Calcineurin inhibitors and variation in the performance of interferon-gamma release assays used to detect TB infection

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Running head: Calcineurin inhibitors compromise IGRA performance

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INTRODUCTION

A key strategy of TB control programs in high-resource countries is identification of latent TB infection (LTBI) and preventive therapy to avert progression to TB disease (1). Currently only tuberculin skin tests (TSTs) and interferon-γ release assays (IGRAs) are used for LTBI screening (2). IGRAs are functional blood-based assays that detect interferon-γ produced by memory T cells after stimulation with mycobacterial antigens (2). Currently two IGRAs are available, the T-SPOT.*TB* and the more widely used QuantiFERON-TB Gold (QFT) assay (3).

Globally, the number of hematopoietic stem cell transplant (HSCT) and solid organ transplant (SOT) recipients is rising steadily. Transplant recipients require long-term immunosuppression, and consequently have a much greater risk of developing TB disease than the general population (4). Furthermore, mortality associated with TB disease is higher (4-6).

Calcineurin inhibitors, including cyclosporin and tacrolimus, are the most commonly used immunosuppressive agents after transplantation (7). They reduce T cell activation, thereby inhibiting production of various cytokines, including interferon-γ and interleukin-2 (IL-2) (8). Both cytokines play crucial roles in human anti-mycobacterial immune responses (9, 10).

TB screening in patients receiving immunosuppressive medication is complex (4, 11-13). Considerable evidence shows that the sensitivity of TSTs is reduced in immunocompromised individuals (2, 14). Previous studies investigating IGRAs in the transplant setting have reported conflicting results, some suggesting they are reliable,

others concluding that their performance is impaired (15-18). The key limitation of all previous clinical studies is that no gold standard for LTBI exists (2). Therefore, the interpretation of negative IGRA results in immunosuppressed patients is difficult, as it is currently impossible to distinguish true absence of TB infection from a false-negative result caused by immunosuppression.

This study aimed to determine the impact of calcineurin inhibitors on the performance of QFT assays using an *ex vivo* model. Additionally, we investigated their impact on recently identified biomarkers of TB infection, mycobacteria-specific IL-2, interferon-γ inducible protein 10 (IP-10), and tumor necrosis factor-α (TNF-α) responses (9, 10).

METHODS

Study population

Adults with a previous positive IGRA result or recent TB exposure were recruited at a TB clinic after written informed consent. Potential participants with known immunodeficiency or receiving immunosuppressive medication were excluded. The study was approved by the National Research Ethics Service Committee (13/SC/0043).

Interferon-gamma release assays

From each participant, three sets of QuantiFERON-TB Gold in-Tube assays (Cellestis/Qiagen, Carnegie, Australia) comprising an antigen-stimulated, a positive (mitogen) control and a negative control tube were obtained. No reagents were

added to the first set ('standard assay'). In the second set, cyclosporin (Sandimmun; Novartis, Camberley, UK) was added to each tube to a final concentration of 200 ng/mL, a common target level in the HSCT setting (19). In the third set, tacrolimus (Prograf; Astellas, Killorglin, Ireland) was added to each tube to a final concentration of 10 ng/mL, a typical target level in the SOT setting (20). Drugs were added within 4 hours of phlebotomy, and samples were immediately transferred into a 37°C incubator. After 24 hours, supernatants were harvested, as per manufacturer's instructions, followed by cryopreservation.

Cytokine measurements

Cytokine concentrations in supernatants were determined with ProcartaPlex xMAP assays (Affymetrix eBioscience, Hatfield, UK) measuring interferon-γ, IP-10, IL-2 and TNF-α according to manufacturer's instruction. Their broad dynamic range allows accurate measurement of the high interferon-γ concentrations that often occur in QFT assays, which exceed the upper limit of QFT ELISAs (13). Assays were read with a Luminex 100 Bioanalyzer with xPONENT™ software (Luminex Corporation, Austin, TX, U.S.).

Interpretation of QFT results

QFT results were interpreted according to the latest version of the manufacturer's package insert (UK version). Briefly, a positive result was defined as a background-corrected interferon-γ response ≥0.35 IU/mL and simultaneously ≥25% of the nil control sample interferon-γ concentration. A negative result was defined as a response below this threshold in the presence of a valid positive control (i.e. background-corrected interferon-γ concentration ≥0.5 IU/mL). An indeterminate

assay result was defined as a sample set in which the negative control failed (i.e. interferon-γ concentration >8.0 IU/mL), or in which the positive control failed (background-corrected interferon-γ concentration <0.5 IU/mL).

