A CD4+ TNF+ monofunctional memory T-cell response to BCG vaccination is associated with *Mycobacterium tuberculosis* infection in infants exposed to HIV

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Summary

Background The immunologic correlates of risk of *Mycobacterium tuberculosis* (Mtb) infection after BCG vaccination are unknown. The mechanism by which BCG influences the tuberculin skin test (TST) remains poorly understood. We evaluated CD4+ T-cell responses in infants exposed to HIV and uninfected (HEU) who received BCG at birth and examined their role in susceptibility to Mtb infection and influence on TST induration.

Methods HEU infants were enrolled in a randomised clinical trial of isoniazid (INH) to prevent Mtb infection in Kenya. We measured mycobacterial antigen-specific Th1 and Th17 cytokine responses at 6–10 weeks of age prior to INH randomisation and compared responses between Mtb infected and uninfected infants. Outcomes at 14 months of age included TST, QuantiFERON-Plus (QFT-Plus), and ESAT-6/CFP-10-specific non-IFN-γ cytokines measured in QFT-Plus supernatants.

Findings A monofunctional mycobacterial antigen-specific TNF+ CD4+ effector memory (CCR7-CD45RA-) T-cell response at 6–10 weeks of age was associated with Mtb infection at 14 months of age as measured by ESAT-6/CFP-10-specific IFN-γ and non-IFN-γ responses (Odds Ratio 2.26; Confidence Interval 1.27–4.15; $P = 0.006$). Mycobacterial antigen-specific polyfunctional effector memory Th1 responses at 6–10 weeks positively correlated with TST induration in infants without evidence of Mtb infection at 14 months, an association which was diminished by INH therapy.

Interpretation Induction of monofunctional TNF+ CD4+ effector memory T-cell responses may be detrimental in TB vaccine development. This study also provides mechanistic insight into the association of BCG-induced immune responses with TST induration and further evidence that TST-based diagnoses of Mtb infection in infants are imprecise.

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Research in context

Evidence before this study

In multiple independent trials and meta-analyses, BCG vaccination has been shown to prevent disseminated pediatric TB with variable efficacy in preventing pulmonary TB. We searched Pubmed without date restrictions using the terms "BCG" AND ("Tuberculosis" OR "*Mycobacterium tuberculosis*" OR "Latent tuberculosis") AND ("CD4+" OR "Cytokines" OR "Immunoprofiling") AND ("Prospective Study" OR "Cohort Study" OR "Case-Control Study") up to January 8th, 2022, without language restrictions. Of 240 results, only 3 prior prospective studies were identified that examined mycobacteria-specific immune responses following BCG vaccination and development of Mtb infection or TB disease. The first study from South Africa examined T-cell cytokine responses to BCG at 10 weeks of age following BCG vaccination at birth and followed infants for 2 years for the development of TB disease. No differences were found between T-cell cytokine responses in infants who developed TB and those who did not. A second study evaluated mycobacterial whole blood cytokine responses at 1 year of age in BCG-vaccinated Ugandan infants and development of Mtb infection measured by T-SPOT.TB at 5 years of age, with no associations found. A third trial in South African infants found that CD4+ HLA-DR expression at 6 months of age was associated with risk of developing TB disease by 2 years of age. BCG-specific IFN-γ secretion as measured by Elispot and Ag85A-specifc IgG levels were associated with reduced risk of TB disease. Taken together, there remains a paucity of evidence regarding BCG-induced immune correlates of risk and protection for Mtb infection and TB disease.

Added value of this study

Among BCG vaccinated infants who were exposed to HIV but uninfected, we found that 6–10 week mycobacterial antigen-specific monofunctional TNF+ effector memory (CCR7- CD45RA-) CD4+ responses were associated with an increased risk of Mtb infection at 14 months of age as measured by ESAT-6/CFP-10-specific cytokine responses. This study is the first to utilise a combination of TST induration, commercial IFN-γ release assay, and non-IFN-γ Mtb-specific responses to identify Mtb infection in young children. It is also the first to utilise memory characterisation of T-cell responses to BCG vaccination, a measure that proved critical to identification of a correlate of risk. Furthermore, 6–10 week polyfunctional mycobacterial antigen-specific CD4+ memory T-cell responses were associated with TST induration at 14 months of age. This association was blunted by isoniazid therapy, suggesting that BCG viability plays an underappreciated role in durability of mycobacterial T-cell responses following vaccination.

Implications of all evidence available

Development of monofunctional TNF+ memory T-cell responses to BCG vaccination at 6–10 weeks of age is detrimental to protection against Mtb infection. Previously identified markers of BCG-specific IFN-γ and CD4+ HLA-DR expression (evaluated at 6 months of age in other studies and assessed at 6–10 weeks of age in this study) were not associated with increased or decreased risk of Mtb infection in this study, and further research is needed to identify their role in Mtb infection versus progression to TB disease. Characterisation of mechanisms of induction of monofunctional TNF+ memory T-cell responses is warranted and should be considered in TB vaccine candidate trials. Furthermore, use of non-IFN-γ markers of Mtb sensitisation increases sensitivity and detection of Mtb infection in young children and improves the ability to evaluate the efficacy of BCG and future TB vaccine candidates. Finally, this study also provides mechanistic insight into the association of BCG-induced immune responses with TST induration and further evidence that TST-based diagnoses of Mtb infection in infants are imprecise.

Introduction

Mycobacterium bovis bacille Calmette-Guérin (BCG) is the only implemented vaccine for *Mycobacterium tuberculosis* (Mtb), one of the leading infectious causes of mortality globally.[1,2] In multiple randomised controlled trials (RCT), BCG vaccination has been shown to prevent severe tuberculosis (TB) disease in children.[3,4] Observational studies also suggest that BCG is associated with protection from Mtb infection.[4] A recent RCT in adolescents showed that BCG revaccination prevented sustained IFN-γ release assay (IGRA) conversion after 2 years follow-up.[5] Despite the established efficacy of BCG, no immune correlate of protection has been discovered.

Even with widespread implementation of BCG in endemic settings, TB remains a leading cause of global childhood morbidity and mortality especially among infants less than 1 year of age.[6] Use of IGRAs for TB diagnosis in children is hindered by reduced sensitivity, especially in children <2 years of age.[7] Administration of a tuberculin skin test (TST) is standard in the evaluation for pediatric TB, though interpretation of results is complicated by cross-reactivity with environmental mycobacteria as well as BCG. Whole blood cytokine responses to purified protein derivative (PPD) in BCG-vaccinated infants are associated with TST induration at 4 months of age, with a correlation identified between the IFN-γ:IL-10 ratio and induration size.[8] Prospective studies of BCG-induced immune responses and TST induration are limited, and the exact nature of the BCG immune response that leads to TST induration remains unknown.

Infants who are HIV-exposed and uninfected (HEU) have higher rates of Mtb infection and TB disease compared to infants who are HIV-unexposed and uninfected

(HUU).[9,10] Infants who are HEU demonstrate altered T-cell responses to BCG following vaccination, with increased proliferation but decreased Th1 and Th2 cytokine production compared to infants HUU.[11] Despite the known differences in T-cell function and higher rates of Mtb clinical outcomes, T-cell responses to BCG in infants who are HEU have never been assessed for associations with Mtb infection.

