

Innate and Adaptive Immune Responses during Acute *M. tuberculosis* Infection in Adult Household Contacts in Kampala, Uganda

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Abstract. Contacts of active pulmonary tuberculosis (TB) patients are at risk for *Mycobacterium tuberculosis* (MTB) infection. Because most infections are controlled, studies during MTB infection provide insight into protective immunity. We compared immune responses of adult household contacts that did and did not convert the tuberculin skin test (TST). Innate and adaptive immune responses were measured by whole blood assay. Responses of TST converters (TSTC) were compared with persistently TST negative contacts (PTST⁻) and contacts who were TST⁺ at baseline (TST⁺). TLR-2, TLR-4, and IFN- γ R responses to IFN- γ did not differ between the groups, nor did $\gamma\delta$ T cell responses. T cell responses to MTB antigens differed markedly among TSTC, PTST⁻, and TST⁺ contacts. Thus, no differences in innate responses were found among the three household contact groups. However, adaptive T cell responses to MTB antigens did differ before and during MTB infection among PTST⁻, TSTC, and TST⁺ contacts.

INTRODUCTION

Little is known about immune changes in humans during acute *Mycobacterium tuberculosis* (MTB) infection. Because most healthy persons successfully control MTB infection, analysis of innate and adaptive immune responses during acute infection provides insight and may identify markers of protective immunity.

Most immune studies compare cross-sectional responses of TB patients with those with or without latent MTB infection (LTBI). These studies assume a steady state of immune responses in persons with LTBI. Persons with LTBI are a heterogeneous group because timing of their MTB exposure is unknown. Identifying persons before and during acute MTB infection requires a prospective study. Investigation of household contacts (HHC) of persons with active pulmonary TB identifies persons at different stages of MTB infection and disease. Longitudinal follow-up can differentiate those with acute MTB infection, from those exposed but uninfected, and those already infected. Serial measurements of immune responses in persons undergoing acute MTB infection compared with those who do not become infected and those already infected can provide insight into immune responses required to control MTB.

Immunity to MTB consists of innate and adaptive responses. Innate responses are mediated by macrophages and dendritic cells that use a variety of mechanisms, including toll-like receptors (TLR), complement receptors, and mannose receptors to recognize MTB.^{1–3} Adaptive immune responses to MTB are mediated by CD4⁺ and CD8⁺ T cells. Non-classical T cells, such as CD1 restricted and V δ 2⁺ T cells ($\gamma\delta$ T cells), recognize MTB and bridge innate and adaptive responses.^{4–7} These different immune responses are measurable with whole blood assays using specific ligands to elicit

cytokine production. Overnight tumor necrosis factor α (TNF- α) production in response to ligands for TLR2, TLR4, and interferon gamma receptor (IFN- γ R) measures innate responses. The $\gamma\delta$ T cell function can be measured as IFN- γ response to bromohydrin pyrophosphate (BrHPP).⁸ The IFN- γ production in response to whole MTB bacilli, MTB culture filtrate (CF), and 30 kDa 85B antigen (Ag 85B) serves as a marker for T cell responses.

This study was part of a longstanding TB household contact studies in Kampala, Uganda.^{9,10} In this urban hyperendemic environment, 20–25% of contacts of pulmonary TB patients are tuberculin skin test negative (TST⁻) at baseline, with a substantial proportion converting their TST within 3–6 months. A small proportion of these contacts remain TST⁻ upon repeat testing over 12–24 months. These persistent TST⁻ persons (PTST⁻) provide a natural control group for TST converters (TSTC) and contacts already TST⁺ at baseline (TST⁺). This study sought to characterize innate and adaptive immune responses of adult TSTC in comparison to PTST⁻ and TST⁺ contacts of pulmonary TB patients.

SUBJECTS, MATERIALS, AND METHODS

Study area and population. Between April 2002 and December 2006, 1,508 persons with either active pulmonary TB or their HHC were enrolled in a prospective cohort study of MTB infection and disease in households in the Kawempe Division of Kampala, Uganda (Figure 1). The HHC were followed for 12–24 months through December 2008. The study was patterned after an earlier TB transmission study in Kampala.¹⁰ In brief, adults with pulmonary TB, living in Kawempe, with one or more HHC were recruited. The diagnosis of TB was based on clinical findings, a positive chest x-ray, and positive sputum culture. The HHC were defined as individuals who had resided in the household of the TB index case for at least 7 consecutive days during the previous 3 months. Active TB was treated with standard short-course therapy and LTBI was treated with isoniazid for 9 months in young children, human immunodeficiency virus (HIV)-infected

