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Elevated IgG Responses in Infants Are Associated With Reduced Prevalence of *Mycobacterium tuberculosis* Infection

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Background: It is unclear whether antibodies can prevent *Mycobacterium tuberculosis* (*Mtb*) infection. In this study, we examined the relationship between total plasma IgG levels, IgG elicited by childhood vaccines and soil-transmitted helminths, and *Mtb* infection prevalence, defined by positive QuantiFERON (QFT) test.

Methods: We studied 100 *Mtb* uninfected infants, aged 4–6 months. Ten infants (10%) converted to positive QFT test (QFT+) within 2 years of follow-up for *Mtb* infection. Antibody responses in plasma samples acquired at baseline and tuberculosis investigation were analyzed by enzyme-linked immunosorbent assay and ImmunoCAP® assay.

Results: QFT– infants displayed a significant increase in total IgG titers when re-tested, compared to IgG titers at baseline, which was not observed in QFT+ infants. Bacille Calmette-Guérin (BCG) vaccine-specific IgG2 and live-attenuated measles vaccine-specific IgG were raised in QFT– infants, and infants who acquired an *Mtb* infection did not appear to launch a BCG-specific IgG2 response. IgG titers against the endemic helminth *Ascaris lumbricoides* increased from baseline to QFT re-testing in all infants.

Conclusion: These data show raised IgG associates with a QFT-status. Importantly, this effect was also associated with a trend showing raised IgG titers to BCG and measles vaccine. Our data suggest a possible protective association between raised antibody titers and acquisition of *Mtb* infection, potentially mediated by exposure to antigens both related and unrelated to *Mtb*.

Keywords: *Mycobacterium tuberculosis* infection, antibody, enzyme-linked immunosorbent assay, Bacille Calmette-Guérin, vaccine, helminth

INTRODUCTION

Mycobacterium tuberculosis (*Mtb*) infection and tuberculosis (TB) disease represents one of the greatest global infectious disease burdens (1). The only licensed TB vaccine, Bacille Calmette-Guérin (BCG), may partially protect against *Mtb* infection and provides protection against disseminated forms of active TB disease (2–5), but protection against pulmonary TB disease varies widely with age and mycobacterial exposure (6). Development of a vaccine that is more effective than BCG is crucial for the ultimate control of both *Mtb* infection and TB disease (7, 8).

Protection conferred by most clinically efficacious vaccines against their target pathogens requires a protective antibody response (9–11). Antibody responses against *Mtb* do correlate with, and may provide protection against, TB disease (12–17). However, there is no clinical evidence to support the hypothesis that an effective TB vaccine might be based on induction of an antibody response that controls *Mtb* infection.

Our current understanding of how antibodies could protect against *Mtb* infection or TB disease is limited, when compared with our understanding of, for example, T cell-driven immunity to *Mtb*. However, classical functions of antibody such as neutralization, opsonization, and antibody-dependent cellular cytotoxicity (ADCC) have all been demonstrated to contribute to protective immunity against *Mtb*. Antibody opsonization can enhance *Mtb* uptake by (for example) macrophages (12–14), while both antibody neutralization (12, 13) and ADCC-mediated control of *Mtb* have been shown to inhibit bacterial growth (18). The intrinsic features of an antibody also contribute to their ability to control *Mtb*. For example, variation in immunoglobulin glycosylation can influence antibody-mediated control of bacterial replication (18). Different roles for antibody classes in the control of *Mtb* infection have also been demonstrated. In active TB disease, IgG has been shown to impair control and IgA enhance control of disease (19). Therefore, the full range of antibody functions can influence host immunity to *Mtb*, but whether these effects enhance or impede control of infection is likely to vary, depending on whether the host is responding to a latent infection or pathogenic disease.

Any antibody-mediated protection against *Mtb* may also be influenced by host vaccination status and/or history of coinfections unrelated to *Mtb*. Childhood vaccinations, such as live attenuated measles, have been suggested to alter *Mtb*-associated immune responses (20, 21). Additionally, in Sub-Saharan Africa, the potential effect of common endemic infections, such as helminths, on immune responses to concurrent or subsequent *Mtb* infection is an important consideration due to the substantial overlap in prevalence of these diseases (22). Clear evidence exists that helminths can alter host immunity to *Mtb* (23, 24). However, our overall understanding of the influence of bystander antigen/pathogen challenges and immunity to *Mtb* remains unclear, warranting further investigation.

Our study participants live in a region endemic for both TB and soil-transmitted helminths (STH) (25). They receive routine Expanded Programme on Immunization (EPI) vaccinations, which provides a platform to investigate the potential interplay between vaccines, helminth exposure, and *Mtb* immunity. In this study, we addressed whether plasma antibody levels associate

with risk of acquiring *Mtb* infection in infants. Additionally, we sought to identify if associations existed between exposure to both related and unrelated antigens/pathogens and altered risk of *Mtb* infection.

