Key Role of Effector Memory CD4⁺ T Lymphocytes in a Short-Incubation Heparin-Binding Hemagglutinin Gamma Interferon Release Assay for the Detection of Latent Tuberculosis

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The treatment of latent tuberculosis infection (LTBI) in target populations is one of the current WHO strategies for preventing active tuberculosis (TB) infection and reducing the Mycobacterium tuberculosis reservoir. Therefore, powerful LTBI screening tools are indispensable. A gamma interferon release assay (IGRA) in response to the stimulation of peripheral blood mononuclear cells by the latency antigen native heparin-binding hemagglutinin (nHBHA-IGRA) has proven its potential for this purpose. We have evaluated its possible optimization through a reduction of incubation time from 96 to 24 h, while compensating for this by adding interleukin 7 (IL-7) to the medium. We have also investigated the phenotypes of the gamma interferon (IFN-γ)-producing cells after both short and long incubation times. One hundred thirty-one nonimmunocompromised patients were recruited from 3 Brussels-based university hospitals. They were divided into 1 of 4 subgroups according to their M. tuberculosis infection status (LTBI, TB infection, undetermined M. tuberculosis infection status, and noninfected controls). The novel 24-h nHBHA-IGRA was performed for all subjects, and a simultaneous 96-h classical HBHA-IGRA was performed for 79 individuals. The results showed a good correlation between the two tests, and the novel 24-h nHBHA-IGRA maintained the principal advantages of the classical test, namely, a high specificity for LTBI diagnosis, an absence of interference of Mycobacterium bovis BCG vaccination during infancy, and a relative discrimination between LTBI and TB infection. Whereas the commercialized IGRA show a greater sensitivity for recent than for remote M. tuberculosis infections, the 24-h nHBHA-IGRA appears to have comparable diagnostic powers for recent and remote LTBI. The IFN-γ detected by the 24-h nHBHA-IGRA was mainly secreted by effector memory CD4⁺ T lymphocytes, a finding suggestive of continuous HBHA presentation during latency.

The screening and treatment for LTBI in target populations in order to prevent TB and reduce the Mycobacterium tuberculosis reservoir are some of the main strategies of the WHO's Global Plan to Stop TB (http://www.who.int/tb/publications/global_report/en). However, a major obstacle to the instauration and effectiveness of these preventive measures resides in the lack of a gold standard LTBI screening tool.

For several decades, the tuberculin skin test (TST) has been the main screening test for LTBI despite its lack of both sensitivity and specificity (1). Subsequently, T-cell-based gamma interferon release assays (IGRAs) in response to antigens encoded in the M. tuberculosis genomic region of difference 1 (RD-1) and RD-11 were developed and commercialized (QuantiFERON-TB Gold In-Tube [QFT-GIT] and T-SPOT.TB tests), with the objective of offering a more powerful diagnostic tool for LTBI. These tests offer a higher specificity than TST particularly in countries with high Mycobacterium bovis BCG vaccination coverage (2). However, recent studies suggest that these short-incubation RD-1-based IGRA may have suboptimal sensitivities (3, 4).

An alternative IGRA in response to the native mycobacterial antigen heparin-binding hemagglutinin (nHBHA-IGRA) that uses a longer incubation time than the commercialized IGRA has been validated in immunocompetent adults in the screening for LTBI (5). This assay not only demonstrates a high sensitivity and specificity for LTBI diagnosis but also a capacity to detect remote M. tuberculosis infections, a substantial advantage over the commercialized IGRA (3–7).

Remote M. tuberculosis infections are generally believed to be identified through central memory T-cell (Tcm) responses detected with long-incubation IGRA (3, 4). Here, however, we demonstrate that both recent and remote M. tuberculosis infections can be identified through effector memory T-cell (Tem) responses using a short-incubation nHBHA-IGRA, the upgraded assay presented in this study. Our results suggest that the detection of IFN-γ-producing CD4⁺ Tem in response to nHBHA reflects the persistence of M. tuberculosis antigens and therefore a true state of latency.

