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Biomarker Changes Associated with Tuberculin Skin Test (TST) Conversion: A Two-Year Longitudinal Follow-Up Study in Exposed Household Contacts

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Abstract

Background: A high prevalence (50–80%) of Tuberculin Skin Test Positivity (TST+ ≥10 mm indurations) has been reported in TB endemic countries. This pool forms a huge reservoir for new incident TB cases. However, immune biomarkers associated with TST conversion are largely unknown. The objective of this study was to identify immune biomarkers associated with TST conversion after acute Mycobacterium tuberculosis (MTB) exposure.

Methodology/Principal Findings: A 24 month longitudinal study was carried out in a recently MTB exposed cohort of household contacts (HC = 93; 75% TST+). Control group consisted of unexposed community controls (EC = 59; 46% TST+). Cytokine secretion was assessed in whole blood cultures in response to either mycobacterial culture filtrate (CF) antigens or mitogens (PHA or LPS) using Elisa methodology. Compared to the EC group, the HC group at recruitment (Kruskal-Wallis Test) showed significantly suppressed IFNγ (p = 0.0001), raised IL-10 (p = 0.0005) and raised TNFα (p = 0.001) in response to CF irrespective of their TST status. Seventeen TST-HC, showed TST conversion when retested at 6 months. Post-TST conversion (paired t tests) significant increases were observed for CF induced IFNγ (p = 0.0038), IL-10 (p = 0.001) and IL-6 (p = 0.006). Cytokine responses were also compared in the exposed HC group with either recent infection (TST converters [N = 17]) or previous infection (TST+ HC [N = 54]) at 0, 6, 12 and 24 months using ANOVA on repeated measures. Significant differences between the exposed HC groups were noted only at 6 months. CF induced IFNγ was higher in previously infected HC group (p = 0.038) while IL-10 was higher in recently infected HC group (p = 0.041). Mitogen induced cytokine secretion showed similar differences for different group.

Conclusions/Significance: Our results suggest that TST conversion is associated with early increases in IFNγ and IL-10 responses and precedes latency by several months post exposure.

Introduction

Pakistan ranks 8th in terms of global tuberculosis (TB) disease burden [1]. One third of the world population is considered to be latently infected as assessed by Tuberculin Skin Test positivity (TST+ ≥10 mm of indurations) [2] resulting in a huge reservoir for new incident cases. In a high transmission setting such as Pakistan the rate of TST positivity can be as high as 50% in the community [3] and household contacts [4] increasing up to 80% in recently exposed household contacts [5,6]. PPD administered in TST is a mixture of proteins prepared from M. tuberculosis (MTB) culture filtrate and is highly cross reactive with other mycobacterial antigens including Mycobacterium bovis. Pakistan has wide BCG vaccination coverage at birth (>70%) through the expanded program for immunization (EPI) [7] and the high prevalence of TST+ in the community may be due to either previous BCG vaccination [8] or booster effects with environmental mycobacteria [9]. ESAT-6, induced interferon (IFN γ) is considered to be a MTB specific marker for infection and disease as it is present only in the M. tuberculosis complex [10]. Commercial IFNγ release assays (IGRA) using ESAT-6 stimulated whole blood cultures are increasingly available for detection of recent infection [11] or disease [12]. However, longitudinal studies suggest IGRA test cannot replace TST in high TB incidence areas for detection of latent infection [13]. The high incidence of HIV (~11%) in 8.8 million new TB cases [14] further confounds the immune correlates associated with latency as immune suppression results in lower TST diameters in HIV+ individuals with latent TB infection. There is an urgent need to carry out such studies in TB endemic areas with a low HIV prevalence to understand unmitigated immune correlates associated with TST conversion. Immune activation after a recent exposure may be associated with activation and modulation of several immune markers and the magnitude and timing of pro- and down-regulatory cytokines may be very important for TST conversion during a natural exposure. IFN γ is a known correlate of delayed type hypersensitivity as well as TST skin test diameter [15]. Early activation of IFN γ is therefore linked to TST conversion and development of effective
immunity. TNFα in conjunction with IFNγ has been shown to play a role in granuloma formation [16] and in maintenance of granuloma [17]. IL-10 is a down-regulatory cytokine and plays a key role in limiting pathology by inhibiting overproduction of pro-inflammatory cytokines [18,19] particularly in the chronic phase of infection [20,21]. IL-6 is a pro-inflammatory cytokine secreted by both macrophages and T cells and plays a critical role in driving the differentiation of B cells [22]. In the mouse gene knockout model IL-6 has also been shown to participate in the early induction of IFNγ production [23]. We have therefore opted to analyze these biomarkers post acute exposure due to their identified roles in control of mycobacterial infections. Due to slow replication of mycobacteria, immune responses are usually slow to evolve requiring serial testing over several months post infection. Longitudinal studies for immune correlates of tuberculosis infection and disease have been carried out in African countries with relatively high HIV prevalence [24–27]. Pakistan still has a relatively low HIV (0.8/100000) prevalence [1] compared to 1–3% in eastern Africa and 10.8% in South Africa [28] and therefore provides a useful setting for such studies. The objective of the current study was to evaluate multiple interrelated biomarkers serially in a MTB exposed cohort to identify biomarkers associated with TST conversion and to analyze differences in the evolution of biomarkers in household contacts with either recent or past infection post acute exposure.