We sought to determine immune correlates of risk of Mtb infection following BCG vaccination in infants who are HEU. We examined mother-infant pairs who were part of the Infant TB Infection Prevention Study (iTIPS), a RCT of isoniazid (INH) therapy to prevent Mtb infection in HEU infants in western Kenya, an area of high HIV and TB burden.[2] TST, QuantiFERON-TB Gold Plus (QFT-Plus), and ESAT-6/CFP-10-specific non-IFN-γ cytokines measured in QFT-Plus supernatants, were used as measures of Mtb infection at 14 months of age. We hypothesised that BCG-vaccinated HEU infants with reduced CD4+ T-cell polyfunctionality are at increased risk of Mtb infection compared to those with higher polyfunctionality. We also hypothesised that 6–10 week BCG-induced T-cell responses are associated with TST induration at 14 months of age and diminished by INH therapy. Our findings provide evidence in the search for immune correlates of risk of Mtb infection and bring additional clarity to the poorly understood relationship between BCG vaccination and TST induration.

Methods

Study design

300 mother-infant pairs were recruited as part of the iTIPS trial, a RCT of INH to prevent Mtb infection in infants who are HEU in western Kenya. Details of trial design and procedures have been previously published including the study power calculations (ClinicalTrials.gov NCT02613169).[12,13] Eligible infants 6–10 weeks of age, born to mothers living with HIV, with birth weight >2·5 kilograms, and >37 weeks gestation, were enrolled from prevention of maternal-to-child transmission of HIV (PMTCT) clinics. Infants were provided prophylactic antiretrovirals through PMTCT clinics. Infants were randomised 1:1 upon enrollment (without stratification) to receive INH (10 mg/kg daily) or no INH for 1 year. The primary trial endpoint was Mtb infection defined as positive QFT-Plus or TST after 1 year following enrollment. Infants were ineligible for enrollment if there was a known TB exposure (including maternal TB diagnosed in past year), or they were enrolled in other TB prevention studies. Infants with HIV seroconversion at any point during the study were excluded from analysis. Infants received BCG vaccination at birth per the Kenyan MOH universal administration program and infants with lack of vaccination per maternal report were excluded from analysis. Infant and maternal PBMCs were collected at enrollment (infant age 6–10 weeks) for T-cell immune profiling using flow cytometry. In brief, CD4+ memory phenotypes and Th1 and Th17 cytokine responses to Mtb whole cell lysate, ESAT6/CFP10 peptide pools, and Ag85A/Ag85B/TB10.4 peptide pools were measured (additional assay information is provided in the Supplemental Material). QFT-Plus testing was performed at 1 year follow-up and processed at Kenya Medical Research Institute (KEMRI) where supernatants were stored for non-IFN-γ cytokine testing. Maternal HIV viral load testing was performed for the Kenyan MOH by KEMRI and abstracted from maternal PMTCT clinic charts. Maternal CD4:CD8 ratio was determined using maternal PBMCs (collected at enrollment) on a subset of mothers using flow cytometry.

Defining Mtb infection outcomes after one year follow-up

QFT-Plus results were considered positive with either TB1-Nil or TB2-Nil >0.35 IU/mL per manufacturer's recommendations. Induration of ≥10 mm at 48–96 h post administration of TST was considered positive.

Defining positivity of QFT-Plus non-IFN-γ cytokines

Non-IFN-γ cytokine responses to Mtb-specific proteins ESAT-6 and CFP-10 were measured in QFT-Plus supernatants using Luminex (multiplexed microbead assay). We measured IL-2, IP10, TNF, IL-5, and IL-15, cytokines which have shown promise in the diagnosis of Mtb infection in prior studies.[14] A test was considered positive if the following criteria were met: 1. TB1-nil >90th percentile for that cytokine and TB1-nil ≥25% of sample nil; and 2. Nil <2x average nil for cytokine (negative control). Non-IFN-γ ESAT-6/CFP-10 cytokine responses were considered negative if the patient did not meet the positivity criteria as defined above, had an appropriate negative control, and had an appropriate positive control as defined as mitogen ≥ 4·8 pg/mL (lower limit of detection of Luminex). Otherwise, a sample was considered indeterminate.

Ethics statement

Written informed consent was obtained from caregivers. Trial procedures were approved by University of Washington Institutional Review Board (STUDY00000341), University of Nairobi/Kenyatta National Hospital (P571/08/2015), Jaramogi Oginga Odinga Teaching and Referral Hospital Ethics and Research Committees, and Kenya Pharmacy and Poisons Board.

Statistical analysis

Initial flow cytometry analysis was performed using SPICE software. CD4+ T-cell functionality was further analysed using COMPASS (Combinatorial polyfunctionality analysis of antigen-specific T-cells subsets) to

generate posterior probabilities of antigen-specific responses, functionality scores, and polyfunctionality scores.[15] Statistical testing comprised of Mann-Whitney U tests, permutation tests, and logistic regression, was performed on infant 6–10 week flow cytometry measurements, maternal TB and HIV clinical variables, and Mtb infection outcomes at 14 months of age using SPICE 6·0, R version 4·0 and Stata 14·1 software. The remainder of all experimental procedures, including sample handling and preparation, Luminex assay, PBMC stimulation assay, and flow cytometry, are described in detail in the Supplemental Materials.

Role of funders
Funders had no role in study design, data collection or analysis, interpretation, manuscript writing, or the decision to publish.

Results

Cohort characteristics
To assess immune correlates of risk of Mtb infection, we examined 176 infants enrolled in the iTIPS trial (Table 1, Supplemental Table 2 and Supplemental Figure 1). Median age at enrolment was 6·3 weeks with a median birth weight of 3·4 kg. 100% of infants were breastfed, 98·3% received antiretroviral prophylaxis, and 49% of infants were randomised to INH as part of the RCT. Maternal HIV viral load was undetectable (<20 RNA copies/mL) in 74·3% of mothers. 98·8% of mothers received combination ART and 69·3% received IPT previously. At 1 year of follow-up (14 months of age), 1·1% of infants were QFT-Plus positive, and 9·7% of infants were TST positive (Table 1, Supplemental Figure 1). Given the reduced sensitivity of IGRAs in young children, we also measured non-IFN-γ cytokines (IL-2, IP10, TNF, IL-5, and IL-15) in QFT-Plus supernatants and found an additional 5·1% of infants with positive tests (Table 1, Supplemental Figure 1).[7,14] No TST positive infants were positive by QFT-Plus or non-IFN-γ cytokines, and only one QFT-Plus positive infant was also ESAT-6/CFP-10-specific non-IFN-γ cytokine positive.

