Monokine induced by interferon gamma and IFN-γ response to a fusion protein of *Mycobacterium tuberculosis* ESAT-6 and CFP-10 in Brazilian tuberculosis patients

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Abstract

IFN-γ responses to *Mycobacterium tuberculosis* antigens ESAT-6 and CFP-10 have been proposed as specific markers of *M. tuberculosis* infection. Monokine induced by gamma interferon (MIG/CXCL9) has been shown to be expressed by IFN-γ stimulated mononuclear cells and to attract activated T-cells through the chemokine receptor CXCR3. Since MIG is induced early in the response to IFN-γ, measuring MIG may provide an interesting marker to assess downstream IFN-γ induced responses, in contrast to assays that mainly focus on quantifying production of IFN-γ per se. We, therefore, investigated MIG and IFN-γ responses to a fusion protein of ESAT-6 and CFP-10, and compared responses to the conserved mycobacterial antigen 85B (Ag85B) and purified protein derivative (PPD) of *M. tuberculosis*, in 29 BCG vaccinee controls and 24 TB patients. IFN-γ secreting cells were determined by ELISPOT, and MIG production was measured by ELISA and flow cytometry. Production of MIG in response to ESAT-6/CFP-10, Ag85B and PPD correlated overall with increased numbers of IFN-γ secreting cells (r = 0.55, P < 0.0001). A significant increase was noted among patients compared to controls in the secretion of IFN-γ and MIG following stimulation with ESAT-6/CFP-10 or PPD (P < 0.05). Moreover, MIG intracellular expression was higher in TB patients compared to BCG vaccinees (P < 0.05) in response to ESAT-6/CFP-10 or PPD. We conclude that MIG production correlates significantly with enhanced T-cell IFN-γ production induced by *M. tuberculosis*-specific antigens ESAT-6/CFP-10. These results point to MIG as a potential novel biomarker that may be helpful in assessing downstream responses induced by IFN-γ in TB.

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1. Introduction

Tuberculosis (TB) affects millions of people worldwide, second only to AIDS among infection-related causes of mortality. A third of the world population is thought to be infected with *Mycobacterium tuberculosis* and 9 million new TB cases occur every year, resulting in 2–3 million deaths [1]. The disease affects both BCG-vaccinated and non-vaccinated people, and susceptibility to infection and disease is related to decreased host immunity. Definitive TB diagnosis is chiefly based on microscopy or culture-based detection of microorganisms. Most patients, however, are only diagnosed at an advanced disease stage. Around 15–20% patients with pulmonary TB are negative on bacilloscopy [2], and microbiological diagnosis is often hampered by the long time needed for the cultures to yield *M. tuberculosis*, along with the scarcity of technically competent facilities in endemic regions.
Purified protein derivative (PPD) is a crude protein mixture, composed of more than 200 proteins, obtained from the supernatant of *M. tuberculosis* culture. The PPD tuberculin skin test is still useful for the detection of active tuberculosis, but false-negative results are frequently associated with extensive disease or advanced HIV-1 infection [3]. Furthermore, false-positive results occur in BCG-vaccinated individuals or in those in contact with environmental mycobacteria [4,5]. The resulting low specificity of the T-cell and skin test response to PPD limits its use for diagnosis of TB [6,7].

A more specific diagnostic test should be based on antigens that are preferentially found in *M. tuberculosis* and not in the *M. bovis* BCG vaccines. Six-kDa early-secreted antigenic target (ESAT-6) [8] and culture filtrate protein 10 (CFP-10) [9] meet this demand, since both are encoded by the region of difference 1 (RD1) which is present in *M. tuberculosis* and in virulent *M. bovis*, but absent from *M. bovis* BCG substrains and most environmental mycobacteria [8,9]. ESAT-6 and CFP-10 are co-secreted proteins that form a 1:1 complex in vitro, which seems to be critical for their biological activity [10]. Several studies have shown that ESAT-6 and CFP-10 are immunodominant T-cell-stimulatory antigens that induce peripheral blood mononuclear cells (PBMC) from TB patients to produce high levels of IFN-γ [11,12], a typical Th1 cytokine produced by T-cells and NK cells. Detection of IFN-γ or IFN-γ-producing cells after antigen stimulation is frequently used as an indicator of cellular effector activity [13,14]. IFN-γ has a central role in several immunological functions in tuberculosis, including macrophage-stimulated chemokine production. Through their ability to recruit discrete leukocyte populations, chemokines may enhance antigen-specific immune responses [15].

