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IMMUNOLOGICAL ASPECTS

Helminths and skewed cytokine profiles increase tuberculin skin test positivity in Warao Amerindians

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SUMMARY

The immune regulatory mechanisms involved in the acquisition of *Mycobacterium tuberculosis* infection in children are largely unknown. We investigated the influence of parasitic infections, malnutrition and plasma cytokine profiles on tuberculin skin test (TST) positivity in Warao Amerindians in Venezuela. Pediatric household contacts of sputum smear-positive tuberculosis (TB) cases were enrolled for TST, chest radiograph, plasma cytokine analyses, QuantiFERON-TB Gold In-Tube (QFT-GIT) testing and stool examinations. Factors associated with TST positivity were studied using generalized estimation equations logistic regression models. Of the 141 asymptomatic contacts, 39% was TST-positive. After adjusting for age, gender and nutritional status, TST positivity was associated with *Trichuris trichiura* infections (OR 3.5, 95% CI 1.1–11.6) and low circulating levels of T helper 1 (Th1) cytokines (OR 0.51, 95% CI 0.33–0.79). *Ascaris lumbricoides* infections in interaction with Th2- and interleukin (IL)-10-dominated cytokine profiles were positively associated with TST positivity (OR 3.1, 95% CI 1.1–8.9 and OR 2.4, 95% CI 1.04–5.7, respectively). A negative correlation of QFT-GIT mitogen responses with Th1 and Th2 levels and a positive correlation with age were observed (all $p < 0.01$). We conclude that helminth infections and low Th1 cytokine plasma levels are significantly associated with TST positivity in indigenous Venezuelan pediatric TB contacts.

1. Introduction

It is estimated that one third of the world's population is infected with *Mycobacterium tuberculosis* and that each year about 9 million people develop tuberculosis (TB), 1 million (11%) of whom are children under 15 years of age.[1] Children who are in close contact with adult pulmonary TB have a high risk of being infected and developing active TB disease. It is generally accepted that 30–50% of household contacts of adults with infectious forms of pulmonary TB will have a positive tuberculin skin test (TST).[2] Age, proximity of exposure, malnutrition, household crowding and the contact's Bacille Calmette-Guérin (BCG) vaccination scar status have been found to influence TST positivity among childhood

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contacts of sputum smear-positive index cases.[3–6] A unique aspect of TB in children is the rapid progression to disease, typically within the first year following infection.[7] The risk of developing disease is determined by a combination of factors, including age, virulence of the TB strain, genetic factors, magnitude of the initial infection and host immunity.[8] The cytokine-mediated immune response to *M. tuberculosis* infection is an important determinant for the development of TB: the cell-mediated immunity, involving activation of macrophages and T helper 1 (Th1) cells, plays a protective role, whereas a Th2 response undermines the efficacy of immunity and contributes to immunopathologic conditions.[9–11]

Parasitic infections share the same 'developing world niche' as TB. One-third of the world's population harbors at least one species of intestinal parasite with children between 5 and 15 years of age bearing the greatest burden in terms of morbidity and mortality.[12,13] The Th2 skewing or immunomodulation induced by helminth infections can decrease the development of a Th1 response after infection with *M. tuberculosis* which may favor persistence of the *M. tuberculosis* infection.[14] Furthermore, helminth infections affect the efficacy of BCG vaccination[15] and helminth infections are associated with undernutrition in endemic populations.[16] As the cell-mediated immunity is influenced by nutritional status of the host,[17] the negative effect of helminth infections on cell-mediated immunity against *M. tuberculosis* may be partly explained by malnutrition accompanying helminth infections.

While many investigators have evaluated cytokine production by stimulated whole blood or peripheral blood mononuclear cells (PBMCs) recovered from patients with *M. tuberculosis* infection or disease,[18–20] few of them have measured circulating serum cytokine concentrations. As the *ex vivo* antigen-stimulated production of cytokines only reflects the cytokine response to a specific stimulus, it may not provide sufficient insight into the actual status of the cytokine network *in vivo*, as this is the result of many different antigen stimulations. Most studies examining cytokine responses have focussed on the comparison of TB patients with healthy controls[21,22] and cytokine plasma levels have been considered as biosignatures of TB disease.[23] Understanding the immune regulatory mechanisms in *M. tuberculosis*-infected children may provide novel insights into their increased susceptibility to progression to disease compared with adults.[8,24]