	All Participants n = 176
Characteristic	**Median (IQR) or n (%)**
Infant Characteristics	
Infant age at enrollment (weeks)	6·3 (6·0 - 6·6)
Birthweight (kg)	3·4 (3·0–3·7)
Male	91 (51·7%)
Breastfed	176 (100·0%)
Received prophylactic PMTCT ARVs	173 (98·3%)
BCG vaccination scar	168 (95·5%)
Infant INH	86 (48·9%)
Maternal Characteristics	
Maternal age	27 (24–31)
Maternal ARVs	
Initiated before pregnancy	128 (72·7%)
Initiated during pregnancy	46 (26·1%)
Initiated after pregnancy	2 (1·1%)
Maternal HIV RNA (copies/ml)	0 (0–47)
HIV viral load undetectable	128 (72·3%)
HIV viral load >1000 (copies/ml)	8 (4·5%)
Maternal history of TB	17 (9·7%)
Maternal history of IPT	122 (69·3%)
Infant Mtb infection outcomes	
QFT-Plus	
Pos at 1 year	2 (1·1%)
Neg at 1 year	145 (82·4%)
TST	
Pos at 1 year	17 (9·6%)
Neg at 1 year	117 (66·5%)
ESAT-6/CFP-10-specific non-IFN-γ *	
Pos at 1 year	10 (5·7%)
Neg at 1 year	135 (76·7%)

***Table 1*: Participant characteristics.**
*Defined as TNF, IL-2, or IP10 >90th percentile. Only one infant was positive by both QFT-Plus and non-IFN-γ cytokines. No TST positive infants were positive by QFT-Plus or non-IFN-γ cytokines.
PMTCT = prevention of mother to child transmission; ARV = anti-retroviral; INH = Isoniazid; IPT = isoniazid prophylactic therapy.

BCG-vaccinated infants HEU demonstrate robust CD4+ T-cell responses to Mtb whole cell lysate at 6–10 weeks of age
To examine whether early CD4+ T-cell responses to mycobacterial antigens are associated with subsequent risk of Mtb infection, we stimulated infant 6–10 week PBMC specimens with media (containing DMSO, the peptide diluent), Mtb whole cell lysate (TBWCL), peptide pools of Mtb-specific antigens ESAT-6 and CFP-10 (ESAT6/CFP10), and peptide pools of shared BCG and Mtb antigens Antigen 85A, 85B, and TB10·4 (Ag85/TB10·4; Figure 1a and Supplemental Figure 2). CD4+ T-cell Th1 cytokine responses to TBWCL were significantly higher than DMSO controls, ESAT6/CFP10 peptides, and Ag85/TB10·4 peptides (Figure 1b, Supplementary Table 3). CD4+ T-cell IFN-γ and IL-2 responses to Ag85/TB10·4 were also significantly higher than DMSO controls. ESAT6/CFP10 responses were not higher than DMSO control for any cytokine. IL-17 responses did not differ between DMSO controls and any of the mycobacterial antigen stimulations.

Similarly, the proportional make-up of CD4+ Th1 responses were significantly different for TBWCL

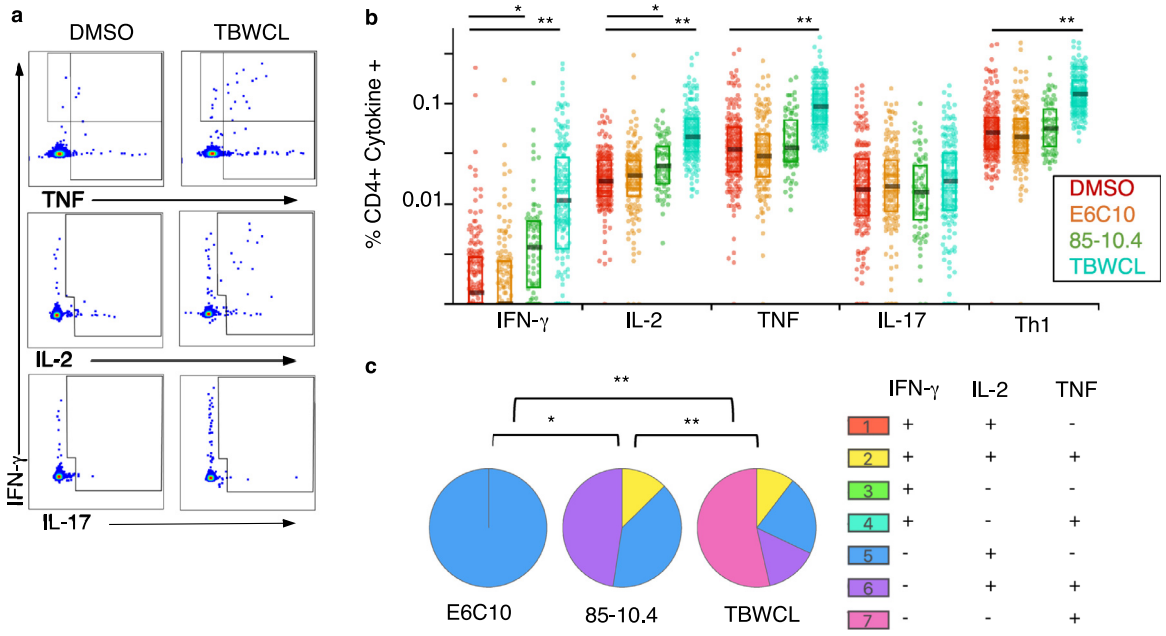

Figure 1. CD4+ T-cell responses to mycobacterial antigens in HIV-exposed BCG-vaccinated infants. PBMCs were stimulated with DMSO, ESAT-6/CFP-10 peptide pools, Ag85/TB10·4 peptide pools, or TBWCL with anti-CD28/CD49d and Brefeldin A for 6 h, then fixed and permeabilised. (a) Representative flow cytometry plots of CD4+ T-cell expression of Th1 and IL-17 cytokines following DMSO and TBWCL stimulation. (b) Percent of IFN-γ, IL-2, TNF, and IL-17 cytokine positive CD4+ T cells in response to negative control and mycobacterial antigens ESAT-6/CFP-10 (n = 145), Ag85/TB10·4 (n = 76), and TBWCL (n = 176) (the percentage of positive cells regardless of co-expression of other cytokines is shown). Th1 column represents the percentage of CD4+ cells with any IFN-γ, IL-2, or TNF response. Bars and boxes represent medians and interquartile ranges, respectively. Statistical significance determined using Mann-Whitney U test. (c) Cytokine composition of Th1 responses to each antigen (background subtracted medians are represented). Statistical significance determined using SPICE permutation testing. *P < 0·05, ** P < 0·01, ***P < 0·001. TNF = tumor necrosis factor; TBWCL = TB whole cell lysate; E6C10 = ESAT-6/CFP-10; 85-10·4 = Ag85/TB10·4.

compared to ESAT6/CFP10 and Ag85/TB10·4 (SPICE permutation test, P < 0·001 for both comparisons; Figure 1c), with a higher proportion of the TBWCL response comprised of TNF. ESAT6/CFP10 responses were composed almost completely of IL-2 responses but were not significantly higher than DMSO controls. When comparing polyfunctional cytokine profiles, Th1 responses were significantly higher in response to TBWCL than to Ag85/TB10·4 for every profile except IFN-γ+IL-2-TNF- and IFN-γ+IL-2+TNF- responses (Supplemental Figure 3).

