$CD8^+$ T cells Provide an Immunologic Signature of Tuberculosis in Young Children

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Online Data Supplement

SUPPLEMENTAL METHODS

Participants and Procedures

Written informed consent was obtained in the local language from each child's parent or guardian prior to study enrollment and the study was approved by the Institutional Review Board of University Hospitals (Cleveland, Ohio), the National Council for Science and Technology, HIV/AIDS Research Committee in Uganda, the Institutional Review Board of Oregon Health Sciences University (Portland, Oregon) and the Institutional Review Board of Makerere University (Kampala, Uganda). Subject recruitment and study coordination was sponsored by the Tuberculosis Research Unit at Case Western Reserve University.

Healthy children exposed to Mtb (HE) and children acutely ill with confirmed or probable pulmonary TB (CP-TB) were enrolled from separate recruitment sites in Kampala, Uganda. For the HE group, we evaluated child household contacts (age < 15 years) of adults with culture-confirmed pulmonary TB who were participants in a larger, prospective cohort study of TB transmission dynamics in Kampala, Uganda. The HE group was enrolled between December 2004 and December 2006 as described previously (1). Briefly, recruitment occurred after an adult family member sought care for TB at the Ugandan National Tuberculosis and Leprosy Control Program in Kampala. At study entry detailed demographic and clinical information for symptoms of TB disease were collected, and a physical exam and anterior chest radiograph (CXR) performed. Nutritional status was determined by comparing individuals' body-mass-index (BMI) to WHO child growth standards to generate a Z-score. Severe malnutrition was defined as a BMI Z-score of ≤-3 (2). A tuberculin skin test (TST) was performed with the Mantoux

method with 5 units of purified protein derivative (Pasteur Méríeux Connaught, Swiftwater, PA) and read within 48-72 hours of placement. A positive test was defined utilizing WHO criteria (3), with induration greater than 5 mm considered positive for severely malnourished children and induration greater than 10 mm considered positive for the remainder of children. TST results were available for all study participants. Children with a positive TST, and all children less than 5 years, were offered 9 months of isoniazid preventive therapy once TB was ruled out. Enrolled subjects had 1-2 ml/kg (maximum 20 ml) of blood drawn at study enrollment, prior to placement of TST. Peripheral blood mononuclear cells (PBMC) were isolated by standard methods and cryopreserved. HIV testing was performed for all children over 18 months by enzyme-linked immunoassay; children less than 18 months old had HIV testing performed if a biological parent was found to be HIV positive and were tested by HIV polymerase chain reaction (PCR). Children were followed clinically for 6-24 months and those with symptoms concerning for TB either at enrollment or during follow-up received a full clinical and diagnostic evaluation by a study physician, including a repeat CXR and mycobacterial smear and culture of at least one gastric aspirate or sputum sample. Specimens were processed by routine methods, underwent fluorochrome staining to detect AFB, and were cultured on Loewenstein-Jensen media as well as in Middlebrook 7H9 broth. All mycobacterial cultures were monitored for growth of AFB for 8 weeks. Microbiologists were blinded to participants' TB classification and the results of TST testing. Children with a history of prior or current TB, children who developed TB within 6 months of enrollment, and children who were HIV-infected or otherwise immunosuppressed (receiving corticosteroids) were excluded.