Materials and Methods

The study cohort comprised of healthy household contacts (HC = 93) from 20 families. Each family had at least one recently diagnosed, untreated pulmonary tuberculosis patient. The intensity and duration of exposure as assessed by history of symptoms and the extent of disease in the index case was similar in all families (Table S1). Inclusion criteria: household contacts who had no previous history of anti-tuberculous treatment (ATT) and who remained disease-free during a 24 month follow up period [5,29]. Exclusion criteria: Children under the age of five or subjects with underlying diseases which could result in immune suppression or individuals on steroids. Epidemiologic controls (EC, N = 59) were employees working in various capacities at The Aga Khan University with no history of recent exposure to tuberculosis. Lady Health Visitors (LHV) made home visits, obtained informed written consent from all adult participants or guardians in case of children <18 years. There is no routine testing for HIV in Pakistan because of the low incidence [1].

Tuberculin Skin Tests

Tuberculin Skin Tests (TST) were carried out by injecting 5 tuberculin units intra-cutaneously in the volar surface of the arm. A single tester administered and read the indurations after 48 hours using a caliper. A diameter ≥ 10 mm was considered positive [30]. At intake TST was carried out on all HC (N = 93) and EC (N = 59). Repeat testing was carried out in TST-HC (N = 23). No further TST testing was done on the TST+HC group and no prophylactic treatment was provided for latent tuberculosis infection.

Reagents

Purified protein derivative from Mycobacterium tuberculosis (MTB) was obtained from Statens Serum Institute (batch RT47; Copenhagen, Denmark). LPS and PHA were purchased from Sigma (St. Louis MO, USA). MTB Culture Filtrate (CF) proteins were prepared in-house at Case Western Reserve University, Cleveland, Ohio and provided by Dr Robert Wallis as described previously [31]. Briefly MTB strain H37 Rv was grown in Prouskeur Beck medium. After 8–10 weeks of culture, bacilli were removed by sedimentation and filtration. The preparation contained minimal amounts of endotoxin as determined by inhibition with polymyxin B [32]. Pairs of mouse monoclonal antibodies specific for each cytokine were purchased from Pharmingen (San Diego, CA) for the assessment of cytokines. All reagents were used according to manufacturer’s instructions.

Whole blood stimulation assay

Whole blood stimulation assay has been described in detail previously [33]. Briefly, 5 ml of blood collected by venipuncture from each donor was mixed with sodium heparin (20 U/ml; Leo pharmaceutical, Ballerup, Denmark) in 15 ml plastic centrifuge tubes (BD Falcon). Blood was further diluted 1:10 with RPMI 1640 tissue culture medium containing 100 U/ml of penicillin+100 µg/ml of streptomycin (Sigma Chemicals St. Louis, MO, USA) and 2 mM of L-glutamine (Sigma Chemicals). Diluted blood (900 µl/well) was dispensed in 24-well tissue culture plates (Flow laboratories, Irvine, CA) within two hours of collection. The blood was subsequently stimulated with 100 µl of PHA [5.0 µg/ml] or LPS [1.0 µg/ml] or CF [5.0 µg/ml] in tissue culture plates and further incubated at 37°C in 5% CO2. Blood cultures were stimulated in separate wells for either 2 days (TNFα, IL-10 and IL-6) or 5 days (IFNγ). Supernatants of whole blood cultures were collected and stored as 4×200 µl of aliquots at −35°C until use. Selection of mitogen/antigen concentrations and optimal days for collection of supernatants for various cytokines has been reported previously [33].

