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Interpretation of *Mycobacterium tuberculosis* antigen-specific IFN-γ release assays (T-SPOT.TB) and factors that may modulate test results

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Summary *Background:* Data about T cell antigen-specific (ESAT-6 and CFP-10) IFN-γ release assays (IGRAs) during and after completion of anti-tuberculous (TB) treatment are limited and highly discordant. Thus, the utility of IGRAs as a surrogate marker of mycobacterial burden remain unclear.
Methods: To investigate factors that modulate IGRA responses during anti-TB treatment we used a standardised assay (T-SPOT.TB) in 33 patients with culture positive tuberculosis.
Results: Significantly more patients in the early ($\leq$4 months of anti-TB treatment) rather than the late phase (>4 months or completed anti-TB treatment) had positive IGRA responses [10/12 (83%) vs 4/21 (19%); $p \leq 0.01$]. Thus, 17/21 (81%) in the late phase or who had completed treatment (mean duration of treatment = 8.7 months) were IGRA negative, despite having robust antigen-specific recall proliferative responses. In these 17 patients prolonged incubation (5 days vs overnight), use of different antigen preparations (protein vs peptide) and addition of endotoxin, failed to elicit positive responses.
Conclusions: In treated TB patients the discordant IGRA data remain unexplained by variation in laboratory protocols and are more likely due to host or environmental factors. In a low burden setting IGRAs may be a promising surrogate marker of mycobacterial disease burden.

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Introduction

In low incidence countries the standardised antigen-specific (ESAT-6 and CFP-10) T cell IFN-γ release assay (IGRA) is a sensitive and specific test for tuberculosis (TB) infection (reviewed in Refs. 1,2). It has also been suggested that IGRAs may be useful surrogate markers of mycobacterial disease burden as, in some studies the frequency of T cell responses progressively decreases or becomes negative with anti-TB treatment.[3] This precept, if borne out, is important for 3 reasons. First, IGRA may be a putative surrogate marker of mycobacterial sterilisation, which may enhance the evaluation of new TB-related therapeutic interventions. Second, as there is no 'gold standard' for the detection of latent TB infection, the post-treatment negativisation of an initially positive result supports the notion that only effector memory T cells are being detected by the assay i.e. a positive result is not due to remote memory in the absence of existing infection. Third, IGRA may be a valuable adjunct to management of complicated cases, including drug-resistant and smear-negative TB. Thus, the study of post-treatment responses are relevant to clinical interpretation and the use of IGRAs as a research tool.

However, analysis of longitudinal treatment responses is not clear-cut, as findings in treated TB patients are highly inconsistent. Some studies have found decreasing or negative responses,[3–5] while others describe increased or persistently positive IGRA responses during or after anti-TB treatment.[6–11] Notably, no study used a standardised proprietary assay, used variable incubation times (overnight vs several days; the longer being more likely to detect central rather than only effector memory T cells) and different antigenic preparations (synthetic peptides vs recombinant protein, which theoretically, if contaminated with endotoxin, might trigger the production of IFN-γ in the absence of existing infection). Interestingly, studies that describe increasing or robust IGRA responses after anti-TB treatment predominantly used long incubation times and recombinant protein antigens.[7–11] We hypothesised that endotoxin contamination of recombinant antigens or long incubation times, which are more likely to activate central memory T cells and thus generate false positive results, might explain existing study inconsistencies. Other possible explanations for these apparent discrepancies include environmental and host T cell heterogeneity. In an attempt to dissect out these possibilities and to test our hypotheses we evaluated IGRA responses, utilising different protocols, in 33 patients with culture proven TB, during various stages of anti-TB treatment using a standardised assay (T-SPOT.TB, Oxford Immunotec, England).

Methods

Patients and samples

Thirty-three adults (55% black-African; 24% Asian; 61% male; median age = 36 years) with culture positive tuberculosis (61% pulmonary [PTB] and 39% extrapulmonary [EPTB]) were recruited during the early phase ($\leq$4 months of treatment; $n = 12$), the continuation phase (>4 months; $n = 7$; median duration of treatment = 10.4 months) or after completion of anti-TB treatment ($n = 14$; median duration of treatment = 7.7 months). Seven of the 12 patients in the early phase and 7 of the 21 patients in the continuation/post-treatment phase had EPTB. Three patients were followed longitudinally, all the patients were HIV negative and 4 patients had isoniazid-resistant tuberculosis (3 with PTB and 1 with EPTB).

