Differential T-cell responses to *Mycobacterium tuberculosis* in Children

By

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Certificate of Approval

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ABSTRACT

Tuberculosis continues to be a leading infectious cause of morbidity and mortality worldwide. The impact of this disease on children is particularly severe, as they are more likely to develop active disease after infection with *Mycobacterium tuberculosis* (MTB) compared to adults. In addition, active disease tends to be more severe and rapidly progressive in children, rendering this population a higher likelihood of developing disseminated disease. The differences in the natural history of tuberculosis between adults and children may be due to differences in the function of the immune system related to age.

This study examined differences in T-cell immunity among children infected with MTB. MTB antigen specific cytokine production was used as a marker of T-cell responses: TH1 type CD4+ T-cell responses were represented by IFN-γ production; TH2 type responses, by IL-5 production; and anergic responses were marked by IL-10 production. Lymphoproliferation assays were used to confirm the presence of a T-cell response to MTB antigens, and ELISA tests were used to measure cytokine production to a panel of recombinant mycobacterial antigens. T-tests were used to compare the mean cytokine production between disease groups.

No differences were found in mean MTB antigen specific IFN-γ, IL-5, or IL-10 production between children with LTBI (n=24) and active disease (n=13). This was apparently not due to poorly functioning immune systems, as the lymphoproliferative assays were positive for both disease groups. The lack of differences between T-cell responses to MTB among children has generally not been reported in the literature. The age and general state of health of our sample population may have played a role. The null findings may reflect an underrepresentation of very young children and overall healthy state of our sample population compared to other populations previously studied. Further studies are warranted in children to elucidate any immunological differences.

INTRODUCTION

Tuberculosis continues to be a leading cause of morbidity and mortality worldwide. The impact of this disease on children is particularly severe. Compared to adults, children are more likely to have active disease (TB) after exposure to *Mycobacterium tuberculosis* (MTB) rather than containing the disease in a form of latent tuberculosis infection (LTBI). Children are also more likely to have rapidly progressive disease, with a greater propensity to disseminate throughout the body (like tuberculous meningitis, miliary TB). The reason behind these differences in susceptibility to TB infection may be due to differences in the responses of the immune systems between children and adults. These potential differences in the immune system responses of children and adults as well as the extent of disease will be further examined in this study.

BACKGROUND AND SIGNIFICANCE

Tuberculosis: a global problem

It is a grim reality that TB is a leading infectious cause of mortality worldwide and contributes greatly to the morbidity of many others. Currently, approximately one third of the world's population, nearly 1.7 billion people, is infected with this contagious and largely pulmonary disease. The World Health Organization (WHO) has estimated that annually, over eight million people become sick with active TB, and nearly three million succumb to this disease[1], including 450,000 children under the age of fifteen.[2] Projecting these figures over the next twenty years, the WHO estimates that approximately one billion people will be newly infected, over 150

million will become sick with active TB, and approximately 36 million will die from this curable disease.[3]

Although since 1992, the incidence in the United States is no longer increasing, many developing countries face an increasing burden of this disease. This global resurgence results from recent changes in health care systems, demographics, and overall health status. In the United States, breakdown of public health infrastructures have led to decreased surveillance; as a result, outbreaks have occurred in various locations including hospitals, skilled care facilities, correctional facilities, and homeless shelters.[4] Increased globalization and travel have allowed the spread of TB out of the traditional TB endemic countries. The HIV/AIDS pandemic has rendered millions of people worldwide with compromised immune function, leaving them more susceptible to infectious diseases like TB. Peter Piot, former executive director of UNAIDS summarized the challenge, "The AIDS epidemic and the TB epidemic are locked in a vicious circle of mutual reinforcement. We can unlock them with a dual strategy of TB control and HIV prevention."[5]

As a result of these trends, the WHO declared TB a global emergency in 1993. The resulting increases in efforts to control TB were effective in curtailing its spread within some American urban settings.[6] Yet this is a global epidemic, and the disease continues to infect and kill an excessive amount of people worldwide.

Current strategies for TB control

Historically, the most common method worldwide of attempting to prevent TB disease is the administration of the Bacillus Calmette-Guerin (BCG) vaccine.

Although it is one of the most widely used vaccines, the efficacy against the spread of TB infection is only partial, with estimates for preventing meningeal TB ranging between 50 and 80%.[7] Meningeal and disseminated forms of TB are especially prevalent and severe in the pediatric population. Thus, despite being immunized, thousands of children continue to suffer from severe TB infections making the need for more research towards effective vaccines most imperative.

Other mechanisms to control the spread of TB infection include antimicrobial therapy for people with LTBI or active disease and directly observed therapy (DOT), both of which are endorsed by the WHO. Today, this is the most effective TB control program; it is estimated to reach more than 60% of the world's population.[8] While this method is effective in treating those with active disease, it does not adequately address the reservoirs of TB—people with LTBI. Unfortunately, this strategy may not justly serve the pediatric population because traditionally the diagnosis of active TB in children is a difficult one to make. Early signs and symptoms of disease in young children are those that are common to a variety of illnesses, including failure to thrive, weight loss, fever, and lethargy.[9] These manifestations lack the specificity needed in making timely diagnoses; as a result, the initiation of appropriate treatment may be delayed. These difficulties are compounded by the fact that, in young children, TB infection can rapidly progress to severe disease including meningeal and disseminated forms, which may cause significant morbidity and mortality despite proper antimicrobial intervention. The grave consequences discussed here highlight the need for improved strategies to control the spread of TB infection in the pediatric population.

An improved vaccine is of utmost importance in preventing the spread of this infection. One that is effective and safe for infants and young children can only be derived from a solid foundation and understanding of the immunology behind pediatric tuberculosis.

Tuberculosis: child-related risks

As mentioned above, there are nearly half a million children per year who die prematurely from TB infection. Since young children typically do not have the capability of generating a cough forceful enough to spread infectious MTB-containing respiratory droplets to others, afflicted children are likely exposed to MTB via infected adults. One theory postulates that the young children are more likely to have prolonged intimate contact with contagious caregivers, allowing for larger respiratory inoculum of MTB and therefore predisposing to more severe disease. While this theory may be plausible, it fails to explain why similar predispositions to severe disease are not consistently found amongst spouses (or other close contacts) of infected people. Another theory links the child-related susceptibilities to differences in the immature immune system compared to that of adults.

Younger children are at greater risk of developing active TB after exposure compared to adults. When examining people who recently converted to having a positive tuberculin skin test (TST), Gedde-Dahl found that those less than six years of age were more likely to develop active TB compared to adults (73% compared to 25%).[10] Not only are children more likely to develop active disease, but they are also more likely to suffer from more severe disease. Tuberculosis in young children

does not always remain within the pulmonary system as is common in adults, but often disseminates throughout the body in the forms of miliary tuberculosis and meningitis.

In 1985, the Centers for Disease Control and Prevention (CDC) found that 60% of all pediatric tuberculosis cases occurred in children under the age of five; those who had disseminated disease were much younger (median age=2 years).[11] Age appears to be a major factor in the varying extent of disease from MTB: children under the age of two are more likely to have active disease following exposure and carry a disproportionately high burden of disseminated disease. Similarly, preschool aged children also are very likely to develop active disease following MTB exposure; however, disseminated forms of disease are much less likely compared to children under age two. Interestingly, school-aged children have the lowest risk of developing active disease after MTB exposure, less so than adolescents and adults.[12] Despite these unique attributes of the young population, research around the immunology of pediatric tuberculosis has largely been understudied.

Immune systems of adults

The human body has developed three broad types of defense against any pathogens encountered: physical barriers to prevent pathogens from entering the body; the innate immune response, a hard-wired mechanism that is quickly deployed to attack any foreign substances from the beginning; and the adaptive immune response, a more sophisticated and elaborate mechanism to attack various pathogens in a highly specific manner. The latter two mechanisms often work

together to ward off infections. It is when there is a deficiency or a muted response that active disease is allowed to flourish.

Table 1. Cell source of cytokine production (partial list)[13]

Source	Cytokines produced
TH1	IFN-γ IL-2 IL-3 IL-12 TNF-alpha TNF-beta
TH2	IL-3 IL-4 IL-5 IL-10 IL-13
Macrophage	IL-1 IL-6 IL-8 IL-12 TNF-alpha

Specific to TB immunology, inappropriate immune control can turn an asymptomatic and non-infectious form of infection, latent tuberculosis infection (LTBI), to an active form of disease. While the immune response generated after infection with MTB is typically successful in containing the bacteria, it is not always capable of eliminating the pathogen. In the general population, each infected person carries a 10% lifetime risk of progressing to active disease. However, the lifetime risk may be altered based upon the individual's immunologic capabilities.

Adequate and effective TB control requires a coordinated effort between the innate and adaptive immune systems. Innate immunity and cell mediated immunity, a sub-division of the adaptive immune response comprised of various T lymphocytes including $CD4^+$T cells, will be further discussed here as they are most relevant to the understanding of the proposed study.