In Venezuela, the average annual national incidence rate of TB is moderate (25–50 per 100,000 inhabitants[25]), but an extraordinary high incidence rate (3190 per 100,000) has been reported in children among Warao Amerindians.[26] The Warao people, one of the largest indigenous populations in the South American lowland, live in the Orinoco Delta in northeastern Venezuela. Warao Amerindians live in extreme poverty and high prevalence rates of intestinal helminth and protozoan infections and malnutrition have been described in Warao children.[27,28] To explore whether parasitic infections and malnutrition influence the risk of *M. tuberculosis* infection and to what extent this risk is modulated by an immunological shift in Th1/Th2 profiles, we investigated circulatory plasma cytokine levels, intestinal parasitic infections and nutritional status in asymptomatic TST-positive and TST-negative Warao pediatric TB contacts.

2. Methods

2.1. Study design and setting

This study was conducted over a period of 12 months, from May 2010 to May 2011, in the Warao Amerindians, an indigenous population living in wooden houses raised on stilts along the Orinoco river banks. Household contacts living with sputum smear-positive pulmonary TB patients in the municipalities Antonio Diaz and Pedernales were asked to enroll in the study. All household contacts aged between 1 and 15 years were eligible. Contacts of patients who registered for TB treatment in the Venezuelan National TB Control Program in the study period were included within one month after registration of the index case. We defined household contacts as children who slept in the same home as the index case, ate with the index case and whose parents identified a common household head. Children who had previously been treated for TB were excluded ($n = 1$).

2.2. Sample collection

Blood was collected from each participant into 1 EDTA tube and 3 heparin-containing tubes for the QuantiFERON-TB Gold In-Tube assay (QFT-GIT), including a positive control (mitogen), a negative control (heparin), and a TB-antigen tube (containing antigens ESAT-6, CFP-10, and TB-7.7). The QFT-GIT assay was performed using QFT-GIT kits (Cellestis) according to manufacturer's instructions by a single experienced laboratory technician who was blinded to all clinical information.[29] The Hemocue Hb201+ and Hemocue WBC (Hemocue AB, Ängelholm, Sweden) were used to assess hemoglobin levels and total white blood cell (WBC) count, respectively. A peripheral blood smear was stored for microscopic leukocyte differentiation. Human immunodeficiency virus (HIV) antibody testing was done after appropriate counseling using the Determine (Abbott Laboratories) HIV 1/2 rapid test. Plasma was stored at −70 °C until cytokine analysis. Subsequently, TST was performed by following standard procedures: 0.1 mL of tuberculin purified protein derivative of *M. tuberculosis* strain RT-23 (Statens Seruminstitut, Copenhagen) was injected intradermally into the volar surface of the left forearm. A palpable transverse induration with a diameter of ≥10 mm measured 48–72 h after injection was considered positive.

In addition, a stool sample was requested from all participants. Stool samples were preserved in sodium acetate–acetic acid–formalin (SAF) preservative[30] and stored at 4 °C until examination by experienced laboratory technicians for the presence of helminths and intestinal protozoa. An aliquot of the unpreserved sample was mixed with ethanol 96%. Feces samples in ethanol were stored at −70 °C until DNA isolation with the High Pure PCR template preparation kit (Roche, Germany). Real-time polymerase chain reaction (PCR) of fecal samples was conducted on the Roche LightCycler® 480 system for detection of *Entamoeba histolytica*, *Dientamoeba fragilis*, *Giardia lamblia* and *Cryptosporidium parvum*.[31,32] The diagnosis of parasitic infections was based on both microscopy and PCR.

Standard antero-posterior and lateral chest radiographs (CXRs) were taken. Two independent experts, blinded to all clinical information, evaluated the CXRs and documented their findings on a standard report form. Where the two experts disagreed, a third expert was consulted and final consensus was achieved. A sputum sample was collected from all children who could expectorate with gastric aspirates taken from all children under 6 years of age. Specimens were cultured on Middlebrook (7H9) liquid broth-based media and on Ogawa solid media. Confirmed TB was defined as isolation of *M. tuberculosis* on culture. PCR-restriction analysis of the hsp65 gene (PRA) was performed to differentiate *M. tuberculosis* from nontuberculous mycobacteria. Probable TB was defined as clinical and radiographic findings consistent with intrathoracic TB as defined by Marais et al.[33] and either (1) a positive TST or QFT-GIT or (2) histopathologic findings compatible with TB, without positive mycobacterial culture results. Possible TB was defined as the presence of abnormal CXR findings not consistent with intrathoracic TB[33] and either (1) a positive TST or QFT-GIT or (2)

histopathologic findings compatible with TB, without positive mycobacterial culture results. Latent TB infection (LTBI) was defined as a TST $\geq$10 mm in the absence of microbiological, radiological and clinical evidence of TB disease.