ELISPOT and proliferation assays

IFN-γ ELISPOT responses to ESAT-6/CFP-10 peptide pools were performed according to manufacturer's instructions (T-SPOT.TB) using peripheral blood mononuclear cells (PBMC, 250,000 cells/well). The number of IFN-γ spot forming T cells (SFC) per million PBMC was enumerated by an operator, blinded to the results, using an ELISPOT reader. In an attempt to evoke memory T cell responses, in a subset of patients, PBMC were co-cultured with antigens for varying incubation times (overnight, 5 days and 12 days; the latter in the presence of recombinant human IL-2), in the presence of LPS at different doses (0.1, 0.5 and 5 μg/ml) and with different antigens [peptide (T-SPOT.TB) vs recombinant ESAT-6 protein, 10 μg/ml, State Serum Institute, Denmark]. Antigen-specific proliferative responses were evaluated using the [^{3}H] thymidine incorporation assay at day 5. Data comparisons were performed using the Mann–Whitney test (GraphPad Prism version 4).

Results

IGRA responses in the early and late phases of anti-TB treatment are different

It has been proposed that IGRA may serve as surrogate markers of mycobacterial burden; hence the magnitude of responses during different phases of treatment is of interest. Significantly more patients in the early ($\leq$4 months of anti-TB treatment) rather than the late phase (>4 months or completed anti-TB treatment) had positive IGRA responses [10/12 (83%) vs 4/21 (17%); $p \leq 0.01$, Fig. 1A]. Thus, 17/21 (81%) of those with late phase/completed treatment were IGRA negative. Responses were persistently positive in 3 patients followed longitudinally (initially tested at 4, 4 and 6 months after commencing treatment and then again at 17, 8, and 18 months, respectively).

Long incubation times fail to elicit positive IGRA responses

We hypothesised that prolonged incubation times of several days, but not overnight incubation, may induce IFN-γ production by central memory T cells[12] and hence explain the inconsistent post-treatment data. However, among 17 patients with late phase or completed treatment and who were IGRA negative, prolonged incubation of 5 days failed to elicit positive responses (Fig. 1B). At least 12 days of incubation were required to elicit modestly positive IGRA responses (2 of 4 became IGRA positive by CFP but not ESAT-6 spot counts). Patients who were IGRA positive had generally stronger responses with time (Fig. 1B).