Monokine induced by IFN-γ (MIG or CXCL9) and interferon-induced protein (IP-10) are related chemokines of the CXC subfamily. The *mig* and *ip-10/cxcr3* genes are adjacent in the human and the mouse genomes, and both are induced in monocytes/macrophages, endothelial cells, keratinocytes, fibroblasts and polymorphonuclear neutrophils specifically in response to IFN-γ [16–18]. IP-10 and MIG induce T-cell adhesion to the vascular endothelium via CXCR3, which is selectively expressed on activated T-cells and eosinophils [17,19,20]. During a kinetic study of normal human bronchial epithelial cells, MIG mRNA was detected one to 2 h after IFN-γ stimulation, reaching a peak at 8 h and slowly decreasing thereafter [21]. MIG production reaches a peak during the early recruitment period in lungs with granuloma induced by agarose-bead-immobilized PPD. Interestingly, in this model, IFN-γ neutralization profoundly abrogated MIG expression, whereas expression of IP-10, which is 37% identical to MIG, was essentially unchanged [22]. Thus, MIG expression may provide an interesting marker that may be helpful in assessing downstream responses induced by IFN-γ.

We, therefore, decided to assess IFN-γ and MIG production in TB since type-1 cytokines are known to play an essential role in controlling infection [23,24]. PBMC from TB patients and BCG-vaccinated controls were stimulated with a fusion protein of ESAT-6/CFP-10, PPD or the crossreactive mycobacterial antigen Ag85B, a secreted protein from *M. tuberculosis* which can stimulate strong humoral and cell-mediated immune responses and confer significant protection against experimental tuberculosis in mice [25]. We measured MIG and IFN-γ production, and next analyzed whether MIG and IFN-γ secretion correlated and also whether IFN-γ and MIG production differed between TB patients and BCG-vaccinated controls.

2. Materials and methods

2.1. Population studied

Twenty-four patients undergoing treatment for pulmonary TB in the Respiratory Diseases Division of the Central Public Health Clinic of Juiz de Fora, State of Minas Gerais, Brazil, were selected (≥ 15 days of chemotherapy; mean treatment-time = 2.16 months). Only those patients with detectable acid-fast bacilli in the sputum bacilloscopy or culture-confirmed disease and who had undergone clinical and chest X-ray examinations as prescribed by the Brazilian Ministry of Health, were included in the study. AIDS, diabetes, hepatitis, hypertension, pregnancy, and alcoholism were exclusion criteria. Twenty-nine BCG-vaccinated controls were included in the control group of medical students without prior history of mycobacterial infection. All patients and controls gave informed consent for blood sampling after written information was provided. The Medical Ethics Committee of the Federal University of Juiz de Fora, approved, the study protocol (UFJF-0020/02-8).

2.2. Separation of PBMC

PBMC were separated by density centrifugation on a Ficoll–Paque Plus (Amersham Bioscience, Uppsala, Sweden), resuspended in 10% DMSO in fetal calf serum (FCS; Sigma, St Louis, MO), aliquoted into 1.8 ml tubes (Nunc) and frozen stepwise to −70 °C overnight and preserved under liquid nitrogen until assayed. Cryopreserved cells were thawed, washed in medium and adjusted to a concentration of 2 × 10⁶ cells per ml in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 µl/ml non-essential aminoacids and 10% heat-inactivated normal human AB plasma (Hemominas, Juiz de Fora, Brazil).