2.3. Anthropometric measurements

Anthropometric measurements were transformed into weight-for-age, height-for-age, and BMI-for-age Z scores based on WHO standard reference populations[34,35] using WHO anthro software.[36] Children under 5 years of age with height-for-age or weight-for-age *Z* scores < -2 standard deviations (SD) were defined as malnourished. Children aged 5–15 years with height-for-age or BMI-for-age *Z* scores < -2 SD were defined as malnourished, as weight-for-age is inadequate for monitoring growth beyond childhood.[34]

2.4. Cytokine measurements

Eleven cytokines (interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17, interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α)) were measured using Luminex multiplex bead-based technology with a Bioplex 11-plex cytokine assay (Biorad Life Science, CA, USA) according to the manufacturer's instructions. Cytokine measurements were performed on a Luminex 100 (Biorad Life Science) and Luminex software was used to calculate the amount of cytokines (in pg/mL plasma).

2.5. Ethical considerations

The nature and objectives of the study were explained to the parents of exposed children in Spanish and/or in their native language. The study was approved by the ethical committee of the Instituto de Biomedicina, the Regional Health Services, and the Indigenous Health Committee of the Ministry of Health and Social Welfare. Children were enrolled if their parents or primary caregivers provided written informed consent. If parents or primary caregivers were illiterate, consent forms were read to them in Spanish and/or in their native language and they were signed by means of a thumb print.

Children diagnosed with confirmed, probable or possible TB were treated with a standard six month anti-TB regime as recommended by the Venezuelan National TB Control Program. Anti-TB treatment is free of charge in Venezuela and was supplied by the Delta Amacuro department of the National TB Control Program. The study team transported treatment to the communities where it was given to the community nurses that are responsible for the delivery of anti-TB treatment to patients. Anti-helminthic and anti-protozoal treatment based on the feces microscopy results was provided free of charge by the study team.

2.6. Statistical analysis

Categorical variables were analyzed using Chi-square test or Fisher's exact test, as appropriate. For continuous variables, the unpaired Student's *t* test or nonparametric Mann–Whitney's test was used depending on whether or not the variables were normally distributed (Kolmogorov–Smirnov's test, $p > 0.05$).

Generalized estimating equations (GEEs) were used to fit a multivariable logistic regression model aimed at identifying possible associations between TST positivity (dependent variable) and circulating cytokine levels and parasitic infections (independent variables). GEEs account for correlation and lack of independence of responses for contacts with an index TB case in common (clusters within households) using quasi-likelihood methods and robust variance estimators.[37] To avoid multicollinearity, a principal component analysis (PCA) was performed to transform the highly intercorrelated cytokine levels into uncorrelated or orthogonal principal components (PCs).[38] The PCA was performed with the correlation matrix (variables standardized to zero mean and unit standard deviation) so that the contribution of individual variables was not influenced by their variance. Kaiser–Meyer–Olkin's (KMO) measure of sampling adequacy and Bartlett's test of sphericity provided the minimum standards to conduct the PCA. The orthogonal varimax rotation[38] was applied to associate each original cytokine variable with only one PC. Six PCs, representing the cytokine profiles dominated by Th1 (IL2, IL12p70, IFNγ and TNFα), Th2 (IL4, IL5 and IL13), IL-1β, IL-6, IL-10, and IL17 were thus generated. Variables univariately associated with TST positivity with a *p*-value $\leq$0.25 were considered for inclusion in the multivariable model. A backward stepwise variable selection procedure was applied: variables showing a *p*-value >0.05 were dropped, but if their exclusion resulted in a substantial change (usually 10–30%) in the coefficients of the included covariates, they were considered as a potential confounder and retained in the model. All models were adjusted for age, sex and nutritional status. The quasi-likelihood under the independence model information criterion (QIC)[39] was used to guide the selection of the most parsimonious model and the best fitting within-group working correlation structure. All possible two-way interaction terms between parasitic infections and cytokine profiles were constructed using the partial Gram–Schmidt orthogonalization procedure.[40] This method has the advantage that the interaction term is orthogonal with respect to the original variables; thus, the assumption of covariate independence is not violated.[40] Model estimates were expressed as odds ratios (OR) and 95% confidence intervals. For simplicity, only the results of the final, parsimonious model were presented.

