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,

79 others concluding that their performance is impaired (15-18). The key limitation of all
80 previous clinical studies is that no gold standard for LTBI exists (2). Therefore, the
81 interpretation of negative IGRA results in immunosuppressed patients is difficult, as it
82 is currently impossible to distinguish true absence of TB infection from a false-
83 negative result caused by immunosuppression.
84
85 This study aimed to determine the impact of calcineurin inhibitors on the performance
86 of QFT assays using an *ex vivo* model. Additionally, we investigated their impact on
87 recently identified biomarkers of TB infection, mycobacteria-specific IL-2,
88 interferon-γ inducible protein 10 (IP-10), and tumor necrosis factor-α (TNF-α)
89 responses (9, 10).
90
91
92 **METHODS**
93 ***Study population***
94 Adults with a previous positive IGRA result or recent TB exposure were recruited
95 at a TB clinic after written informed consent. Potential participants with known
96 immunodeficiency or receiving immunosuppressive medication were excluded. The
97 study was approved by the National Research Ethics Service Committee
98 (13/SC/0043).
99
100 ***Interferon-gamma release assays***
101 From each participant, three sets of QuantiFERON-TB Gold in-Tube assays
102 (Cellestis/Qiagen, Carnegie, Australia) comprising an antigen-stimulated, a positive
103 (mitogen) control and a negative control tube were obtained. No reagents were

104 added to the first set ('standard assay'). In the second set, cyclosporin (Sandimmun;
105 Novartis, Camberley, UK) was added to each tube to a final concentration of 200
106 ng/mL, a common target level in the HSCT setting (19). In the third set, tacrolimus
107 (Prograf; Astellas, Killorglin, Ireland) was added to each tube to a final
108 concentration of 10 ng/mL, a typical target level in the SOT setting (20). Drugs
109 were added within 4 hours of phlebotomy, and samples were immediately
110 transferred into a 37°C incubator. After 24 hours, supernatants were harvested, as
111 per manufacturer's instructions, followed by cryopreservation.
112
113 ***Cytokine measurements***
114 Cytokine concentrations in supernatants were determined with ProcartaPlex xMAP
115 assays (Affymetrix eBioscience, Hatfield, UK) measuring interferon-γ, IP-10, IL-2
116 and TNF-α according to manufacturer's instruction. Their broad dynamic range
117 allows accurate measurement of the high interferon-γ concentrations that often
118 occur in QFT assays, which exceed the upper limit of QFT ELISAs (13). Assays
119 were read with a Luminex 100 Bioanalyzer with xPONENT™ software (Luminex
120 Corporation, Austin, TX, U.S.).
121
122 ***Interpretation of QFT results***
123 QFT results were interpreted according to the latest version of the manufacturer's
124 package insert (UK version). Briefly, a positive result was defined as a background-
125 corrected interferon-γ response ≥0.35 IU/mL and simultaneously ≥25% of the nil
126 control sample interferon-γ concentration. A negative result was defined as a
127 response below this threshold in the presence of a valid positive control (i.e.
128 background-corrected interferon-γ concentration ≥0.5 IU/mL). An indeterminate

129 assay result was defined as a sample set in which the negative control failed (i.e.
130 interferon-γ concentration >8.0 IU/mL), or in which the positive control failed
131 (background-corrected interferon-γ concentration <0.5 IU/mL).
132
133 ***Statistical analyses***
134 All cytokines were analyzed in pg/mL, except interferon-γ, which was measured in
135 pg/mL and then converted to IU/mL (the units used in QFT assays) for analysis, as
136 previously described (21). Statistical comparisons were done in Prism (V6.0;
137 GraphPad, La Jolla, CA, U.S.) using Wilcoxon matched-pairs signed-rank tests.
138
139
140 **RESULTS**
141 A total of 18 participants were recruited, of which 13 had positive QFT results. For
142 the analyses of antigen-stimulated cytokine responses only data from these 13
143 participants were included, while for the analyses of positive control responses, data
144 from all 18 were included.
145
146 ***Interferon-γ responses and categorical QFT results***
147 Both cyclosporin and tacrolimus caused considerable reductions in background-
148 corrected interferon-γ concentrations in the antigen-stimulated samples in all
149 participants (Figure 1). Compared with the standard assay (3.84 IU/mL; IQR: 0.74–
150 10.9) the median interferon-γ concentrations were significantly lower in the
151 cyclosporin- and tacrolimus-treated assay sets (0.0 IU/mL, IQR: -0.12–0.18; $p<0.001$
152 and 0.02 IU/mL, IQR: -0.006–0.13; $p<0.001$, respectively) (Figure 2A). In the
153 cyclosporin- and tacrolimus-treated positive control samples the median interferon-γ

