Oregon Health & Science University
School of Medicine

Scholarly Projects Final Report

Title *(Must match poster title; include key words in the title to improve electronic search capabilities.)*

Differential CD4+ and CD8+ T cell recognition of mycobacterial antigens in pediatric versus adult TB

Student Investigator's Name

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Date of Submission *(mm/dd/yyyy)*

03/16/22

Graduation Year

2023

Project Course *(Indicate whether the project was conducted in the Scholarly Projects Curriculum; Physician Scientist Experience; Combined Degree Program [MD/MPH, MD/PhD]; or other course.)*

MD

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Project/Research Question

Is the capacity of Mtb-specific CD4+ and CD8+ T cells to produce three key Th-1 cytokines compromised among Ugandan children with TB as compared to Ugandan adults with TB? Does use of novel Mtb-antigens (as compared to antigens utilized in commercial blood tests for Mtb-infection) identify additional Th-1 Mtb-specific T cells among Ugandan children with TB as compared to Ugandan adults with TB?

Type of Project *(Best description of your project; e.g., research study, quality improvement project, engineering project, etc.)*

Research study

Key words *(4-10 words describing key aspects of your project)*

Tuberculosis, Pediatrics, Immunology, T-cells, Mycobacterial antigens

Meeting Presentations

If your project was presented at a meeting besides the OHSU Capstone, please provide the meeting(s) name, location, date, and presentation format below (poster vs. podium presentation or other).

American Association of Immunology, Portland OR, May 9th, 2022 (poster)

Publications *(Abstract, article, other)*

If your project was published, please provide reference(s) below in JAMA style.

Manuscript in process

Submission to Archive

Final reports will be archived in a central library to benefit other students and colleagues. Describe any restrictions below (e.g., hold until publication of article on a specific date).

No restrictions

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Next Steps
What are possible next steps that would build upon the results of this project? Could any data or tools resulting from the project have the potential to be used to answer new research questions by future medical students?

More research is needed to understand the vulnerability of young children to TB disease. Longitudinal study of Mtb-exposed children who do and do not progress to TB disease will be needed to identify immunologic characteristics that are either protective versus permissive to development of clinical disease. Future investigators may be interested in further studying our data on pediatric immune response to the 5 mycobacterial antigens for purposes of vaccine development and improved TB diagnostics for children.

Please follow the link below and complete the archival process for your Project in addition to submitting your final report.

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Student's Signature/Date *(Electronic signatures on this form are acceptable.)*
This report describes work that I conducted in the Scholarly Projects Curriculum or alternative academic program at the OHSU School of Medicine. By typing my signature below, I attest to its authenticity and originality and agree to submit it to the Archive.

X _____
Student's fu name

Erin Morrow

Mentor's Approval *(Signature/date)*

Christina L. Lancioni, MD
March 15, 2023

Scholarly Project Final Report

Report*: Information in the report should be consistent with the poster, but could include additional material. Insert text in the following sections targeting 1500-3000 words overall; include key figures and tables. Use Calibri 11-point font, single spaced and 1-inch margin; follow JAMA style conventions as detailed in the full instructions.*

Introduction *(≥250 words)*

Despite being both preventable and treatable, Tuberculosis disease (TB) remains a leading cause of death from infectious disease worldwide[1]. Tuberculosis is also a leading cause of childhood morbidity and mortality due to infectious disease, causing illness in one million children each year and killing nearly a quarter of children affected[2]. The majority of pediatric morbidity and mortality occurs in low- and middle-income countries with a high prevalence of adult TB, such as Uganda. Unlike HIV-uninfected, immunocompetent adults, who have only a 5-10% lifetime risk of developing TB following exposure to *Mycobacterium tuberculosis* (Mtb), 30-40% of infants will develop pulmonary and 10-20% disseminated TB if exposed to Mtb during their first year of life[3]. Despite children's increased vulnerability to TB infection and propensity to develop more severe forms of the disease such as TB meningitis and miliary TB[4], studies focused on identifying mechanisms permissive to development of TB in young children have been limited[5].