MATERIALS AND METHODS

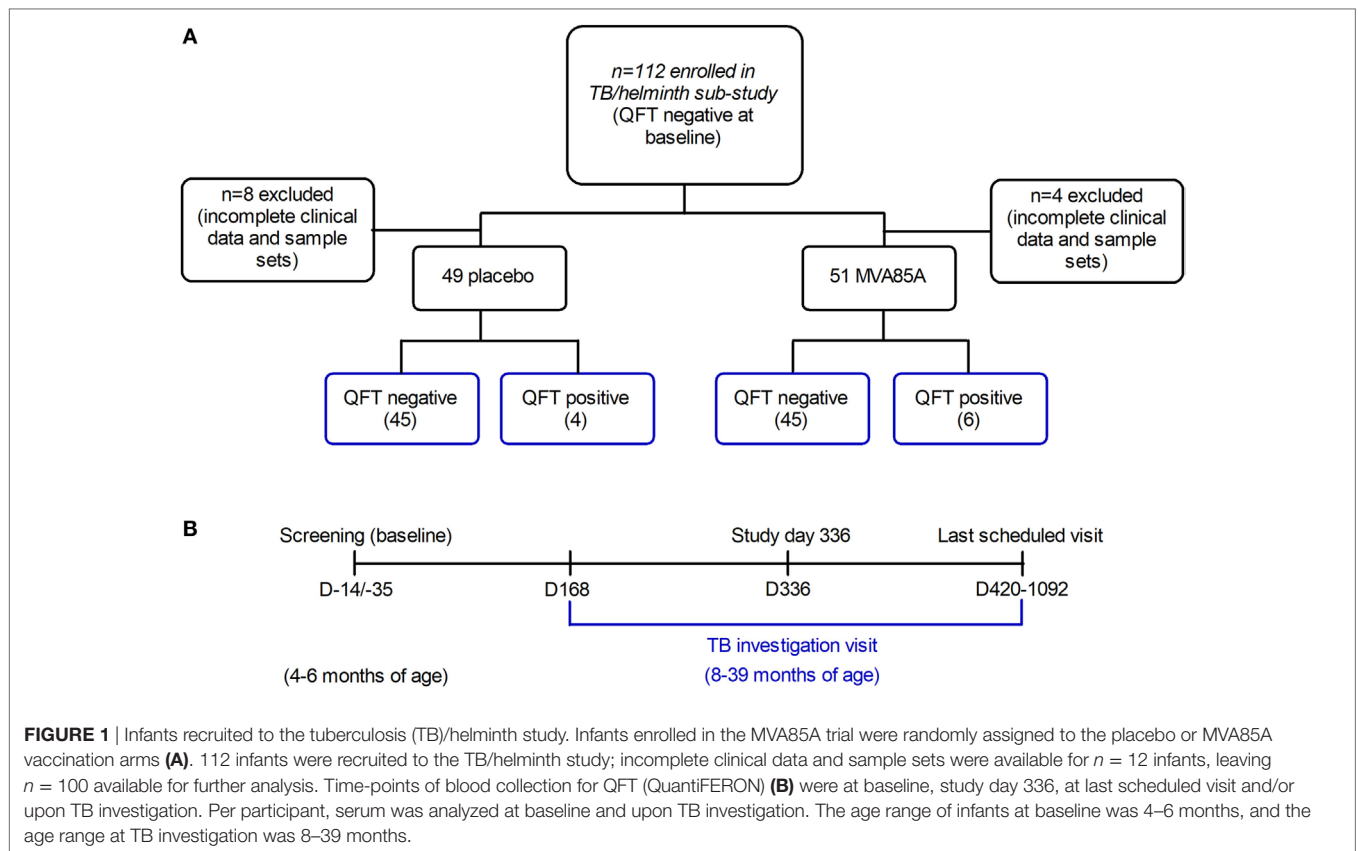
Study Participants

This is a sub-study of a double-blind, placebo-controlled efficacy trial of the MVA85A vaccine candidate, performed in the Western Cape Province of South Africa, and which has been described previously (7). Infants aged 4–6 months, who had received BCG vaccine within 7 days of birth, were enrolled. Inclusion criteria at baseline were a negative HIV enzyme-linked immunosorbent assay (ELISA) result; negative QuantiFERON-TB Gold In-tube (QFT) test (used to detect *Mtb* infection by measuring IFN γ responses to specific mycobacterial proteins); and no known exposure to an adult with active TB disease. Infants were expected to have received all routine vaccinations, per the EPI schedule. The trial showed that MVA85A boost vaccination did not provide additional protection against *Mtb* infection or TB disease, compared to BCG vaccination alone in newborns (7). Therefore, this sub-study combines infants from the placebo and MVA85A arms in the same analysis. 112 infants were sequentially recruited into the sub-study at the time of TB investigation (**Figure 1A**). However, complete clinical data and sample sets were not collected for 12 participants, thus leaving clinical data and samples from 100 participants (89.29%) available for analysis in this study (**Figure 1A**).

The sub-study and the parent trial (C-020-485) were approved by the University of Cape Town Faculty of Health Sciences Human Research Ethics Committee (032/2010 and 291/2008 respectively); and written informed consent was obtained from the infants' parents or legal guardians prior to participation.