Statistical analyses

All cytokines were analyzed in pg/mL, except interferon-γ, which was measured in pg/mL and then converted to IU/mL (the units used in QFT assays) for analysis, as previously described (21). Statistical comparisons were done in Prism (V6.0; GraphPad, La Jolla, CA, U.S.) using Wilcoxon matched-pairs signed-rank tests.

RESULTS

A total of 18 participants were recruited, of which 13 had positive QFT results. For the analyses of antigen-stimulated cytokine responses only data from these 13 participants were included, while for the analyses of positive control responses, data from all 18 were included.

Interferon-γ responses and categorical QFT results

Both cyclosporin and tacrolimus caused considerable reductions in background-corrected interferon-γ concentrations in the antigen-stimulated samples in all participants (Figure 1). Compared with the standard assay (3.84 IU/mL; IQR: 0.74–10.9) the median interferon-γ concentrations were significantly lower in the cyclosporin- and tacrolimus-treated assay sets (0.0 IU/mL, IQR: -0.12–0.18; $p<0.001$ and 0.02 IU/mL, IQR: -0.006–0.13; $p<0.001$, respectively) (Figure 2A). In the cyclosporin- and tacrolimus-treated positive control samples the median interferon-γ

concentrations were also significantly lower (5.1 IU/mL, IQR: 1.6–18.9 and 14.3 IU/mL, IQR: 3.5–39.1, respectively) than in the standard assays (66.6 IU/mL; IQR: 28.0–103.3), but still considerably above the cut-off classifying positive controls as failed (Figure 2B).

Of the 13 participants with a positive QFT result in the standard assay, 10 converted to a negative result in the cyclosporin-treated set, and two to an indeterminate result; one (participant 4) continued to have a positive result despite a markedly reduced antigen-stimulated interferon-γ response (0.76 vs 6.59 IU/mL in the standard assay). In the tacrolimus-treated set, 10 individuals converted to a negative, and two to an indeterminate result; one (participant 1) remained positive, again with markedly reduced response (0.43 vs 13.1 IU/mL).

IL-2, IP-10 and TNF-α responses

Background-corrected IL-2 and IP-10 concentrations were significantly lower in the antigen-stimulated samples in the cyclosporin- and tacrolimus-treated assay sets than in the standard assay (Figure 2A). In contrast, there was no significant difference in background-corrected TNF-α concentrations. TNF-α responses in the positive control samples were also largely maintained, although statistically there was a significant reduction in concentrations in tacrolimus-treated samples (Figure 2B).

DISCUSSION

This study provides robust evidence that calcineurin inhibitors have a significant adverse effect on the performance of IGRAs. Our results suggest that the majority

of patients with LTBI who are on treatment with cyclosporin or tacrolimus would have false-negative IGRA results when screened for TB, for example in the context of contact screening following exposure to a case with pulmonary TB. Importantly, the *ex vivo* model used in this study cannot capture the long-term impact of calcineurin inhibitors on T cells, which may be even more pronounced.

The marked impact of calcineurin inhibitors on IGRAs is consistent with their known mechanism of action. A key property of this drug class is inhibition of T cell activation and suppression of pro-inflammatory cytokines, including interferon-γ and IL-2, in T cells (8, 22, 23), the main source of interferon-γ in functional assays determining anti-mycobacterial immune responses, including QFT assays (2). The observed reduction in IP-10 responses is also predicted, since IP-10 production is primarily induced by interferon-γ (24). It is unlikely that those observations are due to cytoxicity, as previous data show that even at a 100-fold greater concentration than used in this study cyclosporin has no significant cytotoxic effects on T cells (25).

In contrast, TB antigen-induced TNF-α responses were not suppressed by cyclosporin or tacrolimus. This suggests that calcineurin inhibitor have only limited effect on macrophages, the principal source of TNF-α in immune responses directed against mycobacteria, consistent with published data (26). Furthermore, this observation suggests that in patients receiving calcineurin inhibitors novel TB assays based on TNF-α responses, which are currently in development (9, 10), may prove more robust than IGRAs.

In conclusion, considering our results together with previous data showing that the performance of TSTs is also impaired in immunosuppressed patients, both currently used LTBI screening tests should be regarded as unreliable in patients receiving calcineurin inhibitors. Although a positive IGRA result remains useful in this patient population, a negative result provides no meaningful information regarding the TB infection status.

Contributor statement: M.T. conceived of the study. E.B. and M.T. designed the research. E.B., Y.G., D.B. and M.T. performed the laboratory work. All authors contributed to the data analysis and data interpretation. E.B., N.C., P.E. and M.T. drafted the manuscript. All authors provided input into the manuscript and approved the final version for submission.