Numerous studies have suggested that young children harbor several deficiencies in both their innate and adaptive immune systems that may be permissive to development of TB following exposure. For example, Mtb infects host macrophages and dendritic cells and several studies have suggested that these innate cells have impaired anti-microbial capacity in infants. Neonatal dendritic cells are present in reduced numbers as compared to adults[6], have reduced cell surface expression of MHC and cell adhesion molecules[7], and have been shown to be less-efficient at inducing T-cell activation. Monocytes, the precursors to tissue macrophages, have limited chemotaxis during infancy[8] and are limited in their capacity to produce the pro-inflammatory cytokine TNF-α in response pathogen-derived danger signals detected using Toll-like receptor (TLR)[9]. Key to initial containment of Mtb following respiratory exposure are alveolar macrophages that phagocytose Mtb and release key signaling cytokines, including TNF-α, to initiate a granulomatous response within the lung[10]. Although not specific to Mtb, alveolar macrophages in neonates have been shown to be impaired, with animal studies demonstrating decreased macrophage killing and clearance of microbes in neonates[11].

The adaptive, specifically T cell, components of the infantile immune systems are distinct in several ways that may predispose young children to TB. The majority of infant CD4+ and CD8+ T cells exhibit a naïve phenotype and have increased co-stimulatory requirements for activation as compared to memory T cells[12]. Compared to adults, neonatal T cells also express less CD40 ligand, a ligand essential for immune response amplification and coordination[8]. Once activated through the T cell receptor and CD28 co-stimulatory receptor, CD4+ T cells from neonates and young infants are limited in capacity to produce the pro-inflammatory cytokine interferon-gamma (IFN-γ), but rather generate IL-4 and the anti-inflammatory cytokine IL-10[4, 13-18]. Moreover, regulatory T cells are expanded during early infancy and have been shown to have enhanced suppressive function as compared to regulatory T cells in adults[19, 20]. These barriers to mounting a Type-1 helper CD4+ T cell response characterized by IFN-γ production, are believed to contribute to the vulnerability of young children to TB[21].

Although the bias against Th-1 responses during early infancy are well established, the majority of these prior investigations were performed using polyclonal or mitogen-based stimulation conditions and did not examine pathogen-specific T cell responses. Numerous studies have demonstrated that T cells from infants who have been vaccinated with the live mycobacterium bacillus Calmette-Guérin (utilized in BCG-vaccination), as well as infants and young children with asymptomatic Mtb infection and those ill with TB, are capable of mounting Mtb-specific Th-1 responses characterized by robust IFN-γ production[22-26]. Here, we aimed to further probe the capacity of Mtb-specific CD4+ and CD8+ T cells from HIV-uninfected Ugandan infants and young children with confirmed TB to produce three key pro-inflammatory cytokines (IFN-γ, IL-2, and TNF-α) in response to well-characterized Mtb-specific antigens used in current commercial diagnostic assays (ESAT-6 and CFP-10), as well as five novel mycobacterial antigens previously shown to be immunogenic in Mtb-infected adults[27]. Importantly we have compared cytokine production to that elicited from Ugandan adults with confirmed TB, as well as age-matched Ugandan children with non-TB pneumonia and unconfirmed TB. Our findings further contribute to the understanding of immune responses to Mtb during early life that can be applied to development of improved blood-based diagnostics and next-generation vaccines that aim to reduce the burden of TB related morbid ty and mortality for young children.

Methods *(≥250 words)*

Ugandan children aged <5 years admitted to Mulago Hospital in Kampala, Uganda, with c ltu e-confirmed TB (n=12), clinically diagnosed unconfirmed TB (n=41), and non-TB lower respiratory tract infection (LRTI; n=39) were enrolled following provision of written informed consent from a parent/gua dian between 2011-2014 Children with history of prior TB and those receiving 7+ days of TB treatment were excluded HIV-uninfected children were included in this current analysis (Table 1).

Ugandan adults ages 18-65 years old with culture-confirmed TB (n=41) were recruited from TB treatment centers in Kampala, Uganda, and enrolled following provision of written informed consent between 2001-2014. Adults with history of prior TB and those receiving 7+ days of TB treatment were excluded HIV-uninfected adults were included in this current analysis (Table 1).