Mtb Infection

Infants were followed up at 3-monthly intervals for symptoms consistent with TB disease, which if detected, triggered admission for standardized investigation, including QFT re-testing. Infants with symptoms of weight loss or failure to thrive would routinely receive empirical anthelmintic therapy, in addition to regular mass deworming campaigns. Information about prior anthelmintic therapy was not collected. Asymptomatic infants were admitted if an individual with TB disease became a new household contact (7). Plasma samples were analyzed at baseline and at admission for TB disease investigation (**Figure 1B**). One sample was analyzed per participant, per time-point. *Mtb* infection was defined by a single positive QFT test (i.e., interferon-gamma release assay conversion) at the time of TB investigation. Infants who were QFT positive, but did not show evidence of probable or definite TB disease were classified as *Mtb*-infected. None of the infants, irrespective of QFT status, were diagnosed with active TB disease. Infants were stratified by QFT negative (*Mtb*-uninfected), and QFT positive (*Mtb*-infected) status in the subsequent analyses; none of the infants had active TB disease.



Antigen Preparation for ELISA

Vaccine Antigens

Bacille Calmette-Guérin (Danish strain 1331, Statens Serum Institut, Denmark), live-attenuated measles (Rouvax, Sanofi Pasteur), and tetanus toxoid (Tetavax, Sanofi Pasteur) vaccines were reconstituted in 1× phosphate-buffered saline (PBS) to produce antigen stocks (500 µg/ml); aliquots of stocks were stored at -80°C until required.

Helminth Antigen

Whole *Ascaris lumbricoides* worms were treated with a 10× penicillin/streptomycin solution in 1× Amphotericin B (Thermo Fisher Scientific), following which they were washed in filter-sterilized (FS) 1× PBS. Worm sections were then homogenized in FS 1× PBS and centrifuged to remove cellular debris. The protein concentration of the soluble fraction was measured and adjusted to a stock concentration. Aliquots of the stock solution were frozen at -80°C until required.

Non-Specific and Antigen-Specific ELISA

The coating IgG antibody used for the total IgG ELISAs was used at 1:5,000 (anti-human IgG Fc-specific; Sigma-Aldrich). All vaccine and helminth coating antigens were used at 5 µg/ml for antigen-specific IgG or 10 µg/ml for antigen-specific IgG subtypes. Initial plasma sample dilutions were 1:50 (total IgG) and 1:20 (antigen-specific), followed by serial 1:5 dilutions of the initial dilutions. Secondary IgG (Southern Biotech) was used at 1:1,000, and secondary IgG subtypes (Southern Biotech)

were used at 1:500. All secondary antibodies were alkaline phosphatase-linked.

Antibody ELISAs were performed similarly to methods described previously (19, 26). Briefly, Nunc Immuno Maxisorp 96-well plates (Thermo Fisher Scientific) were coated with antibody or antigen and incubated at 37°C for 3 h, following which plates were blocked overnight at 4°C . Sample was added (50 µl/well) and plates were incubated at 4°C overnight. Secondary antibody was added (50 µl/well) after which the reactions were developed using PNP substrate (Sigma Aldrich). Reactions were stopped with 1 M NaOH and plates read on a Versamax 96-well plate reader (Molecular Devices) at 405 nm (492 nm reference filter). Arbitrary antibody responses were determined from sample titration curves (optical density vs. sample dilution) as previously described (27), enabling a relative assessment of antibody levels in serum. According to this analysis, antibody levels in certain samples and for certain antibody types fell below the detection limit and are observed at 0 on the y -axes. This does not preclude the possibility of specific antibody responses within those samples, but may require detection with a more sensitive assay.

ImmunoCAP® Test

The ImmunoCAP® *in vitro* assay was used to detect antigen-specific IgE responses in serum samples and was used according to the manufacturer's instructions. An *Ascaris suum*-derived antigen that is cross-reactive with *A. lumbricoides* was used to detect antigen-specific IgE. Responses to this antigen are described as *Ascaris* specific.

Statistical Analysis

Dot plot graphs are represented with the median and interquartile range where appropriate. Antibody responses in matched sample pairs were compared using the Wilcoxon matched-pairs signed rank test; the Mann–Whitney test was utilized for the analysis of unpaired two-group data. Grouped analyses were investigated by the Kruskal–Wallis test, with the Dunn’s multiple comparison posttest used to compare all pairs of columns. Correlations were investigated using the Spearman correlation test. Where appropriate, analyses were two-tailed. Significance was accepted at $p \leq 0.05$. Analyses of immunological data were performed using the GraphPad Prism software (v. 5.03).

RESULTS

Cohort Description

The median age of infants at TB investigation was 20 months (interquartile range 16–25.63 months). Of the 100 infants analyzed, 10 (10%) tested QFT positive upon re-testing (Table 1).

TABLE 1 | Infant sociodemographic and clinical characteristics at tuberculosis (TB) investigation.