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### MATERIALS AND METHODS

**Ethics statement.** The study protocols (P2007/175 and P2011/113) received approval from the ethics committee ULB–Hôpital Erasme (aggregation no. OMO21), and each participant signed an informed consent form.

**Study population.** Immunocompetent adults were recruited from 3 Brussels-based hospitals and divided into 4 subgroups according to their *M. tuberculosis* infection status: LTBI subjects, TB patients, noninfected controls, and patients with undetermined infection status.

### TABLE 1 Characteristics of subjects with known *Mycobacterium tuberculosis* infection status

| Subject characteristics
|-------------------------|-------------------------|--------------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
|                        | LTBI individuals         |                         | M. tuberculosis infection | TB patients               | Treatment duration | Noninfected controls |
|                        | All                      | Recent
d | Remote
c | Unknown | All          | ≤5 days | >5 days | All |≤5 days | >5 days |
| n                       | 32                       | 9                       | 14                       | 9                   | 23 | 17 | 6 | 24 |
| Median age (range) (yr) | 35 (23–66)              | 30 (24–44)              | 45 (25–63)               | 27 (23–66)          | 32 (20–57) | 30 (20–53) | 39 (20–57) | 33 (25–61) |
| Male/female             |                           |                           |                           |                     | 0.68 | 0.80 | 0.75 | 0.50 |
| No./total no.           | 13/19                    | 4/5                      | 6/8                      | 3/6                | 16/7 | 11/6 | 5/1 | 7/17 |
| Ratio                   |                           |                           |                           |                     | 2.29 | 1.83 | 5   | 0.41 |
| Ethnic origin (no. [%]) |                           |                           |                           |                     |                  |                  |                  |                  |
| Caucasian               | 16 (50.0)                | 5 (55.6)                 | 7 (50.0)                 | 4 (44.4)           | 8 (34.8) | 5 (29.4) | 3 (50.0) | 21 (87.5) |
| North African           | 8 (25.0)                 | 3 (33.3)                 | 2 (14.3)                 | 3 (33.3)           | 4 (17.4) | 3 (17.6) | 1 (16.7) | 3 (12.5) |
| Sub-Saharan African     | 7 (21.9)                 | 1 (11.1)                 | 5 (37.5)                 | 1 (11.1)           | 9 (39.1) | 7 (41.2) | 2 (33.3) | 0 |
| Other                   | 1 (3.1)                  | 0                        | 0                        | 1 (11.1)           | 2 (8.7)  | 2 (11.8) | 0   | 0   |
| M. *leprae* BCG vaccination status
| (no. [%])               |                           |                           |                           |                     |                  |                  |                  |                  |
| Vaccinated              | 19 (59.4)                | 5 (55.6)                 | 11 (78.6)                | 3 (33.3)           | 4 (17.4) | 3 (17.6) | 1 (16.7) | 4 (16.7) |
| Unvaccinated            | 8 (25.0)                 | 3 (33.3)                 | 2 (14.3)                 | 3 (33.3)           | 9 (39.1) | 7 (41.2) | 2 (33.3) | 18 (75.0) |
| Unknown                 | 5 (15.6)                 | 1 (11.1)                 | 1 (7.1)                  | 3 (33.3)           | 10 (43.5) | 7 (41.2) | 3 (50.0) | 2 (8.3) |
| TST (median diam of induration (range)) (mm) | 17 (10–30) | 16 (10–20) | 17 (10–22) | 17 (15–30) | NTa | NT | NT | <5 (0–4) |
| QFT-GIT result          |                           |                           |                           |                     |                  |                  |                  |                  |
| Positive                | 9                        | 2                        | 5                        | 2                  | NT | NT | NT | NT |
| Negative                | 16                       | 5                        | 9                        | 2                  | NT | NT | NT | NT |
| Unknown                 | 7                        | 2                        | 0                        | 5                  | NT | NT | NT | NT |