With respect to the innate response, the macrophage is the cell that plays an essential role in MTB control. The macrophage is a mature form of monocyte cells that has left the blood stream and awaits action in the tissues. When invading pathogens enter the body, the innate immune system is called to action to engulf and kill the foreign substances, and the macrophage is one type of cell capable of accomplishing this necessary task. Macrophages work in conjunction with cell-

mediated immune system, comprised of T-Helper 1 (TH1) $CD4^+$ cells and T-Helper 2 (TH2) $CD4^+$ cells. These cells are partly defined by the cytokines that they release. Cytokines are secreted proteins that interact with various cells of the immune system, usually via membrane receptor proteins on the cell surface. Cytokines provide various signals to their target cells which serve to coordinate the immune response. Each type of cell (TH1 and TH2) gives rise to distinct patterns of cytokine release (see Table 1). A TH1 type response is dominated by the cytokines interleukin-2 (IL-2), IL-12, and interferon-gamma (IFN-γ); a TH2 type response, IL-4, IL-5, and IL-13.[14]

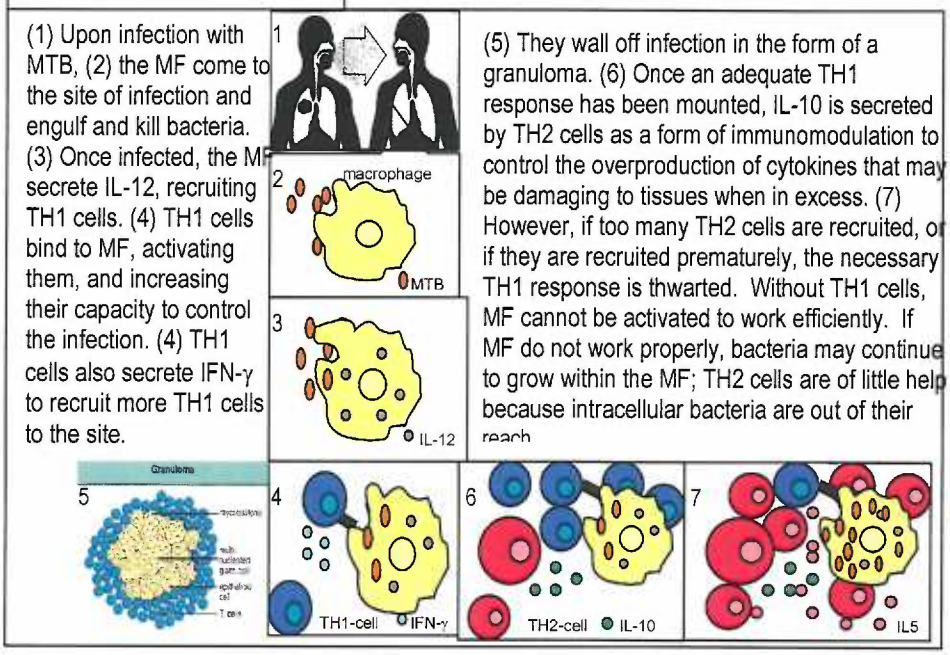
Macrophages are one of the host cells for MTB infection. Once infected, the macrophage is stimulated by the mycobacterial proteins to mount mycobacteriocidal responses. This inflammatory state is mediated by various mechanisms that induce chemical reactions or cell death. For the purposes of this study, cytokine mediated responses will be explored.

Cytokines are essential in the propagation of the innate immune system as well as the promotion of appropriate T-cell functions. Infection of a macrophage will induce the production of interleukin-12 (IL-12), initiating interferon gamma (IFN-γ) production.[15] The latter cytokine, produced by TH1 cells, activates macrophages, allowing them to better inhibit MTB growth via the above-mentioned mechanisms. IFN-γ is a key cytokine for successful containment of MTB. Humans with defective IFN-γ receptors or diminished overall production are prone to infection with mycobacterial pathogens, including *M. tuberculosis.*[16] Mice with deficient or absent IFN-γ production have also been found to be extremely susceptible to active TB.[17]

However, IFN-γ is necessary but not sufficient for the complete containment of MTB.[18]

Once the TH1 T-cells are called to the site of infection to develop a long-standing adaptive immune response, another cytokine, interleukin-10 (IL-10) is produced.

Figure 1: TB infection



Although it is produced by TH2 cells, its ability to inhibit CD-4^{+} cell responses confers IL-10 an immunomodulatory role under normal conditions. It signals to the body that TH1 T-cells have addressed the MTB infection and works to prevent over-stimulation. However, a balance of cytokines is needed. If IL-10 is overproduced, the result may be the unwanted effect of downregulating IL-12 production, and subsequent IFN-γ production, thus prematurely thwarting adequate containment. A state of non-responsiveness results, commonly referred to as an anergic response.[19] In attempts to evade containment, the MTB bacterium has developed a mechanism to induce the production of IL-10 from infected macrophages, thus encouraging an anti-inflammatory, anergic state.

Another subtype of $CD4^+$ T-cells are type 2 Helper T-cells (TH2), defined by the production of various cytokines (interleukin-4, interleukin-5, and interleukin-13). Specific to MTB immunity, TH2 cells work in drastically different ways compared to the TH1 cells. The former have little to do with macrophage cells, and are unable to reach any bacteria that have been engulfed within a macrophage. If a TH2 response dominates, the unchecked MTB growth within the macrophage cell would cause much tissue destruction, resulting in significant morbidity and possible mortality. However, TH2 cells do have an important role in MTB immunity, it is simply not the most appropriate response in the initial phases of controlling infection. TH2-type cells are capable of promoting B-cell immunity and antibody production, which is not relevant for the initial TB control. Additionally, TH2-type responses downregulate the immune response.

Immune systems of children

Smith, Jacobs and Wilson have examined the developing immune system and have found that complete development of the immune system is not reached until five years of age.[20] Namely, they have found general differences in monocyte and macrophage recruitment and activation, and T-cell stimulation in young children compared to adults. Thus, relevant to MTB immunity, the immature immune system may control TB infection differently than the adult immune system, rendering children to increased risk of active tuberculosis.

With respect to monocytes, decreased recruitment of these cells occurs in response to stimulation among neonates. Monocytes are thought to reach full adult-

level capabilities by six to ten years of age[21]. While the overall number and actual antimicrobial activity of these monocytes are generally thought to be equivalent between neonatal and adult populations, any age-related actions specifically against MTB are not known.

The macrophages within the alveoli of the lungs are typically the first cells to be infected with MTB, and are given the first opportunity to contain the bacteria. However, this is not fully seized in young children, as these alveolar macrophages are thought to be less adept at clearing and killing bacteria. Evidence for the deficiencies in alveolar macrophage function, including impaired chemotaxis (use of chemical proteins to signal the recruitment of cells), phagocytosis (ingestion by cells), and intracellular killing of pathogens, during the neonatal period is derived from animal data.[22,23] Additionally, the ability of macrophages from young children to respond to MTB infection with appropriate production of and response to various cytokines may be diminished compared to similar cells from adults.[24] One common qualitative difference found between the immune responses of children compared to adults is the diminished production of IL-12.[25] These deficiencies pose grave consequences, as the successful enactment of the TH-1 response depends on the activation and stimulation of macrophages.

Additional immunologic differences may be found in the baseline cytokine milieu. Studies on animals and humans suggest a bias toward a TH2 profile in the neonatal immune system under neutral conditions and upon antigenic stimulation.[26,27] The mechanism behind the TH2 bias is unclear and is likely multifactorial, with contributions from the TH2 cytokine milieu during pregnancy as

well as altered interactions with different cytokines and effector cells. TH1 cytokine production is dampened during pregnancy, which directly or indirectly favors TH2 cytokines.[28] The endogenous cytokine milieu has tremendous influence on the subsequent priming of T-cells and subset (TH1 or TH2) development. Animal and human data suggest the relative levels of IL-12 and IL-4 (secreted by TH2 cells) determine the overall phenotype of the T-cells.[29,30] Although a TH2 bias may exist, the ability to produce IFN-γ, thus generating a TH1 response, remains and increases with age. However, this TH2 bias may be a major factor in the more severe manifestations of TB in children.

Summary

Children are more susceptible to active disease after MTB infection. In addition, the manifestation of MTB infection is often more severe as the bacteria have an increased tendency spread out of the pulmonary system and disseminate. There are general differences in the adult and child immune systems, most notably a potential for TH2 bias by the very immature immune system. By further studying the specific responses to MTB infection in children, greater knowledge behind the immunology of pediatric tuberculosis will be learned. A better understanding of the immunologic deficiencies related to active TB in young children can aid in the development of a more targeted, effective, and much needed vaccine against tuberculosis.

Study Objectives

To elucidate the immunologic differences between children and adults, this study seeks to answer the question: Does the magnitude and type of immune response differ among children with active TB compared to those with latent TB infection (LTBI), and do these differences mirror those seen in adults. The underlying hypothesis tested in this study is that the magnitude and type of MTB-specific T-cell responses in children ≤18 years of age differ among those with active TB infection and those with LTBI. Overall, these patterns of immune response are different than those expressed by adults who have been exposed to MTB.