Conflict of interest disclosure: M.T. received QuantiFERON-TB Gold assays at reduced cost for another research project from the manufacturer (Cellestis/Qiagen). The manufacturer had no influence on the study design, the data interpretation, the writing of the manuscript or the decision to submit the data for publication. The remaining authors have nothing to disclose.

Figure 1. Background-corrected interferon-γ concentrations in antigen-stimulated (left) and positive control (right) samples in individual participants in the standard assay set compared with sets with added cyclosporin (upper panel; n=13) and tacrolimus (lower panel; n=13). Dotted lines indicate the cut-off for a positive test result in antigen-stimulated samples (0.35 IU/mL), and the cut-off for a valid positive control response (0.5 IU/mL).

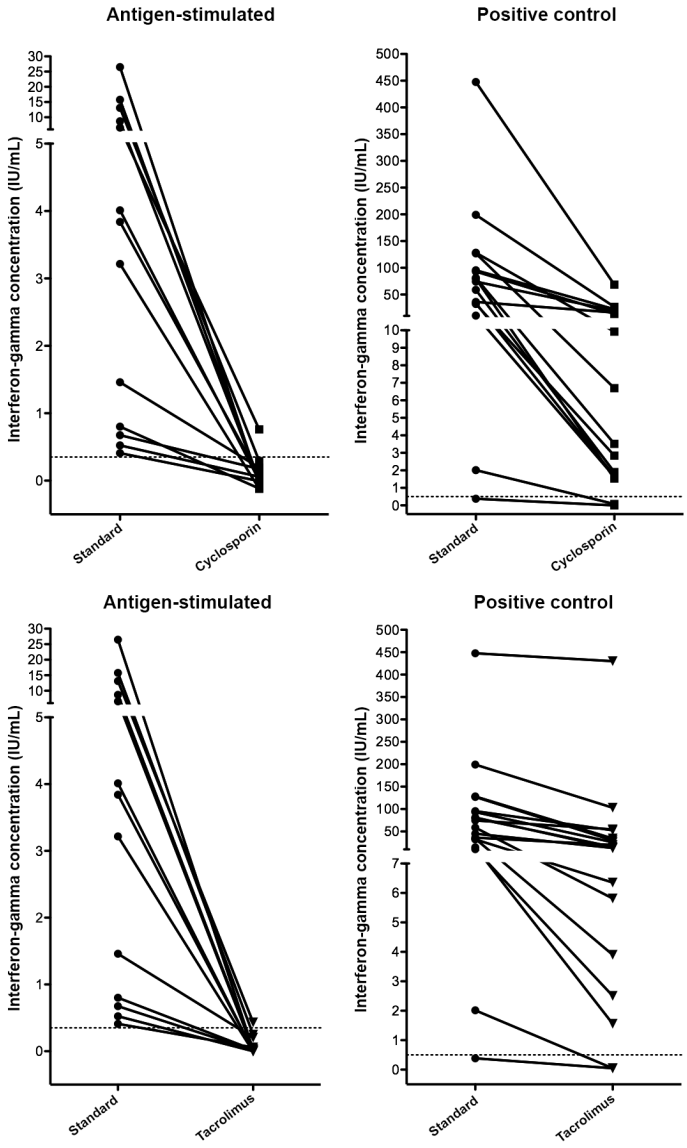
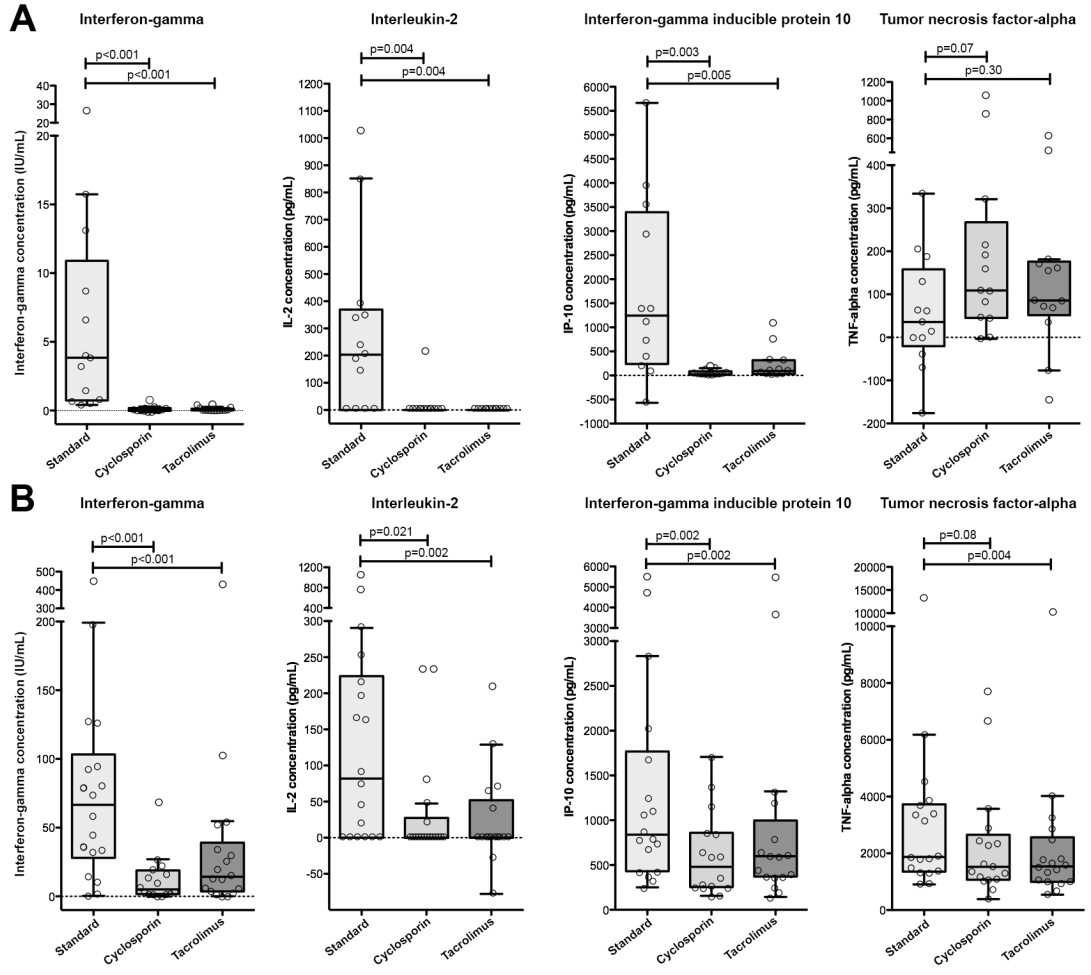