All participants underwent blood draw at enrollment for isolation of peripheral blood mononuclear cells (PBMC) and plasma. PBMC were cryopreserved for batch analysis

CD4+ and CD8+ T cell pro-inflammatory cytokine responses (IL-2, IFN-γ, TNF-α) to peptide pools representing 6 mycobacterial antigens (Table 2), as well as Staphylococcal enterotoxin B (SEB) and resting condition were quantified in PBMC by intracellular flow cytometry and analyzed using FlowJo.

The proportions of children and adults with a positive cytokine response (defined as ≥0.05% of CD4+ or CD8+ T cells producing a cytokine following background correction) to each peptide pool were compared using Fisher-Exact test. The overall frequencies of CD4+ and CD8+ T cell cytokine responses to each peptide pool (fo lowing background correction) were compared by Kruskal-Wallis test. Post-hoc comparisons were performed using Dunn's test of multiple comparisons Adjusted (Dunn's test of multiple comparisons) p-values <0.05 were considered significant.

Table 1. Demographic Characteristics of Study Populations

	Age (median IQR)	Sex (% female)	BCG scar present	Weight-for-age Z-score (median IQR)
Confirmed pediatric TB (n=12)	30 8 months (28 17)	50%	33 3%	-1 55 (2 1)
Unconfirmed pediatric TB (n=41*)	18 months (30 9)	38 5%	69 2%	-1 76 (1 13)
Non-TB pediatric LRTI (n=39)	12 3 months (13 1)	33%	87%	-1 30 (1 19)
Adult confirmed TB (n=41*)	23 years (8)	57 5%	Not collected	Not collected

*clinical data unavailable on 2/41 children with uncon irmed B and 1/41 adults with con irmed B

Table 2: Antigens utilized in ICS Assay

Antigen	Rv Number
ESAT6/CFP10	*Rv3875/Rv3874*
EsxJ Family	*Rv1038c*, Rv1197, Rv2347, Rv3620c
PE12 : PE13	*Rv1172c*(32) : *Rv1195*(18)
PE3	*Rv0159c*(50)
PPE15	*Rv1039c*(50)
PPE51	*Rv3136*(46)

Results *(≥500 words)*

We examined the proportion of children and adults (cross four cohorts with detectable CD4+ and CD8+ T cell cytokine response to 6 mycobacterial antigens (Figure 1). The pro-inflammatory CD4+ and CD8+ T cell response to antigens ESAT6/CFP10 were not significantly different between children with confirmed TB versus adults with TB (adjusted p value = 1). We observed that the proportion of peds confirmed TB pro-inflammatory response to ESAT6/CFP10 did differ significantly from peds unconfirmed TB for the majority of cytokines.