The objectives of this study were to:

1. Use the data collected by the Pediatric TB Study to generate a central database of demographic and immunologic ELISA data.
2. Determine if children with active TB disease were more likely to mount stronger TH2-type $CD4^+$ response and if children with LTBI were more likely to have a stronger TH1-type $CD4^+$ response.
3. Make qualitative inferences between the findings from this pediatric sample and the MTB antigen specific T cell responses found in adults.

RESEARCH DESIGN AND METHODS

Design

The proposed secondary data analysis utilizes a cross-sectional design, examining the correlation between types of immune responses and outcomes of MTB infection via the analysis of MTB-specific antigen responses from T cells. These

laboratory data approximate the individual immune responses of subjects at the time of blood sample collection. The rationale for this type of study design is influenced by practical limitations. Although a prospective cohort study design may be more appropriate to assess causality, this would not be feasible due to the extremely large sample of children needed to observe a sufficient number of pediatric tuberculosis cases, given that the United States has relatively low incidence of disease. A case-control study design cannot easily be used to answer this specific question, as there are no retrospective details to be assessed. However, if the study sought to examine the effect of a previous exposure (like BCG vaccination) and how it relates to current disease status, this design would be appropriate. A randomized controlled trial assessing immune response after infection with MTB versus another bacteria or placebo would not be possible due to the unethical nature of intentionally exposing subjects to MTB or other pathogens. The cross sectional study design is most appropriate manner of assessing the type and magnitude of immune response to TB at the time of clinical presentation.

The length of time between initial MTB exposure and presentation to medical attention can only be estimated for some subjects. The varying lengths of time since exposure may affect the magnitude of the cytokine response, but the overall nature of the immune response should not be affected. Since all subjects in the pediatric cohort are known to have been exposed to MTB, their $CD4^+$ T cells have been primed by MTB antigens and have differentiated into TH1 or TH2 effector cells. When mycobacterial antigens were added to the peripheral blood mononuclear cells (PBMC) derived from whole blood samples, the production of IFN-γ, IL-5 and IL-10

cytokines was stimulated from previously primed T-cells. The measured cytokine response illustrate the immune response to MTB.

Sample population

The data used comes from a prior pilot study conducted by Dr. Ann Loeffler and Dr. Deborah Lewinsohn, in which 52 children (ages 0-17) previously exposed to MTB were enrolled based on the following criteria. Pediatric subjects were included based upon: being ≤18 years of age and previously healthy, and documented to have a positive tuberculin skin test (TST, defined below). Exclusion criteria were based upon an immunocompromised health status (defined by congenital immunodeficiencies, uncontrolled diabetes mellitus, nephrotic syndrome, HIV seropositive status, presence of malignancy, or having any condition treated with steroids or other immuno-suppressants). Dr. Ann Loeffler enrolled study participants from Children's Hospital (Oakland, CA) or from contact investigation of other active TB cases. The institution had Institutional Review Board (IRB) approval for patient enrollment and the study. All unique identification information for enrolled subjects was removed from the dataset used for secondary analysis. By utilized de-identified information, the proposed study was not deemed to require IRB approval.

Data used in this analysis are derived from 37 subjects from the pilot study who were found to have LTBI or active disease. Control subjects were not included because of uncertainty around the possibility that many, but not all, may have been household contacts of people with active TB. Household contacts have likely been exposed and may have mounted an immune response, whereas controls in the

traditional sense have a much lower likelihood of ever being exposed to TB, reflecting a truly naïve immune system with respect to MTB infection. Since the distinction between household contacts and true controls could not be deciphered, the research question focused on the immunologic differences between children with active and latent forms of infection.

The pediatric study participants were diagnosed with either LTBI or active TB infection based on clinical criteria, additional radiographic tests, and documented positive TST results as defined by the criteria set by the American Academy of Pediatrics (AAP). TST guidelines include: induration ≥5mm is a positive result for household contacts of adult cases of active TB, induration ≥10mm is a positive result for children ≤ 4 years of age or in any child in high-risk exposure groups (increased exposure to others with a high prevalence of TB).

By definition, those with the LTBI diagnosis had no clinical manifestations of disease and a chest radiograph negative for signs of infection, but did have a positive TST, indicating previous exposure or previous BCG vaccination. They were largely found as a result of contact investigations for others with active disease. Subjects found to have active disease were symptomatic, had a chest radiograph consistent with active TB infection, and a positive TST. Traditionally, when making the diagnosis of active TB in adults, tests are done to isolate and confirm the bacterial agent. Such tests like blood cultures or gastric aspirates were performed, but not used as enrollment criteria because the lower bacillary load associated with pediatric MTB infection contributes to the relative insensitivity of these bacterial cultures, diminishing the overall yield of isolation tests. Those who were found to have active

disease were started on appropriate therapy, and those with LTBI were recommended to initiate prophylactic therapy. Information on the clinical course of the subjects was updated throughout their duration of therapy.

Data Collection of Primary Study

Laboratory data collection: Once entry criteria were met and informed consent was obtained, each subject's disease status was determined and demographic data were collected. The disease groups included active TB, latent infection, and a control group. A blood sample was obtained and peripheral blood mononuclear cells (PBMC), which include the lymphocytes under study, were isolated and cultured in triplicate in the presence of an antigen from from a panel of mycobacterial antigens, or positive control (tetanus toxoid) to which all subjects are assumed to have been exposed at some time (through routine childhood immunization). The panel included recombinant antigens known to be recognized by adults with either LTBI or active disease, including: ESAT-6, CFP-10, Ag85B, and 38KD Ag. Of note, ESAT-6 and CFP-10 are two antigens that have demonstrated high sensitivity and specificity for infection with MTB and *Mycobacterium bovis.* These antigens are encoded within a gene that is not a part of the BCG vaccine, therefore minimizing the potential bias that previous BCG vaccination may introduce.[31]

Lymphoproliferative assays were performed to evaluate whether the subject's $CD4^+$ T-cells were being stimulated by the added antigen, and responding appropriately by proliferating. These assays are useful in detecting a crude immunologic response to the added antigens. Although each response is antigen-

specific, no specific information about the type of immune response (i.e. TH1 versus TH2) can be gleaned from these tests. However, they do confirm the presence or absence of primed antigen-specific T cell response; if proliferation is present, this indicates previous exposure to MTB and serves as a semi-quantitative measure of the strength of the response. Lymphoproliferation was assessed by adding ^{3}H thymidine to PBMCs that had been cultured for four days. Cells that are actively proliferating incorporate this labeled thymidine into their DNA. The amount of ^{3}H thymidine incorporated into PBMCs was then measured to quantitate proliferation.

Enzyme-linked immunosorbent assay (ELISA) tests were used to qualitatively assess the type of immune response mounted upon addition of recombinant antigens. These tests yield information about the type and magnitude of the immune response by measuring the concentration of cytokine produced by lymphocytes after the addition of recombinant antigens. ELISA tests were also performed on supernatants of PBMC alone (without addition of antigens) to assess background media production of each of the three cytokines ("media-only measurements"). The remainder of the samples had mycobacterial antigens added to the wells. After culturing the blood with the mycobacterial specific antigens for four days, the supernatants were collected and tested for the concentration of antigen specific production of cytokines (IFN-γ, IL-5, IL-10) via ELISA tests.

Methods of Secondary Analysis

Data Assessment: As mentioned above, the production of three specific cytokines (IFN-γ, IL-5, and IL-10) by $CD4^{+}$ T cells have been examined. Each of these three

cytokines is primarily produced by different classes of $CD4^+$ T cells, representing three different types of immune responses. Namely, IFN-γ production reflects a TH1 response; IL-5 represents a TH2 response; and IL-10, an immunomodulatory or anergic response. The presence of increased levels of each cytokine in the supernatant indicates the presence of specific subtypes $CD4^+$ T cells that have previously been primed to the specific antigen presented. The amount of cytokine measured is proportional to the magnitude of this primed $CD4^+$ T cell response; a higher measurement reflects a greater proportion of primed $CD4^+$ T cells and/or increased cytokine production per primed cell. For example, the presence of IFN-γ detected by ELISA in PBMCs cultured with a TB-specific antigen reflects the presence of TH-1 type CD 4^+ T cells specific for that TB-related antigen. Likewise, the presence of IL-5 detected in each culture reflects the presence of the subject's TH-2 type CD 4^+ T cells for the specific TB antigen; and the IL-10 detected is a marker of an anergic response (see Table 1). In addition, each subject had a certain background level of cytokine production when PBMCs were incubated with media alone (without the specific addition of MTB-related antigens); these background values ("media-only measurements") were measured at the same time as the antigen-induced measurements.