For the confirmed and probable TB group (CP-TB), we enrolled acutely ill children (age < 10 years) meeting WHO criteria for either confirmed or probable intrathoracic TB (Table S1; (4)) from the pediatric wards of Mulago Hospital, a referral hospital in Kampala, Uganda. The CP-TB group was enrolled between July 2007 and May 2008. Children hospitalized with symptoms of TB (suspect TB, (4)) were evaluated with full clinical assessment, CXR, TST, and HIV enzyme-linked immunoassay if older than 18 months or HIV PCR if less than 18 months. TST was performed and interpreted exactly as for the HE children. Detailed demographic and clinical information was collected prospectively and surviving children were evaluated at a two month follow-up visit. Exactly as for the HE cohort, children had nutritional status assessed. All children had a mycobacterial smear and culture of one induced sputum sample performed. In some cases lymph node aspirates were obtained for pathology and/or mycobacterial smear and culture. CP-TB subjects had blood drawn at study enrollment after informed consent and within 72 hours of TST placement. PBMC were isolated and cryopreserved. Children with suspected TB were initiated on a standard four drug regimen of isoniazid, rifampin, pyrazinamide, and ethambutol according to Ugandan national treatment guidelines. Children received a final designation of confirmed TB, probable TB, or not TB based on final results of their diagnostic work-up and response to treatment at two-months. Children classified as not having TB disease, and children with HIV infection, were excluded from analysis. Investigators assigning TB classification were blinded to enzyme-linked immunosorbent spot (ELISPOT) assay results.

Media and reagents

Culture medium consisted of RPMI 1640 supplemented with 10% human sera, 5 X 10^{-5} M 2 ME (Sigma-Aldrich,http://www.sigmaaldrich.com/), and 2 mM glutamine (GIBCO BRL, http://www.invitrogen.com/). Peptides were synthesized by Genemed Synthesis (http://www.genemedsyn.com/). A single synthetic peptide pool consisting of 15-mers overlapping by 11 aa, representing Mtb-specific proteins, CFP-10 and ESAT-6, was synthesized. Peptides were resuspended in DMSO, and 43 peptides were combined into one pool such that each peptide in the pool was at a concentration of 1 mg/ml. Peptide pools were stored at 8°C.

IFN-γ ELISPOT assay

To measure Mtb-specific T cell responses in the HE and CP-TB- study groups, overnight IFN-γ enzyme-linked immunosorbent spot (ELISPOT) assays were performed as described previously (5). Assays were performed in batches on cryopreserved PBMC from both the HE and CP-TB cohorts. PBMC preparation, cryopreservation, and IFN-γ ELISPOT assays were performed at the Joint Clinical Research Center immunology laboratory, Kampala, Uganda, under the auspices of the Tuberculosis Research Unit. For determination of frequency of ESAT-6/CFP-10-specific $CD8^+$ T cells, $CD8^+$ T cells, negatively selected from PBMCs using a combination of CD4 and CD56 magnetic beads (Miltenyi Biotec), were used as the source of responding T cells. This $CD8^+$ T cell assay is referred to as a CD8 ELISPOT. While we have found peptide-pulsed monocyte-derived dendritic cells (DC) to be the most sensitive antigen presenting cell (APC) to enumerate $CD8^+$ T cell effectors ex vivo (6), it requires sufficient PBMC to generate DC. For these studies with Ugandan samples, the quantity of blood available precluded this approach. As a result, magnetic-bead depletion was used, allowing the use of

endogenous monocytes as the APC. In preliminary experiments, CD4 depletion alone resulted in a high background in the medium alone samples (negative controls) that was eliminated through additional depletion of $CD56^+$ cells. When directly compared to DC as APC, this method is approximately 80% as efficient in enumerating antigen specific $CD8^+$ T cells (data not shown). Flow cytometric analysis of viable, $CD3^+$ cells revealed a $CD4^+$ T cell contamination rate of <2%, and $CD8^+$ T cell purity of >85%. A population of $CD3^+$ CD4/CD8/$CD56^{negative}$ cells (5-8%) that were not further characterized were also noted. The remaining viable, $CD3^{negative}$ cells were comprised primarily of monocytes and B cells (data not shown). Unfractionated PBMC were used as the source of responding T cells to determine the total T cell response to ESAT-6/CFP-10. This assay is referred to as a PBMC ELISPOT.

