

Lack of an inverse association between tuberculosis infection and atopy: By T-cell-based immune assay (RD1–ELISpot)

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The association between mycobacterial exposure, vaccination with bacillus Calmette-Guerin (BCG) in early life and atopy remains controversial. Distinguishing between environmental mycobacterial exposure, TB infection and BCG-vaccination is not possible with the tuberculin skin test (TST) but new accurate blood-tests for TB infection present an opportunity to differentiate TB infection from environmental mycobacterial exposure and BCG-vaccination. We used a new blood test in parallel with TST to investigate whether *Mycobacterium tuberculosis* infection and/or BCG vaccination are associated with development of atopy in children with prior household TB contacts. All children who had contact with adult active pulmonary TB during the last 6 months underwent TST, chest radiography, and RD1–ELISpot assay. The presence of a BCG scar was documented, and assessment of atopy was carried out by International Study of Asthma and Allergies in Childhood questionnaire, allergy skin prick testing (SPT) and evaluation of serum total IgE. Among 361 children enrolled 39 (11%) had a positive SPT, 236 (63%) positive TST, and 189 (52%) positive RD1–ELISpot. The frequency of SPT positivity, ever wheezing, allergic rhinitis, doctor-diagnosed asthma, high serum IgE level, and median total serum IgE levels did not differ significantly different by TST or RD1–ELISpot status. On the other hand, presence of BCG scar was associated with lower median total serum IgE level ($p = 0.01$) and lower frequency of high IgE ($p = 0.003$). *M. tuberculosis* infection whether measured by TST or RD1–ELISpot, was not associated with atopy in children with household TB contact. Presence of a BCG vaccination scar was inversely associated with atopy, as measured by serum IgE.

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In earlier studies, the development of atopy in humans was found to be inversely correlated with *Mycobacterium tuberculosis* (1, 2). Th2 cells play a central role in the development of an atopic disease while Th1 cells are characterized by the production of interleukin (IL)-2 and interferon (IFN)- γ which counteracts the Th2 response (3–5). *M. tuberculosis* infection and bacillus Calmette-Guerin (BCG) vaccination induce Th1-type immune responses and

cause suppression of Th2-type cytokines which are the characteristics of atopic disorders (2, 6, 7). In 1997, Shirakawa et al. demonstrated an inverse association between delayed hypersensitivity to *M. tuberculosis* and atopy among Japanese school children. This inverse association provided epidemiologic evidence in favor of the hygiene hypothesis and mycobacterial exposure (2). These results have been debated in an attempt to determine to what extent the

results are explained by exposure to mycobacteria, BCG vaccination, or unknown host factors (8–10). In those previous studies, determination of mycobacterial infection relied solely on the tuberculin skin test (TST). However, TST has many drawbacks which include the accuracy and sensitivity of the test being influenced by several factors. The technique of administering, placement, and interpretation, as well as the characteristics of each patient such as age and skin can also introduce some degree of variability. In addition, false-positive reactions can occur because of cross-reactivity related to previous exposure to a non-tuberculosis mycobacteria or prior immunization with BCG. Furthermore, TST may give false-negative results in many conditions including poor nutrition, overwhelming acute illness, immunosuppressive medications, malignancy, HIV infection, and vaccination with live-attenuated viruses (11–13). Therefore, a need arose for a better screening test than TST to discriminate between positivity due to BCG vaccination and infection with *M. tuberculosis*. For that purpose, novel T-cell-based immune assays have been developed recently. These tests use antigens specific to *M. tuberculosis*, such as an early secreted antigenic target-6 kDa (ESAT-6) and a culture filtrate protein 10 kDa (CFP-10). These proteins are products of a genomic region (RD1) that is present in *M. tuberculosis* complex, but absent from all strains of *Mycobacterium bovis* BCG and almost all environmental mycobacteria (14–16). RD1-ELISpot is the *ex vivo* enzyme-linked immunospot (ELISpot) assay which enumerates the IFN- γ secreting T-cells specific for the same antigens. In active TB, the sensitivity and specificity of the RD1-ELISpot assay were found to be 96% and 92%, respectively (17). In close contact cases of active tuberculosis (TB), the association between test positivity and the extent of exposure has been shown to be significantly higher with RD1-ELISpot than with TST (18, 19). This suggests superiority over TST for the detection of latent TB infection, leading to the recommendation in current American and British guidelines (20, 21).