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* TST, tuberculin skin test; QFT-GIT, QuantiFERON-TB Gold In-Tube assay.
* Recent *M. tuberculosis* infections indicate infections of <2 years.
* Remote *M. tuberculosis* infections indicate infections of ≥2 years.
* NT, not tested.
Technologies, Ghent, Belgium), with only concentrations of >25 pg/ml being considered to ensure accuracy and reproducibility. Control values of >200 pg/ml in response to SEB and <50 pg/ml in the negative control were also required. If detectable, the IFN-γ concentrations obtained under nonstimulated conditions were subtracted from those obtained for the nHBHA-stimulated cells.

Phenotypes of IFN-γ-producing cells for the 24-h and 96-h nHBHA-IGRA. A proportion of the suspended PBMC were stimulated as described above except for the concentration of nHBHA used, which was increased to 10 μg/ml. The culture medium was enriched with the costimulatory antibodies anti-CD28 and anti-CD94 (2 μg/ml each; BD Biosciences, Erembodegem, Belgium). For the 24-h assay with an IL-7-enriched medium, 10 μg/ml brefedlin A (Sigma-Aldrich, Diegem, Belgium) and a 1:1,000 dilution of Monensin (BD Biosciences, Erembodegem, Belgium) were added for the last 4 h to block cytokine secretion. For the 96-h assay without IL-7 in the culture medium, the brefedlin A (3 μg/ml) and Monensin (1:2,000 dilution) were added on day 3 for overnight incubation.

To evaluate cell viability, single color staining using the LIVE/DEAD fixable dead cell stain kit (Life Technologies, Belgium) was performed for 10 min at 4°C. The cells were then stained for 30 min at 4°C with antibodies directed to the surface molecules: CCR7-phycoerythrin (PE), CD8-peridinin chlorophyll protein (PerCP), CD45RA-PE-Cy7, and CD4- allophycocyanin (APC)-H7. Surface staining with CD3-Horizon Violet 450 (HV450) and intracellular staining with IFN-γ-APC were performed for 30 min at room temperature after fixation and permeabilization of the cells using the BD Cytotox/Cytoperm fixation/permeabilization kit. All antibodies were mouse IgG1 kappa chains and were obtained from BD Biosciences, except anti-CCR7, which was obtained from R&D Systems Europe (United Kingdom), and anti-IFN-γ, which was obtained from BioLegend (Imtec Diagnostics, Antwerp, Belgium).

Flow cytometric analysis was performed using a fluorescence-activated cell sorter (FACS), Canto II (Becton, Dickinson) and FlowJo software (Tree Star, Ashland, OR). IFN-γ-positive T cells were gated using the unstimulated control to determine the gate location. For results to be considered interpretable, the IFN-γ-positive cell frequency in response to the nHBHA had to be at least twice that obtained under nonstimulated conditions, with a minimum of 18,000 CD3+ cells acquired. The respective percentages of central memory (CCR7+CD45RA−), effector memory (CCR7−CD45RA−), effector (CCR7−CD45RA+), and naive or stem cell memory (CCR7+CD45RA+) T cells among the IFN-γ-positive CD4+ and CD8+ T cells were then determined (Fig. 1).

Statistics. A Mann-Whitney U test or a Kruskal-Wallis test was used to compare continuous variables between the groups, while a Fisher exact test or a chi-square test was used for categorical variables. Correlations between the 96-h and the 24-h assays were analyzed by a nonparametric Spearman test, and their degree of agreement was assessed by the Cohen’s kappa coefficient. All results were obtained using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, CA). A P value of <0.05 was considered significant.

RESULTS

Overview of the study protocol. An overnight nHBHA-IGRA was developed (24-h nHBHA-IGRA) and compared to the validated 96-h nHBHA-based IGRA (96-h nHBHA-IGRA). This 24-h HBHA-IGRA was performed on all enrolled patients. When the number of isolated PBMC was sufficient, the validated 96-h nHBHA-based IGRA was performed in parallel. The two assays were compared in terms of sensitivity, specificity, detection of remote infections, influence of BCG vaccination, and phenotypes of IFN-γ-producing cells.