154 concentrations were also significantly lower (5.1 IU/mL, IQR: 1.6–18.9 and 14.3
155 IU/mL, IQR: 3.5–39.1, respectively) than in the standard assays (66.6 IU/mL; IQR:
156 28.0–103.3), but still considerably above the cut-off classifying positive controls as
157 failed (Figure 2B).
158
159 Of the 13 participants with a positive QFT result in the standard assay, 10 converted
160 to a negative result in the cyclosporin-treated set, and two to an indeterminate result;
161 one (participant 4) continued to have a positive result despite a markedly reduced
162 antigen-stimulated interferon-γ response (0.76 vs 6.59 IU/mL in the standard assay).
163 In the tacrolimus-treated set, 10 individuals converted to a negative, and two to an
164 indeterminate result; one (participant 1) remained positive, again with markedly
165 reduced response (0.43 vs 13.1 IU/mL).
166
167 ***IL-2, IP-10 and TNF-α responses***
168 Background-corrected IL-2 and IP-10 concentrations were significantly lower in the
169 antigen-stimulated samples in the cyclosporin- and tacrolimus-treated assay sets than
170 in the standard assay (Figure 2A). In contrast, there was no significant difference in
171 background-corrected TNF-α concentrations. TNF-α responses in the positive control
172 samples were also largely maintained, although statistically there was a significant
173 reduction in concentrations in tacrolimus-treated samples (Figure 2B).
174
175
176 **DISCUSSION**
177 This study provides robust evidence that calcineurin inhibitors have a significant
178 adverse effect on the performance of IGRAs. Our results suggest that the majority

179 of patients with LTBI who are on treatment with cyclosporin or tacrolimus would
180 have false-negative IGRA results when screened for TB, for example in the
181 context of contact screening following exposure to a case with pulmonary TB.
182 Importantly, the *ex vivo* model used in this study cannot capture the long-term
183 impact of calcineurin inhibitors on T cells, which may be even more pronounced.
184
185 The marked impact of calcineurin inhibitors on IGRAs is consistent with their known
186 mechanism of action. A key property of this drug class is inhibition of T cell
187 activation and suppression of pro-inflammatory cytokines, including interferon-γ and
188 IL-2, in T cells (8, 22, 23), the main source of interferon-γ in functional assays
189 determining anti-mycobacterial immune responses, including QFT assays (2). The
190 observed reduction in IP-10 responses is also predicted, since IP-10 production is
191 primarily induced by interferon-γ (24). It is unlikely that those observations are due to
192 cytoxicity, as previous data show that even at a 100-fold greater concentration than
193 used in this study cyclosporin has no significant cytotoxic effects on T cells (25).
194
195 In contrast, TB antigen-induced TNF-α responses were not suppressed by cyclosporin
196 or tacrolimus. This suggests that calcineurin inhibitor have only limited effect on
197 macrophages, the principal source of TNF-α in immune responses directed against
198 mycobacteria, consistent with published data (26). Furthermore, this observation
199 suggests that in patients receiving calcineurin inhibitors novel TB assays based on
200 TNF-α responses, which are currently in development (9, 10), may prove more robust
201 than IGRAs.
202

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

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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.

222 **Figure 1.** Background-corrected interferon-γ concentrations in antigen-stimulated

223 (left) and positive control (right) samples in individual participants in the standard

224 assay set compared with sets with added cyclosporin (upper panel; n=13) and

225 tacrolimus (lower panel; n=13). Dotted lines indicate the cut-off for a positive test

226 result in antigen-stimulated samples (0.35 IU/mL), and the cut-off for a valid positive

227 control response (0.5 IU/mL).

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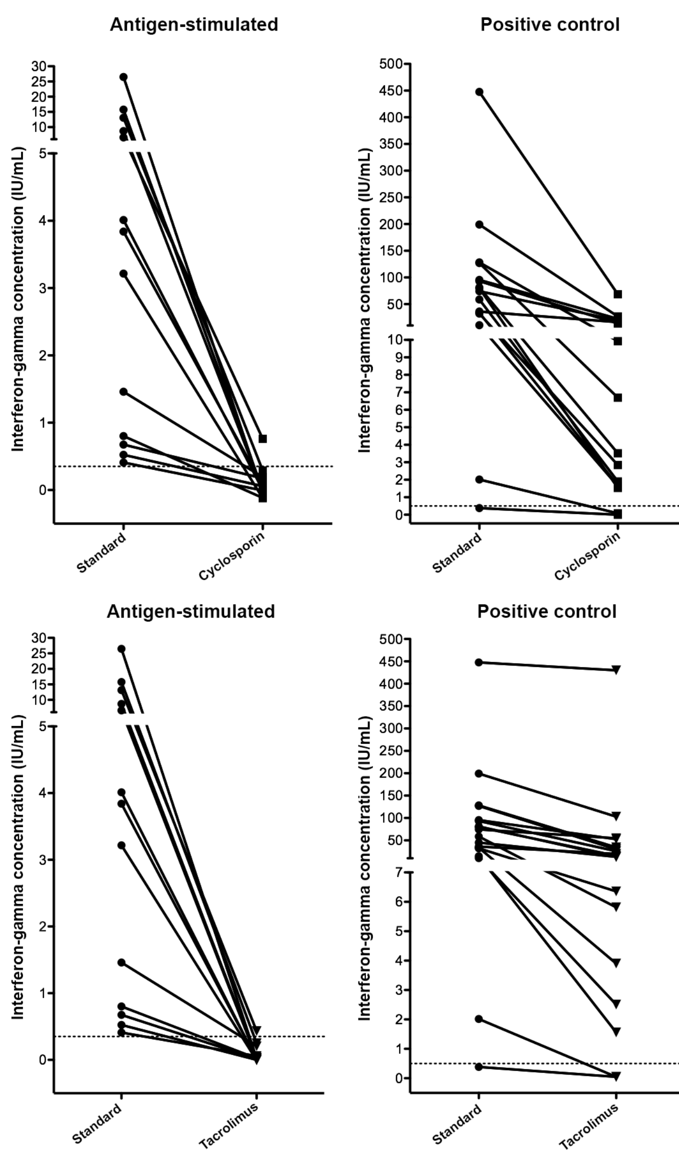