Data Organization: Wells of PBMC were cultured with each antigen (or media alone) in triplicate to minimize error. The data included in the dataset used for this analysis reflects the mean of the triplicate measurements. Using the raw mean data from the ELISA tests, the values were recoded into net values, reflecting antigen-specific

responses (see Table 2). For each antigen-cytokine combination, the value of the background cytokine production (media-only) was subtracted from the value of the antigen-specific cytokine response. Doing this yielded some negative values, thought to be a result of laboratory measurement errors.

Table 2: Example of dataset showing media, antigen-induced, and antigen-specific measurements.

	Media IFN-γ	**TT IFN-γ**	**Net TT- IFN-γ**	**Media IL-5**	**TT IL-5**	**Net TT- IL-5**	**Media IL-10**	**TT IL-10**	**Net TT-IL-10**
$Subject_{(n)}$	$A_{(n)}$	$B_{(n)}$	$B_{(n)}$-$A_{(n)}$	$C_{(n)}$	$D_{(n)}$	$D_{(n)}$-$C_{(n)}$	$E_{(n)}$	$F_{(n)}$	$F_{(n)}$-$E_{(n)}$
$Subject_{(n+1)}$	$A_{(n+1)}$	$B_{(n+1)}$	$B_{(n+1)}$-$A_{(n+1)}$	$C_{(n+1)}$	$D_{(n+1)}$	$D_{(n+1)}$-$C_{(n+1)}$	$E_{(n+1)}$	$F_{(n+1)}$	$F_{(n+1)}$-$E_{(n+1)}$
$Subject_{(n+...+1)}$	...	...	...	...	...	...	...	...	...

TT= tetanus toxoid, positive control.

The first goal was to compare the mean cytokine responses to recombinant antigens between disease groups. Secondly, inferences were made about the actual type of immune response represented by the cytokine production. Individual subjects were not given a phenotype, rather one disease group was labeled as having a stronger phenotype in relation to the other. Each cytokine was assessed separately, and no analyses were done to assess the differences between cytokines. To examine the actual type of immune response mounted by each disease group, the mean results of the ELISA assays were categorized into phenotypes according to the table below (see Table 3). As a marker for a TH1-type of $CD4^+$ T-cell response, IFN-γ was used. A significantly higher group mean IFN-γ value in response to the recombinant antigens correlates with a TH-1 dominant type of immune response. To detect a TH2-type of response, IL-5 was used. Therefore, if one disease group mounts a significantly higher mean antigen-specific IL-5 response, that disease group will be categorized as having a stronger TH2-type of immune response for that

Table 3: Phenotype of immune response by cytokine response

Phenotype:	cytokine response
TH-1	IFN-γ
TH-2	IL-5
Anergic	IL-10

disease groups in a parametric manner. However, if a non-normal distribution was detected, the use of parametric tests would not be appropriate as they rely upon certain assumptions and characteristics of the dataset. These criteria include normally distributed results, a homogeneous variance of each group, and a sample size large enough to represent the population.[32] Thus, in these situations non-parametric tests were used, namely the Wilcoxon rank score to compare the median values between the two disease groups. An alpha of 0.05 was used for all tests.

Data Management

Primary Data: Each clinical site investigator was responsible for compiling data for local subjects enrolled and maintaining a secure Microsoft Access file. Unique identifiers for use in the registry were assigned to each subject, revealing only the study site of origin. The clinical investigator kept the codes linking subjects to unique identifiers separately.

Secondary Data: The demographic information of subjects previously enrolled in the Pediatric TB Study were initially compiled using Epi Info (Center for Disease Control and Prevention, version 6) and Microsoft Access (Windows 1998 version); the pediatric immunologic data was stored in a Microsoft Excel database. The information relevant to this study was imported from the respective datasets into a central database using JMP software (SAS Institute Inc, version 4.0.4). As this study focuses on the initial immune response after exposure to MTB, only data from the subjects' first clinical visit were used.

When creating the central database, the information from the Access database was electronically imported into JMP in order to minimize any error introduced by manual data entry. Once in JMP, the accuracy and completeness were checked by comparing relevant variables against each other, like disease group and PPD size. Missing data were found for a minimal number of subjects; this information was noted, but no alterations were made in the final analysis.

Effect size determination

Effect size estimates for this fixed sample size of 13 subjects with active disease and 24 subjects with LTBI were calculated using PASS software (NCSS & PASS, 2003) to assess the effect size that this study is capable of detecting given standard power requirements. Using a two-sided T-test, and a probability for type I error fixed at a level of α=0.05 revealed the following range of power and beta values. For IL-5 and IL-10 responses, these estimates were obtained in order to detect a response difference between 15.0 and 25.0 ug/mL, a value that is deemed clinically meaningful by experts in the field. The standard deviation used in this estimate is 20 to 35 (see Table 4 and Figure 2).

Figure 2: Power versus standard deviation for IL5 and IL10 responses in pediatric group

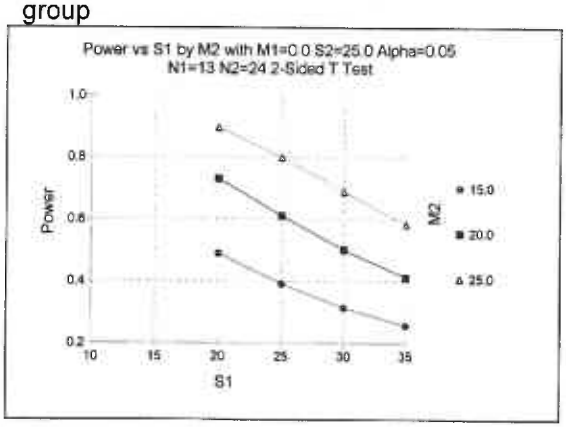


Table 4: Power and beta calculations for IL-5 and IL-10 responses in pediatric group.

Power	N1	N2	Alpha	Beta	Mean1	Mean2	Std Dev1	Std Dev2
0.56277	13	24	0.05	0.43723	0.0	15.0	20.0	20.0
0.39546	13	24	0.05	0.60454	0.0	15.0	25.0	25.0
0.29230	13	24	0.05	0.70770	0.0	15.0	30.0	30.0
0.22756	13	24	0.05	0.77244	0.0	15.0	35.0	35.0
0.80604	13	24	0.05	0.19396	0.0	20.0	20.0	20.0

0.61757	13	24	0.05	0.38243	0.0	20.0	25.0	25.0
0.46927	13	24	0.05	0.53073	0.0	20.0	30.0	30.0
0.36481	13	24	0.05	0.63519	0.0	20.0	35.0	35.0
0.94163	13	24	0.05	0.05837	0.0	25.0	20.0	20.0
0.80604	13	24	0.05	0.19396	0.0	25.0	25.0	25.0
0.65290	13	24	0.05	0.34710	0.0	25.0	30.0	30.0
0.52282	13	24	0.05	0.47718	0.0	25.0	35.0	35.0

Using an acceptable power of 80.6%, this group sample size of 13 active TB subjects and 24 LTBI subjects can detect a difference of 25.0 ug/mL, and the standard deviation of 25.0; the β value associated with this is 0.19.

Effect size estimates for IFN-γ responses were calculated based on a greater difference in mean response because of the fact that T-cells produce more IFN-γ on a per cell basis than they do IL-5 or IL-10 (see Table 5 and Figure 3). Experts in the field deem a response difference near 100 ug/mL clinically meaningful.

Figure 3: Power versus standard deviation for IFN-γ responses in pediatric group

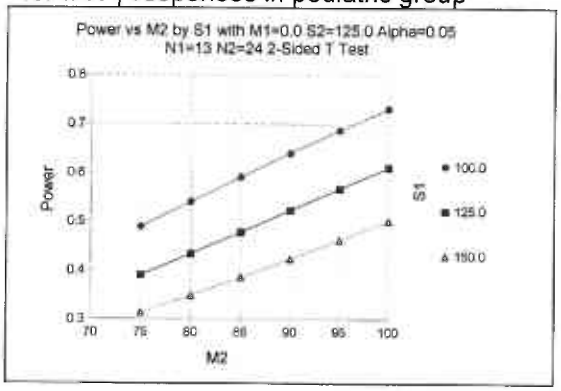


Table 5: Power and beta calculations IFN-γ responses in pediatric group.