Study population. Overall, 131 adults were recruited: 32 LTBI subjects, 23 TB patients, 24 noninfected controls, and 52 patients with undetermined M. tuberculosis infection status. Among the 32 LTBI subjects, the M. tuberculosis infection was dated for 23 patients based on their period of contact with an infectious TB case and/or TST conversion. Nine subjects were infected within the 2 years preceding their enrollment (recent LTBI), and 14 were infected for a period of ≥2 years (remote LTBI). For the subjects with a known M. tuberculosis infection status, their demographic and clinical characteristics are summarized in Table 1, including the QFT-GIT results when tested.

Development of the 24-h nHBHA-IGRA. The performance of a 24-h incubation nHBHA-IGRA was initially evaluated on 7 LTBI subjects and 7 noninfected controls (5 BCG−, 2 BCG+). As short-incubation IGRA have been shown to induce lower IFN-γ concentrations in response to various antigens (3, 11), we evaluated the performance of the test with and without additional IL-7, a cytokine known to increase the sensitivity of IFN-γ-based assays (12). With a 24-h incubation period and without additional IL-7, the IFN-γ concentrations released in response to nHBHA were, for most LTBI subjects, very low, close to the detection limit of the ELISA (median IFN-γ concentration, 36 pg/ml). In contrast, the use of an IL-7-enriched medium resulted in high IFN-γ secretion levels in response to nHBHA, despite the short incubation time. Under these conditions, the IFN-γ concentrations were higher in LTBI than in noninfected controls (Fig. 2). Importantly, the IL-7-enriched medium did not induce IFN-γ secretion in the absence of antigenic stimulation. IL-7 concentrations between 0.63 and 1.25 ng/ml were shown to be optimal for accurate discrimination between LTBI and noninfected controls (P < 0.001). Therefore, this protocol of 1 ng/ml of IL-7 was used.

To establish cutoffs for the novel 24-h nHBHA-IGRA, receiver operating characteristic (ROC) curves were performed using the results obtained for LTBI subjects and for the noninfected controls. Both nontreated (n = 26) and treated LTBI subjects (n = 6) were considered, as we found no significant difference between their IFN-γ responses (median and 25th to 75th percentiles, 248 pg/ml and 61 to 1,777 pg/ml versus 103 pg/ml and 25 to 1,249 pg/ml, respectively).

Evaluation of the 24-h nHBHA-IGRA. With a cutoff established at 50 pg/ml, a specificity of 91.7% was obtained (2 of the 24 noninfected controls had a positive nHBHA-IGRA result). The sensitivity for the identification of LTBI was 75%, as 8 of the 32 LTBI subjects were not detected. Thirteen out of the 14 remote infections were detected. Similar specificities and sensitivities were found when testing with the 96-h nHBHA-IGRA (94.4% and 78.6%, respectively).

The 24-h and 96-h nHBHA-IGRA were performed side by side for 79 patients with known and unknown M. tuberculosis status. The nHBHA-induced IFN-γ levels were not significantly different between the two tests and were strongly correlated (Spearman’s rank correlation coefficient, 0.85; P < 0.0001) (Fig. 3A). When applying the corresponding cutoffs for each test, the degree of agreement of the two tests measured by kappa was almost perfect (kappa, 0.9; Fig. 3B), with only 4 out of the 87 pairs being discordant. Whether it was the 24-h or the 96-h nHBHA-IGRA that was correct could not be determined for 3 of the patients with discordant results, as they belonged to the group of patients with undetermined M. tuberculosis infection status. The last discordant result (highly positive in the 24-h assay and borderline negative in the 96-h assay) was from a patient with treated TB infection.