Concordance between TST and QFT-GIT was assessed using the Kappa (κ) coefficient from respective cross-tabulation analyses. Correlations between mitogen control response and age, cytokine plasma concentrations and WBC count were assessed using the Spearman's rank correlation coefficient (r).

Stata Program version 11 (Stata Corp, College Station, TX) and SPSS software for windows, version 16.0 (SPSS Inc.), were used for statistical analysis. Statistical significance was set to *p*-value <0.05.

3. Results

Thirty-four TB patients and their 154 household contacts were eligible. However, three patients and their contacts were untraceable and in one household, parents were not willing to enroll their children in the study. Finally, TST and CXR were performed in 145 household contacts of 30 adult patients (94%). In none of the 30 households, previous index cases had been registered in the Venezuelan National TB Control Program. Four children (3%) were diagnosed with TB, of which two were confirmed TB and two were probable TB. No child was diagnosed with possible TB. The 141 asymptomatic children were included in the analyses. Blood samples were collected from 140 of 141 children (99%); feces samples were collected from 111 children (79%).

3.1. Associations of cytokine levels and parasitic infections with LTBI

Characteristics of TST-positive and TST-negative children are shown in Table 1. All children were HIV-negative. Male contacts were more often TST-positive than female contacts (66% vs. 34%, $p = 0.03$). Children with positive TST results were older than children with negative TST results (mean age 9.1 vs. 7.2, $p < 0.01$). There were no significant differences between the two groups regarding nutritional status, hematologic values, intensity of contact or presence of a BCG scar (Table 1).

Table 1
Characteristics of TST-negative and TST-positive Warao Amerindian childhood TB contacts.

	TST-negative (n = 86, 61%)	TST-positive (n = 55, 39%)	P value
Demographic figures			
Gender, *n* (%)			0.03
Male	40 (47)	36 (66)	
Female	46 (53)	19 (34)	
Age (years), mean (SD)	7.2 (3.8)	9.1 (3.0)	<0.01
Number of people in household, median (IQR)	6 (6–7)	6 (5–8)	0.76
Nutritional status			
Weight-for-age *Z* score in children <5 years, mean (SD)	−1.69 (0.95)	−0.14 (1.83)	0.16
BMI-for-age *Z* score in children ≥5 years, mean (SD)	0.01 (1.07)	0.12 (0.85)	0.52
Malnourished, *n* (%)	36 (42)	16 (29)	0.13
Contact factors, *n* (%)			
Relationship with index case			0.86
Mother	12 (14)	5 (9)	
Father	12 (14)	8 (15)	
Sibling	6 (7)	4 (7)	
Other	56 (65)	38 (69)	
Duration of contact (h/day)			0.42
1–4	24 (28)	13 (24)	
5–8	13 (15)	7 (13)	
9–12	10 (12)	3 (6)	
13–24	39 (45)	32 (58)	
Sleeping site relative to index case			0.18
Same bedroom	48 (56)	37 (67)	
Different bedroom	38 (44)	18 (33)	
BCG scar, *n* (%)	72 (84)	49 (89)	0.37
Parasitic infections, *n* (%)			
Helminth infections	16 (26)	29 (59)	<0.01
Hookworm	2 (3)	7 (14)	0.04
Ascaris lumbricoides	10 (16)	13 (27)	0.18
Strongyloides stercoralis	1 (2)	3 (6)	0.32
Trichuris trichiura	9 (15)	21 (43)	<0.01
Hymenolepsis nana	5 (8)	4 (8)	1.00
Protozoan infections	47 (76)	44 (90)	0.06
Dientamoeba fragilis	8 (13)	13 (27)	0.07
Giardia lamblia	29 (47)	23 (47)	0.99
Blastocystis hominis	19 (31)	33 (67)	<0.01
Endolimax nana	8 (13)	8 (16)	0.61
Entamoeba coli	21 (34)	23 (47)	0.16
Iodamoeba butschlii	4 (7)	7 (14)	0.21
Entamoeba dispar	1 (2)	1 (2)	1.00
Cytokine concentrations (pg/mL), median (IQR)			
Interleukin-1β	144.6 (87.7–184.8)	108.2 (0.0–145.2)	0.01
Interleukin-2	0.0 (0.0–6.1)	0.0 (0.0–0.0)	0.40
Interleukin-4	89.0 (54.5–122.5)	64.4 (0.0–95.4)	0.01
Interleukin-5	351.5 (201.4–479.9)	247.7 (0.0–399.9)	0.02
Interleukin-6	386.2 (257.9–479.6)	289.9 (142.0–407.6)	0.03
Interleukin-10	262.7 (164.4–357.7)	189.6 (77.1–296.0)	<0.01
Interleukin-12p70	420.5 (208.2–582.7)	329.1 (0.0–485.2)	0.03
Interleukin-13	93.1 (29.2–130.0)	79.1 (0.0–110.0)	0.14
Interleukin-17	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.76
Interferon-γ	1989.8 (1133.5–2483.9)	1337.9 (315.5–1940.0)	<0.01
Tumor necrosis factor-α	1337.2 (722.9–1774.9)	948.8 (0.0–1435.8)	0.01
WBC values (× 10^9 cells/L), median (IQR)			
Total WBC count	11.0 (9.0–14.8)	11.0 (8.8–13.0)	0.36
Neutrophils	5.7 (4.1–8.5)	6.3 (4.4–8.0)	0.66
Lymphocytes	4.0 (3.0–5.7)	3.7 (2.9–4.9)	0.10
Monocytes	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.81
Eosinophils	0.7 (0.2–1.9)	0.6 (0.3–1.5)	0.78
Basophils	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.43
Anemia, *n* (%)*	10 (12)	4 (8)	0.40