	N = 100
Infant characteristics	
Age—months [median (interquartile range—IQR)]	20 (16–25.63)
Gender (male)	45 (45%)
Weight at admission—kg [median (IQR)]	9.34 (8.26–10.29)
TB symptoms (e.g., weight loss and failure-to-thrive) YES	60 (60%)
Positive QuantiFERON	10 (10%)

Infants Displayed an Age-Related Increase in Total IgG Titers

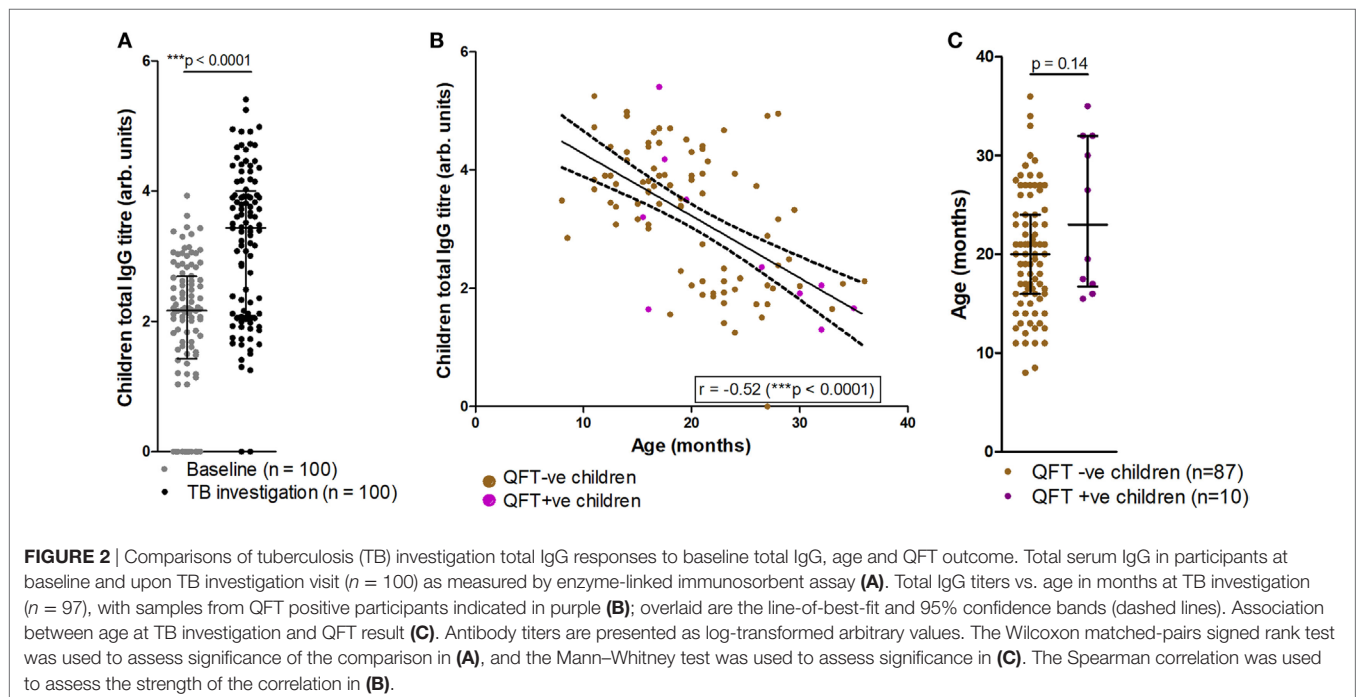
Analysis of total IgG titers revealed a significant increase in total IgG detected at the TB investigation visit, when compared to baseline (Figure 2A). At TB investigation, total IgG titers showed a clear bimodal distribution approximately around the median; further analysis revealed a significant reduction in detected total IgG in older participants (Figure 2B). No significant difference in the median age between QFT– and QFT+ participants was found (median age in months 20 vs. 23, respectively; $p = 0.14$) (Figure 2C).

