EDITORIAL

T-cell interferon- γ release assays: can we do better?

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Accurate diagnosis of latent tuberculosis (TB) infection (LTBI) is scientifically challenging because of the low burden of dormant tubercle bacilli, which are not directly detectable or quantifiable. However, the strong cellular immune response triggered by LTBI serves as an amplified signal for the presence of these dormant bacilli. The first measure of the cellular immune response to be exploited as a marker of *Mycobacterium tuberculosis* infection, developed at the end of the 19th century, was the tuberculin skin test (TST), which measures a delayed type hypersensitivity response to tuberculin purified protein derivative. The development and validation of T-cell-based interferon (IFN)- γ release assays (IGRAs) over the past decade represents a 100-yr upgrade in the diagnosis of LTBI [1, 2]; the amplified signal measured is the *ex vivo* release of T-cell-derived IFN- γ . IGRAs' clearest advantage is increased specificity for detection of *M. tuberculosis* infection thanks to their utilisation of *M. tuberculosis*-specific antigens encoded in region of difference (RD)1, a genomic segment absent from the Bacille Calmette-Guérin (BCG) vaccine and most environmental mycobacteria.

Currently, there are two forms of IGRA: either IFN- γ secretion is measured in whole-blood by ELISA or IFN- γ -secreting T-cells are directly enumerated by enzyme-linked immunospot (ELISpot) assay. The two assays are commercially licensed as QuantiFERON-TB Gold In-Tube (Cellestis Inc, Carnegie, Australia) and T-SPOT.TB (Oxford Immunotec Ltd, Abingdon, UK). Because these tests measure IFN- γ released in response to the RD1-encoded antigens early secretory antigenic target (ESAT)-6 and culture filtrate protein (CFP)-10 (and TB7.7 (Rv2645) in QuantiFERON-TB Gold In-Tube), specificity in populations at very low risk for LTBI is higher than that of TST, approaching 100% [2, 3]. Because there is no gold standard test for LTBI, surrogate markers are used to estimate the diagnostic sensitivity of IGRAs. When using active TB as a surrogate for LTBI, the sensitivity of QuantiFERON-TB Gold In-Tube is 70% (95% confidence interval (CI) 63–78%) and of T-SPOT.TB is 90% (95% CI 86–93%) [4]. The clinical utility of these tests hinges crucially on diagnostic sensitivity. In LTBI, sensitivity is especially important because targeted testing policies for LTBI are predicated on accurate diagnosis and

preventive treatment of those at highest risk of developing active TB, *i.e.* immunocompromised patients (*e.g.* HIV co-infected or iatrogenically immunosuppressed) and young children [5, 6]. For evaluation of patients with suspected active TB, tests of infection cannot be used to rule in TB disease as they cannot differentiate between active TB and LTBI. Rather, the predominant contribution of a test of infection is to enable clinicians rapidly to rule out a possible diagnosis of TB, which requires a test with very few false-negatives, *i.e.* high sensitivity. Although the diagnostic sensitivity of IGRAs is higher than that of the TST, their real-life clinical use demands higher sensitivity. So the key question is whether we can do better.

One approach that has been used for both ELISpot and ELISA to increase diagnostic sensitivity is incorporation of additional antigens of established high specificity [7]. In a recent large prospective study of patients with suspected TB, incorporation of a novel RD-1-encoded antigen, RV3879c, alongside ESAT-6 and CFP-10 (ELISpot^{PLUS}), significantly improved diagnostic sensitivity over the standard ELISpot test [8]. The addition of Rv2645 in QuantiFERON-TB Gold In-Tube significantly improved sensitivity of this assay in diagnosing active TB over QuantiFERON-TB Gold (which, like T-SPOT.TB, contains only ESAT-6 and CFP-10) without compromising specificity [9], in agreement with the observed higher positive results using QuantiFERON-TB Gold In-Tube than QuantiFERON-TB Gold in a cross-sectional study of healthy adults in South Africa [10].

Alternative readouts to measure IFN- γ release by *M. tuberculosis*-specific T-cells have also been explored. For example, using flow cytometry, ESAT-6-specific IFN- γ responses in peripheral blood mononuclear cells were detected in 13 (87%) out of 15 TB patients [11] and measuring IFN- γ mRNA from ESAT-6-stimulated peripheral blood mononuclear cells using quantitative PCR had a sensitivity of 65% in 54 active TB cases and LTBI [12].

Although IFN- γ is a pivotal cytokine in the immune response to *M. tuberculosis*, are there alternative or additional cytokines that could be used to improve detection of *M. tuberculosis* infection? An essential role of IFN- γ in host immunity against *M. tuberculosis* infection is activation of macrophages. Downstream chemokines induced by IFN- γ , namely IFN- γ inducible protein 10 (IP-10 or CXCL10), monocyte chemoattractant protein (MCP)-2 and monokine-inducible protein (MIG or CXCL9) have been found in: delayed type hypersensitivity reactions to tuberculin purified protein derivative [13]; lymph node and lung TB granulomas; pleural effusions of TB patients [14]; and the plasma of TB patients [15].