Power	N1	N2	Alpha	Beta	Mean1	Mean2	Std Dev1	Std Dev2
0.56277	13	24	0.05	0.43723	0.0	75.0	100.0	100.0
0.39546	13	24	0.05	0.60454	0.0	75.0	125.0	125.0
0.29230	13	24	0.05	0.70770	0.0	75.0	150.0	150.0
0.61757	13	24	0.05	0.38243	0.0	80.0	100.0	100.0
0.43947	13	24	0.05	0.56053	0.0	80.0	125.0	125.0
0.32530	13	24	0.05	0.67470	0.0	80.0	150.0	150.0
0.67010	13	24	0.05	0.32990	0.0	85.0	100.0	100.0
0.48425	13	24	0.05	0.51575	0.0	85.0	125.0	125.0
0.35978	13	24	0.05	0.64022	0.0	85.0	150.0	150.0
0.71947	13	24	0.05	0.28053	0.0	90.0	100.0	100.0
0.52924	13	24	0.05	0.47076	0.0	90.0	125.0	125.0
0.39546	13	24	0.05	0.60454	0.0	90.0	150.0	150.0
0.76496	13	24	0.05	0.23504	0.0	95.0	100.0	100.0
0.57387	13	24	0.05	0.42613	0.0	95.0	125.0	125.0
0.43206	13	24	0.05	0.56794	0.0	95.0	150.0	150.0
0.80604	13	24	0.05	0.19396	0.0	100.0	100.0	100.0
0.61757	13	24	0.05	0.38243	0.0	100.0	125.0	125.0
0.46927	13	24	0.05	0.53073	0.0	100.0	150.0	150.0

Using a fixed group sample size of 13 active TB subjects and 24 LTBI subjects, this study has adequate power of 80.6% to detect a difference of 100.0 ug/mL, with a the standard deviation of 100.0; the β value associated with this is 0.19.

RESULTS

Patient Characteristics

A total of 37 children participated in this study (Table 6a). All were enrolled from the Children's Hospital Oakland, although only 17 (46%) were born in the United States. The remainder of participants was from Central and South America (n=8, 22%), Asia (n=6, 16%), Africa (n=3, 8%), Eastern Europe (n=2, 5%), and New Zealand (n=1, 3%). Study subjects ranged from one month of age to 18 years, with a slight preponderance of younger children; the mean and median ages of the entire sample were 7.7 and 7.0 years, respectively. The gender makeup of all participants was essentially equivalent, with 19 males and 18 females overall.

The mean age for the LTBI group was 8.5 years and 6.3 years for the active disease group (t- test, p=0.290). The gender distribution was also not significantly different, with 45.8% females in the LTBI group and 53.8% females in the active disease group (two-tail Fisher's exact test, p=0.737).

Of the 13 subjects (35.1%) with active tuberculosis, the majority (n=11, 84.6%) had localized disease while two subjects (15.4%) had active disease manifested as a disseminated form, miliary tuberculosis. The remaining subjects were diagnosed with LTBI (n=24, 64.9%).

TSTs were performed on all subjects, although results were only recorded for 33 of the 37 subjects. As expected from both diseased categories, there were no subjects that had a TST less than 5 mm of induration. Along with a preponderance of foreign-born study participants, nearly half of the sample (46%) had received the BCG vaccine during childhood. The vaccination history was unknown for three subjects (8%). Based on disease group, equal proportions (46%) of active cases and LTBI cases had prior BCG vaccination.

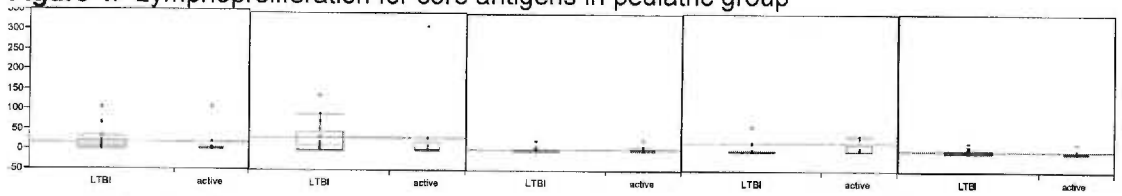
Table 6. Characteristics of pediatric study participants, n=37

CHARACTERISTIC	LTBI	ACTIVE DISEASE	P-VALUE
Age (years)			p=0.290
Range	0-17	0-18	
Mean	8.5	6.3	
Median	9.0	2.0	
25th percentile	4.25	1.0	
75th percentile	12.75	14.5	
Gender	no. (% LTBI)	no. (% actives)	p=0.737
Male	13 (54.2)	6 (46.2)	
Female	11 (45.8)	7 (53.6)	
Country of Origin	no. (% LTBI)	no. (% actives)	p=0.490
United States	11 (45.8)	6 (46.2)	
Central/South America	7 (29.2)	1 (7.7)	
Eastern Europe	1 (4.2)	1 (7.7)	
Africa	2 (8.3)	1 (7.7)	
Asia	3 (12.5)	3 (23.0)	
New Zealand	0 (0)	1 (7.7)	
Clinical Manifestation of Disease	no. (% total)	no. (% total)	no. (% active)
Active Tuberculosis	n/a	13 (35.1)	
Localized disease no. (% of active)			11 (84.6)
Pulmonary			9 (69.2)
Pleural			1 (7.7)
Scrofula			1 (7.7)
Disseminated disease no. (% of active)			2 (15.4)
Latent Tuberculosis Infection no. (% total)	24 (64.9)	n/a	
TST Result	no. (% LTBI)	no. (% active)	p=0.186
0 - 5 mm induration	0 (0)	0 (0)	
6 - 10 mm induration	7 (29.2)	2 (15.4)	
11-15 mm induration	8 (33.3)	6 (46.2)	
> 15 mm induration	8 (33.3)	2 (15.4)	
Not reported	1 (4.2)	3 (23.1)	
BCG Vaccination History	no. (% LTBI)	no. (% active)	p=0.998
Yes	11 (45.8)	6 (46.2)	
No	11 (45.8)	6 (46.2)	
Unknown	2 (8.3)	1 (7.7)	

Lymphoproliferative Assay Data:

Cell proliferation in response to added antigens were measured to assess the presence and strength of the overall antigen-specific $CD4^+$ T cell response. When comparing the median values of immune responses between disease groups, no significant differences were found (Figure 4 and Table 7). Thus, neither the LTBI nor active disease group demonstrated differential ability to mount a general immune response to any of the recombinant antigens tested.

Figure 4: Lymphoproliferation for core antigens in pediatric group



Tetanus Toxin p=0.097 | CSP p=0.233 | CFP-10 p=0.111 | Esat6 p=0.599 | α-Crystalline Protein p=0.679

By infection group, in picograms

Table 7. Summary of Lymphoproliferative data for all antigens in the pediatric population

Antigen	Median Values (Interquartile Range) LTBI	Active	p-values (Wilcoxon, 1-way, χ^2 approx)
TT	8.50 (4.82, 21.35)	3.45 (1.38, 6.77)	0.097
CSP	14.50 (2.40, 49.43)	4.50 (1.55, 23.05)	0.233
Mtb39	1.25 (0.70, 5.68)	1.60 (0.75, 8.30)	0.622
CFP-10	0.95 (0.60, 2.23)	1.60 (1.00, 6.75)	0.111
Ag85b	1.00 (0.70, 1.38)	1.20 (0.75, 2.20)	0.416
Mtb32a (c-term)	1.00 (0.60, 2.18)	1.10 (0.90, 1.40)	0.738
Mtb8.4	1.55 (0.73, 5.75)	0.80 (0.70, 2.25)	0.286
Mtb9.9	1.85 (1.23, 3.50)	1.50 (0.90, 2.15)	0.407
Esat6	1.85 (1.33, 3.88)	2.50 (1.15, 21.10)	0.599
38kd	1.15 (0.70, 1.78)	1.30 (0.95, 2.10)	0.390
α-Crystalline Protein	1.20 (0.63, 4.45)	1.10 (0.95, 1.95)	0.679
Ra1	0.87 (0.63, 1.75)	1.00 (0.80, 1.30)	0.924
Ra11	1.25 (0.57, 2.23)	1.00 (0.70, 2.55)	0.924
Mtb32a (n-term)	0.85 (0.60, 1.40)	0.80 (0.40, 1.00)	0.219
Mtb32a	1.30 (0.63, 3.10)	0.90 (0.55, 1.55)	0.331
Mtb39/CFP-10	1.80 (0.73, 8.70)	3.70 (1.25, 19.65)	0.176
Mtb39/Mtb9.9/CFP-10	3.10 (1.50, 15.50)	5.95 (0.93, 29.33)	0.689
Mtb39/Mtb9.9/CFP-10/Mtb8.4	2.45 (1.13, 15.73)	6.40 (1.00, 18.50)	0.645
Mtb39/Mtb9.9/Mtb8.4/Ag85b	2.80 (1.23, 24.63)	6.30 (1.50, 27.75)	0.567
MSL	0.85 (0.70, 4.78)	0.85 (0.55, 19.05)	0.630
Mtb32a/Mtb39	1.15 (0.70, 3.43)	2.55 (0.85, 5.35)	0.482
α-Cryst. Prot/Mtb8.4/Mtb9.9	1.65 (0.68, 8.73)	0.75 (0.50, 6.73)	0.530

ELISA Data:

IFN-γ production to MTB antigens, a marker of TH1 immunity: When no antigens were added, there were significant differences between the median level of background IFN-γ production between LTBI and active disease groups (figure 5a). Specifically, children with LTBI were found to have greater baseline production of IFN-γ compared to children with active disease (p=0.025). However, when recombinant antigens were added and MTB antigen-specific IFN-γ responses assessed, no significant differences were found among mean responses between the disease groups (figure 5b and table 10).