The aim of this study was to investigate the association between atopy and exposure to mycobacterial antigens by means of either the BCG vaccination or *M. tuberculosis* infection which was evaluated by both TST and RD1-ELISpot test as an unbiased measure of TB-specific T-cell response in children with recent household exposure to active pulmonary TB.

Methods

Study population

The study was conducted at the Division of Pediatric Infectious Diseases and Pediatric Allergy-Immunology of the Marmara University School of Medicine in Istanbul, Turkey. Ethical approval was obtained from the Ethics committee of the university. Between October 2002 and May 2004, children with a recent household contact to an adult patient diagnosed as sputum smear-positive pulmonary TB at any of the seven government-funded TB clinics in Istanbul were enrolled consequently in the study. All participants' caregivers provided a written informed consent. For each patient, a full medical history, BCG vaccination scars were documented as well as a physical examination was performed.

All children underwent TST and chest radiography, and gave 10-ml of venous blood sample for the RD1-ELISpot assay and total serum IgE level. The sera were kept at -20°C until the IgE level analysis. The allergy skin prick test (SPT) was performed following blood sampling. The International Study of Asthma and Allergies in Childhood (ISAAC) questionnaire was filled out by the study pediatricians to gather data on ever-wheezing, allergic rhinitis, doctor-diagnosed asthma, and atopic dermatitis.

Tuberculin skin testing

All patients underwent tuberculin skin testing which was performed on the volar aspect of the forearm by the Mantoux method using 0.1 ml (2 tuberculin units) of purified protein derivative (PPD) RT23 (Statens Serum Institute, Copenhagen, Denmark). The test was performed and evaluated by an experienced nurse who was blinded to RD1-ELISpot results. Intradermal inoculation of PPD was confirmed in all participants by the appearance of cutaneous peau d'orange. Induration was measured after 48–72 h using the ballpoint pen method. The TST response was scored as positive if the induration diameter was ≥ 10 mm (11).

Ex-vivo IFN- γ ELISpot assay. Pre-coated interferon- γ ELISpot plates (Mabtech AB, Stockholm, Sweden) were seeded with 2.5×10^5 peripheral blood mononuclear cells per well: duplicate wells contained no antigen (negative control), phytohemagglutinin (positive control; ICN Biomedical, Aurora, OH, USA) at 5 $\mu\text{g}/\text{ml}$ and a further six pairs of duplicate wells, each containing one of six peptide pools, which incorporated six or seven 15mer peptides from

35 such peptides spanning the length of ESAT-6 or CFP-10, such that the final concentration of each peptide was 10 µg/ml, as previously described (19, 20). After overnight incubation at 37°C in 5% CO₂, the plates were developed with pre-conjugated detector antibody (Mabtech AB) followed by the chromogenic substrate BCIP-NBT^{PLUS} (Moss Inc., Pasadena, MD, USA), as previously described (18, 19).

The ELISpot plates were scored by an automated ELISpot counter (AID-GmbH, Strassberg, Germany). Intensity and spot size settings were pre-defined and the same settings were used throughout. Mean readings from duplicate wells were electronically transferred to a spreadsheet and scored as positive or negative by a customized software program, ELISTAT (AID-GmbH, Strassberg, Germany). Responses were scored as positive if the test wells contained a mean of at least five spot-forming cells more than the mean of the negative control wells, and, in addition, this number was at least twice the mean of the negative control wells. These predefined cut-off points were translated into a detection threshold of 20 peptide-specific T cells per 10⁶ PBMC. Persons performing and reading the assays were blind to all personal identifiers and TST results.

ISAAC questionnaire

The International Study of Asthma and Allergies in Childhood (ISAAC) questionnaire was used to obtain data on ever-wheezing, allergic rhinitis, doctor-diagnosed asthma, and atopic dermatitis from parents, usually the mother.