2.3. IFN-γ ELISPOT

For enumeration of IFN-γ secreting cells, an adaptation of the methodology described by Möller and Borrebaeck [26] and modified by Teixeira and Kaufmann [27] was used. Briefly, filtration plates (Millipore, Bedford, MA, USA) were coated overnight at 4 °C with anti-human IFN-γ monoclonal antibodies (R&D, Minneapolis, MN, USA, 4 µg/ml). Wells
were blocked with 1% BSA-PBS for 2 h at 37 °C. PBMC of the selected TB patients and controls were resuspended in supplemented RPMI medium, added to 96 well plates in a volume of 100 µl (5 × 10⁴–2 × 10⁵ per well) and incubated in the presence or absence of recombinant ESAT-6/CFP-10 (10 µg/ml), Ag85B (10 µg/ml) and PPD (5 µg/ml), for 48 h at 37 °C in an atmosphere of 5% CO₂, in duplicates. Plates were thoroughly washed with PBS-Tween, before adding aliquots of 100 µl per well of biotinylated anti-human IFN-γ mAb (R&D, Minneapolis, MN, USA, 0.5 µg/ml). After incubation at 37 °C for 2 h, plates were washed 15 times with PBS-Tween and three times with alkaline phosphatase buffer, pH 9.5. Then, the substrate, 5-bromo-4 chloro-3-indol phosphate (Life Technologies, Grand Island, NY, USA), and the catalyst, nitroblue tetrazolium chloride (Life Technologies) were added. After 15 min, blue dots had developed and the reaction was stopped with distilled water. Individual spots were counted under the dissecting microscope at 32-fold.

Antigen responses are shown as Δ values (number of IFN-γ spot forming cells (SFC) in the antigen stimulated wells minus IFN-γ SFC in the unstimulated wells). Positive antigen responses were defined as Δ values > 100 SFC/10⁶ PBMC, which is approximately the mean plus two standard deviations of the concentration measured in the non-stimulated control.

2.4. MIG ELISA

For quantification of MIG secretion, cell-free culture supernatants were harvested after 16 h of in vitro stimulation by antigen, in duplicates, and stored immediately (−70 °C) until assayed. MIG production was determined by a standard ELISA technique with commercially available reagents (Pharmingen, San Diego, CA, USA). OD was determined at 450 nm using a spectramax-190 (Molecular Devices, Sunnyvale, CA, USA). Recombinant human MIG (Pharmingen, San Diego, CA, USA) served as standard in all assays. Antigen responses are shown as Δ values (MIG production in the antigen stimulated wells minus IFN-γ SFC in the unstimulated wells). Positive antigen responses were defined as Δ values > 100 pg/ml, which is approximately the mean plus one standard deviation of the concentration measured in the non-stimulated control.

2.5. Flow cytometry

For the quantification of MIG positive cells through intracellular staining, 5 × 10⁵ PBMC were cultured in RPMI medium in a 96 well plate at 37 °C, 5% CO₂ for 48 h. ESAT-6/CFP-10 (10 µg/ml), Ag85B (10 µg/ml) and PPD (5 µg/ml) were added to the initial culture. Cells were then washed with PBS containing 0.1% of bovine serum albumine (washing buffer) and the pellet was stained with anti-CD14-fluorescein isothiocyanate (FITC) for 30 min at 4 °C. Cells were washed two times with washing buffer, resuspended in 100 µl of Cytofix/Cytoperm (BD) and incubated for 20 min at 4 °C. Then, cells were washed two times with perm/wash (BD) solution and the pellet was stained for intracellular MIG by resuspending cells in 50 µl of perm/wash solution containing anti-MIG phycoerythrin (PE) or IgG1 isotype control (clone 107.3) for 30 min at 4 °C. Cells were washed twice in perm/wash solution and fixed with PBS containing 1% paraformaldehyde, kept at 4 °C overnight, read and analyzed using a FACScalibur flow cytometer (BD). Analysis was performed on 10,000 acquired events for each sample. Cells were gated on CD14 (monocytes) and forward scatter-height using the CellQuest® research software (BD). Then the gated cells were presented on two-dimensional dot-plots displaying fluorescence of MIG-PE and CD14-FITC. All reagents were purchased from Becton Dickinson, San Jose, CA, USA.

2.6. Statistical analysis

Comparisons between groups were analyzed with the Kruskal–Wallis or non-paired Wilcoxon (Mann Whitney U) tests, as appropriate. All statistical analyses were 2-sided. Results are reported as means ± standard errors. The relationship between MIG and IFN-γ production was examined using Spearman’s rank correlation coefficient. Statistical significance was set at 5% (P<0.05).