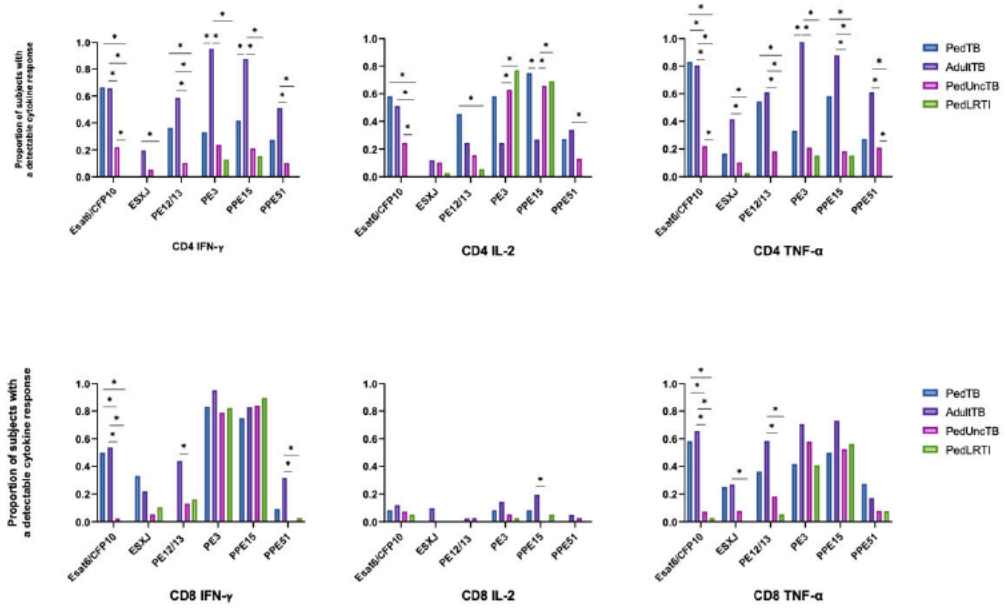

Figure 1. Proportion of children and adults with detectable CD4+ and CD8+ T cell cytokine response to 6 mycobacterial antigens. PBMC isolated from children with confirmed (n=12), unconfirmed TB (n=41), nonTB pneumonia (n=39), and adults with confirmed TB (n=41), were thawed in batches, rested overnight, and CD4+ and CD8+ T cell production of IFN-γ, IL-2, and TNF-α in response to 6 peptide pools representing mycobacterial antigens was quantified by intracellular flow cytometry (ICS). A positive response to each peptide pool was defined as ≥0.05% of CD4+ or CD8+ T cells producing a cytokine following background correction.* indicates adjusted p-value ≤0.05.

Although children with TB demonstrated T cell response to ESAT6/CFP10 antigens, children in the non-TB LRTI cohort did not exhibit significant CD4+ or CD8+ T cell responses to ESAT6/CFP10 (proportion of responders = < 0.05%). However, children with non-TB LRTI did respond to other mycobacterial antigens,

particularly PE3 and PPE15 (Figure 2).

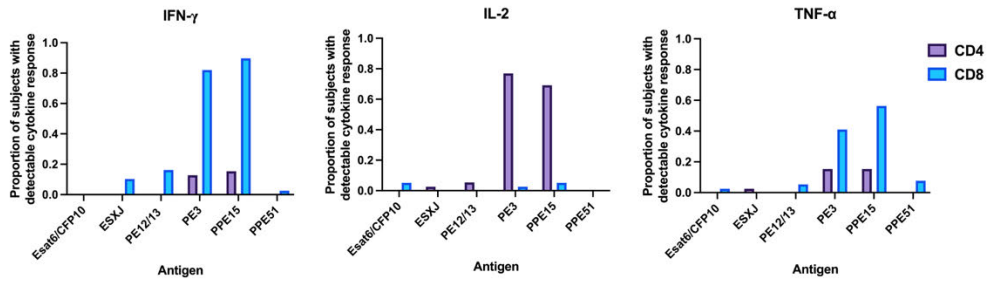

Figure 2. Children with non-TB LRTI do not exhibit CD4+ or CD8+ T cell responses to ESAT6/CFP10 but respond to other mycobacterial antigens. PBMC isolated from children with non-TB LRTI confirmed (n=39) were assessed for CD4+ and CD8+ T cell cytokine responses to 6 mycobacterial antigens as previously described.
*indicates adjusted p-value ≤0.05.

Frequency of CD4+ and CD8+ T cells that produce cytokines in response to mycobacterial antigens were also measured across cohorts (Figure 3). When examining the five novel Mtb antigens, we observed a diminished CD4+ IFN-γ T cell response in children. CD4+ response to antigens PE3, PPE15 and PE12/13 were diminished in children with TB vs adults with TB (p values < 0.0001) suggesting these are not immunodominant antigens in children. We also compared the frequency of T cell response in children with TB vs. children with unconfirmed TB. Here, the frequency of CD4+ and CD8+ T cell response to ESAT/CFP10 were not significantly different (p=.18 and .24 respectively).