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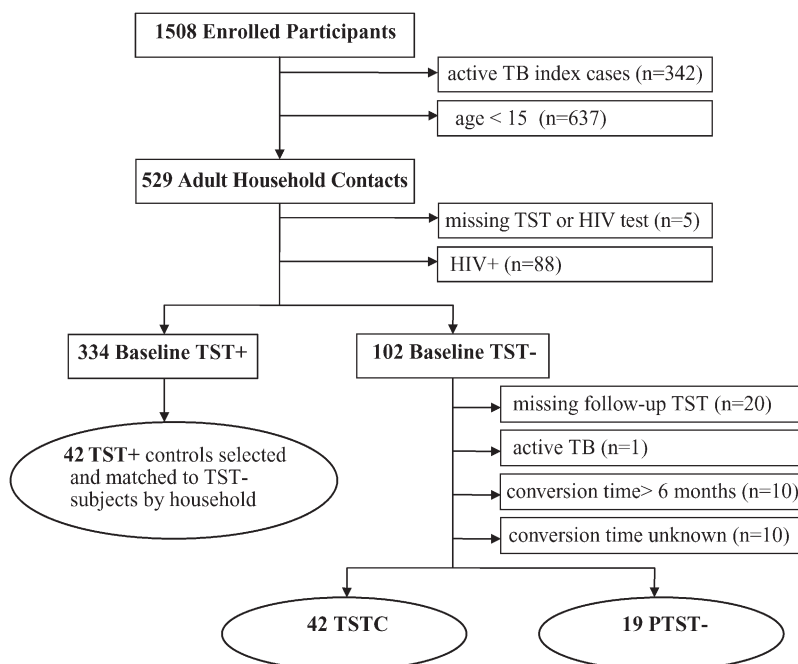


FIGURE 1. Distribution of age, TST, and HIV status of TB index cases and contacts enrolled in a TB household contact study in Kampala, Uganda between 2002–2006. HIV = human immunodeficiency virus; TST = tuberculin skin test; TSTC = tuberculin skin test converter at 3 months; PTST- = persistently TST negative at 12 and 24 months.

contacts, and TST+ HHC. The study protocol was reviewed and approved by the AIDS Scientific Committee of Makerere University, The Uganda National Council on Science and Technology, and institutional review board at University Hospitals Case Medical Center, Cleveland, OH. Written informed consent was obtained from the head of household, all adults, and parents/guardians of children in the household.

Clinical measurements. The HHC were evaluated at baseline, within 3 weeks of the diagnosis of the index case, with standardized questionnaires about TB risk, a limited physical examination that included ascertainment of bacilli Calmette-Guérin (BCG) scar, chest x-ray, HIV-1 serology, and TST. Blood for immune studies was drawn before TST was administered using the Mantoux method (0.1 mL of five tuberculin units of purified protein derivative, Tubersol; Cannaught Laboratories Limited, Willowdale, Ontario, Canada). The TST was administered on the volar aspect of the left forearm and read after 48–72 hours as the diameter (mm) of palpable induration. The same team of home health visitors did skin testing and reading; the intra-reader and inter-reader reliability was high (Pearson's correlation coefficients = 0.00 and 0.97, respectively, $P < 0.001$). The BCG vaccination was ascertained through physical examination and review of medical records. The extent of disease on posteroanterior chest radiographs was graded on a four-category ordinal scale.¹¹

For this study, HHC aged 15 and older, confirmed HIV-, and with negative baseline TST (TST-) reactions, defined as < 10 mm, were recruited for follow-up blood draws at 1, 3, 6, 12, and 24 months; TST was performed at 3, 6, 12, and 24 months if TST conversion had not occurred at earlier time points. The TST conversion for HHC was defined as (1) baseline TST induration < 10 mm, (2) TST reaction of ≥ 10 mm upon subsequent testing, and (3) an increase in TST size of ≥ 6 mm upon retesting. Age-matched controls were recruited from

HIV- HHC with a positive TST (TST+) at baseline and when possible from the same household.

Reagents. The phytohemagglutinin (PHA) and lipopolysaccharide (LPS) were purchased from Sigma (St. Louis, MO). The 19 kDa LpqH protein (Rv3673; 19 kDa) was purified from MTB H37Ra in Dr. Boom's laboratory as described.¹² The MTB culture filtrate proteins (CF) and 30 kDa 85B antigen (Ag85B) were obtained from John Belisle, Colorado State University (Fort Collins, CO) through the Tuberculosis Vaccine Testing and Research Materials Contract (NIAID, HHSN266200400091C). The MTB H37Ra bacilli were cultured in Dr. Boom's laboratory, colony forming units (CFU) determined, and stock aliquots stored at -80°C . The BrHPP was a gift from Innate Pharma (Marseille, France). Recombinant interleukin 2 (IL-2) and IFN- γ were purchased from R&D Systems (Minneapolis, MN) and Endogen (Woburn, MA). The IFN- γ and TNF- α ELISA kits were from Endogen.