* Anemia was defined by hemoglobin levels <6.8 mmol/L (11 g/dL) for children <6 years of age, <7.1 mmol/L (11.5 g/dL) for children 6–11 years of age and <7.7 mmol/L (12.5 g/dL) or <7.3 mmol/L (11.8 g/dL) for respectively males and females >11 years of age, in accordance with the criteria of the Centers for Disease Control and Prevention.[78]

TST-positive children showed a tendency toward more helminth and protozoan infections, some of which (*Hookworm*, *Trichuris trichiura* and *Blastocystis hominis*) being statistically significant. Cytokine plasma concentrations of IL-1β, IL-4, IL-5, IL-6, IL-10, IL-12p70, IFN-γ and TNF-α were significantly lower in TST-positive compared to TST-negative children (Table 1).

Multivariable analysis adjusting for age, gender and nutritional status showed a significantly lower Th1-dominated profile (IL-2, IL-12p70, IFN-γ, TNF-α) in TST-positive compared to TST-negative children (OR 0.51, 95% CI 0.33–0.79, Table 2). Infection with *T. trichiura* was significantly more likely to be present in TST-positive compared to TST-negative children (OR 3.5, 95% CI 1.1–11.6). Interactions of Th2- and IL-10-dominated profiles with *Ascaris lumbricoides* infection were both significantly positively associated with TST positivity (OR 3.1, 95% CI 1.1–8.9 and OR 2.4, 95% CI 1.04–5.7, respectively).

Table 2
Factors predicting TST positivity in Warao Amerindian childhood contacts. Odds ratios and 95% confidence intervals (CI) from the final GEE multivariable logistic regression model are presented.

	Odds ratio* of TST-positive vs. TST-negative contacts	95% CI
Helminth infections		
Trichuris trichiura	3.5	1.1–11.6
Ascaris lumbricoides	0.97	0.37–2.6
Cytokine profiles		
Th1-dominated profile (IL2, IL12p70, IFNγ, TNFα)	0.51	0.33–0.79
Th2-dominated profile (IL4, IL5, IL13)	0.87	0.52–1.4
IL-10-dominated profile	0.64	0.34–1.2
Interaction between cytokine profiles and helminth infections		
Th2-dominated profile × *A. lumbricoides*	3.1	1.1–8.9
IL-10-dominated profile × *A. lumbricoides*	2.4	1.04–5.7

* Adjusted for age, gender and nutritional status.