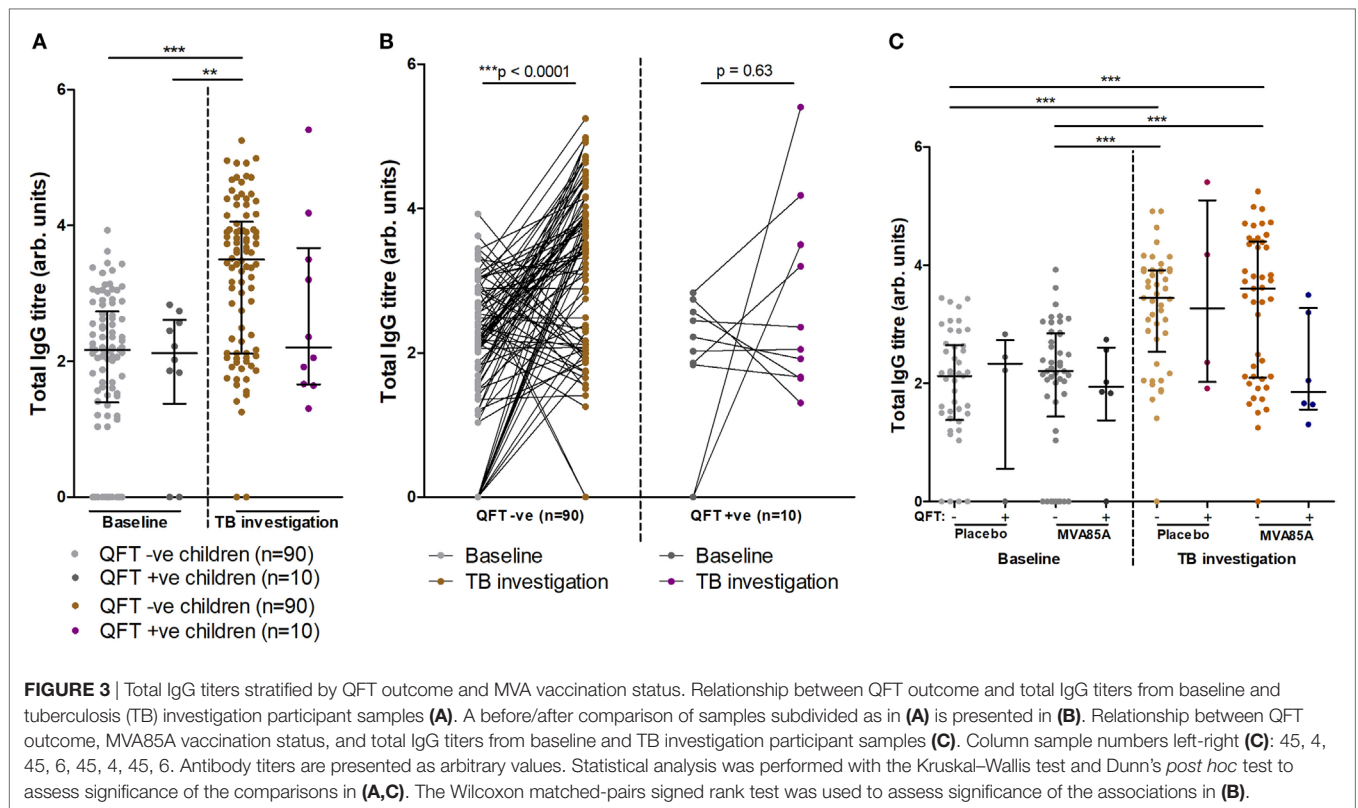
Total IgG Titers Increase Significantly in QFT Negative Infants

At baseline, infants who did or did not progress to *Mtb* infection had similar total IgG titers (Figure 3A). Analysis of total IgG responses at TB investigation revealed that infants who remained QFT– showed significant increases in total IgG titers from baseline compared to infants who became *Mtb*-infected (QFT+) (Figure 3A). However, there was no significant difference in total IgG titers between the QFT– and QFT+ groups at TB investigation (Figure 3A).

Furthermore, longitudinal analysis of infant IgG responses at baseline and at TB investigation showed significantly increased total IgG titers compared to baseline in infants who remained QFT– on the level of individual participants (Figure 3B). Infants who were QFT+ at TB investigation did not demonstrate significant increases in total IgG (Figure 3B).

The potential influence on total IgG responses of vaccination with MVA85A or placebo (Candin®) was also addressed. In agreement with data presented in Figure 3A, we found no significant differences in total IgG between placebo and MVA85A vaccinated





study participants at baseline or TB investigation. Moreover, no differences were found in IgG titers between QFT– or QFT+ placebo and MVA85A vaccinated infants (Figure 3C).