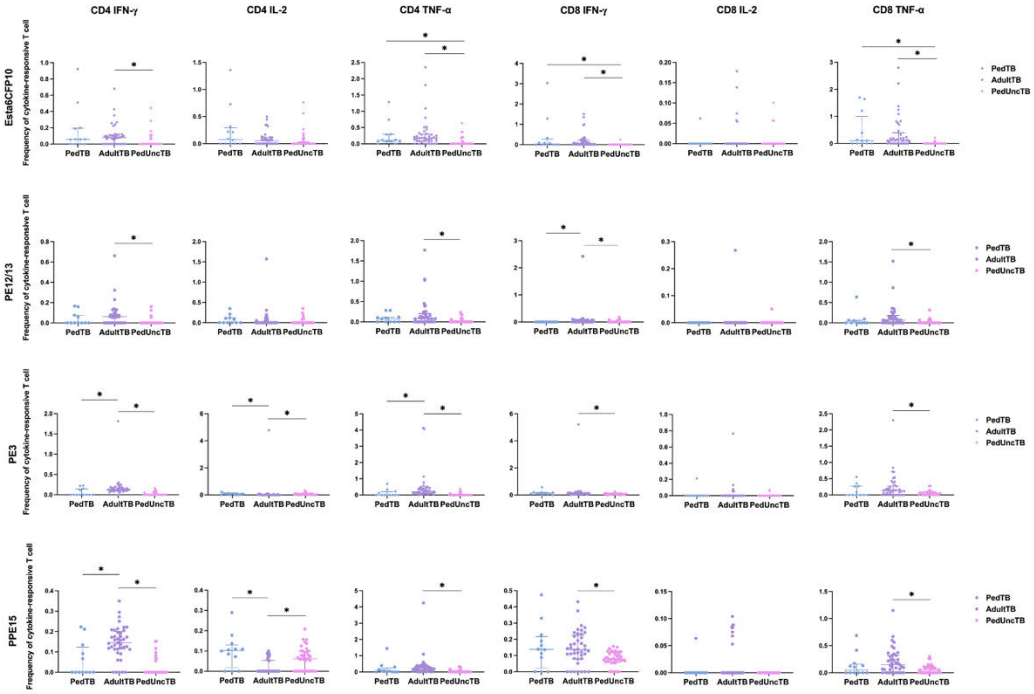

Figure 3. Frequency of CD4+ and CD8+ T cells that produce cytokines in response to mycobacterial antigens.
Frequency of CD4+ and CD8+ T cells that produce IFN-γ, IL-2, or TNF-α in response to 6 mycobacterial antigens were quantified by ICS. Background correction (subtraction of the unstimulated/resting responses) was performed, and responses <0.05% set to zero. Comparisons among cohorts were performed using Kruskal-Wallis test; post-hoc comparisons were performed using Dunn's test of multiple comparisons. Shown are medians with IQR. Only peptide pools where significant differences among cohorts were identified (4 of 6 tested pools) are shown. *indicates adjusted p-value ≤0.05.

When examining the frequency of CD4+ and CD8+ T cells that produce cytokines in response to Staphylococcal enterotoxin B (SEB), we observed significant differences in children with TB compared to adults with TB (Figure 4). Adjusted p-value for peds TB vs. adult TB was < 0.0001 for all cytokines in both CD4+ and CD8+ T cells. Among all peds cohorts (peds TB, peds unconfirmed TB and peds LRTI) there were

no significant differences in the frequencies of T cell response to SEB (all adjusted p-values >0.05).

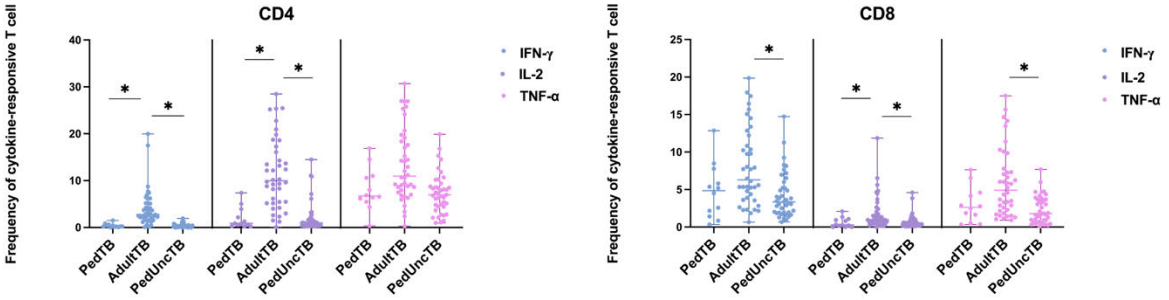

Figure 4. Frequency of CD4+ and CD8+ T cells that produce cytokines in response to Staphylococcal enterotoxin B (SEB). Frequency of CD4+ and CD8+ T cells that produce IFN-γ, IL-2, or TNF-α in response to SEB were quantified by ICS. Background correction (subtraction of the unstimulated/resting responses) was performed, and responses <0.05% set to zero. Comparisons among cohorts were performed using Kruskal-Wallis test; post-hoc comparisons were performed using Dunn's test of multiple comparisons. Shown are medians with IQR. *indicates adjusted p-value ≤0.05.