3.2. Associations of QFT-GIT results with parasitic infections and malnutrition

QFT-GIT results were negative in 80 children (57%), positive in 48 (34%) and indeterminate in 12 children (9%). The concordance between TST and QFT-GIT when excluding indeterminate results was substantial (agreement 88%; $\kappa = 0.74$, 95% CI 0.62–0.86). The 12 children with indeterminate QFT-GIT results were more often infected with helminths than children with negative QFT-GIT results (67% vs. 25%, $p = 0.02$). This association was small and not statistically significant for children with positive QFT-GIT results (67% vs. 56%, $p = 0.72$). No significant associations were found between protozoan infections, malnutrition or age and QFT-GIT results, although there was a tendency toward more indeterminate results in malnourished children ($p = 0.21$ and $p = 0.18$ for indeterminate results vs. negative and positive results, respectively). Of the 12 indeterminate results, 10 (83%) were due to a low mitogen response. Mitogen responses in all children were significantly positively correlated with age (Spearman's r 0.29, $p < 0.01$) and there was a significant negative correlation of mitogen response with total WBC count (Spearman's r −0.28, $p < 0.01$). Mitogen responses were also negatively correlated with all cytokine plasma concentrations and this was significant in all but 2 (IL-17 and IL-2) cytokines with Spearman's r varying from −0.23 to −0.39.

4. Discussion

Helminth infections increased the risk of TST positivity in Warao Amerindian pediatric TB contacts. Circulating levels of all measured cytokines were generally lower in TST-positive children compared to TST-negative children, but only a Th1-dominated cytokine profile was significantly and negatively associated with TST positivity in multivariable analysis.

Chronic helminth infections are associated with the production of regulatory T cells that suppress T cell-mediated immune responses.[41] This immunosuppression could lead to a greater risk of infection with *M. tuberculosis* which may be associated with a positive TST. In studies from the Middle East no differences in serum cytokine levels between TST-positive and TST-negative individuals were found.[42,43] One Canadian study reported higher levels of TNF-α, IL6-, IL-1β and IL-4 in serum of TST-positive individuals compared to TST-negative individuals, whereas no differences were found in circulating levels of IL-17, IFN-γ or IL-12p70.[44] There are several possible explanations for the low Th1 circulating cytokine levels in TST-positive children in our study. First, the localization of the primary infection in the lung and compartmentalized expression of Th1 cytokines leading to lower systemic cytokine concentrations could have played a role.[45–47] Second, since PBMCs of Warao TB patients and controls showed lower Th1 responses compared to Creoles upon stimulation with TB antigens,[48] and prevalence rates of TB disease in Warao children are extraordinary high compared to national estimates,[25,26] we speculate that our finding might reflect an inherent high susceptibility to TB in the Warao population. The evidence for a genetic component in TB susceptibility is incontrovertible.[49] For instance, mutations affecting the expression of IFN-γ receptors, IL-12 or IL-12 receptors have been associated with disseminated mycobacterial infections in children.[50,51] An unusual immune reaction to *M. tuberculosis* leading to increased vulnerability and an extremely high prevalence of active TB (6400 per 100,000) has also been described in Brazilian Yanomami Indians.[52] Finally, we used a multivariable modeling approach taking into account other putatively influencing factors, such as parasitic infections, demographic characteristics, nutritional status, other cytokine profiles, and the clustering at the household level. The univariate approach that was used in most other studies[42–44,53] could have contributed to insufficient insight into the actual status of the cytokine network.

We observed that the interactions between *A. lumbricoides* infection and IL-10- and Th2-dominated profiles were significantly associated with TST positivity. In contrast, the simple effects of Th2 and IL-10 levels, without concurrent *A. lumbricoides* infection, were not significantly associated with TST outcome. A possible explanation for this finding comes from the animal model. In mice co-infected with *M. tuberculosis* and the helminth *Nippostrongylus brasiliensis*, a mouse prototype of *A. lumbricoides*, an increase in Th2 response alone did not impair the development of *M. tuberculosis*-specific immune responses. However, the helminth-induced Th2 environment resulted in the accumulation of alternatively activated macrophages in the lung that enhanced the intracellular persistence of *M. tuberculosis*.[54] For IL-10, similar results were seen in transgenic mice overexpressing IL-10 that were infected with TB; IL-10 triggered aspects of alternative macrophage activation and promoted *M. tuberculosis* survival independent of overt effects on anti-TB immunity.[55] The interactions of *T. trichiura* infection with any of the cytokine levels were not associated with TST outcome. Possibly, the influence of *T. trichiura* infection on systemic cytokine profiles is less pronounced than for *A. lumbricoides* because *T. trichiura* remains localized in the gut area whereas *A. lumbricoides* larvae undergo an extensive migration through the circulatory system, liver and lungs.[56]