Cytokine assessment

The Elisa assay was optimized in-house and details of the assay have been reported previously [33]. Briefly Immulon 4 plates were coated with capture antibodies and subsequently incubated with 100 µl of un-stimulated or stimulated culture supernatants overnight at 4°C. Subsequently the probing and revealing antibodies were added [100 µl] stepwise for appropriate incubations. The plates were washed between incubations. All probing antibodies were biotin labeled and the revealing antibodies were labeled with avidin bound to horse- radish peroxidase (HRP) Sigma Chemicals. HRP substrate was added and color development was stopped with 1N NaOH after 30 minutes incubation. The optical densities were read using an Elisa reader (Biorad microplate reader 680). Mitogen stimulated cytokines were assessed at a dilution of 1:10 and 1:100 and antigen stimulated cytokines were assessed at neat and a dilution of 1:10 of the supernatants. Optical density values falling in the midrange of the standard curve were used to calculate the concentration of cytokine in the supernatants and final values were obtained after multiplying by the dilution at which the cytokines were assessed. In case of PHA stimulated cultures occasionally (IFNγ in 3 donors) a dilution of 1:1000 was needed to calculate the final value. Results were expressed as δ pg/ml, after deducting secretion in the absence of stimulus to normalize the expression of mitogen/antigen specific secretion. The sensitivity and range of cytokine detection was as follows: TNFα (7.8–1000 pg/ml); IL-6 (7.8–1000 pg/ml); IL-10 (15–250 pg/ml); Interferon γ (50–2000 pg/ml).

Statistical analysis

SPSS software (version 16.0) was used for the statistical analyses. Kruskal-Wallis analysis was carried to compare overlapping test groups with control group. Paired student t tests were applied to compare two related samples. Analysis of variance (ANOVA) on repeated measures was carried out using General Linear Model (GLM) for comparison of group cytokine profiles.
Ethics statement

The study protocol received approval of the Ethical Review Committee of The Aga Khan University and Hospital.

Results

Study subjects

Table 1 shows the characteristics of the study groups at recruitment (0 months). The EC group was healthy and had no co-morbid conditions that could compromise the immune response. The intensity and duration of Mycobacterium tuberculosis (MTB) exposure was similar in the household contacts (HC) (Table S1). Although it is difficult to exclude TB exposure in high TB burden countries, none of the community controls (EC) gave a history of recent exposure to a tuberculosis patient and were considered unexposed. TST diameter in the TST+HC and the TST+EC groups was comparable (Table 1). The TST positivity in the EC group in the absence of MTB exposure is therefore most likely due to either previous BCG vaccination or exposure to environmental mycobacteria. The TST positivity in the HC group at recruitment is more likely due to previous infection as these individuals were from a high transmission pocket [6]. We have therefore, considered TST+HC group as having latent infection at the time of recruitment for the current study. The TST-HC group showed slightly higher TST diameter (mean indurations 3.5±3.0) compared to the TST-EC group (mean indurations 0.8±2.3). The absence of TST positivity in the HC group (23/93) was not related to BCG vaccination as there was no difference in the presence of BCG scar in TST-HC (40%) compared to TST+HC (39.5%) (Table S1). The slightly higher TST diameter in the TST-HC is probably due recent exposure. The TST-HC group was therefore considered a recently infected group.

Cytokine secretion in recently exposed HC and non exposed EC in mycobacterial antigen (CF) stimulated whole blood cultures

To assess early changes in biomarkers associated with recent exposure to MTB, we first compared CF induced cytokine responses in the exposed HC group (TST-HC = 23; TST+HC = 70) with the unexposed EC (TST-EC = 32; TST+EC = 27) group (Figure 1 and Table 2). Kruskal-Wallis ANOVA tests were applied to determine the significance of differences in cytokines in the exposed HC groups compared to the non exposed EC groups. The distribution of CF induced cytokines is shown as a box plot (Figure 1) with the median and inter-quartile ranges (IQR) shown in Table 2. CF induced IFN \( \gamma \) was significantly suppressed (\( p = 0.0001 \)) in the HC group irrespective of the TST status. Increased CF induced TNF \( \alpha \) (\( p = 0.001 \)) and CF induced IL-10 (\( p = 0.0005 \)) were observed in the HC group compared to the EC groups (Table 2).

Comparison of mycobacterial antigen (CF) and mitogen (PHA or LPS) stimulated cytokine levels pre- and post TST conversion in exposed disease free household contacts (HC)

Repeat skin testing was carried out in 17/23 TST-HC donors at 6 months. TST conversion was noted in all donors \( (N = 17) \); TST diameters pre-conversion, mean = 3.18±3.55 SD; post conversion, mean = 11±0.6 SD mm). The remaining donors (6/23) could only be restested at 24 months and were excluded from these analyses as the time of TST conversion could not be ascertained. To assess biomarker changes associated with TST conversion, we analyzed mycobacterial antigens or mitogen induced cytokine secretion pre- and post TST conversion.