CD4+ T-cell polyfunctional responses are driven by memory T-cell populations

To further characterise BCG-induced T-cell responses to TBWCL, we utilised COMPASS to examine memory and naïve CD4+ T-cell responses defined by expression of CCR7 and CD45RA (Figure 2a).[16] Naïve cells (CCR7+CD45RA+) demonstrated a TBWCL-specific TNF+IL-2-IFN-γ-IL-17- subset response in a majority of infants (87.5%, Figure 2b). CD4+ memory T cells including effector memory (CCR7-CD45RA-), CD45RA+ effector memory (CCR7-CD45RA+), and central memory (CCR7+CD45RA-) accounted for all polyfunctional antigen-specific subsets detected by COMPASS (Figure 2c). Accordingly, COMPASS polyfunctional scores were significantly higher amongst memory (CCR7-CD45RA-, CCR7-CD45RA+, and CCR7+CD45RA-), compared to naïve (CCR7+CD45RA+) cells (median 0·128 vs 0·019, Mann-Whitney P < 0·001; Figure 2d). When examining T-cell responses to Mtb-specific antigens ESAT6/CFP10 at 6–10 weeks of age, the only detectable response by COMPASS were naïve cells (CCR7+CD45RA+) secreting IL-2 (Supplemental Figure 4). No ESAT6/CFP10 antigen specific responses were detected amongst memory T cells. Naïve IL-2 responses did not correlate with any TBWCL cytokine responses (Supplementary Table 4). Together, these data suggest that infants have a robust memory T-cell response to TBWCL following BCG vaccination which does not appear to be related to Mtb infection.

Infant BCG-induced CD4+ T-cell IFN-γ responses are increased amongst mothers with viral load suppression in pregnancy

To better characterise the effects of maternal HIV infection and TB history on infant BCG-induced immunity,

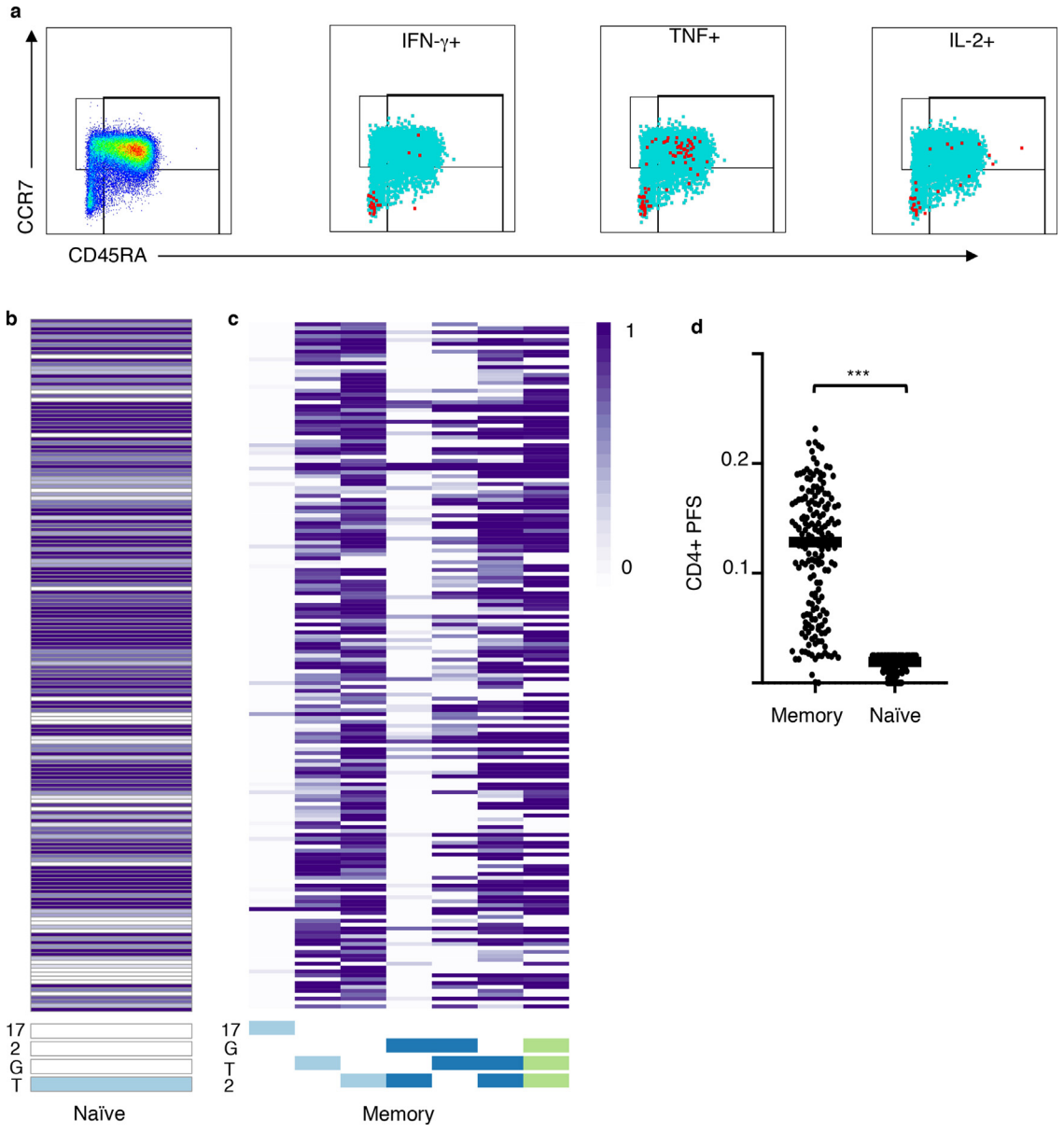

Figure 2. Polyfunctionality of memory and naïve CD4+ T-cell responses in HIV-exposed BCG-vaccinated infants. (a) Gating strategy for CCR7 and CD45RA to classify memory vs. naïve CD4+ T cells. IFN-γ, IL-2, and TNF positive cells are overlaid CCR7 and CD45RA expression to give a representation of memory cytokine responses to TBWCL. (b) COMPASS heatmap of CD4+ naïve and (c) memory responses to TBWCL. Naïve T cells defined as positive for both CCR7 and CD45RA. Memory T cells defined as negative for one or both CCR7 and CD45RA. Darkening shades of purple represent increasing posterior probability of an antigen-specific response ranging from 0 to 1. Cytokine profiles without an antigen specific response detected by COMPASS are not shown. Heatmaps are ordered vertically by participant ID (n = 176). (d) COMPASS polyfunctional scores (PFS) of CD4+ memory and naïve responses. Statistical significance determined using Mann-Whitney U test. * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$, *** $P < 0{\cdot}001$. TNF = tumor necrosis factor; T = TNF; 17 = IL-17; G = IFN-γ; 2 = IL-2; TBWCL = TB whole cell lysate; COMPASS = Combinatorial polyfunctionality analysis of antigen-specific T-cells subsets.

we examined the influence of maternal HIV viral load during pregnancy, CD4:CD8 ratio (obtained post-partum at study enrollment), prior TB disease, and prior isoniazid prophylactic therapy (IPT) on infant T-cell cytokine responses to TBWCL. Mothers were classified as having viral load suppression in pregnancy (n = 70), new HIV diagnosis in pregnancy (n = 42) or diagnosed before pregnancy but with an elevated viral load in pregnancy (>1,000 RNA copies, n = 14). Infants with mothers diagnosed with HIV during pregnancy had