QFT– Status and Vaccine-Specific IgG titers After BCG and Measles Vaccination

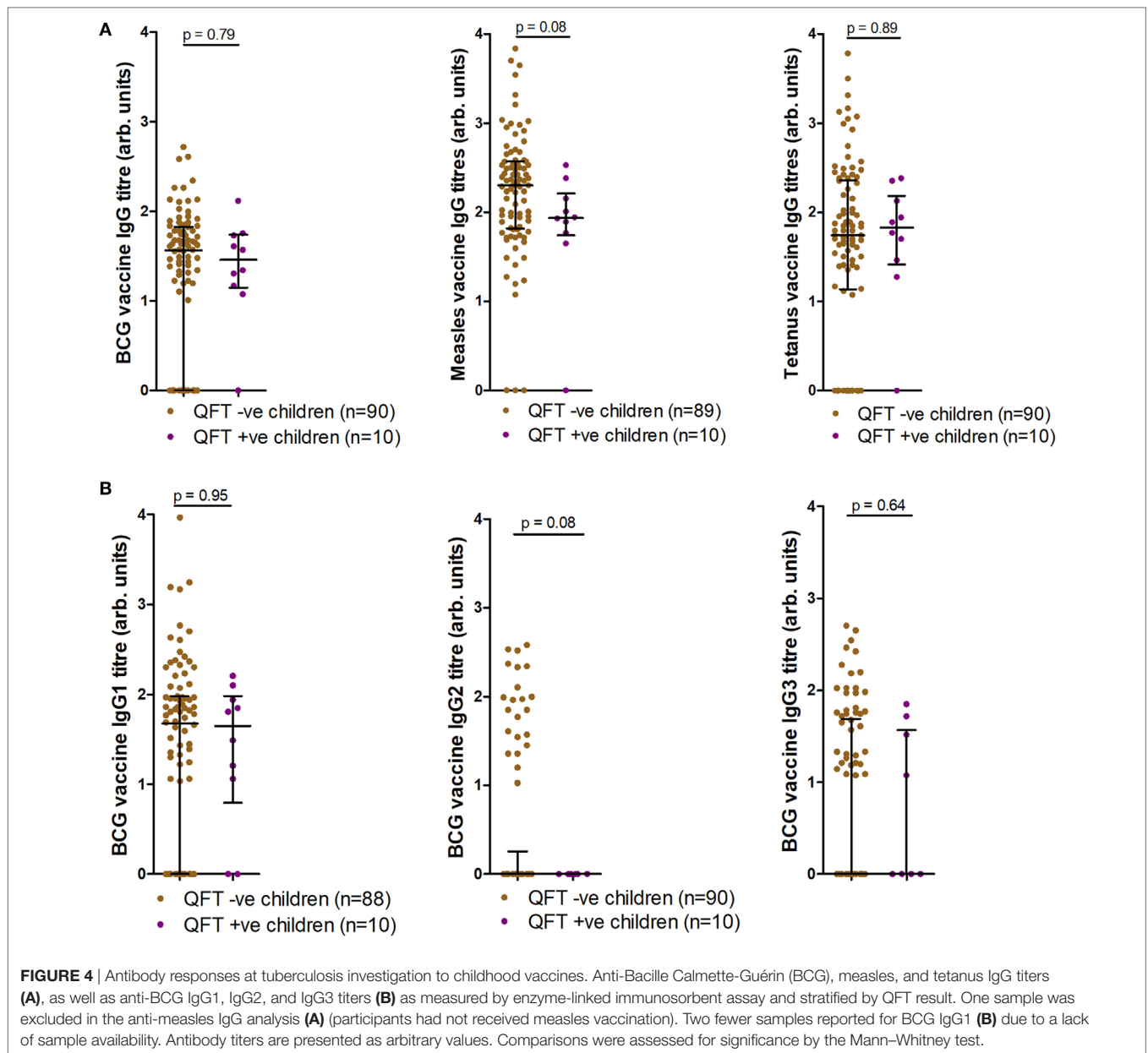
We next tested whether QFT status associated with antibody responses to BCG or to heterologous antigens. Antigen-specific antibody responses to BCG, live-attenuated measles, and tetanus toxoid vaccines were also measured at TB investigation and compared to infant *Mtb* infection and outcome. Analysis of IgG responses to BCG, measles, and tetanus toxoid revealed a trend for increased measles-specific IgG titers ($p = 0.08$) in infants who did not acquire *Mtb* infection, but no association between BCG or tetanus toxoid IgG and *Mtb* infection outcome (Figure 4A). Analysis of BCG IgG subtype-specific responses revealed a trend for increased BCG-specific IgG2 titers ($p = 0.08$) in infants who did not acquire *Mtb* infection, but no association between BCG-specific IgG1 or IgG3 and *Mtb* infection outcome (Figure 4B). This finding was related to significant inverse correlations between measles-specific IgG and quantitative QFT values, and between BCG-specific IgG2 and quantitative QFT values (Figures S1A,B in Supplementary Material). No associations were found with BCG-specific IgG/IgG1/IgG3 or tetanus-specific IgG titers and quantitative QFT values (Figures S1C–F in Supplementary Material). However, it is important to note that all participants who had *Mtb* infection but not disease had low levels of detectable IFN γ . As expected (28), tetanus toxoid-specific IgG titers

significantly correlated with age at follow-up (Figure S2F in Supplementary Material). No relationship with age was found with anti-BCG or live-attenuated measles IgG titers at follow-up (Figure S2 in Supplementary Material).

Soil-Transmitted Helminth-Specific IgG Titers Were Similar in QFT– and QFT+ Infants

Since helminth exposure can alter risk and outcome of *Mtb* disease (29–31), we examined if any relationship existed between infant exposure to *A. lumbricoides* and *Mtb* infection. Of 91 stool samples obtained, none tested positive for active helminth infection (Table S1 in Supplementary Material). However, anti-*Ascaris* IgE and IgG, indicative of prior exposure to the parasite, were found in infants (Figure 5A). At TB investigation, 2/50 (4%) infants tested for IgE responses presented with raised *Ascaris*-specific IgE, as detected by ImmunoCAP® (Figure 5A). Additionally, 85/100 (85%) infants at baseline and all infants at TB investigation had detectable anti-*A. lumbricoides* IgG, with these levels increasing significantly from baseline to TB investigation (Figure 5A). Analysis of IgG subtype responses revealed that 6/100 (6%) infants at baseline and 48/100 (48%) infants at TB investigation had detectable levels of anti-*A. lumbricoides* IgG4 (Figure 5A). Anti-*A. lumbricoides* IgG nor IgG4 titers were not significantly different between QFT– infants and QFT+ infants (Figure 5B).