Table 1. Characteristics of the study group at intake.

| Group ID | n | Age (X±1SD) | Male/Female | Diameter of indurations (X±1SD) |
|----------|---|------------|-------------|---------------------------------|
| TST+HC   | 70 | 25.8±15.9  | 34/36       | 17.6±6.6                        |
| TST-EC   | 23 | 20.0±10.7  | 11/12       | 3.5±3.2                         |
| TST+EC   | 27 | 31.3±9.4** | 10/17       | 16.6±4.1                        |
| TST-EC   | 32 | 24.0±5.0   | 18/14       | 0.8±2.3                         |

\( \text{HC} = \text{Healthy household contacts. EC = Community controls.} \)

\( \text{TST}^+ = \text{Positive Tuberculin Skin Test diameter } \geq 10 \text{ mm.} \)

\( \text{TST}^- = \text{Negative Tuberculin Skin Test diameter } <10 \text{ mm.} \)

**One way ANOVA, \( p = .008 \).

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was only 2–3 fold higher compared to CF induced TNFα. These results are in line with previously published reports that mycobacterial antigens are potent stimulators of macrophages [31,34]. The overall cellular activation was parallel to mycobacterial antigen stimulated cytokine secretion.

Longitudinal assessment of CF and mitogen induced cytokine secretion post exposure in recently infected and previously infected HC groups over a 2-year period

To identify the cytokine dynamics post exposure in the HC group with recent infection (TST-HC = 17) compared to the HC group with previous infection (TST+HC = 54) analysis of variance (ANOVA) was carried on repeated measures over a two year period. The reference group (TST+HC) had similar MTB exposure and demographics at recruitment including the presence of BCG scar (Table S1). Only donors sampled at all time points (54/70) were included in the analysis. Figure 3 (IFNγ and IL-10) and Figure 4 (TNFα and IL-6) show comparative profiles for mycobacterial antigen (CF) or mitogen (PHA or LPS) induced cytokine secretion.

Both groups showed dynamic changes in cytokines after acute exposure. Not surprisingly, CF induced IFNγ (Figure 3, panel A) showed significantly lower responses in recently infected compared to previously infected group. Table 2 shows the results of cytokine secretion in recently exposed HC and non exposed EC in mycobacterial antigen induced whole blood cultures.

**Table 2.** Cytokine secretion in recently exposed HC and non exposed EC in mycobacterial antigen induced whole blood cultures.

| Groups     | TST- HC | TST+HC | TST-EC | TST+EC | p =     |
|------------|---------|--------|--------|--------|---------|
| Biomarker (pg/ml) | median (IQR) | median (IQR) | median (IQR) | median (IQR) |         |
| IFNγ       | 397(1009)| 283 (758)| 873(2724)| 1720(2619)| 0.0001  |
| TNFα       | 544(452)| 541 (574)| 216(420)| 441(750)| 0.001   |
| IL-10      | 212(351)| 263(472)| 141(212)| 195(258)| 0.0005  |
| IL-6       | 2078(2913)| 2232(3932)| 2918(3697)| 3429(3351)| >0.1    |

Note: Values in bold signify significant up regulation and values underlined indicate significant suppression Kruskal Wallis analysis were applied for statistical significance.

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to previously infected HC group at 6 months [TST-HC; lower IFN γ (\(p = 0.038\)). In response to PHA (Figure 3, panel C), the magnitude of IFN γ response did not differ between the two HC groups, but the timing of peak responses were different. The previously infected HC group showed peak IFN γ response at 6 months, while the recently infected HC group peaked at 12 months (Figure 3, panel C) suggesting that TST conversion may be related to the strength of mycobacterial specific T cell response rather than the overall strength of T cell activation. The trend with CF induced IL-10 (Figure 3, panel B) in the two groups was opposite to that observed with CF induced IFN γ with IL-10 responses being significantly higher in recently infected HC at 6 months (\(p = 0.041\)). Parallel trends were observed with LPS induced IL-10 (Figure 3, panel D). Both LPS and CF induced IL-10 showed a much more dynamic profile with greater variability in recently infected HC group (Table S2) compared to a relatively flat response over time in the previously infected HC group indicating that immune responses in newly infected HC take at least a year to stabilize.