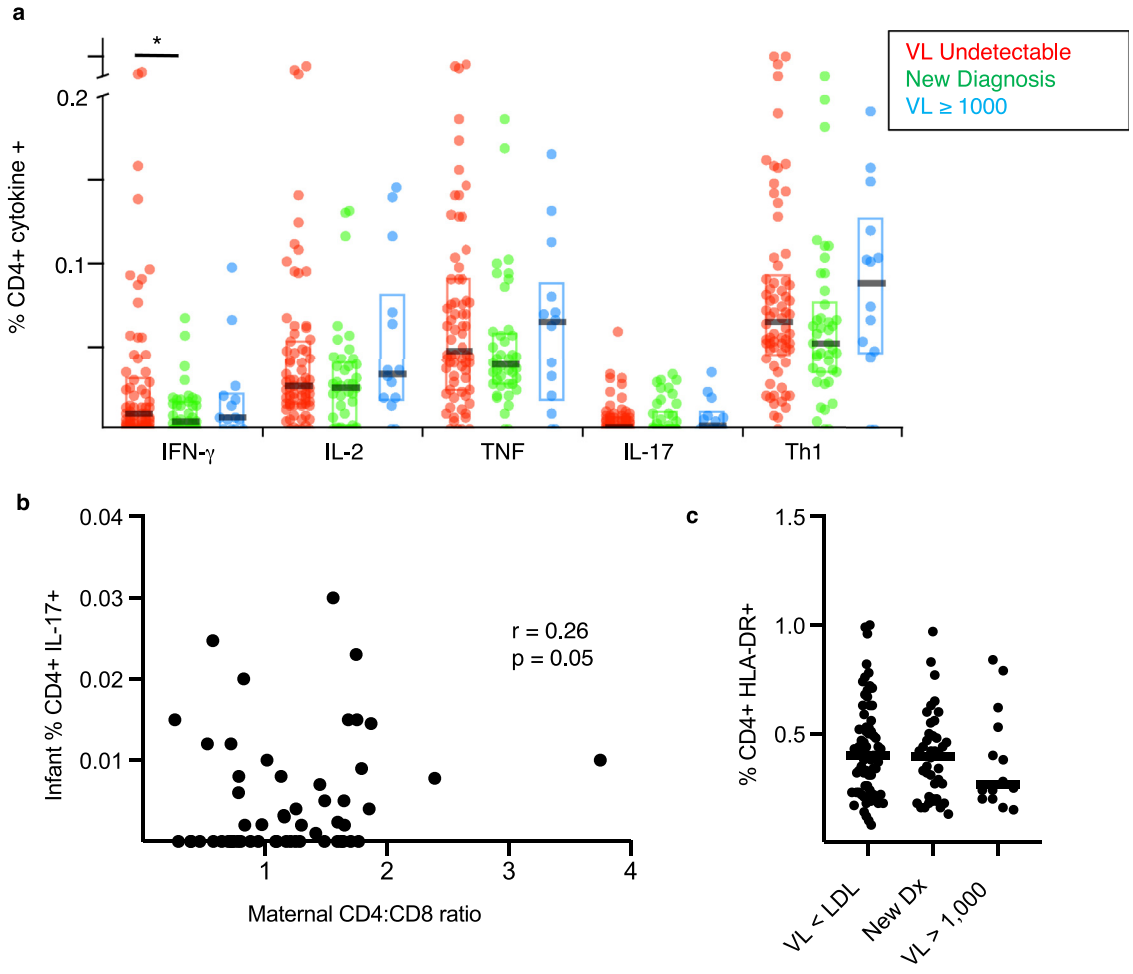

Figure 3. Association of maternal HIV disease with infant mycobacterial antigen-specific CD4+ T-cell responses. Timing of maternal HIV diagnosis, VL, and ART regimen were obtained from Kenyan Ministry of Health clinic records. CD4:CD8 ratio was determined at enrollment 6–10 weeks postpartum on a subset of mothers using flow cytometry (n = 56). (a) Percentage of background subtracted CD4+ T cells positive for IFN-γ, IL-2, and TNF in response to TBWCL compared between infants of mothers with undetectable VL in pregnancy (n = 70), newly diagnosed with HIV in pregnancy (n = 42), or mothers previously diagnosed and VL in pregnancy >1,000 RNA copies (n = 14). (b) Correlation plot of CD4:CD8 ratio and percent of background subtracted CD4+ IL-17+ responses to TBWCL. (c) Percent HLA-DR expression in DMSO control condition compared between mothers with undetectable VL, new HIV diagnosis, or mothers previously diagnosed with VL >1,000 RNA copies. Statistical significance determined using Mann-Whitney U test. * $P < 0{\cdot}05$. TNF = tumor necrosis factor; TBWCL = TB whole cell lysate; VL = viral load; LDL = lower detection limit.

significantly lower CD4+ IFN-γ+ responses to TBWCL than infants of mothers with suppressed viral loads in pregnancy (median 0·005 vs 0·010, Mann-Whitney P = 0·048; Figure 3a). IL-2, TNF, and IL-17 responses did not differ. Infants of mothers with HIV diagnosed before pregnancy but with an elevated viral load in pregnancy did not have significantly different cytokine responses compared to mothers with suppressed viral load or mothers newly diagnosed in pregnancy. Postpartum CD4:CD8 ratio had a borderline association with infant IL-17 responses to TBWCL (Spearman's r = 0·26, P = 0·050; Figure 3b, n = 56). CD4:CD8 ratio was not associated with any of the Th1 cytokines (Supplemental Figure 5). Maternal viral load (Figure 3c) and CD4:CD8 ratio (data not shown) were not associated with infant CD4+ HLA-DR expression. Lastly, maternal TB factors including self-reported history of TB disease, any prior IPT, and IPT post-partum were not associated with infant TBWCL T-cell responses (Supplemental Figure 6).

TBWCL-specific CD4+ polyfunctional Th1 responses at 6–10 weeks of age are associated with TST size at 14 months of age and negated by INH therapy

We next analysed the possible effects of BCG-induced T-cell responses on TST induration at 14 months of age. Amongst all infants with TST measurements, TBWCL-specific CD4+ COMPASS PFS at 6 weeks of age

correlated with TST size at 14 months of age (Figure 4a, Spearman's $r = 0{\cdot}18$, $P = 0{\cdot}04$). For infants who did not have Mtb infection (negative by TST, ESAT6/CFP10-specific IFN-γ and non-IFN-γ cytokines at 14 months) and who did not receive INH, TST size was most strongly correlated with the percentage of TNF and any Th1 cytokine response to TBWCL (Figure 4b, $r = 0{\cdot}45$, $P = 0{\cdot}01$; $r = 0{\cdot}42$, $P = 0{\cdot}02$, respectively). In contrast, there was no correlation among infants randomised to receive INH (Figure 4b).

To identify the specific T-cell subsets that contributed most to this association, we utilised COMPASS posterior probabilities. Two antigen-specific subsets were associated with TST induration at 14 months of age in infants without Mtb infection or INH treatment (Table 2). The CCR7- CD45RA- polyfunctional subset expressing TNF and IL-2 and the CCR7- CD45RA- polyfunctional subset expressing TNF, IL-2, and IFN-γ showed similar levels of correlation to TST induration (Spearman's $r = 0{\cdot}37$, $P = 0{\cdot}006$, q = 0·027; Spearman's $r = 0{\cdot}34$, $P = 0{\cdot}013$, q = 0·029, respectively). COMPASS PFS and FS score were also significantly associated with TST induration (Spearman's $r = 0{\cdot}35$, $P = 0{\cdot}012$, q = 0·036; Spearman's $r = 0{\cdot}37$, $P = 0{\cdot}006$, q = 0·054, respectively).