SPT and measurement of total serum IgE level

Skin prick testing was performed with 15 common aeroallergens including *Dermatophagoides farinae*, *Dermatophagoides pteronyssinus*, *Alternaria*, *Aspergillus* mix, *Cladosporium*, *Penicillium* mix, *Candida albicans*, Betulaceae, mixture of 4 cereals, mixture of 12 grasses, Salicaceae, Composites, feathers mixture, cat hair, and dog hair (Stallergens S.A., Antony Cedex, France). Histamine and dihydrochloride saline were used as positive and negative controls. A wheal size of ≥3 mm was considered as positive. Subjects with one or more positive reactions were considered atopic. Total serum IgE level for each subject was determined by the Immulite method (Euro/DPC, Llnberis, UK). A high serum total IgE level was accepted when total serum IgE level was >45 IU/ml in children between 1 and 5 yrs old, >100 IU/ml in children above 6 yrs old and older (22, 23).

Statistical analysis

Statistical analyses were performed by the STATA version 7.0 software package program (STATA, College Station, TX, USA). Differences between groups were tested for significance with chi-square test, Fisher's exact test, *t*-test, or Mann-Whitney *U*-test as appropriate. A *p*-value of <0.05 was considered to be significant.

Results

We enrolled 361 household contacts of adult patients with sputum smear-positive pulmonary TB. RD1-ELISpot, TST, SPT, and ISAAC questionnaire results of each participant were available. Total serum IgE was measured in 347 contacts. The mean (±s.d.) age of the contacts was 8 ± 4 yrs, of whom 53% were male and 47% were female. Of those contacts, 290 (80%) had a BCG vaccine scar. TST was positive in 236 (63%) contacts in whom the TST induration size (mean ± s.d.) was 12.8 ± 9 mm (95% CI 11.9–13.8). The diagnosis of an active TB disease was made in six (1.6%) contacts. SPT and RD1-ELISpot tests were positive in 39 (11%) and 189 (52%) contacts, respectively. The median total serum IgE level was 8 [95% CI 7–10; min–max (1–944) IU/ml] in the whole study group. The total serum IgE level was higher than the age-specific normal limit in 22 (6%) contacts. The median total serum IgE level was significantly higher in contacts with a positive SPT [16.5 (95% CI 9.5–33); (min–max 2–180) IU/ml] compared with children with a negative SPT [8 (95% CI 6–9); (min–max 1–944) IU/ml] (*p* = 0.0043). Also, the frequency of a high total serum IgE level (higher than age-specific normal) was found to be higher in contacts with a positive SPT compared with contacts with a negative SPT (*p* = 0.004). According to the results of the ISAAC questionnaire, the frequency of ever-wheezing, allergic rhinitis, doctor-diagnosed asthma, and atopic dermatitis were 25%, 2.5%, 13%, and 8%, respectively.

Association between the BCG scar and atopy

The SPT was positive in 31 out of 290 (11%) BCG scar-positive contacts and 8 out of 71 (11%) BCG scar-negative contacts and the difference was not significant (*p* = 0.83). The median (min–max) total serum IgE level [8 (95% CI 6–9); (min–max 2–944) IU/ml] was significantly lower in BCG-vaccinated contacts compared with unvaccinated (scar-negative) contacts [12 (95% CI 8–18); (min–max 1–398) IU/ml]

($p = 0.01$). The high total serum IgE level (higher than age-specific normal) was observed in 12 out of 276 (4%) BCG scar-positive contacts compared with 10 out of 71 (14%) scar-negative contacts and difference was statistically significant ($p = 0.003$). On the other hand, according to the results of the ISAAC questionnaire, the frequency of ever-wheezing, doctor-diagnosed asthma, allergic rhinitis, and atopic dermatitis were not significantly different between BCG scar-positive and -negative contacts (Table 1).

Association between atopy and tuberculosis infection

With respect to TST. The mean ($\pm$ s.d.) TST induration size was 12.4 ± 9.2 mm (95% CI 9.4–15.4) in SPT-positive contacts and 12.9 ± 8.9 mm (95% CI 11.9–13.9) in SPT-negative contacts. The difference between the two groups was not statistically significant ($p = 0.75$). The frequency of SPT positivity was similar in TST-positive and -negative contacts ($p = 0.59$) (Table 1). The median total serum IgE level and high total serum IgE levels for age were also similar in TST-positive and -negative contacts (Table 1). The frequency of ever-wheezing, doctor-diagnosed asthma, and allergic rhinitis were not significantly different between TST-positive and -negative contacts, whereas the frequency of atopic dermatitis was significantly lower in TST-positive contacts compared with TST-negative contacts ($p = 0.02$) (Table 1).