No significant differences were noted with TNFα (Figure 4, panel A and C) or IL-6 (Figure 4, panel B and D) in response to CF or mitogens between recently infected compared to previously infected HC (Figure 4). The only exception was LPS induced IL-6 (Figure 4D) which was significantly higher (\(p = 0.039\)) in the previously infected HC group at 0 months compared to the HC group with recent infection at recruitment.
Discussion

This is the first report on serial assessment of multiple biomarkers to identify changes associated with TST conversion in a recently MTB exposed cohort in a low HIV setting. Natural evolution of biomarkers post exposure was possible in those with recent and past infection as no prophylaxis was given for latent tuberculosis. The cohort remained disease free over a 4 year period suggesting that these biomarker profiles may represent protective profiles in the HC group post exposure. The strength of the study was therefore, careful selection of a cohort with no history of TB or of anti-tuberculous treatment in the past.

Variability in the duration and intensity of exposure can change the dynamics of immune parameters particularly with respect to innate responses that are activated early during infection. Both these parameters are very difficult to control in a high transmission setting. We have tried to limit this variability by including families where the symptoms in the index cases initiated no more than 3 months and the pulmonary disease was restricted to moderate lung disease. We have used MTB culture filtrate antigens which are comparable in activity to PPD antigens used in TST. In household contacts IFN-γ secretion in response to culture filtrate antigens in stimulated whole blood have been recommended for early diagnosis of latent infection especially in a resource strapped countries [25]. When cytokines responses were compared in the exposed and the non exposed groups, elevated responses were observed with mycobacterial antigen induced TNFα and IL-10 which were most likely derived from innate cells. This was not surprising as mycobacterial proteins are known to be potent stimulators of macrophages [24, 33, 35, and 36]. However, suppressed mycobacterial antigen induced IFN-γ responses in the exposed HC group compared to the EC group was surprising. Pakistan has wide BCG vaccination coverage at birth ([70%]) through the expanded program for immunization (EPI) [7] and a high prevalence of TST+ in the non exposed community control group may be due to either previous BCG vaccination [8] or booster effects with environmental mycobacteria [9]. As to why we did not observe such a booster effect in the recently exposed cohort may lie with CF induced IL-10 which was selectively raised in the exposed HC groups but not in the non exposed EC group. IL-10 is a down-regulatory cytokine and inhibits pro-inflammatory cytokines such as IFN-γ [18,19] and may be the reason for the observed lower CF induced IFN-γ responses in the exposed HC group. The HC groups had been exposed to MTB for at least 3

![Figure 3. CF and mitogen induced IFN-γ and IL-10 in TST+HC and TST-HC groups.](image-url)
months, which may be sufficient to activate the innate arm of the immune responses. CF induced IL-6 was the only cytokine which did not show differences between the HC and the EC groups. The HC groups stratified on the basis of TST status did not show significant differences (Mann Whitney U tests) in CF induced cytokine responses (Figure 3 &4). The differences between the non exposed EC and the exposed HC groups are therefore most likely due to recent MTB exposure.

In relation to TST conversion, the HC group did show an increase in CF induced IFNγ secretion post TST conversion. Similar increases in IFNγ to mycobacterial antigens have been reported post exposure [37] and post TST conversion to ESAT-6 which is a MTB specific antigen [27]. Antigen induced IFNγ has been reported to correlate with TST diameter [38], but the coordinate expression of CF induced IL-10 was surprising. A similar LPS induced IL-10 secretion was noted, suggesting an overall activation of macrophages post TST conversion. IL-10 may be elevated to reduce collateral tissue damage [19] in response to a heightened proinflammatory response during the early phase of infection. The time frame of TST conversion (6 months) and establishment of stable cytokine responses is also the time frame where the highest reported incidence of disease post MTB exposure was observed in this cohort [5]. Disease progression in this cohort (N = 7) was associated with depressed IFNγ/IL-10 ratio [5]. Surprisingly there was no concurrent elevation of TNFα post TST conversion. TNFα is prototypic marker of classically activated macrophages (CAMφ) while alternatively activated macrophages (AAMφ) secrete high levels of IL-10. [39]. Mycobacterial antigens can signal macrophages via the Toll like receptors [40] and recently TLR9 has been implicated in the activation of alternatively activated macrophages (AAMφ) [41]. It is therefore tempting to speculate that post TST conversion there is concurrent elevation of Th1 and AAMφ which down regulate CAMφ. Increase in IL-6 post TST conversion was also intriguing. Although the role of IL-6 in human tuberculosis is not clear, IL-6 has been shown to be elevated in established disease [33]. In the experimental IL-6 gene knockout mouse, a delay in early induction of IFNγ [23] as well a delay in the initial differentiation of Th1 cells, but not in expansion, has been reported [42].