To evaluate the discriminatory power of the 24-h nHBHA-IGRA, we compared the IFN-γ concentrations obtained for the LTBI and the pretreatment TB patients (≥5 days of therapy). Al-
though an overlap was found between the values obtained in each group, the median IFN-γ concentrations were significantly higher for the LTBI group ($P = 0.006$; Fig. 4).

To assess the possible influence of a previous BCG vaccination on the results, we compared the median IFN-γ concentrations of the BCG-vaccinated ($n = 24$) and non-BCG-vaccinated ($n = 11$) individuals among those with a positive 24-h nHBHA-IGRA result and known BCG status. There was no significant difference ($P = 0.384$). In addition, the 2 noninfected controls with a positive 24-h nHBHA-IGRA result had not been vaccinated, while all 4 noninfected controls with past BCG vaccination had a negative test result. Of note, a much higher proportion of BCG-vaccinated

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**FIG 1** Gating strategy applied to evaluate the phenotypes of IFN-γ-producing T cells. Sequential gating was made on time of acquisition (A), live cells (B), single cells (C), lymphocytes, including blasts (D), nonautofluorescent cells (E), CD3⁺ T cells (F), and CD4⁺ or CD8⁺ T cells (G). The respective percentages of central memory (Tcm) (CCR7⁺ CD45RA⁻), effector memory (Tem) (CCR7⁻ CD45RA⁻), effector (Te) (CCR7⁻ CD45RA⁻), and naive or stem cell memory (Tn or Tscm, respectively) (CCR7⁺ CD45RA⁻) T cells among the CD4⁺ (H) and CD8⁺ T cells (K) were determined. In turn, the IFN-γ-positive CD4⁺ (I) and CD8⁺ T cells (L) were specifically selected and overlaid into these 4 phenotypes (J and M).
individuals was found in the LTBI group (59%) than in the non-infected controls (17%) (Table 1; \( P = 0.004 \)).

**Phenotypes of the IFN-γ-producing T lymphocytes.** The phenotype of IFN-γ-producing T lymphocytes in the 24-h nHBHA-IGRA setting (IL-7-enriched medium) and the 96-h nHBHA-IGRA setting were characterized in 11 LTBI subjects. Among these subjects, 8 had remote LTBI infections. For each patient, as expected, the majority of the IFN-γ-producing T lymphocytes in response to nHBHA stimulation were CD4+ T cells. We therefore focused on these lymphocytes and characterized their subpopulations by an analysis of the expression levels of the surface markers CD45RA and CCR7. Of note, however, a low-level CD8+ response toward nHBHA was detected after a 24-h incubation in 10 out of 11 subjects, confirming their implication in the response to nHBHA. The majority of IFN-γ-producing CD4+ T lymphocytes displayed a phenotype of memory cells, with a major contribution of Tem, especially in the 24-h assay (Table 2, Fig. 5A and B). The results were more heterogeneous in the 96-h assay, with a predominant contribution of Tcm for 7/11 subjects (Table 2, Fig. 5C and D). The contribution of CD4+ Te to the nHBHA-induced IFN-γ secretion after a 24-h stimulation was low (median, 1.1%; 25th to 75th percentiles, 0.7% to 4.2%), as was the contribution of CD45RA+ CCR7+ T cells (median, 1.6%; 25th to 75th percentiles, 0.0% to 5.5%). The latter phenotype classically defines naive T lymphocytes. However, in this context, it probably indicates a subset of memory stem cells, as was recently demonstrated in the literature (13).

**FIG 2** Dose-response curves to determine optimal IL-7 concentrations for the optimization of a 24-h nHBHA-IGRA. PBMC isolated from 7 LTBI subjects (circles) and 7 noninfected controls (triangles) (5 BCG+; 2 BCG−) were stimulated with (filled symbols) or without (open symbols) nHBHA in the presence of a 2-fold serial dilution of IL-7. IFN-γ secretion was measured by ELISA after 24 h of incubation. Each data point represents the median value obtained for the included subjects, and the dotted lines symbolize the 25th to 75th percentiles, when informative.