3. Results

3.1. IFN-γ and MIG production in response to M. tuberculosis antigens in TB patients

PBMC from TB patients and from BCG-vaccinated controls were stimulated in vitro with PPD, Ag85B, and ESAT-6/CFP-10 fusion protein. MIG and IFN-γ production were assessed through ELISA and ELISPOT, respectively. All antigens were observed to foster a significant increase (P<0.05) in IFN-γ secretion and MIG production as compared with non-stimulated culture (data not shown). As can be seen in Fig. 1, a significant correlation existed between IFN-γ SFC and MIG production in response to the in vitro stimulation by the three antigens considered together in all paired subjects (n = 100; Spearman’s r = 0.55; 95% CI, 0.38–0.69; P < 0.0001). This correlation was also significant when considering the ESAT-6/CFP-10 (n = 34; Spearman’s r = 0.58; 95% CI, 0.29–0.77; P < 0.0003) and PPD (n = 34; Spearman’s r = 0.69; 95% CI, 0.45–0.83; P < 0.0001), but not antigen 85B responses (n = 32; Spearman’s r = 0.21; 95% CI, −0.16–0.53; P = 0.2495).

Fig. 2 shows that IFN-γ and MIG production was greater in TB patients’ cells stimulated with PPD and ESAT-6/CFP-10 as compared with BCG-C (P < 0.05). This difference was not observed, either for IFN-γ or MIG, when the cultures were stimulated with Ag85B (Fig. 2).
Table 1 shows that adopting one hundred SFC/10^6 cells as cut-off level for detection of IFN-γ producing cells by ELISPOT, 72% of BCG-vaccinated controls responded to PPD (mean, 327 SFC/10^6 cells; 25–75th percentile, 80–478 SFC/10^6 cells) and 50% to ESAT-6/CFP-10 (mean, 214 SFC/10^6 cells; 25–75th percentile, 26–328 SFC/10^6 cells, P = 0.1249 versus PPD). As for MIG production (cut-off level 100 pg/ml), 28% of vaccinated controls responded to PPD (mean, 182 pg/ml; 25–75th percentile, 0–212 pg/ml) and 14% to ESAT-6/CFP-10 (mean, 72 pg/ml; 25–75th percentile, 0–73 pg/ml, P = 0.6272 versus PPD). In TB patients, about 90% produced IFN-γ to both PPD (mean, 692 SFC/10^6 cells; 25–75th percentile, 187–1206 SFC/10^6 cells) and ESAT-6/CFP-10 (mean, 552 SFC/10^6 cells; 25–75th percentile, 191–689 SFC/10^6 cells). MIG was produced by PBMC’s from nearly 50% TB patients to both PPD (mean, 221 pg/ml; 25–75th percentile, 18–331 pg/ml) and ESAT-6/CFP-10 (mean, 174 pg/ml; 25–75th percentile, 1–252 pg/ml). Ag85B induced in TB patients and in vaccinated controls a similar response regarding IFN-γ production (72%, mean 382 SFC/10^6 cells; 25–75th percentile, 93–250 SFC/10^6 cells and 61%, mean 390 SFC/10^6 cells; 25th–75th percentile, 59–520 SFC/10^6 cells, respectively) and MIG production (50%, mean 167 SFC/10^6 cells; 25–75th percentile, 18–194 SFC/10^6 cells and 47%, mean 158 SFC/10^6 cells; 25–75th percentile, 0.5–221 SFC/10^6 cells, respectively). In spite of both MIG and IFN-γ enabling discrimination between TB patients and controls, the MIG ELISA was less sensitive in this regard compared to the IFN-γ ELISPOT assay in this first analysis. Further work needs to be done to see whether the sensitivity of this new assay can be improved or whether other more sensitive assays can be developed to detect MIG.