To further understand the evolution of cytokines post MTB exposure, we compared the HC group with recent infection (TST converters) or past infection (TST-HC at recruitment). Again significant differences post exposure in the two groups, were noted only at 6 months. These results suggest that TST conversion may precede latency and stable granuloma formation as cytokine responses in the two groups (recent infection Vs previous infection) become comparable beyond 12 months. The cytokines likely to play a role in TST conversion such as IFNγ show a plateau beyond 12 months while IL-10 continues to increase in both groups beyond 12 months and may play a greater role in establishing dormancy and latency. These findings therefore
supports the notion that latent tuberculosis actually represents a subclinical spectrum which can only be defined by the host immune response [43] and is supported by the variability of responses in recently infected groups while previously infected groups represent a more stable host response. Our studies therefore suggest that while IFN γ may play an important role in resolution of infection and TST conversion, IL-10 may be critical in dampening the pro-inflammatory arm of the immune response as well as in initiation of dormancy during the later phase of infection. The current study therefore, raises important questions with regards to the source of various biomarkers and their role in establishing TST conversion, dormancy and latency.

Supporting Information

Table S1 Intensity and duration of exposure to M. tuberculosis in Household Contacts at Recruitment. a Cough and or low grade fever and or weight loss. b Intensity of Acid Fast Bacilli in sputum smear. c Determined by radiology [According to Crofton et al 1990][30] d ATT (days) started prior recruitment. TST+HC were not given prophylactic ATT. e # HC in the family. # TST-HC in the HC within each family is shown in brackets. * repeat TST available at 24 months only. Cytokine levels were available at all time points (0, 6, 12 and 24 months) on 77 HC (TST+ = 54; TST- = 23). Secondary cases (N = 8) diagnosed over 4 years follow up, and contacts previously treated (N = 5) were excluded. § One co-prevalent case (on ATT) in the family BCG scar was present in 40% (38/94) HC. Among BCG scar positive HC, 71% were TST positive, and in BCG scar negative HC 70.5% were TST positive. Reference: Crofton, J (1990) Clinical features of tuberculosis. In: Seton D, Gordon A, editors. Crofton and Douglas Respiratory Diseases. London: Blackwell Scientific. pp. 395–421.

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Table S2 Repeated measure on TST+ HC and TST- HC. Intergroup comparison of cytokine profiles in the HC group with previous infection (TST+HC at intake) with recent infection (TST- HC at intake) in response to mycobacterial antigens (CF) and mitogens (PHA or LPS). This is a companion table for Figures 3 and 4. ANOVA test for repeated measures using GLM was carried to assess the differences in the two groups. The cytokine values are log n transformed and the estimated marginal means, SEM, β estimates, p value and 95% CI for each of the stimulant and cytokines shown in Figure 3 and Figure 4. A p value<0.05 was considered significant.

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Author Contributions
Conceived and designed the experiments: RH. Performed the experiments: NT FS. Analyzed the data: RH NT. Contributed reagents/materials/analysis tools: RH. Wrote the paper: RH. Statistical analysis and data presentation: NT. Data entry and logistical support: FS. Patient recruitment, diagnosis and clinical follow-up: GD.

