UCL) [30]. Because of restricted availability, these were not tested on the same individuals, and thus we cannot directly compare the relationship between them in the present study. However, in comparison with *M. tuberculosis* PPD, the association was weaker with the UCL antigen than with the SSI antigen. This is reflected in Spearman’s rank correlation coefficients (*r* = .59 and .86 for *M. tuberculosis* PPD with *M. kansasii* UCL and *M. kansasii* SSI, respectively). The *M. kansasii* and *M. vaccae* antigens from UCL produced the lowest IFN-γ responses observed in this study. It is possible that, because these preparations are obtained following ultrasonication, without trichloroacetic acid precipitation, the antigens are less degraded than the low-molecular-weight antigens and peptides present in PPD preparations and thus require more antigen processing. It is not easy to compare the constituents of such antigens, since clear protein profiles are not obtained using SDS-PAGE electrophoresis, but one would predict that the balance of somatic and secreted antigens will vary between the 2 types of preparations, and a greater concentration of secreted antigens or epitopes, which are often immunodominant in the immune response [34], may be present in the PPDs. The strong association between the median IFN-γ response to *M. tuberculosis* PPD-RT48 and the DTH response to *M. tuberculosis* RT23 tuberculin has been discussed elsewhere [28]. We show here that a similar, very strong positive correlation with DTH is observed for the closely related MAIS complex and *M. marinum* species, but not for antigens from the more distantly related, fast-growing mycobacteria, *M. fortuitum* and *M. vaccae*. In this context, it is interesting that responses to both of the *M. kansasii* antigens were more similar to those of the fast than to those of the slow growers, despite the fact that *M. kansasii* is generally considered to be closely related to the *M. tuberculosis* and MAIS complexes, at least as determined by 16S rRNA analyses [35].

IFN-γ responsiveness was generally higher in male than in female individuals, except for *M. kansasii* and *M. vaccae*. Given that the prevalence of DTH to tuberculin is generally higher among male than among female individuals after the age of puberty, in all populations in which it has been tested, this may reflect a sex difference in immune responsiveness rather than in exposure [36]. Within the Karonga District, there is a gradient in the prevalence of leprosy, with more disease in the north than in the south of the district [15]. Skin test responsiveness to *M. leprae* soluble antigen showed the opposite gradient,
Figure 4. Effect of prevaccination interferon (IFN-\(\gamma\)) responsiveness to mycobacteria of the *Mycobacterium avium-intracellulare-scrofulaceum* (MAIS) complex on changes in IFN-\(\gamma\) responses to *M. tuberculosis* purified protein derivative (PPD) induced by *M. bovis* bacille Calmette-Guérin (BCG) vaccination. Changes in IFN-\(\gamma\) responsiveness to *M. tuberculosis* PPD (RT48) from prevaccination to 1 year after BCG vaccination (right panels) or placebo (left panels) among individuals with low (<62 pg/mL; top panels) or high (>250 pg/mL; bottom panels) prevaccination responsiveness to MAIS antigens are shown. All individuals had a prevaccination IFN-\(\gamma\) response to *M. tuberculosis* PPD-RT48 of <250 pg/mL.

which suggests that skin test positivity (presumably induced by exposure to environmental mycobacteria cross-reactive with *M. leprae*) was associated with protection against leprosy [14]. Analysis of the IFN-\(\gamma\) dataset in the present study showed no significant geographical differences in responses to any of the environmental strains tested. It is likely that individuals living in Karonga are exposed to many additional mycobacterial species: we have evidence from environmental studies using 16S rRNA probes that *M. asiaticum*, *M. chlorophenolicum*, *M. chubbense*, *M. duvalii*, *M. elephantis*, *M. peregrinum*, *M. parafortitum*, and *M. wolinski* are present in Karonga District, along with other cultivable and noncultivable mycobacterial species that do not match current 16S rRNA databases (B. Chilima and P. Hirsch, personal communication).

Our analysis of the implications of prior sensitivity to MAIS antigens for vaccine-induced IFN-\(\gamma\) responsiveness to the *M. tuberculosis* PPD RT48 reflects a unique opportunity provided by this trial. We have used responsiveness to *M. tuberculosis* PPD RT48 as the most relevant outcome, given our evidence that BCG-induced responsiveness to this antigen is higher in the United Kingdom, where BCG is known to provide good protection against tuberculosis, than in Karonga, where we find no evidence of protection by BCG against tuberculosis [2] (G. F. Black, R. E. Weir, S. Floyd, L. Bliss, D. K. Warndorff, A. C. Crampin, B. Ngwira, L. Sichali, B. Nazareth, J. M. Blackwell, K. Branson, S. D. Chaguluka, L. Donovan, E. Jarman, E. King, P. E. M. Fine, and H. M. Dockrell, unpublished data). Because of the strong positive correlations between responsiveness to the *M. tuberculosis* and MAIS complex antigens, this analysis was restricted to individuals with initial low responsiveness to the *M. tuberculosis* PPD-RT48 antigen, in whom we compared vaccine-induced responsiveness between individuals with either low or high initial responsiveness to the MAIS antigens. The result goes in the predicted direction, with higher increases in responsiveness to *M. tuberculosis* PPD-RT48 antigen, after BCG vaccination, among those individuals who had the lowest prior sensitivity to the environmental antigen.

Although the difference fails to reach statistical significance, perhaps because the number of individuals included in this particular analysis was small (only 74, split among 4 groups, despite the large size of the overall study), its direction is consistent with the hypothesis that immune responses attributable to environmental mycobacterial exposure mask protection by BCG. Mycobacteria from the *M. avium* complex, isolated from this same Malawian population, were recently shown to sensitize mice to block the replication of BCG (L. Brandt and P. Andersen, personal communication). The data presented here thus provide strong evidence that exposure to various environmental mycobacteria is highly prevalent in an area where BCG has been found to fail to protect against pulmonary tuberculosis, and provide at least suggestive evidence that prior exposure to such mycobacteria, as reflected in IFN-\(\gamma\) responses to MAIS antigens at the time of vaccination, can influence the immune response to BCG vaccination.

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