**FIG 3** Correlation between the 24-h nHBHA-IGRA and the validated 96-h nHBHA IGRA. The IFN-γ concentrations obtained for the 24-h nHBHA-IGRA and 96-h nHBHA-IGRA are represented in graph A in logarithmic scale for the 79 patients and the 87 comparisons made in total. The Spearman’s rank correlation coefficient is 0.85 (\( P < 0.0001 \)). The dotted lines represent the corresponding cutoffs for each assay. When applying these cutoffs and classifying test results as positive or negative (B), the degree of agreement of the two tests as measured by the kappa coefficient is 0.9.

**FIG 4** The 24-h nHBHA-IGRA results according to M. tuberculosis infection status. The results of the 24-h nHBHA-IGRA (IFN-γ concentrations as measured by ELISA after PBMC stimulation with nHBHA and 24 h of incubation in an IL-7-enriched medium) are shown for three different groups of subjects: 32 subjects with a latent M. tuberculosis infection (LTBI), 17 patients with active tuberculosis (TB) (pretreatment), and 24 noninfected controls. The dotted line represents the positivity cutoff for the assay. For each group, the median value of the results is marked by a horizontal line. The median value obtained for the LTBI is significantly different from those obtained for the two other subgroups. ***, \( P = 0.0006 \); ***, \( P < 0.0001 \).
TABLE 2 Phenotypes of IFN-γ⁺ CD4⁺ T lymphocytes

| Subject | nHBHA-IGRA incubation period (h) | % IFN-γ⁺ among CD4⁺ | % of indicated phenotype of IFN-γ-producing CD4⁺ T cells
|---------|--------------------------------|---------------------|---------------------------------------------------------|
|         | 24                             | 0.57                | Tem 63.4 Tcm 26.9 Tn or Tscm 5.5 Te 4.2               |
|         | 96                             | 0.28                | Tem 30.8 Tcm 55.6 Tn or Tscm 12.1 Te 1.5              |
| 2       | 24                             | 0.51                | Tem 69.9 Tcm 26.9 Tn or Tscm 2.3 Te 0.9              |
|         | 96                             | 0.51                | Tem 16.8 Tcm 80.8 Tn or Tscm 2.4 Te 0.0              |
| 3       | 24                             | 0.56                | Tem 78.2 Tcm 4.9 Tn or Tscm 11.4 Te 5.5              |
|         | 96                             | 1.00                | Tem 97.2 Tcm 0.0 Tn or Tscm 0.0 Te 2.8              |
| 4       | 24                             | 0.25                | Tem 82.2 Tcm 12.0 Tn or Tscm 0.0 Te 5.8              |
|         | 96                             | 2.08                | Tem 5.7 Tcm 90.7 Tn 0.0 Tscm 3.2 Te 0.3              |
| 5       | 24                             | 0.13                | Tem 93.4 Tcm 3.4 Tn or Tscm 1.6 Te 1.6              |
|         | 96                             | 0.27                | Tem 91.4 Tcm 8.6 Tn or Tscm 0.0 Te 0.0              |
| 6       | 24                             | 2.25                | Tem 79.6 Tcm 18.6 Tn or Tscm 1.1 Te 0.7              |
|         | 96                             | 3.58                | Tem 15.4 Tcm 84.1 Tn or Tscm 0.5 Te 0.0              |
| 7       | 24                             | 0.58                | Tem 84.2 Tcm 12.5 Tn or Tscm 2.2 Te 1.1              |
|         | 96                             | 0.49                | Tem 17.2 Tcm 74.1 Tn 7.8 Te 0.8                  |
| 8       | 24                             | 0.19                | Tem 71.4 Tcm 28.6 Tn or Tscm 0.0 Te 0.0              |
|         | 96                             | 0.25                | Tem 40.9 Tcm 30.4 Tn 27.5 Te 1.3                  |
| 9       | 24                             | 0.37                | Tem 80.5 Tcm 19.4 Tn or Tscm 0.0 Te 0.1              |
|         | 96                             | 0.28                | Tem 2.7 Tcm 93.3 Tn 3.2 Te 0.7                  |
| 10      | 24                             | 1.33                | Tem 63.9 Tcm 34.5 Tn or Tscm 0.9 Te 0.7              |
|         | 96                             | 1.33                | Tem 4.2 Tcm 93.3 Tn 2.5 Te 0.1                  |
| 11      | 24                             | 0.38                | Tem 70.8 Tcm 20.8 Tn or Tscm 7.3 Te 1.2              |
|         | 96                             | 0.54                | Tem 49.0 Tcm 49.2 Tn 1.8 Te 0.0                  |