References

1. World Health Organization (2008) WHO Report 2008 - Country Profile Pakistan. Global Tuberculosis Control. pp. 133–136.
2. Dye C, Scheele S, Dolin P, Nathanial V, Raviglione MC (1999) Global Burden of Tuberculosis: Estimated incidence, Prevalence, and Mortality by Country. JAMA 282: 677–686.
3. Hussain H, Akhtar S, Nanand D (2003) Prevalence of and risk factors associated with Mycobacterium tuberculosis infection in prisoners, North West Frontier Province, Pakistan. Int J Epidemiol 32: 794–799.
4. Rathi SK, Akhtar S, Raheem MH, Azam SI (2002) Prevalence and risk factors associated with tuberculosis skin test positivity among household contacts of smear-positive pulmonary tuberculosis cases in Umerkot, Pakistan. Int J Tuberc Lung Dis 6: 851–857.
5. Hussain R, Talat N, Shafid F, Davood G (2007) Longitudinal tracking of cytokines after acute exposure to Tuberculosis: Association of Distinct Cytokine patterns with protection and disease development. Clin Vaccine Immunol 14: 1578–1586.
6. Akhtar S, White F, Hasam R, Rozi S, Younus M, et al. (2007) Hyperendemic incidence population containing a high proportion of BCG-vaccinated persons. Respir Res 8: 77.
7. World Health Organization (2009) Review of National Immunization coverage 1980–2008 (Pakistan).
8. Diet R, Nierhaus A, Lunge C, Meywald-Walter K, Frosenbom M, et al. (2006) Tuberculosis contact investigation with a new, specific blood test in a low-incidence population containing a high proportion of BCG-vaccinated persons. Respirology 7: 77.
9. Menzies D (1999) Interpretation of Repeated Tuberculin Tests. Am J Respir Crit Care Med 159: 15–21.
10. Sorenson AL, Nagai S, Houen G, Andersen P, Andersen AB (1995) Purification and characterization of a low-molecular mass ‘T’ cell antigen secreted by Mycobacterium tuberculosis. Infect Immun 63: 1710–1717.
11. Strent JA, Desem N, Jones SL (1998) Sensitivity and specificity of a gamma interferon blood test for tuberculosis infection. Int J Tubere Lung Dis 2: 443–450.
12. Britton W, Gilbert GL, Wheatley J, Leslie D, Rothel JS, et al. (2001) Sensitivity of human gamma interferon assay and tuberculin skin test for detecting infection with Mycobacterium tuberculosis in patients with culture positive tuberculosis. Tuberculosis 86: 137–145.
13. Hill PC, Jackson-Sillah DJ, Foxx A, Brooks R, de Jong BC, et al. (2008) Incidence of tuberculosis and the predictive value of ELISPOT and Monteux tests in Gambian case contacts. PLoS ONE 3: e1579.
14. World Health Organization (2007) WHO: Global Tuberculosis Control: Surveillance, Planning, Financing.
15. Black GF, Fine PEM, Warnodff DK, Foyd S, Weir RE, et al. (2001) Relationship between IFN gamma and skin test responsiveness to Mycobacterium tuberculosis PPD in healthy, non BCG vaccinated young adults in Northern Malawi. Int J Tuberc Lung Dis 5: 1–9.
16. Kindler V, Sappino AP, Grau GE, Piguet PF, Via LE, et al. (1989) The inducing role of tumor necrosis factor in the development of bacilliardic granulomas during BCG infection. Cell 56: 731–740.
17. Kneze J (2005) TNF-blocking agent and tuberculosis: new drugs illuminate an old topic. Rheumatology 44: 714–720.
18. Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O’Garra A (1991) IL-10 inhibits cytokine production by activated macrophages. J Immunol 147: 3815–3822.
19. O’Garra A, Vieira PL, Vieira P, Goldfeld AE (2004) IL-10 producing and naturally occurring CD4+ Tregs: limiting collateral damage. J Clin Investig 114: 1372–1378.
20. Abebe F, Mustafa T, Nerland AH, Bjune GA (2003) Cytokine profile during latent and slowly progressive primary tuberculosis: a possible role for interleukin-15 in mediating clinical disease. Clin Exp Immunol 143: 189–192.
21. Higgins DA, Sanchez-Campillo J, Rojas-Taraco AG, Lee EJ, Orme IM, et al. (2009) Lack of IL-10 alters inflammatory and immune responses during pulmonary Mycobacterium tuberculosis infection. Tuberculosis (Edinb) 89: 149–157.
22. Laur C, Kyono H, Mcalree JR, Fujihashi K, Kishimoto T, et al. (1991) Recombinant human interleukin 6 (rHu-6) promotes the terminal differentiation of in vivo activated human B cells into antibody secreting cells. Cellular Immunology 132: 423–434.
23. Saunders BM, Frank AA, Orme IM, Cooper AM (2000) Interleukin-6 induces early gamma interferon production in the infected lung but is not required for generation of specific immunity to Mycobacterium tuberculosis infection. Infect Immun 68: 3322–3328.