With respect to RD1–ELISpot. SPT results, median total serum IgE, percentage of high total serum IgE levels, as well as parameters evaluated in the ISAAC questionnaire including wheezing, doctor-diagnosed asthma, allergic rhinitis, and atopic dermatitis were not significantly different in RD1–ELISpot-positive contacts compared with RD1–ELISpot-negative contacts (Table 1).

Discussion

In this study, we investigated the association between exposure to mycobacterial antigens and atopy in children with a high risk of TB infection using the conventional TST and the recently developed RD1–ELISpot assay which is a more accurate determinant of TB infection. There are numerous studies in the English literature investigating the relationship between atopic disorders and either mycobacterial infection or TB disease. The pioneer study reported by Shirakawa et al. showed that positive tuberculin responses were associated with lower IgE levels and dominance of Th1 over Th2 in the peripheral blood cytokine profiles (2). The authors suggested that BCG vaccination and exposure to mycobacteria might reduce the development of atopic disease by promoting Th1 immunity. More recently, a prospective international birth cohort study conducted in children who were vaccinated with BCG at birth evaluated the relationship between tuberculin reactivity with development of atopy, asthma, and allergic diseases at the age of 2 and 5 yrs by administering an ISAAC questionnaire and skin prick testing (24). This study revealed that TST positivity was negatively associated with development of allergic symptoms but was not helpful in discriminating children with positive SPT from negative. In another study, Obihara et al. reported that children with a positive TST was significantly less likely to have recent atopic rhinitis than those with a negative TST. They also demonstrated that positive SPT was significantly more common in children with negative TST who had recent allergic rhinitis, but not in children with positive TST and recent allergic rhinitis (25). Meanwhile, a number of other studies did not confirm this inverse relationship between TST positivity and atopy or atopic diseases (10, 26–31).

Table 1. SPT, median total serum IgE levels, and high serum total IgE levels, with respect to TST, RD1–ELISpot, and BCG scar status

	TST			RD1–ELISpot			BCG scar status		
	Negative (n = 125)	Positive (n = 236)	p-value	Negative (n = 172)	Positive (n = 189)	p-value	≥ 1 scar (n = 290)	No scar (n = 71)	p-value
Positive SPT (n, %)	15 (12)	24 (10)	0.59	22 (13)	17 (9)	0.24	31 (11)	8 (11)	0.83
Median total serum IgE level (IU/ml)	8	9	0.46	9	8	0.95	8	12	0.01
High total serum IgE level* (n, %)	8 (7)	14 (6)	1.00	9 (6)	13 (7)	0.66	12 (4)	10 (14)	0.003
Ever wheezing (n, %)	36 (29)	56 (24)	0.29	43 (25)	49 (26)	0.84	76 (26)	16 (23)	0.52
Doctor-diagnosed asthma (n, %)	16 (13)	32 (14)	0.83	26 (15)	22 (12)	0.33	40 (14)	8 (11)	0.57
Allergic rhinitis (n, %)	4 (3)	5 (2)	0.53	4 (2)	5 (3)	1.00	8 (3)	1 (1)	1.00
Atopic dermatitis (n, %)	16 (13)	14 (6)	0.02	12 (7)	18 (10)	0.39	24 (8)	6 (8)	1.00

TST, tuberculin skin test; ELISpot, enzyme-linked immunospot; BCG, bacillus Calmette-Guerin; SPT, skin prick testing.

In accordance with the negative reports in this study, we found that allergic symptoms with the exception of atopic dermatitis, SPT positivity, median total serum IgE levels and a frequency of higher IgE levels did not differ in tuberculin positive and negative contacts.

Epidemiological studies provide evidence for an inverse association between TB disease and the occurrence of atopic disorders. The relationship between TB notification rates and the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema was investigated in a multicenter study which demonstrated that TB notification rates were significantly inversely associated with a life-time prevalence of wheezing and asthma (32). Another retrospective study in Finish adults revealed that TB in the first two decades of life was inversely associated with allergies and asthma in women but not in men (33). In support of this finding, two other studies reported that an active TB disease itself may have a protective effect on development of atopic sensitization (34, 35). These contradictory results may be explained by the effect of an active TB disease on atopy, rather than infection.