Whole blood assay. Whole blood (10 mL) was collected in heparinized vacutainer tubes (Beckton Dickinson, Franklin Lakes, NJ) and diluted 1:10 with RPMI-1640 supplemented with 20 mM HEPES (Lonza, Walkersville, MD), glutamine (200 mM), penicillin (10,000 U/mL), and streptomycin (10 $\mu\text{g}/\text{mL}$). Diluted blood (1 mL/well) was dispensed into 24-well tissue culture plates (Corning Inc., Corning, NY).

For innate immune responses, whole blood was stimulated in separate wells with CF (3, 10 $\mu\text{g}/\text{mL}$) alone, or 19 kDa LpqH (0.25 $\mu\text{g}/\text{mL}$), or LPS (0.3 and 1 $\mu\text{g}/\text{mL}$) with and without 2-hour pretreatment with IFN- γ (3,000 U/mL). The PHA (5 $\mu\text{g}/\text{mL}$) served as the positive control. Whole blood was incubated for 18 hours at 37°C in 5% CO_2 . Supernatants were harvested and stored at -80°C until batch testing for TNF- α .

For T cell responses, whole blood was stimulated in separate wells with MTB CF (3, 10 $\mu\text{g}/\text{mL}$), MTB H37Ra (10^6 and 10^7 CFU/mL), Ag85B (10 $\mu\text{g}/\text{mL}$), and PHA (5 $\mu\text{g}/\text{mL}$). For

$\gamma\delta$ T cell responses, whole blood was stimulated with BrHPP (1 μ M and 250 μ M) and IL-2 (25 U/mL).⁸ Whole blood was cultured for 7 days at 37°C in 5% CO₂. Supernatants were harvested and stored at -80°C until batch testing for IFN- γ .

Cytokine measurement. Supernatant cytokine concentrations in pg/mL were measured using commercial enzyme-linked immunosorbent assays (ELISAs) for IFN- γ and TNF- α . Sensitivity of IFN- γ ELISA was 2 pg/mL with a range of 25.6–1,000 pg/mL and TNF- α ELISA was 2 pg/ml with a range of 15.6–1,000 pg/mL. Final results reflect stimulated values minus unstimulated controls expressed as cytokine in pg/mL per mL of whole blood, i.e., 10 \times the concentration measured by ELISA to correct for initial whole blood dilution.

Statistical analysis. Baseline data differences between group means or medians for demographic and exposure variables were tested using an appropriate parametric (analysis of variance) or the non-parametric (Kruskal-Wallis) method. The χ^2 or Fisher's exact test were used to test for differences in proportions. Baseline differences in cytokine responses between groups were assessed using the non-parametric Wilcoxon rank-sum test.

In longitudinal analyses, changes in cytokine response levels during follow-up within and between groups were assessed using log₁₀ transformed values. A linear mixed modeling approach was used to adjust for the correlation (unstructured) among repeated measures within subjects over time. Statistical analyses were done using SAS version 9.1 (SAS Institute, Inc, Cary, NC) and GraphPad Prism version 5.02, San Diego, CA.

RESULTS

Rates of TST conversion and persistence of a negative TST among contacts. Among 440 HIV uninfected adult HHC (15 years of age and older), 334 (76%) had a positive TST and 102 (23%) a negative TST when households were first

evaluated (Figure 1). The 102 TST- participants were followed for a minimum of 1 year and 42 (41%) converted the TST (TSTC) from negative to positive by 3 months. Ten contacts converted by 6 months or later, and for 10 persons timing of TST conversion was not determined because of missed visits. Nineteen contacts (19%) remained TST- for at least 12 months and were considered persistent TST- (PTST-). Twenty of 102 contacts lacked serial TST follow-up and one contact developed active TB. No significant baseline demographic or clinical differences were found between contacts that completed follow-up and those with insufficient follow-up (data not shown). Rates of negative TST at baseline and TST conversion were similar to those observed during an earlier (1995–99) TB household contact study conducted in the same urban area.¹⁰

Because most TST conversions occurred by 3 months, we focused on them for epidemiologic and immunologic comparisons with PTST- and TST+ contacts. Demographic characteristics, extent of disease, and degree of exposure to the index case were compared for 3 months; TSTC ($N = 42$), PTST- ($N = 19$), and contacts who were already TST positive (TST+) at baseline ($N = 42$) (Table 1). Baseline TST in mm was significantly different between TST+ (16.8 \pm 3.7 mm) and contacts with negative TST at baseline, but did not differ between TSTC and PTST- (2.8 \pm 3.7 mm versus 0.6 \pm 2.1 mm). The TST+, TSTC, and PTST- were similar in terms of demographic measures and index case disease severity. The TSTC tended to have a closer relationship to index cases, but no factor was significantly different.