Figure 2. Background-corrected interferon-γ, IL-2, IP-10 and TNF-α concentrations in (A) antigen-stimulated (n=13) and (B) positive control (n=18) samples in standard assay sets and sets with added cyclosporin and tacrolimus. Box plot with Tukey whiskers; horizontal lines depict the medians; p-values calculated with Wilcoxon matched pairs signed-rank tests. Negative values are due to background correction (see Methods section).



References

1. World Health Organization. Global tuberculosis report 2017. *World-Health-Organization, Geneva ISBN 978-92-4-156551-6.* 2017.
2. Tebruegge M, Ritz N, Curtis N, Shingadia D. Diagnostic tests for childhood tuberculosis: past imperfect, present tense and future perfect? *Pediatr Infect Dis J* 2015; 34: 1014-1019.
3. Tebruegge M, Ritz N, Koetz K, Noguera-Julian A, Seddon JA, Welch SB, *et al.* Availability and use of molecular microbiological and immunological tests for the diagnosis of tuberculosis in europe. *PLoS One* 2014; 9: e99129.
4. Bumbacea D, Arend SM, Eyuboglu F, Fishman JA, Goletti D, Ison MG, e*t al.* The risk of tuberculosis in transplant candidates and recipients: a TBNET consensus statement. *Eur Respir J* 2012; 40: 990-1013.
5. Benito N, Garcia-Vazquez E, Horcajada JP, Gonzalez J, Oppenheimer F, Cofan F, *et al.* Clinical features and outcomes of tuberculosis in transplant recipients as compared with the general population: a retrospective matched cohort study. *Clin Microbiol Infect* 2015; 21: 651-658.
6. Russo RL, Dulley FL, Suganuma L, Franca IL, Yasuda MA, Costa SF. Tuberculosis in hematopoietic stem cell transplant patients: case report and review of the literature. *Int J Infect Dis* 2010; 14 Suppl 3: e187-191.
7. Ruutu T, Gratwohl A, de Witte T, Afanasyev B, Apperley J, Bacigalupo A, *et al.* Prophylaxis and treatment of GVHD: EBMT-ELN working group recommendations for a standardized practice. *Bone Marrow Transplant* 2014; 49: 168-173.
8. Rao A, Luo C, Hogan PG. Transcription factors of the NFAT family: regulation and function. *Annu Rev Immunol* 1997; 15: 707-747.

9. Clifford V, Tebruegge M, Zufferey C, Germano S, Forbes B, Cosentino L, *et al.* Mycobacteria-specific cytokine responses as correlates of treatment response in active and latent tuberculosis. *J Infect* 2017; 75: 132-145.