Clinical history of helminth infection in this study was unknown. Furthermore, due to the cross-sectional study design, it was unknown whether helminth infection preceded TST positivity or whether helminths were acquired after TST conversion. It is well known that helminth infections exhibit an overdispersed frequency distribution, which results in the helminths aggregating in a small consistent proportion of the population, called 'wormy persons'.[57–59] If the children infected in our study belong to a group of 'wormy' individuals, helminth infection probably preceded TST positivity and the helminth-induced modulation of T cell responses could have led to an increased susceptibility to *M. tuberculosis* infection. Prospective follow-up studies are needed to determine whether helminth-related immune modulation could also predispose to subsequent clinical TB. Longitudinal studies are also needed to determine whether the associations between helminth infections, TST positivity and cytokine levels we observed reflect patterns in 'wormy' individuals and whether anti-helminthic treatment influences the observed associations.

Male contacts were more often TST-positive than female contacts in our study. In most other studies girls are equally or more often TST-positive than boys up to adolescence, after which TST positivity is higher among males.[60–62] The difference we observed may reflect greater exposure among male contacts related to the division of labor by gender. Girls from a young age help their mothers in the cultivation of ocumo outside the household. However, boys go hunting and fishing as soon as they are found to be old enough, generally around ten years of age. Another possibility is that the gender difference in TST positivity reflects a genuine difference in susceptibility to TB infection or predisposition to delayed-type hypersensitivity responsiveness.[63]

IFN-γ assays have been found to be more specific in the diagnosis of LTBI than the TST as false-positive TST results occur due to cross-reactivity with environmental mycobacteria and BCG.[64] However, data on the use of IFN-γ assays in children are scarce and contradictory. Ethnicity, malnutrition, parasitic infections, young age, acute inflammation, immunosuppression, therapy with beta-lactam antibiotics and atopy have been associated with indeterminate QFT-GIT results in children.[65–67] The lack of a gold standard test for LTBI limits firm conclusions on the associations between immune response, helminth infections and *M. tuberculosis* infection when the latter is defined by tests that are known to have flaws. Repetition of our multivariable analysis taking the QFT-GIT positive children as the LTBI group showed the same pattern of associations as for TST-positive children. This is an indication that our results are robust regardless of the LTBI definition used.

As in most studies assessing the performance of the QFT-GIT,[68–70] the majority of indeterminate results in our study was due to a low mitogen response. We observed a positive correlation of age with mitogen response; a finding that has consistently been observed in studies in different populations.[71–73] In other studies, leukocytopenia was a good predictor of indeterminate results caused by low mitogen responses.[68,69] The finding that in our study high WBC counts were correlated with low mitogen responses is, therefore, remarkable. As children in our study live under remote circumstances where they are exposed to multiple pathogens, a low IFN-γ production in response to the mitogen control could be due to T cell exhaustion with less production of cytokines in response to mitogen. This phenomenon has been described in chronic infections, malaria and cancer.[74,75] The finding that the mitogen response decreased with increasing plasma levels of all detectable cytokines supports the hypothesis that T cells were exhausted by continuous infective triggers enhancing cytokine production. Helminth infections could have played a role in T cell exhaustion, as intestinal *A. lumbricoides* and *T. trichiura* infections have been associated with a generalized suppression of Th1 and Th2 cytokine responses to mitogen in children.[76,77] Further investigation of the performance of IFN-γ assays in remote populations where other infections, including helminth infections, are endemic is therefore necessary.

5. Conclusions

In Warao Amerindians in Venezuela, TST positivity in contacts of sputum-smear positive TB patients is significantly associated with low circulating levels of Th1 cytokines. Furthermore, we provide evidence indicating that *T. trichiura* infection and *A. lumbricoides*-modulated Th2 and IL-10 levels can increase the risk of a positive TST after *M. tuberculosis* exposure. Finally, interpretation of IFN-γ assays requires special consideration when performed in children exposed to multiple pathogens, including intestinal helminths, as a negative correlation between mitogen response and an already existing immune activation exists.

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