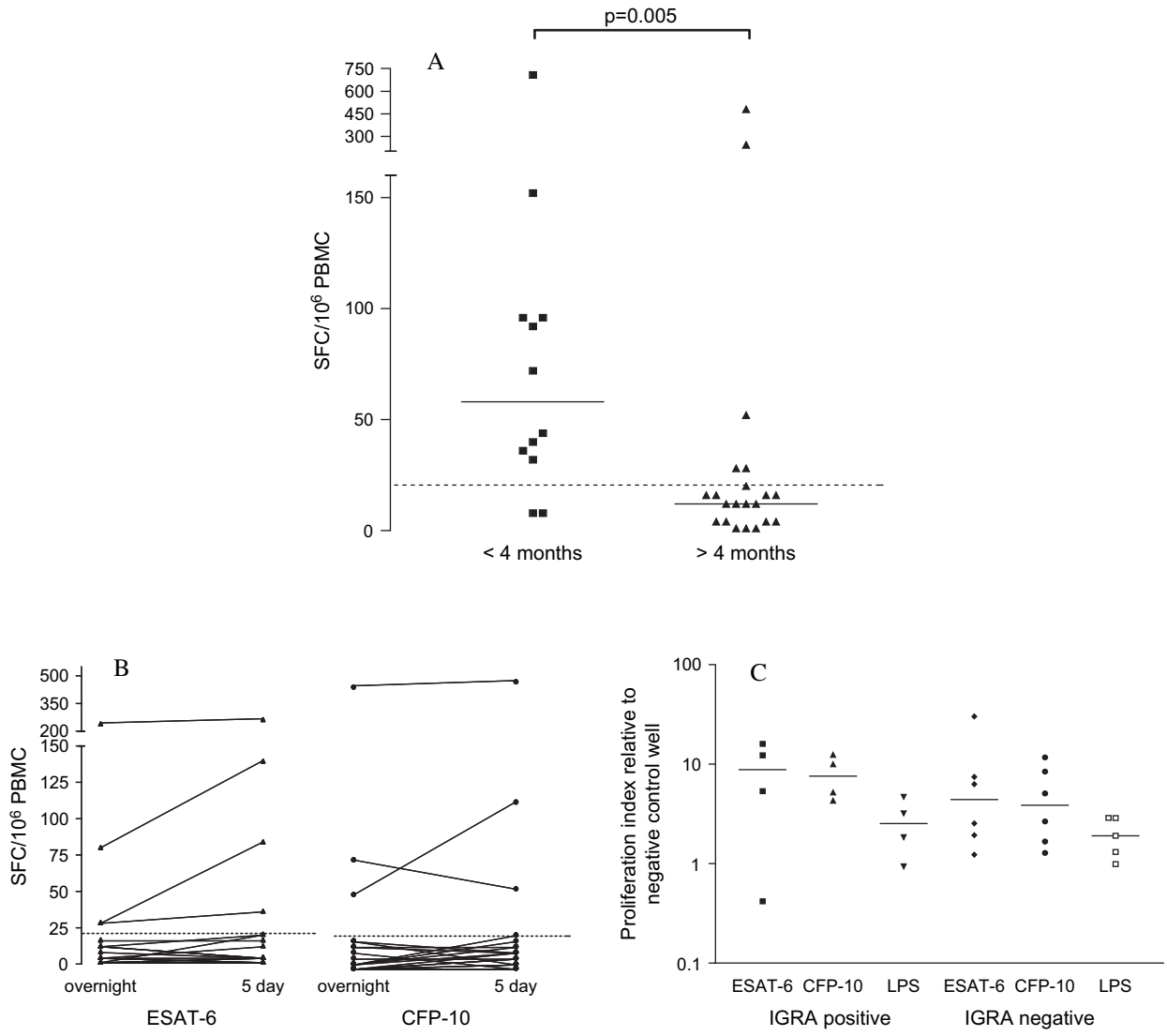

Figure 1 IFN-γ and recall responses in patients during or after anti-TB treatment. Significantly more patients, in the late intensive phase or post-treatment ($n = 21$), were IGRA negative compared to donors in the early phase ($n = 12$; first 4 months of treatment; $p \leq 0.01$, Chi-square; A); spot counts were also significantly lower in the late compared to the early phase of treatment (median of 12 vs 58 SFCs/10^6 PBMCs; $p = 0.005$, Mann–Whitney). Patients who were IGRA negative in the late intensive phase or post-treatment ($n = 17$) remained so despite an incubation period of several days (B). However, these patients had robust antigen-specific recall responses despite their failure to produce IFN-γ (C). Dashed line represents cut-off of 20 SFCs/10^6 PBMCs.

Treated patients with negative responses have strong recall responses

There are limited data about the nature of the recall response in successfully treated patients. Despite, as outlined above, the failure to evoke IFN-γ production after prolonged incubation (5 days), the proliferative recall responses to ESAT-6 and CFP-10 antigens were robust, thus suggesting the presence of non-IFN-γ-producing memory T cells.

Protein antigen fails to convert negative responses

Given the putative membrane lytic function of ESAT-6 protein[13] we hypothesised that protein may activate pathogen recognition receptors or pathways distinct from peptide. Eight patients who were IGRA negative by ESAT-6 peptide (overnight incubation) remained negative when ESAT-6 protein was used (median of 4 vs 1 SFC/10^6 PBMC, respectively).

Endotoxin contamination fails to evoke positive responses

In 5 patients who were IGRA negative post-treatment, addition of LPS at different concentrations to ESAT-6 and CFP-10 peptides or protein failed to evoke positive responses (e.g. for overnight incubation with 5 μg/ml LPS and ESAT-6 peptide: median of 5 vs 4 vs 1 SFC/10^6 PBMC for peptide, peptide + LPS and LPS only, respectively). Similar results were obtained after 5-day incubations. In IGRA

positive patients LPS failed to evoke stronger responses when using different incubation times.

Discussion

For clinicians, scientists and vaccinologists, determining whether IGRAs might serve as proxy markers of mycobacterial burden, is imperative. However, published data evaluating intra- and post-treatment responses are inconsistent.[3–11] For meaningful interpretation it is important to understand factors that might explain these discrepancies. In the current study we focussed on technical factors rather than host or environmental factors. Our data indicate that incubation times (overnight vs several days), type of antigen (protein vs peptide) and endotoxin contamination of recombinant antigen are unlikely to explain the variation across studies. Our data also shed some light on the question of whether IFN-γ release occurs from memory T cells after clearance of infection. Thus, we attempted to define if factors, other than existing infection, might generate positive IGRA test results. In the absence of a 'gold standard' for TB infection, cohorts of treated TB patients provide a good model for studying such factors.