Innate immune responses of PTST-, TSTC, and TST+ contacts. Innate immune responses were compared between PTST-, TSTC, and TST+ contacts at baseline and during follow-up. The hypothesis was that PTST- persons would have enhanced innate responses compared with TSTC and TST+ contacts. Overnight TNF- α production by whole blood stimulated by both broad and specific innate receptor ligands were used to probe innate responses. Whole MTB bacilli are a

TABLE 1

Demographic and *Mycobacterium tuberculosis* exposure characteristics of home household contacts (HHC) who were TST+ at baseline (TST+), remained persistently TST- (PTST-), or TST converted by 3 months (TSTC)

	TSTC N = 42	PTST- N = 19	TST+ N = 42	P value
Demographic factors				
Age in years, mean (SD)	26 (14)	28 (16)	28 (8)	0.64
Male gender, n (%)	11 (26)	6 (32)	16 (38)	0.50
Baseline TST size in mm, mean (SD)	2.8 (3.7)	0.6 (2.1)	16.8 (3.7)	< 0.0001†
Presence of BCG scar, n (%)	24 (57)	7 (37)	24 (57)	0.47
Index case factors				
AFB smear grade \geq 1+, n (%)	39 (92)	16 (84)	40 (95)	0.30
Extent of disease on chest radiograph, n (%)				
Normal/minimal	7 (17)	4 (22)	11 (26)	0.61
Moderately advanced	17 (41)	5 (28)	11 (26)	
Far advanced	17 (41)	9 (50)	20 (48)	
Cough duration in days, median [range]	84 [14–730]	90 [21–210]	90 [21–690]	0.42
Household exposure factors				
Biological relationship to index, n (%)				
Close (sibling, parent, child)	19 (45)	11 (58)	17 (40)	0.35
Distant (cousin, avuncular)	10 (24)	4 (21)	6 (14)	
Unrelated (spouse or other)	13 (31)	4 (21)	19 (45)	
Share bed with index, n (%)	7 (17)	2 (10)	11 (26)	0.30
Care for index, n (%)	35 (83)	12 (63)	31 (74)	0.22
Share meals with index, n (%)	38 (90)	16 (84)	40 (97)	0.18
With index > 7–12 hours/day, n (%)	36 (86)	14 (74)	36 (88)	0.25
With index > 3 days/week, n (%)	40 (95)	15 (79)	37 (90)	0.14

*TST+ = tuberculin skin test positive; PTST- = persistently TST negative; TSTC = TST converters; BCG = bacille Calmette-Guérin; AFB = acid-fast bacilli.

†Significant pairwise comparisons TST+ > TSTC and TST+ > PTST-.

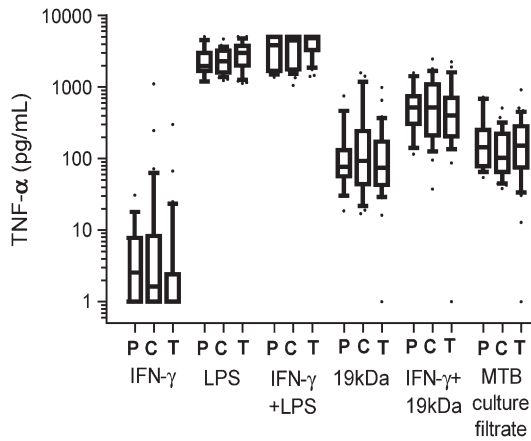


FIGURE 2. TNF- α responses of PTST- (n = 19), TSTC (n = 42) and TST+ (n = 42) contacts at baseline in response to innate receptor ligands. Whole blood was stimulated overnight with either IFN- γ , LPS, IFN- γ pretreatment followed by LPS (IFN- γ + LPS), 19 kDa LpQh (19 kDa), IFN- γ pretreatment followed by 19 kDa LpQh (IFN- γ + 19 kDa) or MTB CF. Supernatants were harvested after 18 h and TNF- α measured by ELISA. Results presented are the mean \pm SD of single measurements for each group of contacts. P are PTST- (N = 19), C are TSTC (N = 42) and T are TST+ (N = 42). Box and whisker plots with the whiskers defining the 90th and 10th percentiles. *There were no statistically significant differences among the 3 groups ($P > 0.05$).