The IFN-γ ELISPOT assay was performed using 250,000 cells/well of PBMC (PBMC ELISPOT) or CD4/CD56-depleted PBMC (CD8 ELISPOT) and peptide pools as antigen (final concentration of each peptide 5μg/ml). Negative and positive controls consisted of cells cultured with medium alone or phytohemagglutanin (PHA, 10 μg/ml; EMD Biosciences, http://www.emdbiosciences.com/), respectively. All determinations were performed in duplicate. In some cases the no antigen (media) control was performed in triplicate. To determine the ex vivo frequency of antigen-specific T cells, the average number of spot forming units (SFU) per well for each duplicate was determined and compared to the average number of SFU in the media control wells. To account for well-to-well variance among technical replicates, a standard deviation of the media control was calculated. A positive ELISPOT assay was defined as one in which the antigen-specific response was at least two standard deviations above the background

control plus 5 SFU. If these criteria were met, the background was subtracted out to determine the antigen-specific response. A positive PHA response was defined as ≥ 20 SFU per well.

Study design and statistical analysis

We performed a cross sectional study examining PBMC (PBMC ELISPOT) and $CD8^+$ T cell (CD8 ELISPOT) responses to Mtb antigens at baseline from two clinical study groups, children with CP-TB and HE children. To examine the effect of age on development of Mtb-specific T cell responses we studied the HE study group independent of the CP-TB group. For this analysis, T cell responses were compared between children less than or equal to 5 (< 5) years and children older than 5 and less than or equal to 15 (5 < 15) years. Next, to compare PBMC and $CD8^+$ T cell responses between CP-TB and HE study groups, we only selected children from the HE group age less than or equal to 10 (< 10) years to adjust for the inherent age differences in the cohorts as the CP-TB study group recruited to age < 10 years. Finally, for stratified analysis between HE and CP-TB cohorts, age less than or equal to 5 (< 5) years and age older than 5 and less than or equal to 10 (5 < 10) years were utilized based on the inverse relationship between age and the risk of TB following exposure (1, 7, 8). ELISPOT SFU data was imported from Excel (Microsoft CORP, Redmond, WA, USA) into a SAS data file and all analysis was performed using SAS version 9.1 (SAS Institute Inc, Cary, NC, USA). Baseline (univariate) comparisons between HE and CP-TB and confirmed TB (C-TB) study groups were performed using Students t test for continuous variables, and chi squared (or Fishers exact test where indicated) for categorical variables. Similarly, categorical comparisons of the frequency of positive ELISPOT assays by clinical study

group were evaluated with a chi squared test, or Fishers exact test where indicated. Due to the non normal distribution, SFU above background were compared using nonparametric analysis for continuous variables (Wilcoxon Rank Sum test).

To study the factors associated with CP-TB, logistic regression models were constructed to examine the independent influence of the $CD8^+$ T cell response (CD8 ELISPOT, categorical), the PBMC response (PBMC ELISPOT, categorical), age group (< 5 and > 5), nutritional status (BMI Z score, continuous), and TST (categorical) on the odds of having TB compared to the reference group (HE). The reference groups for categorical variables were a negative CD8 ELISPOT, a negative PBMC ELISPOT, age < 5 years, and a negative TST. The Z score was entered into the model as a continuous variable. Backwards logistic regression was performed with significance level of 0.05.

SUPPLEMENTAL REFERENCES

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SUPPLEMENTAL FIGURE LEGEND

Supplemental Figure E1. Magnitude of Mtb-specific T cell responses among children age 5 < 10 years with TB or HE children.

The magnitude of Mtb-specific T cell responses are shown. Results of CD8 ELISPOT (A) and PBMC ELISPOT (B) assays among children age 5 < 10 years, are shown. The sample size of the subgroups are: A) HE (*n* = 20), CP-TB (*n* = 22), C-TB (*n* = 7); and B) HE (*n* = 22), CP-TB (*n* = 20), C-TB (*n* = 5). SFU = Spot Forming Units per 250,000 cells above background.