It is thought that central memory T cells, but not effector ones, may take several days (rather than hours) to produce effector cytokines.[12] Thus, use of long incubation times (5–6 days) in several studies, as previously suggested,[14] might explain the inconsistent post-treatment data. We found that in 17 post-treatment IGRA negative subjects, several days of incubation failed to elicit positive responses. Up to 12 days were required to generate modestly positive responses.[5] Furthermore, addition of LPS failed to evoke IFN-γ secretion, despite its potent ability to induce maturation of dendritic cells (DC) that might theoretically enhance conversion of IL-2-producing memory T cells into IFN-γ-producing effector cells. Similarly, the ability of LPS-induced IL-12 to cause IFN-γ secretion by NK cells was clearly not sufficient to cause a measurable ELISPOT response. Indeed LPS is a poor inducer of IFN-γ in human mononuclear cells.[15] In keeping with published data[16] we found that recombinant ESAT-6 antigen did not significantly alter test outcomes, despite the known membrane interactions of this molecule[13] that might, like LPS, provide a ''danger'' or DC-maturation signal. Thus, variable incubation time, type of antigen, and endotoxin contamination all fail to explain the inconsistent published data.[3–11] Another possibility that may explain persistently positive responses in post-treatment patients is re-infection, though this seems unlikely given the low burden setting of the study. Alternatively, circulating T cells may persist for several weeks after the infection is cleared, or responses may persist due to incomplete sterilisation (3 of 4 subjects with persistent responses in the late continuation phase had isoniazid-resistant TB), strain-specific differences[17] and ethnicity-related T cell receptor heterogeneity. The latter point may explain why some patients are negative at diagnosis. Three patients who were followed up longitudinally failed to convert to negative responses. The significance of this finding requires elaboration in prospective cohort studies.

Of note, all the intra- or post-treatment IGRA-negative patients had robust antigen-specific recall responses. These preliminary data imply the presence of non-IFN-γ producing memory T cells. This is in keeping with models of antigen clearance, but not persistence, where central memory T cells are more likely to produce IL-2 rather than IFN-γ.[18] According to this model, proposed by Harari et al.,[18] in the presumed absence of infection, as in treated TB patients, IL-2 producing central memory cells predominante, but there are no IFN-γ producing effector memory cells. It is possible that in different environmental settings or different populations, effector cells may take longer to revert to a central memory phenotype, or, that some individuals retain an IFN-γ producing memory phenotype. Indeed, in India[19] and in The Gambia[6] a high proportion of IGRA responses remain persistently positive, although in these environments there is the additional possibility that an effector memory T cell population is maintained, even after clearance of *Mycobacterium tuberculosis*, by continuing exposure to environmental mycobacteria expressing cross-reactive ESAT-6 or CFP-10 homologues (reviewed in Ref. 2). If this is the case, the utility of these assays in high burden developing countries will be severely compromised. Thus, our data and hypotheses require clarification in larger studies, in both high and low burden settings, using detailed memory cell markers.

Our data have applicability to the important clinical question of whether IGRA test results are a reflection of remote infection that has since been treated or cleared. How specific are *memory* T cell responses for existing infection? The predominantly negative responses in treated, culture positive TB patients, support the specificity of IGRAs for existing infection i.e. proof of principle that the IGRA is detecting effector cells rather than less relevant central memory cells.

A major limitation of our study is the lack of longitudinal follow-up in individual patients. This was not possible for logistical reasons. However, a T-SPOT.TB positivity rate of 84% in the early phase of treatment is consistent with other studies,[20] and contrasts with the 81% negative rate, post-treatment. More importantly, the main objective of this study was to evaluate the effect of technical factors (incubation time, endotoxin contamination and antigen type) on IGRA responses, and not to evaluate longitudinal or specific phenotypes of memory T cells.

In conclusion, these preliminary data suggest that technical factors (incubation time, endotoxin contamination and antigen type) fail to explain persistently positive post-treatment IGRA responses. Rather host or environmental factors may be more important in this regard. Further studies are required to clarify the role of ESAT-6 homologue-producing environmental mycobacteria and differential memory T cell responses in explaining persistently positive responses in treated patients. Such studies, particularly in developing countries, will be just as important as studies reporting performance outcome data.

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