3.2. Detection of intracellular MIG staining in monocytes after culture with M. tuberculosis antigens

MIG production in TB patients and BCG-vaccinated controls to ESAT-6/CFP-10, PPD and Ag85B was also assessed through flow cytometry. PBMCs were labeled with anti-MIG and anti-CD14 antibodies. PPD, Ag85B, and ESAT-6/CFP-10 were observed to stimulate MIG production in comparison with non-stimulated cells (P < 0.05) (Table 2). The TB group again showed a significant increase in the percentage of MIG positive cells in comparison with BCG-vaccinated controls after stimulation with PPD and ESAT-6/CFP-10 (P < 0.05), but not with Ag85B (Table 2 and Fig. 3), in line with the IFN-γ and MIG production results above. These results indicate that tests which use ESAT-6/CFP-10 or PPD
as stimulus can discriminate between TB patients and vaccinated controls, whereas the crossreactive Ag85B cannot.

4. Discussion

The most salient findings of this work are that 1) peripheral blood monocytes of patients with tuberculosis produced MIG in response to ESAT-6/CFP-10, PPD and Ag85B; 2) a significant correlation was found between MIG and numbers of IFN-γ-producing cells; and 3) TB patients produced higher levels of MIG and had higher frequencies of IFN-γ and MIG positive cells than BCG-vaccinated controls in response to the \textit{M. tuberculosis}-specific antigens ESAT-6/CFP-10, similar results were obtained for PPD.

Quantification of \textit{M. tuberculosis}-specific IFN-γ secreting cells using ELISPOT and other techniques has been used for rapid detection of \textit{Mycobacteria}-reactive T-cells [28,29]. Several studies have shown ESAT-6 and CFP-10 to be promising antigens for diagnosing \textit{M. tuberculosis} infection [11,30,31]. In this study, as much as 50% of control subjects produced IFN-γ as determined by ELISPOT in response to ESAT-6/CFP-10 while 90% of TB patients responded to these antigens. In a previous study in Brazil, Cardoso et al. [30] found 60% of pulmonary TB patients and 59% of BCG-vaccinated controls responding to ESAT-6 by ELISA. Lalvani et al. [28], observed 80% of healthy urban Indians responding to ESAT-6 and CFP-10 by ELISPOT; in contrast, among BCG-vaccinated United Kingdom-resident healthy adults, none responded to either antigen, supporting other studies [31] (reviewed in [32]). These findings most likely reflect the fact that many individuals from TB endemic areas harbor latent \textit{M. tuberculosis} infection, leading to a T-cell response to \textit{M. tuberculosis}-specific antigens. Our results reported here confirm and extend these findings: both IFN-γ and MIG production induced by ESAT-6/CFP-10 and PPD are significantly higher in TB patients than in BCG vaccinee-controls. Moreover, the numbers of IFN-γ secreting cells and levels of MIG production, in response to ESAT-6/CFP-10 and PPD, correlate significantly also.

During the immune response to \textit{M. tuberculosis} infection, T-cells and macrophages are recruited to the site of infection, resulting in tissue inflammation and granuloma formation. The mechanism of recruitment of these cells is likely to involve chemokines, which attract neutrophils, lymphocytes, and monocytes [15]. MIG, or CXCL9, is a chemokine pro-

![Fig. 3. Expression of intracellular MIG in monocytes stimulated with \textit{M. tuberculosis} antigens. After 16 h under standard culture conditions, PBMC were analyzed by flow cytometry on the FACScalibur cytometer, which was gated to include monocytes. Cells were stained with fluorescence-conjugated antibodies to MIG (PE) and CD14 (FITC). This figure is representative of one of 17 BCG-vaccinated controls and 18 TB patients studied. Numbers in the upper right corner represent the percentage of MIG positive monocytes. Axes show log fluorescence intensity.]