Discussion *(≥500 words)*

Despite the increased morbidity and mortality associated with TB infection observed in young children, studies investigating mechanisms permissive to development of severe TB in young children have been limited[5]. In this study, we aimed to understand which elements of the pediatric T cell adaptive immune response to TB differ from that observed in adults. To do this we compared CD4+ and CD8+ T cell production of Th-1 cytokines key to a successful immune response to Mycobacterium tuberculosis infection, IFN-γ, IL-2, and TNF-α, in response to Mtb-specific antigens ESAT-6 and CFP-10 (used in current commercial diagnostic assays) and five novel mycobacterial antigens. In our comparison of children with confirmed TB to adults with confirmed TB, we did not observe any significant differences in the proportion of participants who demonstrated a detectable CD4+ or CD8+ T cell response to ESAT6/CFP10, nor were there differences in the frequencies of the CD4+ and CD8+ T cell cytokine response to ESAT6/CFP10. Our finding emphasizes that CD4+ and CD8+ T cells from young children with TB are not deficient in their production of key Th-1 cytokines in response to antigens specific to Mycobacterium tuberculosis, when compared to adults. This finding was in stark contrast to our results when examining the frequencies of CD4+ and CD8+ T cells that produce Th-1 cytokine in response to the mitogen SEB. Here, as in prior studies, the pediatric Th-1 response to SEB was significant reduced as compared to adult. Thus, although the Th-1 response to stimuli such as SEB is limited in children as compared to adults, the capacity of children with confirmed TB to generate a Th-1 response to a Mtb-specific antigen is equivalent.

In this study, we also had access to children with clinically diagnosed, unconfirmed TB (PedUncTB). CD4+ and CD8+ T cells from children with PedUncTB showed numerous significant differences in response to several antigens - most notably ESAT6/CFP10 - as compared to adults and children with confirmed TB. We believe a large proportion of children treated for unconfirmed TB are not actually infected with Mtb, and thus their results should not be combined with those obtained from children with confirmed disease. Indeed, in our own analysis, if we combined children with confirmed and unconfirmed TB and then compared their Th-1 responses to ESAT6/CFP10 to adults with confirmed disease, we would have reported

a significantly diminished response in the combined pediatric cohort (data not shown). This is a key finding as many published studies of the immunobiology of pediatric TB have included a large proportion of children with unconfirmed TB (due to the difficulty in confirming TB in young children using culture and molecular-based approaches)

In this study, we also examined Th-1 cytokine responses to mycobacterial antigens that are not specific to TB, but shared among other mycobacterial species included BCG. Here we noted that the pattern of CD4+ T cell cytokine production in response to PE3 and PPE15 differed between children and adults. This illustrates that pediatric and adult T cells may recognize similar antigens but produce different cytokines. This finding likely represents differences in T cell phenotype and differentiation status in children vs. adults. We noted that these antigens were commonly recognized among children with non-TB LRTI, likely due to presence of these antigens in BCG vaccine.

More research is needed to understand the vulnerability of young children to TB disease, and to identify Mtb-specific immune responses that can be used to improve diagnostic assays for pediatric TB. Longitudinal study of Mtb-exposed children who do and do not progress to TB disease will be needed to identify immunologic characteristics that are either protective versus permissive to development of clinical disease.

Conclusions *(2-3 summary sentences)*

The pro-inflammatory CD4+ and CD8+ T cell response to Mtb-specific antigens ESAT6/CFP10 is not impaired among young children with confirmed TB compared to adults with confirmed TB. In contrast to above, the frequency of pro-inflammatory CD4+ and CD8+ T cells in response to superantigen SEB is impaired in young children with confirmed TB. This is consistent with prior literature. The pattern of CD4+ T cell cytokine production in response to PE3 and PPE15 differed between children and adults, illustrating that pediatric and adult T cells may recognize similar antigens but produce different cytokines responses. This finding likely represents differences in T cell phenotype and differentiation status in children vs. adults.

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