Figure 5a: Background IFN-γ production in pediatric group, p=0.025

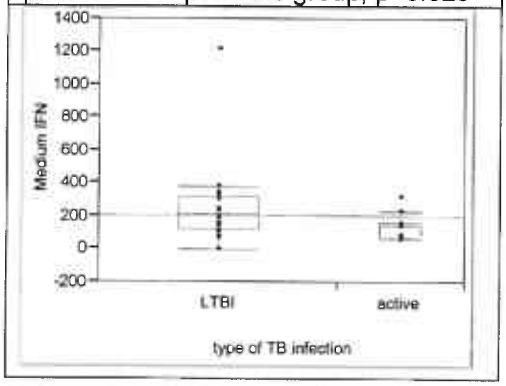
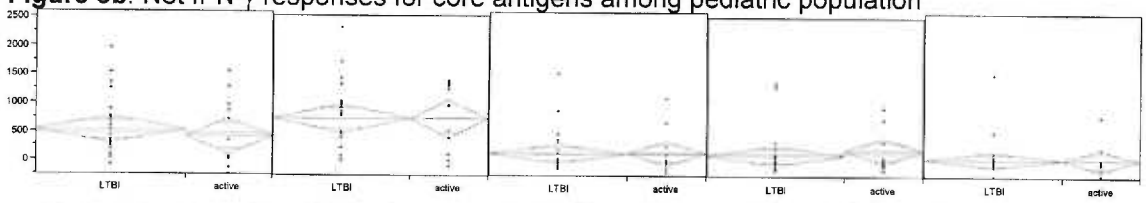


Figure 5b: Net IFN-γ responses for core antigens among pediatric population

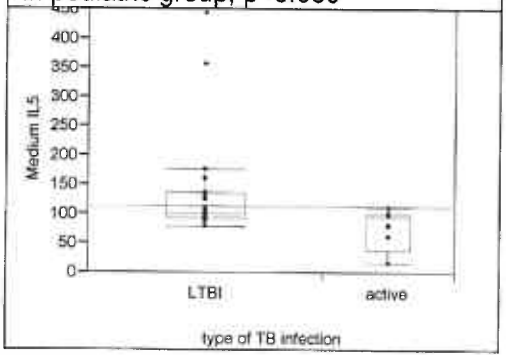


Net Tetanus Toxin IFN p=0.600 | Net CSP-IFN p=0.962 | Net CFP-10-IFN p=0.912 | Net Esat-6-IFN p=0.535 | Net α-Cryst. Protein-IFN p=0.925

By infection group, in picograms

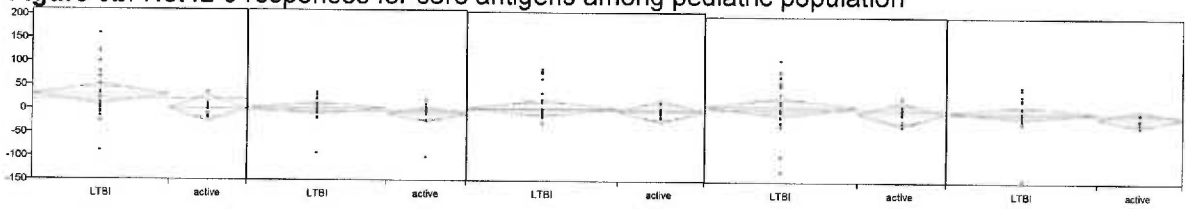
IL-5 production to MTB antigens, a marker of TH2 immunity: The background level of IL-5 production was significantly different between the pediatric disease groups (Figure 6a); children with LTBI had greater median background production of IL-5 compared to the children with

Figure 6a: Background IL-5 production in pediatric group, p=0.030



active disease (p=0.030). When examining the antigen-specific production of IL-5, no significant differences were found in the mean IL-5 production between disease groups (Figure 6b and Table 8).

Figure 6b: Net IL-5 responses for core antigens among pediatric population



Net TT-IL5 p=0.098 | Net CSP-IL5, p=0.223 | Net CFP-10-IL5 p=0.643 | Net Esat6-IL5 p=0.365 | Net α-Crystalline Protein -IL5 p=0.293

By infection group, in picograms

IL-10 production to MTB antigens, a marker of anergy: The median background production of IL-10 did not significantly differ between the pediatric disease groups (figure 7a). The ELISA results of antigen-specific IL-10 production also failed to reveal any statistically significant differences among the mean responses between pediatric disease groups (Figure 7b and Table 8).

Figure 7a: Background IL-10 production in pediatric group, p=0.148

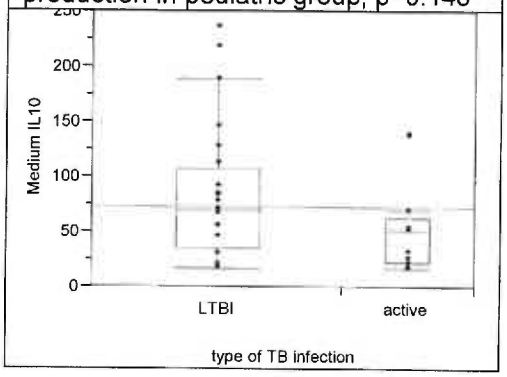
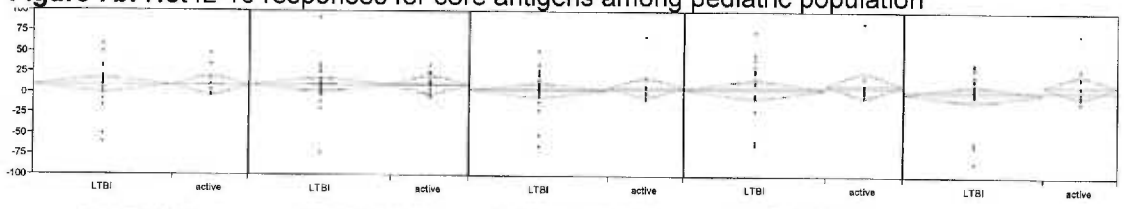


Figure 7b: Net IL-10 responses for core antigens among pediatric population



Net TT-IL10 p=0.965 | Net CSP-IL10 p=0.900 | Net CFP-10-IL10 p=0.652 | Net Esat6-IL10 p=0.608 | Net α-Cryst. Protein-IL10, p=0.290

By infection group, in picograms

Table 8: Antigen-specific cytokine responses for all antigens (n=37)

ANTIGEN & CYTOKINES	MEAN VALUES (95% CI)		P-VALUES (T-TEST)
	LTBI N=24*	ACTIVE N=13*	
	*UNLESS OTHERWISE INDICATED		
TT			
IL-5	30.69 (9.9, 51.48) n=23	1.68 (-25.97, 29.34)	0.098
IL-10	9.58 (-0.10, 19.26)	9.22 (-393, 22.38)	0.965
IFN	529.74 (292.58, 766.90) n=23	426.95 (111.51, 742.40)	0.600
PHA			
IL-5	68.22 (28.11, 108.33) n=23	11.41 (-41.94, 64.77)	0.093
IL-10	39.79 (19.76, 59.81)	19.38 (-7.83, 46.59)	0.228
IFN	775.17 (499.33, 1051.0) n=23	516.93 (150.02, 883.8)	0.261
CSP			
IL-5	2.24 (-9.53, 14.02)	-9.72 (-25.39, 5.95)	0.223
IL-10	10.27 (0.693, 19.84)	11.27 (-1.74, 24.28)	0.900
IFN	773.56 (516.93, 1030.2) n=23	763.35 (421.99, 1104.7)	0.984
Mtb39			
IL-5	2.40 (-10.78, 15.59) n=23	0.95 (-16.59, 18.49)	0.893
IL-10	2.47 (-7.60, 12.53)	7.66 (-6.02, 21.34)	0.539
IFN	83.00 (-3.8, 169.84) n=23	14.09 (-101.4, 129.60)	0.339
CFP-10			
IL-5	5.89 (-11.13, 22.920 n=23	-0.62 (-23.27, 22.02)	0.643
IL-10	4.82 (-4.78, 14.42)	8.45 (-4.59, 21.49)	0.652
IFN	134.13 (-28.08, 296.34) n=23	148.91 (-66.86, 364.67)	0.912
Ag85b			
IL-5	7.13 (-5.99, 20.24) n=23	-1.88 (-19.33, 15.56)	0.401
IL-10	1.93 (-11.57, 15.42)	10.24 (-8.09, 28.57)	0.463
IFN	-26.83 (-120.8, 67.15) n=23	25.82 (-99.2, 150.82)	0.499
Mtb32a (c-)			
IL-5	8.32 (-5.97, 22.60) n=23	2.0 (-16.98, 21.02)	0.594
IL-10	3.58 (-7.97, 15.12)	7.28 (-8.41, 22.97)	0.702
IFN	26.70 (-78.19, 131.58) n=23	58.38 (-81.13, 197.88)	0.715
Mtb8.4			
IL-5	5.68 (-8.59, 19.96)	0.92 (-18.07, 19.90)	0.686
IL-10	13.11 (1.19, 25.03)	15.16 (-1.03, 31.36)	0.837
IFN	93.65 (-59.88, 247.18) n=23	194.96 (-9.25, 399.17)	0.426
Mtb9.9			
IL-5	9.15 (-4.41, 22.71) n=23	-6.58 (-24.62, 11.45)	0.166
IL-10	0.83 (-9.62, 11.28)	9.64 (-4.56, 23.84)	0.317
IFN	22.27 (-64.8, 109.31) n=23	-5.02 (-120.80, 110.74)	0.704
Esat6			
IL-5	15.15 (-4.56, 34.87) n=23	0.32 (-25.90, 26.55)	0.365
IL-10	6.62 (-5.74, 18.97)	11.93 (-4.86, 28.72)	0.608
IFN	138.00 (-37.05, 313.05) n=23	227.81 (-5.03, 460.64)	0.535
38kd			
IL-5	17.88 (-1.35, 37.11) n=23	1.47 (-24.11, 27.05)	0.305
IL-10	3.08 (-9.44, 15.59)	11.41 (-5.59, 28.41)	0.428
IFN	-36.43 (-173.5, 100.62) n=23	108.35 (-73.9, 290.65)	0.206