particulate stimulus expressing not only a wide range of ligands for TLR-2, TLR-9, and NODs, but they also stimulate phagocytic processes and NK cells. The CF, enriched for MTB proteins, also contains cell wall glycolipids that stimulate TLRs¹³; the 19 kDa LpQh and LPS are specific ligands for TLR-2 and TLR-4, respectively.^{1,2} The IFN- γ R responsiveness was determined by pre-treatment with IFN- γ for 2 hours before stimulating with LPS or 19 kDa LpQh.¹⁴

As shown in Figure 2, although there were no significant differences between the three groups, there was a trend toward increased TNF- α production to LPS for TST+ compared with PTST-. The TNF- α responses to 19-kDa LpQh and LPS pretreated with INF- γ resulted in boosted cytokine

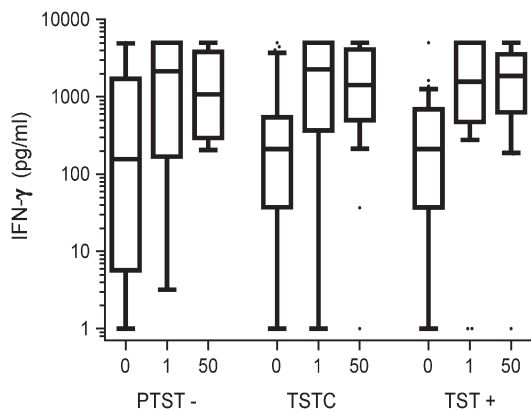


FIGURE 3. V δ 2 T cell responses of PTST-, TSTC and TST+ contacts at baseline in response to BrHPP and IL-2. Whole blood was stimulated with IL-2 (25 U/mL) with and without BrHPP (1 and 50 μ M). Supernatants were harvested after 7 days and IFN- γ measured by ELISA. Results presented are the mean \pm SD of single measurements for each group of contacts. 0 = IL2 (25 units/mL) alone; 1 = IL2 + 1 μ M BrHPP; 50 = IL2 + 50 μ M BrHPP. Box and whisker plots with whiskers defining the 90th and 10th percentiles. *There were no statistically significant differences between the 3 groups ($P > 0.05$).

production that was similar for all three HHC groups. Adjusted analysis comparing TNF- α responses longitudinally at 3, 6, 12, and 24 months did not reveal differences among the three groups of contacts at any time point.

V δ 2+ $\gamma\delta$ T cell responses of PTST-, TSTC, and TST+ contacts. The V δ 2 T cells respond readily to MTB bacilli and their TCR recognizes small mycobacterial phosphoantigens. The MTB-specific V δ 2 T cell responses can be elicited by synthetic phosphoantigens such as BrHPP.¹⁵ We measured V δ 2 T cell responses in response to 7-day stimulation of whole blood with three different concentrations of BrHPP plus IL-2. There was a trend toward increased IFN- γ production by V δ 2 T cells in TSTC but differences did not reach statistical significance (Figure 3). Adjusted analysis was also performed comparing IFN- γ