In infants who were not Mtb infected at 14 months of age, these associations were negated by INH administration and significantly transformed according to Fisher z-transformation analysis (Table 2). In total, these results show mycobacterial T-cell responses following BCG vaccination correlate with TST induration at 14 months of age, and that INH therapy starting at 6–10 weeks of age interferes with the ability to sustain an immunologic response as measured by TST at 14 months of age.

TBWCL-specific monofunctional TNF+CD4+ memory T-cell responses at 6–10 weeks of age are associated with ESAT6/CFP10-specific IFN-γ and non-IFN-γ positivity at 14 months of age but not TST positivity

To discover immune correlates of risk of Mtb infection, we compared mycobacteria-specific T-cell responses at 6–10 weeks of age between Mtb infected infants and uninfected infants at 14 months of age (Figure 5). Mtb uninfected infants were negative by TST, ESAT6/CFP10-specific IFN-γ and non-IFN-γ cytokines at 14 months of age. No CD4+ T-cell subsets were associated with TST positivity (Table 3). Furthermore, percentage of cytokine responses to TBWCL, infant CD4+ HLA-DR expression, and maternal HIV viral load were not associated with infant Mtb infection at 14 months of age (Supplementary Table 5). Amongst infants positive by ESAT6/CFP10-specific IFN-γ and non-IFN-γ cytokines at 14 months of age, a T-cell subset expressing TNF and negative for CCR7 and CD45RA at 6–10 weeks was associated with an increased risk of Mtb infection (Table 3 and Supplemental Figure 7, Odds Ratio (OR) 2·26; Confidence Interval (CI) 1·27–4·15; $P = 0{\cdot}006$; q = 0·054). This association was strongest amongst infants positive by non-IFN-γ cytokines (Supplementary Table 6, OR 2·87; CI 1·45-5·67; $P = 0{\cdot}002$; q = 0·018). Of note, COMPASS FS trended towards an association but was not significant after adjusting for multiple comparisons (OR 2·43, CI 1·09–5·39, $P = 0{\cdot}029$, $q = 0{\cdot}131$). This analysis demonstrates that a mycobacteria-specific monofunctional CCR7- CD45RA- CD4+ TNF+ response is associated with increased risk of Mtb infection, while none of the evaluated T-cell responses were associated with decreased risk.

Discussion

In this study of immune correlates of risk of Mtb infection in BCG-vaccinated infants exposed to HIV, we found that a mycobacterial-specific CD4+ T-cell effector memory monofunctional TNF+ response at 6–10 weeks of age is associated with Mtb infection as determined by ESAT6/CFP10-specific IFN-γ and non-IFN-γ cytokine responses at 14 months of age. We also found that BCG-induced CD4+ Th1 polyfunctional memory T-cell responses at 6–10 weeks of age are correlated with TST induration at 14 months of age. This correlation is negated by INH therapy starting at 6–10 weeks of age among infants without evidence of Mtb infection, demonstrating that a subset of memory T-cell responses to BCG play an important role in the initiation and persistence of TST induration in this age group. Finally, we found that BCG-induced IFN-γ responses were lower among infants whose mothers initiated ART in pregnancy compared to those who were treated pre-pregnancy and virally suppressed.

Only two prior prospective cohort studies have evaluated BCG-induced immune correlates of risk of TB disease as their primary endpoint.[17,18] In a cohort study of South African BCG-vaccinated infants followed for 2 years, T-cell cytokine profiles 10 weeks following vaccination were not associated with TB disease. The MVA85A vaccine trial also included South African BCG-vaccinated infants and found that at 4–6 months of age, CD4+ HLA-DR expression, BCG-specific IFN-γ secretion, and Ag85A-specific IgG levels were associated with TB disease risk after 2 years of follow-up. A third study of Ugandan children, which evaluated mycobacterial whole blood cytokine responses and development of T-SPOT.TB conversion, was limited in sample size and did not identify any correlates of risk.[19]

The importance of T cells in controlling Mtb infection has been established in animal and human studies.[20] IFN-γ and TNF signaling pathways have necessary roles in containing Mtb, but T-cell expression of either cytokine is not sufficient to protect against Mtb infection.[20,21] Despite the MVA85A study showing that IFN-γ responses to BCG were associated with reduced

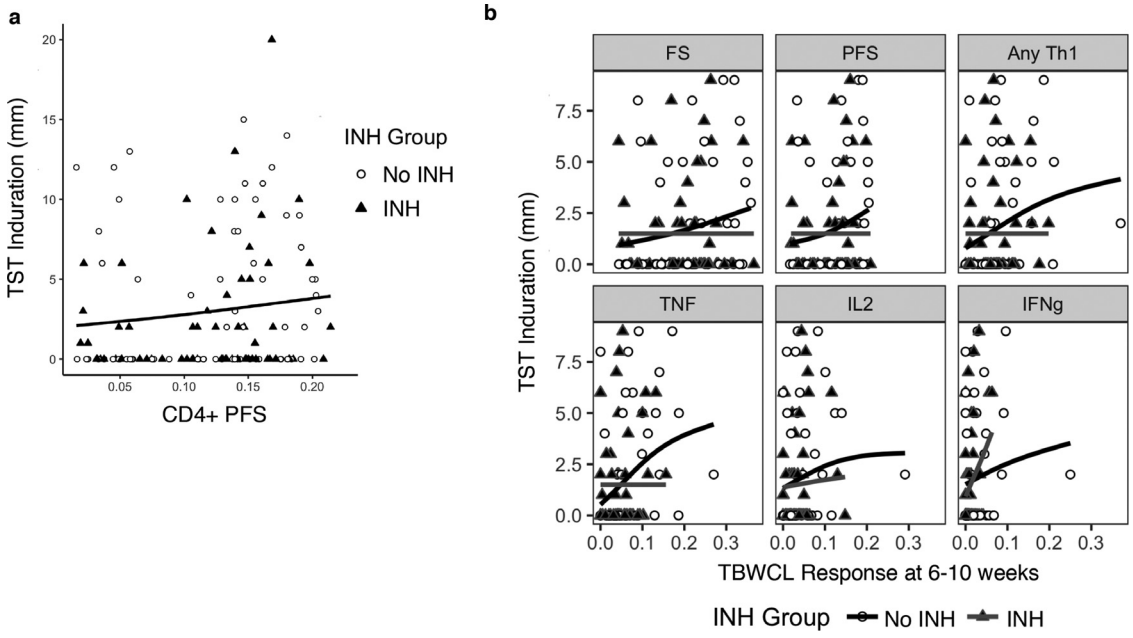

Figure 4. CD4+ Th1 responses to TBWCL at 6−10 weeks of age in BCG-vaccinated HIV-exposed infants are associated with 14 month TST induration and blunted by isoniazid therapy. Plots contain GAM smoother, which are independent of Spearman correlation coefficient. (a) Infant 6−10 week CD4+ T-cell response to TBWCL as measured by COMPASS Polyfunctionality Score (PFS) is positively correlated with 14-month TST size (Spearman r = 0·18, p = 0·04, n = 135) across all infants. (b) The positive correlation between infant 6−10 week CD4+ responses to TBWCL (background subtracted) and 14-month TST size is blunted by INH; Fisher z-transformation p-value (Spearman correlation No INH vs INH), COMPASS Functionality Score (FS) p = 0·09 (r = 0·31 vs -0·06), PFS p = 0·08 (r = 0·32 vs -0·01), any Th1 response p = 0·02 (r = 0·42 vs -0·05), TNF p = 0·01 (r = 0·45 vs -0·06), IL-2 p = 0·07 (r = 0·33 vs 0·11), IFN-γ p = 0·09 (r = 0·31 vs 0·22), n = 110. Infants with TST≥10, QFT-Plus positive, or ESAT-6/CFP-10 non-IFN-γ positive were excluded. TNF = tumor necrosis factor; TBWCL = TB whole cell lysate.