10. Tebruegge M, Dutta B, Donath S, Ritz N, Forbes B, Camacho-Badilla K, *et al.* Mycobacteria-specific cytokine responses detect tuberculosis infection and distinguish latent from active tuberculosis. *Am J Respir Crit Care Med* 2015; 192: 485-499.

11. Clifford V, Tebruegge M, Curtis N. Limitations of current tuberculosis screening tests in immunosuppressed patients. *BMJ* 2015; 350: h2226.

12. Clifford V, Zufferey C, Germano S, Ryan N, Leslie D, Street A, *et al*. The impact of anti-tuberculous antibiotics and corticosteroids on cytokine production in QuantiFERON-TB Gold In Tube assays. *Tuberculosis (Edinb)* 2015; 95: 343-349.

13. Edwards A, Gao Y, Allan RN, Ball D, de Graaf H, Coelho T, *et al.* Corticosteroids and infliximab impair the performance of interferon-gamma release assays used for diagnosis of latent tuberculosis. *Thorax* 2017; 72: 946-949.

14. Richeldi L, Losi M, D'Amico R, Luppi M, Ferrari A, Mussini C, *et al.* Performance of tests for latent tuberculosis in different groups of immunocompromised patients. *Chest* 2009; 136: 198-204.

15. Hadaya K, Bridevaux PO, Roux-Lombard P, Delort A, Saudan P, Martin PY, Janssens JP. Contribution of interferon-gamma release assays (IGRAs) to the diagnosis of latent tuberculosis infection after renal transplantation. *Transplantation* 2013; 95: 1485-1490.

16. Redelman-Sidi G, Sepkowitz KA. IFN-gamma release assays in the diagnosis of latent tuberculosis infection among immunocompromised adults. *Am J Respir Crit Care Med* 2013; 188: 422-431.

17. Sester M, van Leth F, Bruchfeld J, Bumbacea D, Cirillo DM, Dilektasli AG, *et al.* Risk assessment of tuberculosis in immunocompromised patients. A TBNET study. *Am J Respir Crit Care Med* 2014; 190: 1168-1176.

18. Scholman T, Straub M, Sotgiu G, Elsasser J, Leyking S, Singh M, *et al*. Superior sensitivity of ex vivo IFN-gamma release assays as compared to skin testing in immunocompromised patients. *Am J Transplant* 2015; 15: 2616-2624.

19. Peters C, Minkov M, Gadner H, Klingebiel T, Vossen J, Locatelli F, *et al.* Statement of current majority practices in graft-versus-host disease prophylaxis and treatment in children. *Bone Marrow Transplant* 2000; 26: 405-411.

20. Israni AK, Riad SM, Leduc R, Oetting WS, Guan W, Schladt D, *et al*. Tacrolimus trough levels after month 3 as a predictor of acute rejection following kidney transplantation: a lesson learned from DeKAF Genomics. *Transpl Int* 2013; 26: 982-989.

21. Desem N, Jones SL. Development of a human gamma interferon enzyme immunoassay and comparison with tuberculin skin testing for detection of Mycobacterium tuberculosis infection. *Clin Diagn Lab Immunol* 1998; 5: 531-536.

22. Gelfand EW, Cheung RK, Mills GB. The cyclosporins inhibit lymphocyte activation at more than one site. *J Immunol* 1987; 138: 1115-1120.

23. Kino T, Hatanaka H, Miyata S, Inamura N, Nishiyama M, Yajima T, *et al.* FK-506, a novel immunosuppressant isolated from a Streptomyces. II.

316 Immunosuppressive effect of FK-506 in vitro. *J Antibiot (Tokyo)* 1987; 40:
317 1256-1265.
318 24. Neville LF, Mathiak G, Bagasra O. The immunobiology of interferon-gamma
319 inducible protein 10 kD (IP-10): a novel, pleiotropic member of the C-X-C
320 chemokine superfamily. *Cytokine Growth Factor Rev* 1997; 8: 207-219.
321 25. Gertsch J, Guttinger M, Sticher O, Heilmann J. Relative quantification of mRNA
322 levels in Jurkat T cells with RT-real time-PCR (RT-rt-PCR): new possibilities
323 for the screening of anti-inflammatory and cytotoxic compounds. *Pharm Res*
324 2002; 19: 1236-1243.
325 26. van den Bosch TP, Kannegieter NM, Hesselink DA, Baan CC, Rowshani AT.
326 Targeting the monocyte-macrophage lineage in solid organ transplantation.
327 *Front Immunol* 2017; 8: 153.
328