α Cryst.Prot.			
IL-5	4.80 (-8.47, 18.08) n=23	-6.82 (-24.47, 10.84)	0.293
IL-10	1.45 (-9.72, 12.63)	11.43 (-3.75, 26.61)	0.290
IFN	62.65 (-74.6, 199.9) n=23	51.97 (-130.6, 234.52)	0.925
Ra1			
IL-5	17.36 (-1.76, 36.49) n=23	3.35 (-22.08, 28.79)	0.377
IL-10	2.28 (-9.78, 14.33)	12.88 (-3.50, 29.27)	0.297
IFN	-23.04 (-90.1, 44.04) n=23	43.91 (-121.7, 56.80)	0.865
Ra11			
IL-5	7.83 (-9.99, 25.65) n=23	-3.47 (-27.17, 20.24)	0.444
IL-10	5.84 (-6.44, 18.13)	13.62 (-3.07, 30.32)	0.451
IFN	131.0 (-6.4, 268.44) n=23	25..76 (-157.0, 208.57)	0.356
Mtb32a (n-)			
IL-5	13.03 (0.64, 25.42) n=23	-4.22 (-20.70, 12.27)	0.098
IL-10	2.80 (-7.91, 13.51)	9.92 (-4.63, 24.46)	0.423
IFN	42.00 (-43.83, 127.83) n=23	23.12 (-91.05, 137.29)	0.790
Mtb32a			
IL-5	22.87 (4.53, 41.23) n=23	0.19 (-24.21, 24.60)	0.140
IL-10	4.84 (-8.14, 17.81)	12.43 (-5.20, 30.06)	0.486
IFN	148.48 (-5.4, 302.32) n=23	44.00 (-160.6, 248.63)	0.413
Mtb39/CFP-10			
IL-5	31.58 (10.88, 52.28) n=23	4.59 (-22.94, 32.12)	0.121
IL-10	8.34 -5.65, 22.44)	16.30 (-2.78, 35.38)	0.503
IFN	175.83 (3.11, 348.54) n=23	137.65 (-92.08, 367.38)	0.789
Mtb39/Mtb9.9/CFP-10			
IL-5	9.965 (-4.09, 24.02) n=23	-5.74 (-24.44, 12.96)	0.181
IL-10	9.43 (-0.88, 19.73)	10.57 (-3.43, 24.57)	0.895
IFN	319.22 (100.2, 538.24) n=23	247.78 (-43.5, 539.10)	0.693
Mtb39/Mtb9.9/CFP-10/Mtb8.4			
IL-5	17.45 (-1.54, 36.45) n=23	-7.00 (-32.26, 18.27)	0.125
IL-10	34.25 (12.56, 55.95)	13.68 (-15.80, 43.17)	0.262
IFN	383.00 (158.23, 607.77) n=23	326.25 (27.28, 625.21)	0.760
Mtb39/Mtb9.9/Mtb8.4/ Ag85b			
IL-5	27.59 (4.71, 50.47) n=23	-3.15 (-33.58, 27.29)	0.110
IL-10	23.65 (7.36, 39.95)	17.42 (-4.73, 39.56)	0.648
IFN	441.61 (205.12, 678.1) n=23	449.31 (134.75, 763.86)	0.969
MSL			
IL-5	15.38 (2.69, 28.08) n=17	-0.66 (-16.43, 15.13) n=11	0.116
IL-10	12.08 (-2.57, 26.73) n=18	7.75 (-10.99, 26.48) n=11	0.712
IFN	56.06 (-35.70, 147.81) n=17	70.23 (-43.84, 184.29) n=11	0.844
Mtb32a/Mtb39			
IL-5	28.17 (7.90, 48.45) n=17	3.86 (-21.34, 29.07) n=11	0.135
IL-10	22.5 (3.65, 41.37) n=18	7.58 (-16.55, 31.70) n=11	0.326
IFN	97.06 (-18.86, 212.98) n=17	59.94 (-84.17, 204.04) n=11	0.683
α CRYST, PROT/ MTB8.4/MTB9.9			
IL-5	23.31 (9.87, 36.76) n=17	0.55 (-16.17, 17.26) n=11	0.038
IL-10	22.91 (1.52, 44.29) n=18	17.04 (-10.32, 44.39) n=11	0.731
IFN	130.77 (-120.9, 382.43) n=17	68.16 (-244.7, 381.02) n=11	0.751

DISCUSSION

The results of this analysis suggest that there are no significant differences in the nature of the immune responses between children with LTBI or active disease in

this sample. Lymphoproliferative assays, used to detect the presence and magnitude of the overall antigen-specific CD4+ T cell response, and MTB-antigen specific cytokine responses, used to detect specific types of immune responses, found no differences between the groups of children exposed to MTB, implying that although there is some stimulation of the immune system to the added antigens, the magnitude or type of immune response is not different between disease groups. Thus, we were not able to reject the null hypothesis that there are no differences in T-cell responses between children with active and latent tuberculosis.

Interestingly, similar results have been found in one cohort of recently studied adults. Dr. David Lewinsohn and co-investigators assessed the immune responses of 101 adults from the Seattle-King County TB Clinic with the same laboratory methodology as the Pediatric TB Study (manuscript in preparation). The sample population included 50 subjects with LTBI, 25 with active disease, and 26 control subjects. The results found conflicted with other published studies in that the TH1 and TH2 profiles showed no significant differences between adults with LTBI or active disease, suggesting the possibility that differences in the CD4+ T cells studied are not strong in this sample of adults with differing manifestations of disease.

While it may be true that differences in pediatric disease manifestation are not associated with differential T cell responses alone, examination of the published literature highlights other reasons that may explain the negative findings in our study. Some of the possible confounders include age, severity of disease, and prior BCG vaccination. As discussed earlier, differences have been found in neonatal immune systems and complete maturation of the immune system is not reached until six to

ten years of age. Additionally, children under two years of age are at increased risk of having disseminated forms of TB, possibly related to a TH2 bias in the cytokine milieu. Our study population had a limited number of very young children; there were three infants, eight children under two years of age in our sample. However, analyses were performed to examine the effect of age in our study population. When the responses to certain antigens were stratified by age ($\geq$ or < 2 years), no significant differences surfaced between disease groups.

With respect to disease severity, researchers have examined its effect on the strength of the TH1 response among children infected with MTB.[33] In addition to finding that children with active TB had lower IFN-γ production compared to children with LTBI, production was even lower by a subset of moderately- and severely-advanced patients. This inverse relationship between IFN-γ production and severity of disease has also been found in adults. Zhang *et al* found healthy adults with LTBI had strong IFN-γ production, demonstrating a TH1 response. The adults with active forms of TB had markedly diminished TH1 responses but normal TH2 responses, thus concluding that active disease in adults is associated with a relative decrease in IFN-γ production compared to adults with LTBI; no association was found between active TB and strong TH2 responses in this study.[34] However, further differences have been elucidated after examining disease severity. When stratifying by relative state of health, other researchers have found significant TH2 responses in very ill adults with active disease.[35] Thus, the findings in adults with active TB show the immunologic profiles rest on a continuum of cytokine responses. Manifestation of disease may not only be due to relative differences in IFN-γ production, but also the

health status of the subjects included. This suggests a dose-related response with the severity of disease with respect to both IFN-γ and IL-5 production.

Overall, the subjects in this study were fairly healthy. The subjects in our study were originally recruited largely through contact investigation and may have been detected at an earlier stage of disease that other studied populations. Additionally, our ability to examine the effects of disease severity was limited by the fact that only two subjects with disseminated disease were recruited into this study. Therefore, we did not have adequate power to demonstrate potential differences related to severity of disease.