24. Elliott AM, Holdeson WS, Kysvinnme J, Quasley MA, Nakinyong J, et al. (2004) Cytokine responses and progression to active tuberculosis in HIV-1-infected Ugandans: a prospective study. Trans Roy Soc Trop Med & Hyg 98: 660–670.
25. Whalen CC, Chunda A, Zawango S, Nshuti L, Jones-Lopez E, et al. (2006) Immune correlates of acute Mycobacterium tuberculosis infection in household contacts in Kampaala Uganda. Am J Trop Med Hyg 75: 55–61.
26. Demisse A, Wasse L, Abebe M, Acsella A, Rooy G, et al. (2006) The 6 kilodalton early secreted antigenic target-responsive, asymptomatic contacts of tuberculous patients express elevated levels of interleukin-4 and reduced levels of gamma interferon. Infect Immun 74: 2017–2022.
27. Hill PC, Brooks RH, Foxx A, Jackson-Sillah D, Jeffries DJ, et al. (2007) Longitudinal assessment of an ELISPOT test for Mycobacterium tuberculosis infection. PLoS Med 4: e192.
28. [Anonymous] (2009) World Health Organization statistics 2009.
29. Talat N, Shafid F, Davood G, Hussain R (2009) Dynamic changes in Biomarker Profiles Associated with Clinical and Tuberculous Tuberculosis in a...
High Transmission Setting: A Four-Year Follow-Study. Scand J Immunol 69: 537–546.
30. Crofton J (1990) Clinical features of tuberculosis. In: Seton D, Gordon A, eds. Crofton and Douglas Respiratory Diseases. London: Blackwell Scientific. pp 395–421.
31. Hussain R, Shiratsuchi H, Philips M, Ellner J, Wallis RS (2001) Opsonizing antibodies (IgG1) up-regulate monocyte proinflammatory cytokines tumor necrosis factor-alpha (TNF-α) and IL-6 but not anti-inflammatory cytokine IL-10 in mycobacterial antigen-stimulated monocytes-implications for pathogenesis. Clin Exp Immunol 123: 210–218.
32. Thakurdas SM, Hasan Z, Hussain R (2004) IgG1 antimycobacterial antibodies can reverse the inhibitory effect of pentoxifylline on tumour necrosis factor alpha (TNF-α) secreted by mycobacterial antigen stimulated adherent cells. Clin Exp Immunol 136: 320–327.
33. Hussain R, Kaleem A, Shahid F, Dojki M, Jamil B, et al. (2002) Cytokine profiles using whole blood assays can discriminate between tuberculosis patients and healthy endemic controls in BCG-vaccinated population. J Immunol Meth 264: 95–108.
34. Wallis RS, Amir-Tahmasseb M, Ellner JJ (1990) Induction of interleukin-1 and tumor necrosis factor by mycobacterial proteins: The monocyte western blot. Proc Natl Acad Sci USA 87: 3348–3352.
35. Wallis RS, Paranjape R, Philips M (1995) Identification of two dimensional Gel Electrophoresis of a 38-kilodalton tumor necrosis factor introducing protein of mycobacterium tuberculosis. Infect Immun 61: 627–632.
36. Gaikward AN, Sinha S (2008) Determinants of natural immunity against tuberculosis in an endemic setting: factors operating at the level of macrophage-Mycobacterium tuberculosis interaction. Clin Exp Immunol 151: 411–422.
37. Ewer K, Millington KA, Deeks JJ, Alvezar I, Bryant G, et al. (2006) Dynamic antigen-specific T-cell responses after point-source exposure to Mycobacterium tuberculosis. Am J Respir Crit Care Med 174: 831–839.
38. Black GF, Weir RE, Chagulaka SD, Warradoff D, Crampon AC, et al. (2003) Gamma interferon responses induced by a panel of recombinant and purified mycobacterial antigens in healthy, non-Mycobacterium bovis BCG-vaccinated malawian young adults. Clin Diag Lab Immunol 10: 602–611.
39. Kataoka T, Miyazaki M, Kohayashi M, Herndon DN, Suzuki F (2004) CCL17 and IL-10 as effectors that enable alternatively activated macrophages to inhibit the generation of classically activated macrophages. J Immunol 172: 1407–1413.
40. Stenger S, Modlin RL (2002) Control of Mycobacterium tuberculosis through mammalian Toll-like receptors. Curr Opin Immunol 14: 452–457.
41. Ito T, Schaller M, Hogaboam CM, Staniford TJ, Chenase SW, et al. (2007) TLR9 activation is a key event for the maintenance of a mycobacterial antigen-specific pulmonary granulomatous response. Eur J Immunol 37: 1–9.
42. Leal IS, Smedegard B, Andersen P, Appelberg R (1999) Interleukin-6 and interleukin-12 participate in induction of a type 1 protective T-cell response during vaccination with a tuberculosis subunit vaccine. Infect Immun 67: 5747–5754.
43. Young DB, Gideon HP, Wilkinson RJ (2009) Eliminating latent tuberculosis. Trends Microbiol 17: 103–108.