risk of TB disease and HLA-DR with increased risk, neither were associated with infant Mtb infection at 14 months of age in our study.[18] Our finding of a monofunctional TNF response amongst effector memory T cells associated with Mtb infection may indicate terminal differentiation, a functional characteristic of T cells that has been shown to confer poor replicative capacity, Mtb containment, and lung parenchymal homing in mouse models.[22,23] While polyfunctional T-cell responses have been associated with protection from

T-cell Functional Variables	No INH (n = 52)			INH (n = 57)			
	Rho	p-value	q-value	Rho	p-value	q-value	Fisher z p-value
CCR7+CD45RA+TNF+	0·25	0·069	0·124	-0·22	0·105	0·945	**0·015**
CCR7-CD45RA-TNF+	0·25	0·073	0·110	-0·21	0·115	0·518	**0·017**
CCR7-CD45RA-IL2+	0·06	0·659	0·659	-0·11	0·401	0·722	0·370
CCR7-CD45RA-TNF+IL2+	**0·37**	**0·006**	**0·027**	-0·01	0·461	0·692	**0·013**
CCR7-CD45RA-TNF+IFN-γ+	0·21	0·129	0·166	0·09	0·529	0·680	0·506
CCR7-CD45RA-IL2+IFN-γ+	0·08	0·563	0·633	0·05	0·719	0·719	0·866
CCR7-CD45RA-TNF+IL2+IFN-γ+	**0·34**	**0·013**	**0·029**	0·15	0·254	0·762	0·303
PFS	**0·35**	**0·012**	**0·036**	-0·05	0·712	0·801	**0·038**
FS	**0·37**	**0·006**	**0·054**	-0·13	0·321	0·722	**0·008**

***Table 2*: Association of BCG-induced T-cell responses with TST induration at 14 months of age in infants exposed to HIV*.**
*Spearman's Rho for TST induration in correlate analyses of the COMPASS subset specific responses and functionality/polyfunctionality score (FS/PFS) in the iTIPS trial. Spearman's Rho for each variable (COMPASS estimated subset specific probability of response) are divided between infants in the INH and no INH arms. Infants with TST≥10, ESAT6/CFP10-specific IFN-γ and non-IFN-γ positive responses are excluded. Q-values are the FDR adjusted p-values across all considered variables in the table. Variables listed are cytokine positive cell subsets in which probability of response is not zero across all participants.

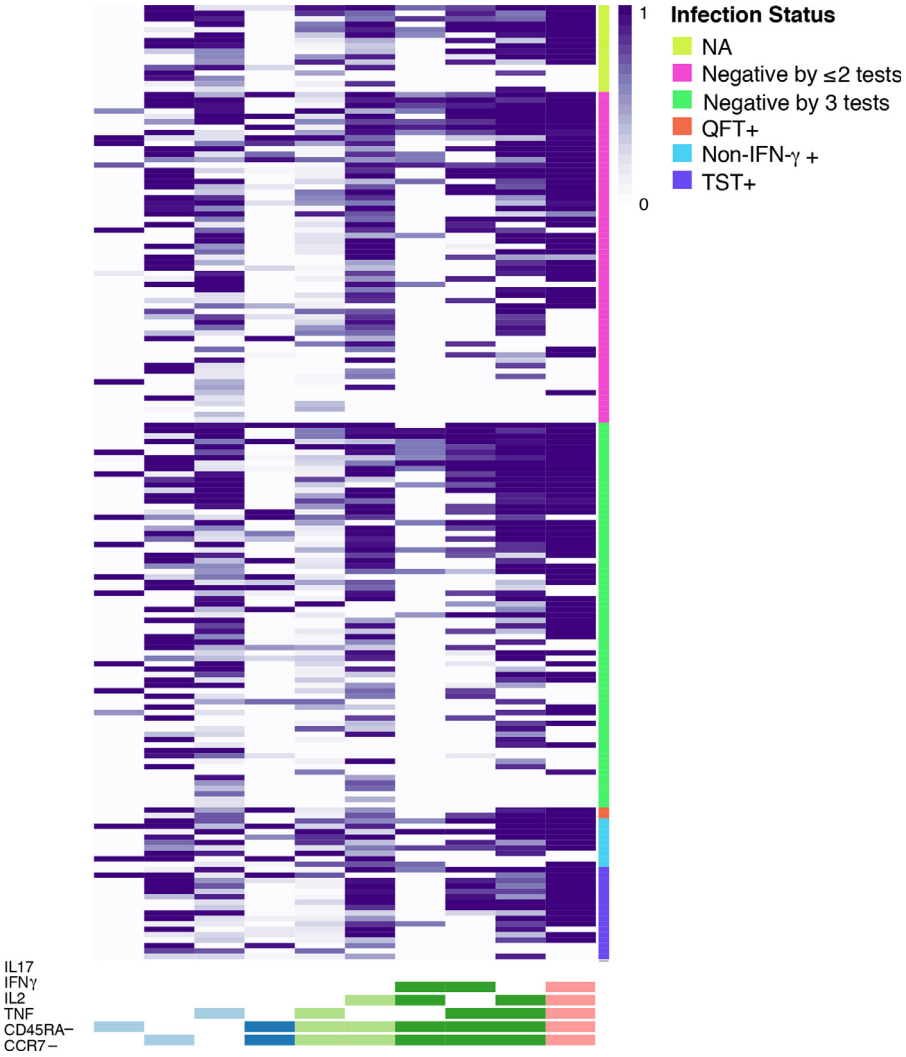

Figure 5. Association of CD4+ T-cell polyfunctional mycobacterial antigen-specific responses at 6−10 weeks in BCG-vaccinated HIV-exposed infants with Mtb infection outcomes at 14 months of age. A COMPASS heatmap of CD4+ responses to TBWCL grouped by Mtb infection status is shown (n = 176). Darkening shades of purple represent increasing posterior probability of an antigen-specific response ranging from 0 to 1. Infant Mtb infection status was classified as (1) not available (2) negative by less than 3 tests (3) negative by all 3 tests (4) QFT-Plus positive (5) positive by ESAT-6/CFP-10-specific non-IFN-γ cytokines (6) TST≥10. TNF = tumor necrosis factor; TBWCL = TB whole cell lysate.

infection in animal and human models, what constitutes a protective and durable memory T-cell response remains poorly understood.[15,24,25] Key differences exist between our data and prior studies of correlates of risk following BCG vaccination which may account for the variability in findings. Both prior prospective cohort studies utilised progression to TB disease as their endpoint, without analysing Mtb infection outcomes. Additionally, neither study examined the function of memory T cells. Lastly, our study focused on a high-risk population comprised of infants who are HEU.