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These inflammatory chemokines, secreted by monocytes/macrophages activated by pro-inflammatory cytokines or infection with a pathogen, traffic activated effector T-cells to inflamed foci. Because they are downstream and induced by IFN- γ , these chemokines may serve as a more amplified readout than IFN- γ itself, thereby yielding higher sensitivity. IP-10 measured with a multiplex bead array identified four out of seven patients with active TB who had either negative or indeterminate QuantiFERON-TB Gold In-Tube results [16]. In another study, intracellular MIG, measured by flow cytometry, was higher in TB patients compared with BCG vaccinees [17].

In this issue of the *European Respiratory Journal*, RUHWALD *et al.* [18] evaluate whether measuring IP-10 and MCP-2 in addition to IFN- γ secretion in response to *M. tuberculosis*-specific antigens enhances test sensitivity for diagnosis of active TB. In this proof-of-principle case-control study with 204 participants IFN- γ , IP-10 and MCP-2 released from whole blood stimulated with ESAT-6, CFP-10 and TB7.7 peptides for 18 h were measured using ELISA for IFN- γ (QuantiFERON-TB Gold In-Tube) or multiplex bead arrays with flow cytometry for IP-10 and MCP-2. Overall, 5, 8 and 11% of assays in TB patients were indeterminate for IFN- γ , IP-10 and MCP-2, respectively. None of the controls had indeterminate assays. Excluding indeterminate results, QuantiFERON-TB Gold In-Tube was positive in 86% out of 80 culture and/or PCR-positive TB patients, IP-10 was positive in 89% of patients and MCP-2 was positive in 80% of patients. Specificity of 100% with QuantiFERON-TB Gold In-Tube test in 124 controls with no risk factors for LTBI (31% of whom were BCG vaccinated) was reduced to 93 and 99% when measuring IP-10 and MCP-2, respectively. Interestingly, decreasing the cut-off value for a positive QuantiFERON-TB Gold In-Tube result from the manufacturer's recommendations to 4 pg·mL⁻¹ increased the sensitivity of this test by 14% whilst maintaining high specificity in this population. In contrast, decreasing the cut-off value for a positive IP-10 result increased sensitivity accompanied by a decrease in specificity. Measuring IP-10 in addition to IFN- γ significantly improved sensitivity by 4% over the QuantiFERON-TB Gold In-Tube test alone ($p < 0.009$) while only slightly compromising specificity, which decreased from 100 to 98%. Measuring MCP-2 in addition to IFN- γ did not significantly improve sensitivity. Importantly, immunosuppression was not significantly associated with negative or indeterminate results for any of the tests.

These results are promising for the combined readout of IFN- γ /IP-10 as a potentially more sensitive marker of *M. tuberculosis* infection than current IGRAs. However, as with the clinical validation of IGRAs, the IFN- γ /IP-10 test should now be prospectively evaluated in large numbers of unselected patients presenting with suspected active TB in routine practice to determine its true diagnostic accuracy and clinical utility, as was recently done for the next-generation ELISpot^{PLUS} [8]. And ideally, such studies should include significant numbers of patients in whom TB is most difficult to diagnose, *i.e.* patients with HIV co-infection and children. Evaluating the performance of the IFN- γ /IP-10 test in LTBI will require correlation of test results with epidemiologically well-defined exposure to TB, as was first carried out for IGRAs in 2001 [19]. Ultimately, the prognostic value of the IFN- γ /IP-10 test for subsequent development of active TB will need to be

established to confirm its validity as a test of LTBI, as was recently done for QuantiFERON-TB Gold In-Tube [20] and ELISpot [21].

Given the complexity of the cellular immune response to *M. tuberculosis* infection, can we infer additional useful clinical and biological information about our patients by measuring other immunological mediators? Simultaneous measurement of interleukin (IL)-2 with IFN- γ at the single T-cell level reveals T-cell cytokine profiles (defined as the relative proportions of antigen-specific T-cells secreting IFN- γ -only, IFN- γ /IL-2 or IL-2-only) that correlate with pathogen and antigen load across a range of viral infections [22]. These functional cytokine profiles also correlate with bacterial and antigen load in TB, shifting dynamically in response to successful treatment [23], suggesting a potentially clinically useful role for this new tool for TB treatment monitoring and test of cure [24]. Furthermore, measuring mRNA expression of several cytokines in response to ESAT-6 stimulation revealed that for a combination of IL-8, FOXP3 and IL-12 β , signatures that discriminated active TB from LTBI could be defined [12]. Whether detection of T-cell responses to specific antigens or peptide epitopes can discriminate active from latent TB is also being actively investigated [25–27].

Thus, current interferon- γ release assays, when viewed in the context of advances in T-cell immunology over the past decade, are in reality an evolving work in progress that embody the state of our knowledge at the end of the 20th century [1]. The study by RUHWALD *et al.* [18] provides a cogent example of taking more recent discoveries about the immune response to tuberculosis and translating them into proof-of-principle human studies in order to establish their potential clinical relevance. It is now widely recognised that interferon- γ release assays were the 100-yr upgrade on tuberculin skin test for diagnosis of latent tuberculosis infection [1, 2, 4, 6]. What studies such as those by RUHWALD *et al.* [18] show us is that the next generation of immune-based diagnostic tests for tuberculosis is not another century away but more likely just around the corner.

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