Longitudinal analysis of individual infant anti-*A. lumbricoides* IgG responses at baseline and TB investigation showed significantly increased total IgG titers in both QFT– and QFT+ infants,



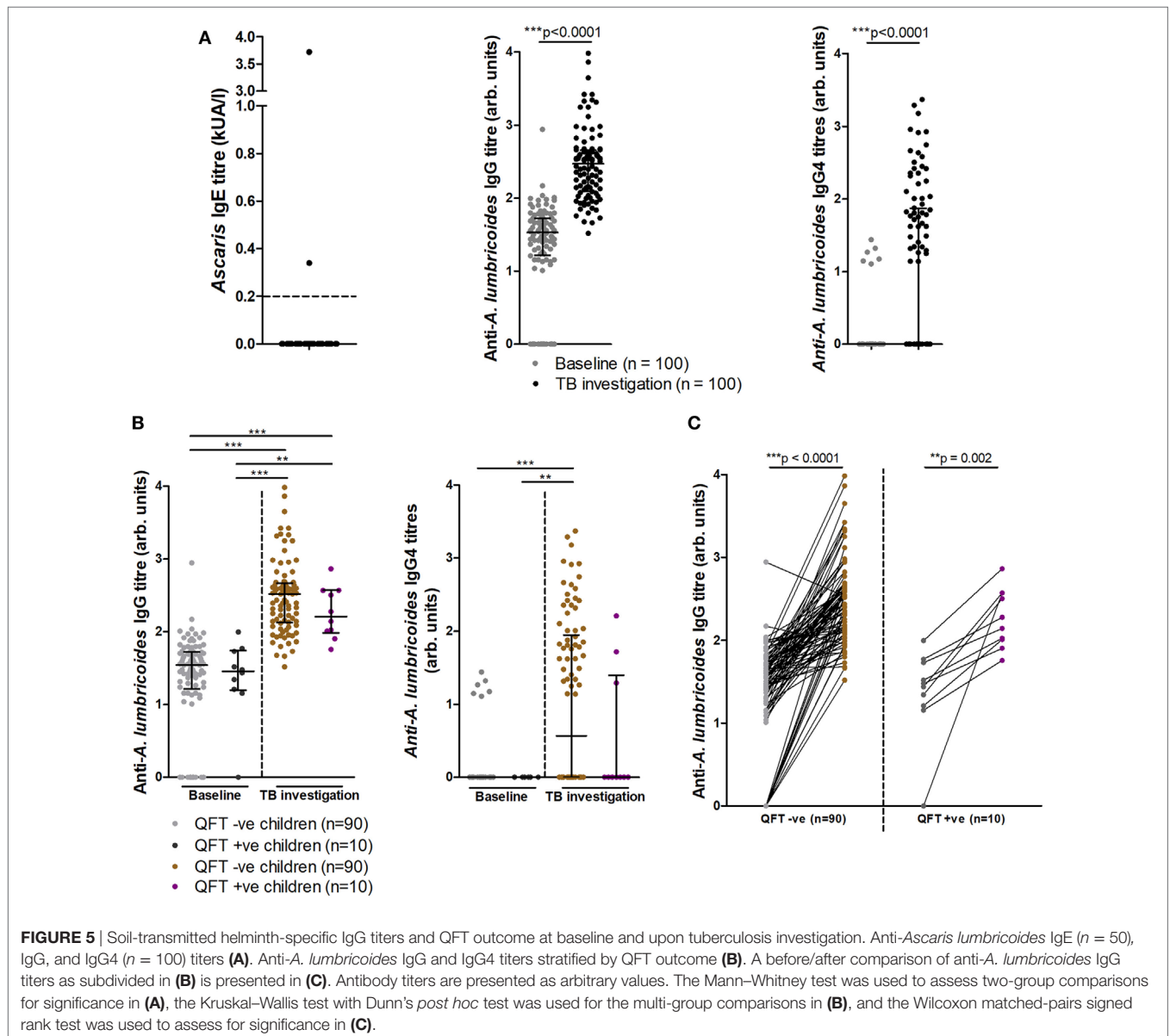
indicating an increase in anti-*A. lumbricoides* IgG responses over time (Figure 5C). Others have suggested that helminth infection negatively influences the magnitude of the QFT IFN γ response, resulting in a higher likelihood of an indeterminate result (32, 33). Although no association was found between anti-*A. lumbricoides* IgG and quantitative IFN γ in the same samples (as detected by QFT), a significant inverse correlation was found between anti-*A. lumbricoides* IgG4 and quantitative IFN γ levels (Figure S3 in Supplementary Material).

DISCUSSION

In this study, we found that infants who did not acquire *Mtb* infection had significantly increased total IgG titers from baseline

to TB investigation, unlike infants who became *Mtb* infected. This effect was related to a trend showing raised IgG titers against BCG and measles vaccination. Together, these findings suggest that raised specific and heterologous IgG titers may play a role in host responses that protect against *Mtb* infection. Therefore, induction of protective IgG responses in QFT– infants may be a feature of immune activation by both pathogen-related and unrelated antigens.

Initial findings indicate that total IgG responses increase significantly from baseline to TB investigation; however, at TB investigation, there was an inverse correlation between total IgG titers and age. It is important to note that the total IgG titers presented are from two distinct age ranges, namely 4–6 months at baseline and 8–39 months at TB investigation, and that the range of antibody titer values is wide. Additionally, older infants tend



to fall within the lower half of the bimodal sample distribution observed at TB investigation, indicating why an inverse correlation between age and antibody titer is possible despite a higher median total IgG titer at TB investigation than at baseline.