Our data also demonstrates that infant TST induration at 14 months is influenced by the strength of BCG T-cell responses at 6−10 weeks of age. This association was driven by CD4+ effector memory responses expressing both TNF and IL-2. Although the impact of exposure to environmental non-tuberculosis mycobacteria (NTM) cannot be completely accounted for, the blunting effect seen with INH therapy strongly suggests that BCG remains viable and stimulates the T-cell immune response well beyond the 6−10 week time point. Ongoing exposure to NTM could also boost BCG-induced immune responses and be blunted by INH therapy, although limited knowledge exists regarding the prevalence of NTM exposure in this age group in western Kenya.[26] The viability and long-term survival of BCG in humans has not been extensively examined. Previous studies using culture and PCR detected BCG

T-cell Functional Variables	TST +			ESAT6/CFP10-specific IFN-γ and non-IFN-γ +		
	Odds Ratio (95% CI)	*p*-value	*q*-value	Odds Ratio (95% CI)	*p*-value	*q*-value
CCR7+CD45RA+TNF+	0·77 (0·43−1·35)	0·359	1	1·00 (0·53−1·91)	0·995	0·995
CCR7-CD45RA-TNF+	**0·81 (0·41−1·57)**	**0·525**	**0·945**	**2·26 (1·27−4·15)**	**0·006**	**0·054**
CCR7-CD45RA-IL2+	1·14 (0·63−2·06)	0·668	0·752	0·98 (0·48−2·00)	0·959	1
CCR7-CD45RA-TNF+IL2+	1·86 (0·68−2·07)	0·550	0·825	1·42 (0·71−2·86)	0·322	0·483
CCR7-CD45RA-TNF+IFNγ+	1·16 (0·69−1·96)	0·567	0·729	1·17 (0·63−2·19)	0·624	0·802
CCR7-CD45RA-IL2+IFN-γ+	0·72 (0·36−1·42)	0·340	1	1·39 (0·81−2·35)	0·230	0·518
CCR7-CD45RA-TNF+IL2+IFNγ+	1·28 (0·71−2·32)	0·403	1	1·52 (0·70−3·29)	0·291	0·522
PFS	1·21 (0·69−2·13)	0·512	1	2·00 (0·89−4·50)	0·094	0·282
FS	1·12 (0·64−1·94)	0·684	0·684	2·43 (1·09−5·39)	0·029	0·131

***Table 3*: Odds ratios for Mtb infection risk for BCG-induced T-cell subsets in infants exposed to HIV*.**
*Odds ratios for Mtb infection in correlate analyses of the COMPASS subset specific responses and functionality/polyfunctionality score (FS/PFS) in the iTIPS trial. Odds ratios for each variable (COMPASS estimated subset specific probability of response) are assessed for both TST positivity ($n = 17$) and ESAT-6/CFP-10-specific IFN-γ and non-IFN-γ positivity ($n = 11$; no infants were positive for both TST and ESAT-6/CFP-10 specific cytokines). Q-values are the FDR adjusted p-values across all considered variables in the table. Variables listed are all cytokine positive cell subsets in which probability of response is not zero across all participants.

in inoculation sites as long as 12 weeks post-vaccination.[27,28] A mouse model of BCG vaccination demonstrated BCG survival in lymph nodes through 16 months post-vaccination, and the quantity of viable BCG was associated with magnitude of IFN-γ secretion.[29] In addition, administration of antibiotics (INH, ethambutol, and rifampicin) reduced CD4+ T-cell polyfunctional responses. In human studies, whole blood cytokine levels (IFN-γ:IL-10 ratio) in response to PPD at 4.5 months of age were associated with TST size.[8] Size of TST induration in BCG-vaccinated children decreases with increasing age as do BCG-specific T-cell responses.[30,31] While these previous animal and human studies collectively suggest that BCG-induced T-cell responses influence TST induration, our prospective study design utilising randomised INH therapy provides the strongest evidence to date that T-cell responses and persistence of viable BCG underlie the association between BCG and TST induration in children.

When investigating the influence of maternal HIV, we found maternal viral load suppression was associated with increased infant IFN-γ expression but not other cytokines, including TNF. Studies of infants HEU have shown decreased capacity of innate cells to produce IL-12 in the first 6 weeks of life compared to infants HUU, a mechanism which may play a role in the decreased IFN-γ production evidenced in our study.[32,33] These and other immunologic effects of HIV exposure may explain differences between our findings and previous cohort studies of correlates of risk.[17,18] While HIV-exposed infants are a high-risk group, INH did not benefit this cohort of infants in preventing Mtb infection and appeared to diminish the immunologic response generated by BCG vaccination.

This study has limitations including a small number of endpoints within the parent trial, in part a reflection of the performance of IGRAs and the currently limited Mtb infection diagnostic capabilities in children.[7] However, we improved detection of Mtb infection by measuring non-IFN-γ cytokines in QFT-Plus supernatants, capturing an additional 5·1% of infants with Mtb sensitisation at 14 months of age.[14] Measurement of non-IFN-γ cytokines as part of QFT-Plus testing is a strength of this study that allows for insights into Mtb infection in young children. Another limitation of this study is that we were not able to measure BCG viability directly in participants. BCG is not easily measured in humans even when using invasive biopsies of inoculation sites.[28] The RCT study design utilising INH therapy provides the best available evidence of BCG viability in humans and its role in TST induration.

In conclusion, our study provides insights into immune correlates of risk following BCG vaccination

and brings further clarity to the mechanism by which BCG affects TST induration. Further studies are needed to determine the mechanisms underlying CD4+ TNF+ effector memory T-cell mediated immunity, identify vaccine designs capable of producing more effective T-cell memory responses, and determine the influence of BCG viability on protection from Mtb infection.

Contributors

GJ-S, JK, TRH, and SML designed the randomised clinical trial. TRH, GJ-S, SCD, and JAS designed the immunologic studies. SML, GJ-S, TRH, LMC, JK, DM, AJW, JM, and EM-O developed the clinical trial protocol. AJW, CA, and FKN performed experiments. TRH, JAS, AJW, CA, and SCD, designed and performed analysis of immune assays. All authors read, edited, and approved the manuscript. AJW and CA verified the underlying data.

Data sharing

Anonymised participant data is available upon request with publication. Study protocol and statistical analysis plan have been previously published (DOI:10.1136/bmjopen-2019-034308). Proposals for data usage may be sent to the corresponding author at alex.warr@bcm.edu.

Declaration of interests

This work was supported by the Thrasher Research Fund (to GJS & TRH), NIH/NIAID K23AI120793 to SML, NIH/NIAID K24AI137310 to TRH, NIH K12 HD000850-36 Pediatric Scientist Development Program grant to CA, NIH/Fogarty R25TW009345 to AJW, NIH/NIAID K23AI143479 to LMC, NIH UL1TR000423 for REDCap, and the University of Washington / Fred Hutch Center for AIDS Research (to SCD), an NIH-funded program under award number AI027757 which is supported by the following NIH Institutes and Centers: NIAID, NCI, NIMH, NIDA, NICHD, NHLBI, NIA, NIGMS, NIDDK. All authors declare no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2022.104023.

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