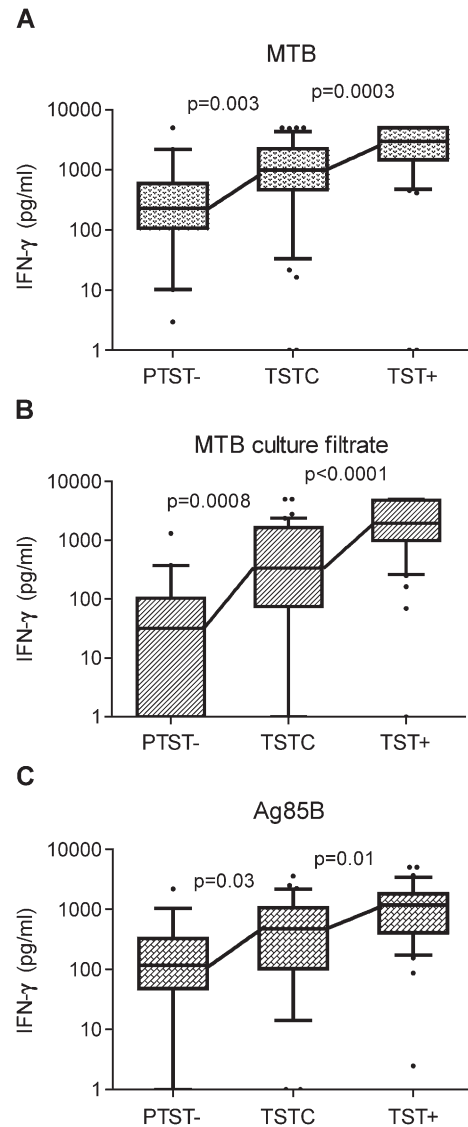


FIGURE 4. Baseline IFN- γ responses of PTST-, TSTC, and TST+ contacts to MTB bacilli, MTB CF and Ag85B. Whole blood was stimulated with: (A) MTB bacilli (10⁶ CFU/mL). (B) MTB Culture Filtrate (10 μ g/mL). (C) Ag85B (10 μ g/mL). Supernatants were harvested after 7 days and IFN- γ measured by ELISA. Results presented are the mean \pm SD of single measurements for each group of contacts. Box and whisker plots with whiskers defining the 90th and 10th percentiles. P values shown are those for comparisons between adjacent groups.

responses among the three groups stimulated with BrHPP at 3, 6, 12, and 24 months (data not shown). There were no differences between groups at other time points. There was little variability within each group at any time point suggesting stability of V δ 2 T cell responses over time (data not shown).

Adaptive T cell responses of PTST-, TSTC, and TST+ contacts. To measure adaptive T cell responses, diluted whole blood was stimulated for 7 days with MTB bacilli, MTB CF, or Ag85B, and supernatants analyzed for IFN- γ production. The MTB bacilli activate not only MHC-restricted CD4+ and CD8+ T cells but also nontraditional T cells such as $\gamma\delta$ and CD1-restricted T cells.⁶ The CF and Ag85B are protein anti-

gens and stimulate primarily MHC-II restricted CD4+ T cells. These diverse stimuli probe different aspects of T cell responses to MTB. Figure 4 shows the distribution of baseline IFN- γ responses to these antigens for PTST-, TSTC, and TST+ contacts and differences in median responses. All three groups had similarly robust responses to stimulation with PHA suggesting intact general immune responsiveness (data not shown). Patterns among the three antigens evaluated were similar, with PTST- having the lowest responses and TST+ contacts the highest. These response patterns were the same for lower concentrations of MTB CF (3 μ g/mL) and higher numbers of MTB bacilli (10⁷ CFU/mL). The highest and most differential responses were produced by stimulation with MTB CF (10 μ g/mL). Converters had intermediate responses suggesting that at the time of household evaluation, these persons already had enhanced T cell responses to MTB antigens. Strong IFN- γ responses to Ag85B and MTB CF suggest that CD4+ T cells had a major role in these responses.

Next, we assessed the evolution of adaptive T cell responses among the three groups of contacts. Using a conditional growth model adjusted for multiple comparisons, IFN- γ responses to MTB bacilli (10⁶ CFU), MTB CF (10 μ g/mL), and Ag 85B (10 μ g/mL) at baseline, 1, 3, 6, 9, 12, and 24 months were compared between PTST-, TSTC and TST+ contacts (Figure 5). Significant differences in IFN- γ production in response to MTB bacilli, MTB CF, and Ag85B at baseline were again found among all three groups. Within 3 months the response to Ag85B was no longer different in the TST+ and the TSTC groups. It took between 9 and 12 months for the responses to MTB bacilli and CF to become equivalent for these two groups. This accelerated increase in response to Ag85B to near TST+ levels may reflect a more rapid stabilization of the CD4+ T cell responses as opposed to slower evolution of heterogeneous components of the immune response stimulated by MTB bacilli and CF. Responses to MTB bacilli, MTB CF, and Ag85B were significantly greater in both TST+ and TSTC as compared with PTST- contacts at all time points. The IFN- γ responses in PTST- and TST+ contacts were remarkably stable over time.