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<tr>
<th>Antigen</th>
<th>BCG-C</th>
<th>TB</th>
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<td>(–)</td>
<td>4.48 ± 1.61&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.12 ± 1.46&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>PPD</td>
<td>7.83 ± 1.79&lt;sup&gt;d&lt;/sup&gt;</td>
<td>14.79 ± 2.46&lt;sup&gt;d,e&lt;/sup&gt;</td>
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<tr>
<td>ESAT-6/CFP-10</td>
<td>11.60 ± 3.04&lt;sup&gt;d&lt;/sup&gt;</td>
<td>18.48 ± 3.59&lt;sup&gt;d,e&lt;/sup&gt;</td>
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<td>Ag85B</td>
<td>15.18 ± 3.00&lt;sup&gt;d&lt;/sup&gt;</td>
<td>20.12 ± 4.16&lt;sup&gt;d&lt;/sup&gt;</td>
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<sup>1</sup> BCG-vaccinated controls (n = 17).
<sup>2</sup> TB patients (n = 18).
<sup>3</sup> Mean ± S.E.M.
<sup>4</sup> P < 0.05 versus (–).
<sup>5</sup> P < 0.05 versus BCG-C.
duced by macrophages and other cells after stimulation with IFN-γ [17]. Induction of MIG expression has been detected in PBMCs from healthy volunteers stimulated with peptides from cytomegalovirus, influenza virus, Epstein-Barr virus and Plasmodium falciparum circumsporozoite protein [16]. As far as we are aware, this work is the first to demonstrate MIG production to *M. tuberculosis*-specific antigens in peripheral blood monocytes of patients with tuberculosis. Our data are consistent with studies in tuberculosis in which other chemokines were described: CD14+ blood monocytes from pulmonary TB patients spontaneously expressed higher levels of monocyte chemoattractant protein 1 (MCP-1) (mRNA and protein) than CD14+ monocytes from healthy tuberculin reactors [33]. Blood monocytes from pulmonary TB patients can also produce MCP-1 after stimulation with PPD [34]. Ferrero et al. [35], detected high levels of IL-8 and MCP-1 in supernatants from *M. tuberculosis* antigen-induced peripheral blood monocytes from healthy subjects, and Verreck et al. described induction of various cytokines and chemokines by human macrophage subsets in response to *M. tuberculosis* ([36] and our unpublished data). Human alveolar macrophages infected with *M. tuberculosis* were able to produce RANTES (regulated upon activation, normal T-cell expressed and secreted), MIP-1α (macrophage inflammatory protein 1 α) and MIP-1β [37]. All these findings suggest that chemokines, including MIG as reported in this study, potentially contribute to the anti-mycobacterial inflammatory response by attracting monocytes, neutrophils and T-lymphocytes, to form well-organized TB granuloma. Among these chemokines, MIG appears to be of remarkable importance because it is mostly induced by IFN-γ [22]. MIG expression, however, can be inhibited by down-regulatory cytokines interleukin (IL)-10 and transforming growth factor (TGF)-β, as described in mice with myocardium infected by *Trypanosoma cruzi* [38]. The precise role-played by other cytokines known to inhibit IFN-γ-mediated macrophage activation and their capacity to also modulate MIG production in TB remains to be determined.

Ag85B is part of the Ag85 complex and encompasses a large portion of the proteins secreted by *M. tuberculosis* culture filtrate. In contrast to ESAT-6 and CFP-10, it shows a high degree of conservation between mycobacterial species. Ag85B vaccination has been shown to confer protection in experimental TB [39], and this correlated with increased IFN-γ production. In accordance, our results showed strong IFN-γ and MIG responses to Ag85B in BCG-vaccinated controls and in tuberculosis patients, presumably correlating with vaccine or natural infection induced immunity against Ag85B. Thus, the immunodominant antigens ESAT-6/CFP-10 and Ag85B are both strong IFN-γ and MIG inducers in TB patients, but Ag85B induces relatively high IFN-γ and MIG responses in BCG-vaccinated controls, probably because Ag85B is shared between virulent strains of mycobacteria, BCG and environmental mycobacteria [26]. In summary, our results show that increased MIG production correlates significantly with enhanced IFN-γ production in response to *M. tuberculosis* antigens ESAT-6/CFP-10, Ag85B and PPD. These findings point to MIG as a potential new biomarker that might be useful to assess IFN-γ induced responses in tuberculosis. Measuring IFN-γ induced responses in TB may provide an important new angle for immunological profiling in TB, next to assays that measure the frequency of IFN-γ producing T-cells or the level of IFN-γ production, since these latter assays cannot assess the integrity of the IFN-γR signaling pathway, which is crucial in controlling human tuberculosis [23,24]. MIG detection at this stage, however, cannot replace IFN-γ detection assays in immunological profiling of TB, given its apparent lower sensitivity compared to IFN-γ detection assays.

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