Table E1. World Health Organization provisional guidelines for the diagnosis of pulmonary tuberculosis in children (4)

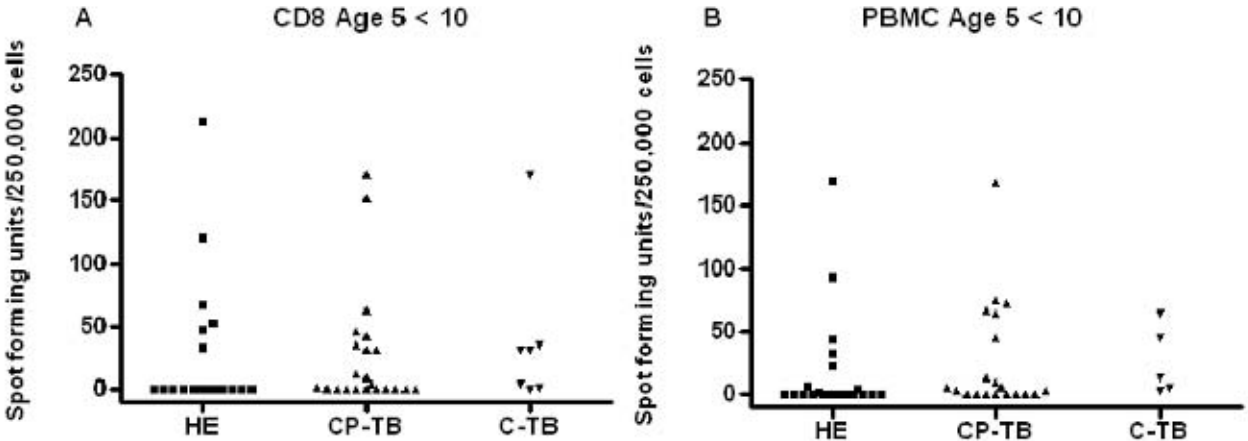
Suspected tuberculosis
An ill child with a history of contact with a confirmed case of pulmonary tuberculosis Any child: 1-Not regaining normal health after measles or whooping cough 2-With loss of weight, cough and wheeze not responding to antibiotic therapy for respiratory disease 3-With painless swelling in a group of superficial nodes
Probable tuberculosis (Prob-TB)
A suspect case and any of the following Positive (>10 mm) induration on tuberculin testing Suggestive appearance on chest radiograph Suggestive histological appearance of biopsy material Favorable response to specific antituberculous therapy
Confirmed tuberculosis (C-TB)
Detection by microscopy or culture of tubercle bacilli from secretions or tissues Identification of tubercle bacilli as *Mycobacterium tuberculosis* by culture characteristics

Supplemental Table E2. Predictors of having TB Disease (CP-TB)

Model	Covariate	e^{β} ($OR_{ADJUSTED}$)	95% CI	p value
CD8	CD8* (positive/negative)	3.7	1.5-9.3	0.005
	Age Group	0.27	0.12-0.65	0.003
	BMI Z Score	0.77	0.6-0.9	0.03
	TST result	0.9	0.4-2.2	0.9
PBMC	PBMC† (positive/negative)	1.4	0.7-2.9	0.36
	Age Group	0.43	0.19-0.9	0.03
	BMI Z Score	0.77	0.6-0.9	0.02
	TST result	1.1	0.5-2.2	0.8
CD8 & PBMC	CD8 (positive/negative)	5.3	1.7-15.8	0.003
	PBMC (positive/negative)	0.5	0.2-1.5	0.2
	Age Group	0.3	0.1-0.6	0.004
	BMI Z Score	0.8	0.6-1.0	0.053
	TST result	1.1	0.4-2.5	0.82

*CD8 = CD8 ELISPOT assay binary result

†PBMC= PBMC ELISPOT assay binary result.


A
CD8 Age 5 < 10
Spot forming units/250,000 cells
250
200
150
100
50
0
HE
CP-TB
C-TB
B
PBMC Age 5 < 10
Spot forming units/250,000 cells
250
200
150
100
50
0
HE
CP-TB
C-TB