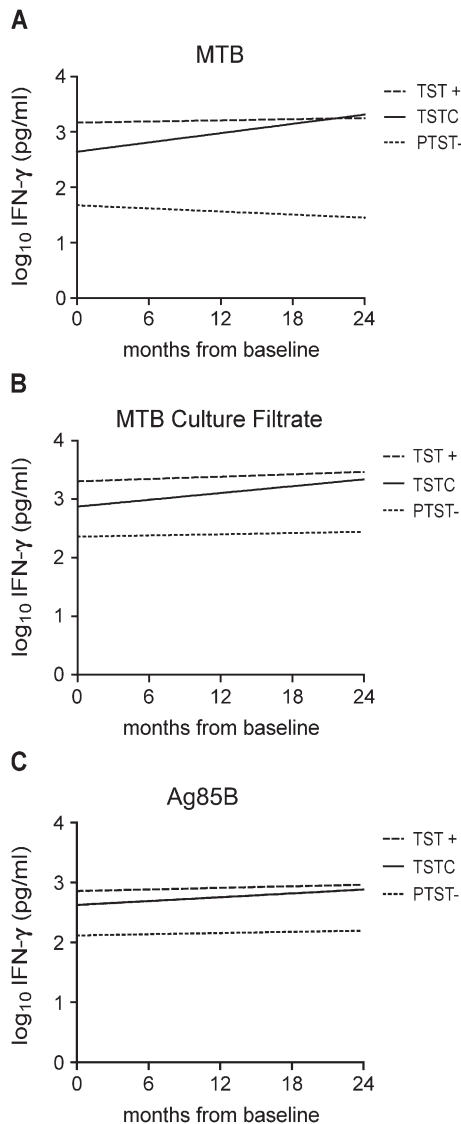


FIGURE 5. Changes in IFN- γ responses of PTST-, TSTC and TST+ contacts to MTB bacilli, MTB CF and Ag85B during 2 years of follow-up. Adjusted conditional growth model evaluating changes in IFN- γ responses over time to MTB antigens in three groups of contacts. (A) MTB bacilli (10⁶ CFU/well). TSTC and TST+ responses remain significantly different until 10–11 months. (B) MTB Culture Filtrate (10 μ g/mL). TSTC and TST remain significantly different ($P < 0.05$) until 9–10 months when values converge. (C) Ag 85B (10 μ g/mL). TSTC and TST+ significant differences are lost after baseline. PTST- responses remain significantly different for all 3 antigens at all time points as compared to the TSTC and TST+ groups ($P < 0.05$).

DISCUSSION

Longitudinal HHC studies allow measurement of changes in host responses to MTB exposure and infection. The TST is currently the screening method of choice in this region to identify persons with MTB infection. The TST conversion reflects recent MTB infection, but fails to fully define the ongoing immunologic changes in persons exposed to MTB. Of particular interest are those persons with heavy exposure to MTB who fail to convert their TST. This study evaluated adult (≥ 15 years of age) HHC of pulmonary TB cases. Of 440 HIV- negative persons, 76% were TST+ upon initial household evaluation and 24% TST-. Of 81 TST- persons at baseline with adequate follow-up, 62 eventually converted their TST and 19 remained persistently TST-. We measured innate and adaptive immune responses to determine if there were differences among PTST-, TSTC, and TST+ contacts. T cell responses to MTB antigens were able to distinguish these three groups, and there was a trend toward differences in innate responses.

Monocytes, macrophages, and dendritic cells recognize MTB through a multitude of mechanisms including pattern

recognition receptors (e.g., TLR, NOD) or they can be activated through the IFN- γ R, leading to enhanced ability to control MTB^{3,16–19}; the hypothesis was that PTST⁻ would have enhanced innate responses compared with TST⁺ and TSTC contacts. We did not find increased innate responses in PTST⁻. In fact, the trend toward increased TNF- α in response to LPS was in TST⁺, not in PTST⁻. Explanations for these results include the small sample size of PTST⁻ contacts, the selection of receptors (study of TLR9, complement, mannose, or NOD-like receptors might reveal differences), and limitations of whole blood stimulation assays. A more extensive study of immune responses of PTST⁻ contacts should determine if there are major innate protective mechanisms against MTB.

The $\gamma\delta$ T cells act as both antigen presenting cells (innate) and effector T cells (adaptive).⁷ Circulating $\gamma\delta$ T cells are increased in active TB and LTBI. The IFN- γ production in response to BrHPP was used to measure $\gamma\delta$ T cell activation.⁸ There was a positive trend toward increased IFN- γ production at baseline in TSTC compared with PTST⁻ contacts that did not reach statistical significance. High rates of BCG vaccination in our population may have led to higher $\gamma\delta$ T cell responsiveness in all three cohorts and dampened the ability to discern differences.^{20,21}