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232 **Figure 2.** Background-corrected interferon-γ, IL-2, IP-10 and TNF-α concentrations
233 in (A) antigen-stimulated (n=13) and (B) positive control (n=18) samples in standard
234 assay sets and sets with added cyclosporin and tacrolimus. Box plot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

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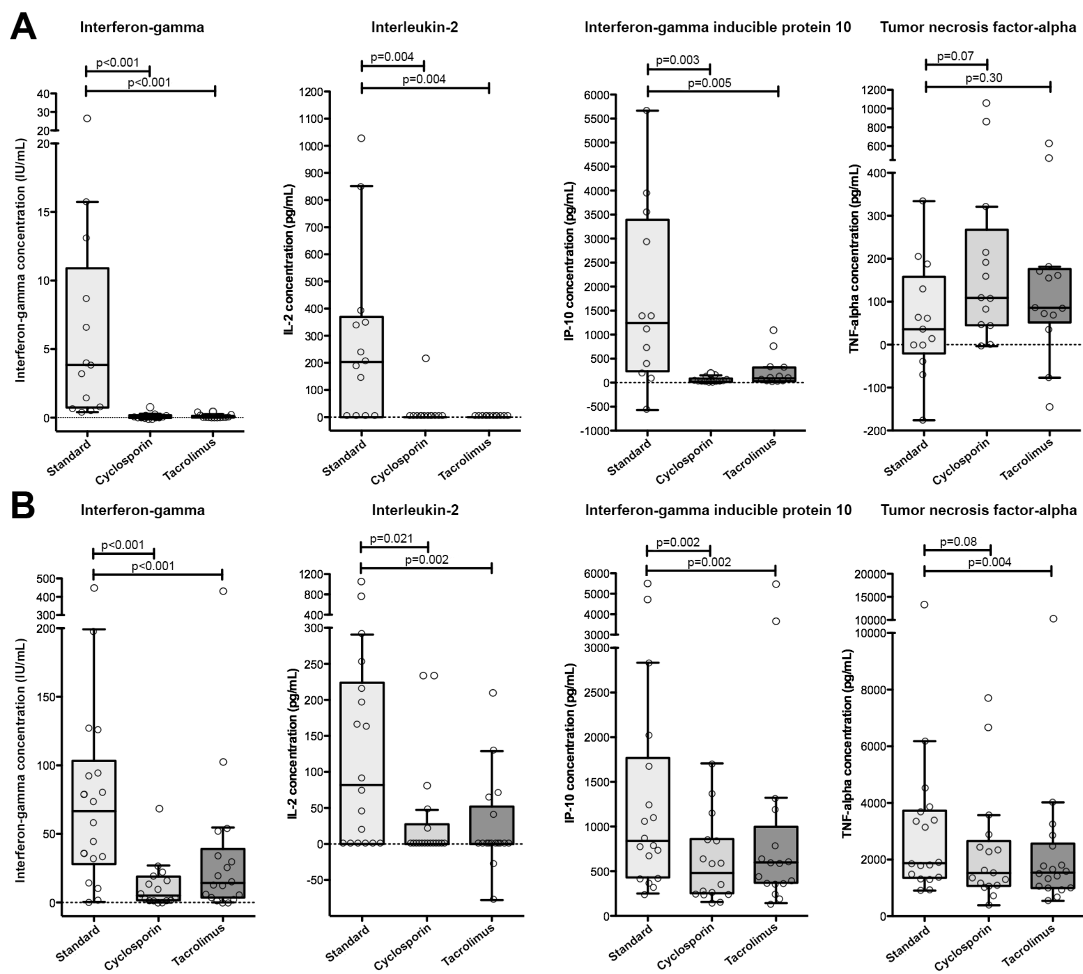

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