Our findings build on recent reports demonstrating that antibody responses differentially contribute to control of *Mtb* infection and disease. Studies have addressed *Mtb* antigen-specific immunoglobulin responses as contributing to a range of classical antibody-mediated responses (18, 19). In this study, we observed that only QFT– infants showed a significant increase in total IgG titers from baseline to TB investigation, suggesting an association between raised IgG and reduced risk of *Mtb* infection, highlighting the potential importance of antibodies in protective responses against *Mtb*.

It has long been known that BCG vaccination stimulates *Mycobacterium*-specific antibody production (34–36). Induction

of IgM and IgG has been observed, with IgG1, IgG2, and IgG3 as the most prevalent IgG subtypes detected (37, 38). Our identification of BCG-induced antibody responses in this study supports these previous studies. Our findings also suggest that infants who became *Mtb* infected did not launch a BCG-specific IgG2 response, which could indicate some impairment of the humoral response to BCG vaccination in these infants, which in turn may be implicated in increased susceptibility to *Mtb* infection. However, the current study lacks sufficient participant numbers and power to address this question adequately and a larger cohort study would be required to analyze the importance of this finding. Nevertheless, to the best of our knowledge, this observation is novel and warrants further investigation.

We also identified a trend toward increased measles vaccine-specific IgG titers in QFT– infants as compared to QFT+ infants. However, since this difference was not statistically

significant, the possibility that measles vaccination might provide heterologous protection against acquisition of *Mtb* infection remains speculative. Measles vaccination has previously been shown to decrease responsiveness to the tuberculin skin test in individuals with a positive reaction to purified protein derivative and/or active TB disease (20, 21). Conversely others have suggested that certain childhood vaccines, such as measles (and BCG), may provide protection against all-cause mortality (39, 40).

We acknowledge that generalizability of the abovementioned results is limited by the sample size and that other infections at the time of TB investigation may have contributed to total IgG titers. However, it is unlikely that other infections would contribute differentially to IgG titers in QFT+ vs. QFT- children, or to BCG-specific or measles vaccine-specific antibody titers.

We found no association between anti-*A. lumbricoides* IgG or IgG4 responses and *Mtb* infection, suggesting that anti-helminth responses do not underlie the differences observed with the total IgG responses in this cohort. Our current understanding of how helminth infection/exposure may influence *Mtb* infection is incomplete. Rodent studies have presented evidence of positive (23), neutral (41–43) and negative effects (30, 44) on host control of *Mtb* infection by helminths. Clinical reports of the relationship between helminth and *Mtb* infections are also not consistent, which is likely to reflect the diverse contexts of both helminth or *Mtb* infection/disease. Similar studies to ours also did not identify positive or negative associations between helminth exposure and *Mtb* infection or BCG efficacy (45–47).

We found that few infants had raised anti-*A. lumbricoides* IgE, in agreement with reports by others that anti-*Ascaris* IgE is primarily detected in older children and IgG4 is primarily detected in younger children (48). We propose that the raised levels of anti-*A. lumbricoides* IgG we observed are reflective of prior exposure to helminths. The unexpectedly low levels of current infection (25) may be attributable to appropriate household sanitation, water provision (Table S1 in Supplementary Material), and mass de-worming campaigns in the study community. Regardless, infants in this study showed evidence of immunological memory to helminth infection.

This study did not address antibody responses to *Mtb*-specific antigens due to a lack of antigen availability, and limited samples could not be used to optimize functional assays for more in-depth analysis of antibody responses. It is also important to consider the impact that small sample size may have had on the findings presented here. Despite a 2-year cumulative incidence of *Mtb* infection of 10%, the number of infants in the study who demonstrated QFT conversion was small. A larger sample size would be required to investigate these initial findings more thoroughly. Notably, antigen-specific antibody responses and their association with *Mtb* infection and/or TB disease have been investigated in a similar cohort; here, antigen 85A-specific IgG responses were associated with a difference in the occurrence of TB disease, but not *Mtb* infection (15). However, a direct comparison between studies would be inaccurate due to the difference in antigen

specificity and design of the assays, as well as the discrepancy in sample size between studies.

The data presented here suggest that both pathogen related and heterologous immune activation of the immature immune system by childhood vaccination may be implicated in reduced risk of acquiring an *Mtb* infection. The central finding that infants who did not acquire *Mtb* infection exhibited significantly increased total IgG titers from baseline to the time of TB investigation warrants further investigation of the specific components of the total IgG response responsible for this observation.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the University of Cape Town Faculty of Health Sciences Human Research Ethics Committee. The protocol was approved by the University of Cape Town Faculty of Health Sciences Human Research Ethics Committee. Written informed consent was obtained from the infants' parents or legal guardians in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

AL, WH, and MH contributed to conceptualization and design of the study; EL performed experiments and data analysis; AL was the site investigator of the helminth study and analyzed clinical and sociodemographic data; MT and MH were principal investigators of the parent trial; HM was the study data manager; CO was the study coordinator; EL, TS, WH, and MH drafted the manuscript; EL, AL, DM, AC, MT, HS, WH, and MH contributed to manuscript revision and critical analysis.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <https://www.frontiersin.org/articles/10.3389/fimmu.2018.01529/full#supplementary-material>.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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