Lastly, prior BCG vaccination may be associated with our null findings in children. Following BCG vaccination at birth, infants develop strong TH1-type $CD4^+$ responses specific to PPD.[36] However, the effects of BCG on subsequent immunity to MTB infection in children has not been studied and it is certainly possible that a pre-existing immune response to BCG could alter the cytokine milieu and immune response to MTB infection. This is of little concern when studying adults with TB because the immunity mounted typically wanes a few years after BCG vaccination. However, if the exposure to mycobacterial products contained in the vaccine predispose children to mounting a certain type of immune response, it would have been more difficult to discern any true differences since nearly half of the subjects in each disease group had been previously exposed to the vaccine. Yet, upon examining our data with this in mind, our conclusions were not affected. When MTB-antigen specific cytokine responses were stratified by BCG vaccination status, no

significant differences were found in the nature of the immune responses between disease groups.

Additionally, there is a possibility that the null hypothesis was erroneously accepted, resulting in a type II error. Possible reasons to explain a type II error include inadequate power and the techniques behind data assessment. Generally, when assessing power, it is calculated before the initiation of the study with the goal of determining the sample size needed to detect a specific change, with a given magnitude of difference, variance of data, and acceptable type I error. A discussion of the post-hoc power calculations may be relevant for determining the detectable effect size. For IL-5 and IL-10 cytokines, this study had adequate power (80.6%) to detect a 25 picogram difference between disease groups, a difference that is generally thought to be of clinical significance by experts in the field. The probability of type II error (β) was also within an acceptable range, at 0.19. For IFN-γ, the clinically acceptable difference is 100 picograms. The power to detect this difference was 80.6% if the standard deviation was 100. However, for some antigens assessed, the standard deviation was greater than this value, which decreased the power to only 61.8%; the associated β of 0.38 was much greater than the generally accepted probability of 0.20. Therefore, we had an increased probability of erroneously rejecting the null hypothesis with regards to the IFN-γ results for some antigens.

Other reasons to explain why a difference that potentially exists may not have been detected involve the techniques behind data assessment, including the ELISA tests, laboratory measurements, initial data entry, and data organization. The

sensitivity and specificity of the measurement tool, the ELISA test, could have contributed to potential measurement error. The actual ability of ELISA tests to detect minute quantities of cytokines is quite good. The kits used in the original study were capable of detecting 3.5 picograms/mL of IL-10 and 15 picograms/mL of IFN-γ and IL-5. Until recently, ELISA tests were the only method of detecting a primed cytokine-producing $CD4^+$ T cell response. Since the conclusion of the original study, better tests have been developed that are capable of detecting the actual primed $CD4^+$ T cells and thus, more accurate quantitative measurements. There is no reason to believe that any differential information or measurement bias occurred in the collection of the data. Although, many of the assays were negative or only detected little cytokine production, leaving the possibility that if more sensitive test were used to detect the presence and magnitude of a primed CD4+ T cell response, more individuals may have had a positive assay, which may have reflected differences between groups in cytokine production.

In the laboratory, there was potential for error when culturing lymphocytes with MTB-specific antigens. The overall number of lymphocytes present in one well correlates with the proportion of primed $CD4^+$ T cells, which in turn, directly correlates with the magnitude of the antigen-specific cytokine production. In creating the wells, approximately 200,000 lymphocytes were measured and added to each. If the wells did not have equal numbers of lymphocytes, the potential to detect the cytokine responses may have been hindered. However, the impact on the data would likely only be of importance if there was a systematic error in the allotment of lymphocytes per well that was consistently different between the active disease group and the

LTBI group. Standard techniques were used by a single trained laboratory technician, thus making the chance of a differential error less likely. Another opportunity for introducing error could involve the interpretation of ELISA results. Differential errors could have been introduced if different technicians interpreted the results, or if the results were not blinded; but again, standard techniques were followed by one laboratory technician who was blinded to the group assignment of each subject.

Further potential for measurement error may come from data entry and data organization. The individual ELISA measurements could have been entered incorrectly, the average of the triplicate measures could have been calculated erroneously, or the antigen-specific cytokine values could have been calculated incorrectly, yielding inaccurate results. However, computer programs were used for these tasks and there is no reason to believe that these potential errors occurred in a systematic fashion.

One area of concern during the analysis phase of this project focused on the negative values obtained when calculating the antigen-specific cytokine responses. A negative mean value for antigen-specific cytokine production is difficult to explain in a biologically plausible manner. When MTB-antigen specific proteins are added to PBMC, one of two things should occur: the ELISA tests should theoretically reveal increased cytokine production or no response, but not a negative response. For example, if an individual's PBMC did not have any $CD4^+$ T cells that were previously primed to the mycobacterial antigen, the addition of that antigen would do nothing to stimulate the production of more cytokines; thus, the level of cytokine measured by

ELISA should not be different than the media-only measurements. Although any potential toxicity the recombinant antigens introduced could confound the results. For example, if the added antigen selectively inhibited cytokine production or was toxic resulting in cell death, cytokine production could be inhibited, and a low level of cytokines would be detected. This could result in the antigen-specific response being less than the "media-only" measurement. However, if indeed the antigens were thought to be toxic to cells, uniform results would be seen. With the lack of uniform results, it was difficult to make a biologically feasible conclusion, which led us to believe that any negative net values were secondary to measurement error.

In handling this measurement error, we unintentionally introduced a systematic bias during the initial data analysis. Since a null response is more biologically understandable, our initial intent was to recode all negative values into zero to reflect a null response, immunologically speaking, but positive values were not changed. This process altered the distribution of results, leaving most cytokine-specific antigens with a non-normal distribution pattern, calling for the use of non-parametric statistical tests. When Wilcoxon rank score tests were performed on the re-coded pediatric data, consistent trends were found between the disease groups in response to the antigen-specific production of IL-5. Among all antigens tested, children with LTBI consistently had stronger TH2 responses compared to those children with active disease. Statistically significant differences ($p<0.05$) were found among one of the core antigens (α-crystalline protein) and seven others in the panel of twenty-three recombinant MTB antigens; three additional antigens stimulated sufficient IL-5 production to approach statistical significance at the $\alpha=0.10$ level (data not shown).

These findings suggested that a TH-2 response may be linked to latent types of TB infection, conflicting with our initial expectations based on the hypothesis that TH2-type of immunity is associated with active TB disease.

Although a null response is more clinically relevant than a negative response, for the final statistical analysis our data were ultimately not changed to reflect this due to concerns of differential treatment of measurement error. The potential for measurement error exists for all our collected data. While it is easy to identify the measurement error in the negative responses, there is no reason to suspect that only those values falling below zero are susceptible. Theoretically, some element of measurement error likely exists for the positive values, as well. Thus, to alter only the negative values would be inappropriate, because an unspecified margin of error would remain unaltered among the positive antigen-specific values. In attempts to quantify the margin of error, the amount of within-subject variation could theoretically have been examined and analyzed with a test of repeated measures. However, this was not possible because the data points, originally performed in triplicate, were only displayed in the aggregate form; individual values were not available.

Lastly, an interesting finding resulted when the data were analyzed as proposed in the methods section. Significant differences were found in the background production of IFN-γ and IL-5. Graphs of the data, illustrate that subjects with LTBI consistently had a greater spread of background values compared to subjects with active disease (see figure 4a and 5a). To explain this finding, we could postulate that those with active disease had greater IL-10 production, causing down-regulation or immunomodulation of the immune system. However, if this were true,

we would expect the background levels of IL-10 production to be significantly greater for the active disease group, which was not seen. Thus, it is difficult to explain biologically why these differences exist. Although small, there is a possibility that the discrepant spread and significant differences in background IFN-γ and IL-5 production may have been due to chance.

SUMMARY

Overall, the question of why some children exposed to MTB are predisposed to severe forms of active disease remains unanswered, as we found no immunologic differences in cytokine production between disease groups. The possibility remains that underlying differences in the immune responses are responsible, however this study of a limited number of children was unable to decipher this. Further study of children, especially those that are younger than two years of age and those with disseminated forms of disease, may be helpful in resolving the gaps in our understanding of pediatric immunology to MTB. Additionally, the role of prior BCG vaccination may also be important in elucidating immunological differences, and future plans are underway to generate a larger pediatric study population to assess the potential contributing effects.

PUBLIC HEALTH IMPLICATIONS

Prevention of TB is an especially important public health concern because of the increasing incidence worldwide. This may have grave consequences for very

young children, as they are more likely to have symptomatic disease after infection, and can rapidly progress to more severe forms of disease.

The factors contributing to the spread of TB, combined with the difficulties in diagnosing TB in children contribute to the need for improved prevention and diagnostics efforts. Understanding the mechanisms behind the different manifestations of TB is important, as it will provide the building blocks for improved vaccine development. A more effective vaccine in protecting against TB during early childhood and beyond is crucial for curtailing the spread of TB, as the currently available BCG vaccine is not effective in preventing localized disease, and only modestly effective in preventing disseminated forms of disease.

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