Even though TSTC and PTST⁻ had similar mean TST at baseline, TSTC had significantly elevated IFN- γ levels in response to MTB bacilli, CF, and Ag 85B compared with PTST⁻. This is consistent with our earlier study indicating that IFN- γ responses to CF were predictive of TST conversion and findings by Hussain and others in their HHC studies.^{10,22} These robust responses to MTB antigens are evidence that early measurable changes in adaptive immunity precede TST conversion. Studies of IFN- γ production to ESAT-6 and CFP-10 in HHC provides further evidence that recent MTB infection is detectable *ex vivo* before TST conversion.²³

T cells of PTST⁻ contacts remained unresponsive to MTB antigens despite extensive exposure in the household. Whether they simply were never infected or cleared infection through innate mechanisms without requiring MTB-specific T cells is difficult to determine with current immunological or microbiologic tools. Whether upper respiratory colonization occurs without MTB infection is unknown. Clearly PTST⁻ contacts were at high risk for infection and in hyperendemic Kampala remained at risk during the 2 years of follow-up. It is likely that PTST⁻ have innate abilities other than the ones measured to control MTB growth before T cell priming and sensitization occur.

T cell responses of TSTC to whole MTB, CF, and Ag 85B were not only greater in TSTC at baseline than in PTST⁻, but when followed longitudinally eventually rose to those of TST⁺ controls between 3 to 12 months after TST conversion. Immune responses were dynamic during MTB infection over several months before reaching a new steady state. The kinetics of responses differed depending on the antigen. Responses to Ag85B of TSTC reached those of TST⁺ by 3 months, whereas responses to CF and MTB bacilli took 9–10 and 10–11 months, respectively. These different time courses suggest that the more complex the antigen the longer it takes for immune responses of TSTC to resemble those of TST⁺. The time course for responses to CF in our cohort is similar to that seen in a HHC study in Pakistan where IFN- γ reached a plateau after 6 months.²⁴ The Ag85B protein is primarily a stimulus for CD4⁺ T cells and its rapid response

may in part reflect boosting of memory response primed by BCG vaccination or environmental mycobacteria that also can express Ag85B. Additionally, NK T cells, part of innate immune responses, can produce IFN- γ in response to CF and whole MTB, but not to defined antigens such as Ag85B.²⁵ Differences in NK T cell activity could contribute to the more prolonged differences between the TSTC and TST⁺ groups in response to CF and MTB shown in Figure 5. The CF is a complex mixture of MTB proteins and peptides that stimulate primarily CD4 T cells, but also CD8 T cells.¹³ The MTB bacilli are the most complex antigen stimulating CD4⁺, CD8⁺, and non-traditional T cells, i.e., $\gamma\delta$ and CD1 restricted T cells.

Limitations of our study include the small number studied hampering our ability to discern small differences in TLR2, TLR4, IFN- γ R, and $\gamma\delta$ T cell responses. The BCG vaccination is routine in Uganda and although rates of vaccination did not differ among the three HHC cohorts, it is possible that some TST conversions represented boosted immune responses rather than MTB infection. Additionally, this study focused on older adolescent and adult contacts (15 years of age and older) who may not be as susceptible to MTB infection as younger children. The impact of prior BCG on the interpretation of TST conversion is controversial, although most still find it a reasonable surrogate marker for MTB infection in high incidence settings.^{23,26–30} False positive TST conversions caused by boosting in our study would over-estimate MTB infection and decrease the number of PTST⁻. Our study tested a limited range of MTB antigens ranging from one defined protein to whole MTB bacilli. The range of MTB antigens recognized by persons with LTBI in Uganda and elsewhere in Africa is broad but also varies by region for host genetic and MTB strain reasons.³¹ Identifying antigens to better differentiate the recently infected from those with LTBI is necessary to identify those at greatest risk for progression to disease. Finally, the natural history of MTB infection and TB disease is also influenced by a number of factors related to clinical characteristics of infectious TB cases that come into contact with an individual genetic variation in MTB, and genetic susceptibility of the human host; this study did not account for these factors, but these shall be the focus of future research.^{9,32–34}

In summary, this study shows that adaptive immune responses can differentiate adult contacts of TB patients who will convert their TST from those already infected and those who remain TST⁻. Furthermore, for those who convert the TST by 3 months, immune responses to MTB antigens are dynamic and reach those of TST⁺ contacts anywhere from 3 to 12 months depending on the complexity of the MTB antigen. Furthermore, 20–25% of contacts TST⁻ at baseline remained TST⁻ during 12–24 months. Thus, PTST⁻ is not rare. Interestingly, probing a limited panel of innate receptors failed to identify differences between PTST⁻ and the other contacts. Further study of immune responses of contacts with acute MTB infection and those who appear to resist infection should provide insight into innate and adaptive immune responses that protect against MTB infection and disease.

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