It is well known that TST is neither specific nor sensitive in the diagnosis of *M. tuberculosis* infection and is subject to significant variations and limitations (11–13). It cannot reliably differentiate between *M. tuberculosis* infection, a BCG vaccination and an exposure to non-TB mycobacteria. Exposure to non-TB *Mycobacteria* in the local environment of where child lives might be important in inhibiting the atopic status in children. This might explain the different results obtained by TST positivity and the atopic state in children in different countries. For those reasons, an improved screening method is needed for the determination of *M. tuberculosis* infection. The RD1–ELISpot assay for the detection of activated T-cells specific for ESAT-6 and CFP10 is a sensitive and specific marker of *M. tuberculosis* infection. This assay provides uniformly negative results in healthy unexposed controls (irrespective of BCG vaccination status), and correlates those results with *M. tuberculosis* exposure among contacts (18, 19). This test is not confounded by exposure to environmental mycobacteria as most of them do not contain a gene encoding ESAT-6 and CFP10 (14–16).

To our knowledge, our study is the first evaluating the association of *M. tuberculosis* infection with atopy by means of a more accurate test, the RD-1 ELISpot assay. Our results demonstrated no statistically significant difference between RD1–ELISpot-positive (*M. tuber-*

culosis infected) and ELISpot-negative (*M. tuberculosis* non-infected) contacts with regard to SPT positivity, median total serum IgE levels and the frequency of higher IgE concentrations. As the RD1–ELISpot test is not confounded by BCG vaccination and exposure to most of the non-TB mycobacteria, we can easily eliminate the effect of BCG and exposure of environmental mycobacteria from our results. Furthermore, we did not observe a significant difference between frequencies of ever-wheezing, doctor-diagnosed asthma, and allergic rhinitis in ELISpot-negative and -positive contacts. These results suggest that *M. tuberculosis* infection may not have preventive effects on the development of atopy based on a more accurate determinant of TB infection besides TST.

As known, BCG vaccination is another form of immunostimulation with mycobacterial antigens. Animal studies have shown that BCG vaccination could suppress allergic-induced airway eosinophilia and hyperreactivity in mice by stimulating a Th1 type response (36, 37). However, evidences on the protective effect of BCG for atopy in human are conflicting. The strong association seen in Japan (2) and Guinea-Bissau (38) were not confirmed by other studies of different countries (10, 26–28). In our study population, the presence or absence of a BCG scar was the most reliable method for determining the vaccination status, as the BCG vaccination records were not available for most participants. Although the presence of a scar confirms a previous BCG vaccination, its' absence does not confirm the lack of a vaccination because not all BCG-vaccinated children develop a detectable scar (39). However, we believe that most children without a scar were unvaccinated, for two reasons. First, the latest available surveillance data for a BCG vaccination in Turkey indicated a rate of 79% in children in 2004 (40), which is the same as the 80% prevalence rate of BCG scars noted in our population. Second, several studies have shown that a neonatal BCG vaccination results in the scar formation in more than 90% of children (41, 42). Therefore, the number of contacts who were vaccinated but unscarred would be small, and would not alter our conclusion. In accordance with previously reported studies, we did not show an association between the presence of a BCG vaccine scar and the lower rate of SPT positivity. Also, we did not observe a significant difference between the frequencies of ever-wheezing, doctor-diagnosed asthma, allergic rhinitis, and atopic dermatitis in BCG scar-negative and -positive contacts. On the other hand, our results reveal

that the presence of a BCG vaccine scar is associated with a lower median total IgE level and a lower frequency of elevated IgE concentration. Although the development of atopy in the newborn is determined by a variety of factors, an intense Th1 stimulus during the early life period could contribute to switching away from the Th2 environment of the placental unit. We may speculate that any mycobacterial inhibition of atopy in humans should have its maximum impact early in life before the establishment of a predominant Th2 response. These may explain why we observed a protective effect of BCG against the serum IgE levels, but not of *M. tuberculosis* infection (diagnosed by TST or RD1-ELISpot assays), as children in our study were exposed to infectious-smear-positive index case has occurred at a later age (mean $\pm$ s.d. 8 ± 4 yrs).

In conclusion, the results of this study provided further evidence for the lack of an inverse association between *M. tuberculosis* infection and atopy by means of the RD1-ELISpot assay which is not confounded by a BCG vaccination and an exposure to most environmental mycobacteria.

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