*Tem, effector memory T cells; Tcm, central memory T cells; Tn, CD45RA⁺CCR7⁻ naïve T cells; Tscm, CD45RA⁻CCR7⁺ memory stem cells; Te, effector T cells.

DISCUSSION

We previously reported the high accuracy of a 96-h nHBHA-IGRA for the detection of LTBI (5). We show here that a 24-h nHBHA-IGRA also provides high specificity for the diagnosis of LTBI among asymptomatic subjects and a relative discrimination between LTBI and TB.

As short-incubation IGRA’s induce lower IFN-γ levels, the 24-h nHBHA-IGRA was developed by adding IL-7 to the culture medium, thus achieving sufficient sensitivity without affecting specificity. Indeed, at low doses, IL-7 does not by itself induce proliferation of resting T cells but helps preserve the function and enhances the survival in isolated T cells (14). Furthermore, IL-7 promotes IFN-γ gene expression in activated T cells and stabilizes IFN-γ mRNA transcripts, resulting in enhanced IFN-γ protein secretion within 3 to 6 h of stimulation (15).

Although QFT-GIT is becoming the main referent screening tool in most equivalent studies, in this study, the diagnosis of LTBI was based on TST results, as it is the first-line screening test recommended in Belgium (16). Indeed, TST remains a reasonable option in this country, which has a low coverage of BCG vaccination and low prevalence of atypical mycobacterial infections (17). Furthermore, the use of high cutoffs for positivity in patients with a low risk of M. tuberculosis exposure as applied here reduces the number of false-positive results induced by environmental mycobacteria (1). Nevertheless, in the absence of a gold standard for LTBI diagnosis, the main obstacle that arose during this study was the classification of the patients into the different M. tuberculosis infection subgroups, which was achievable for only 60% of the overall cohort and resulted in small numbers.

Reflecting the difficulty of LTBI diagnosis and mistrust of the TST, QFT-GIT was requested by the clinicians as a second-line test for 25 out of the 32 LTBI patients identified by TST. Interestingly, only 9 of these 25 patients had a positive test result with the QFT-GIT (36%) compared with 75% (24/32) detection by the 24-h nHBHA-IGRA. These results are comparable to those found with the 96-h nHBHA-IGRA in a study by Hougardy et al. (5). Notably, among the 8 LTBI subjects that were not detected by the 24-h nHBHA-IGRA, 6 had a simultaneous QFT-GIT and 4 had a positive result. Differential IFN-γ responses to nHBHA and to the RD-1 antigens used in the QFT-GIT are believed to reflect the dynamic state that characterizes LTBI (18), the former present in quiescent infections and the latter when subclinical M. tuberculosis replication persists (7). Combining the results of the nHBHA-based IGRA with the results of the RD-1-based IGRA therefore broadens the spectrum of LTBI detected. This might prove to be essential in immunocompromised patients and those requiring immunosuppressive therapy. Sharing equivalent incubation times, the commercialized IGRA and the novel 24-h nHBHA-IGRA can be efficiently tested in parallel, with a reduced burden on laboratory resources compared to that caused by the original 96-h assay.

Among the LTBI patients identified by TST but not by the 24-h nHBHA-IGRA, three had no detectable responses to the in vitro stimulation by PPD. This might be attributed to a possible experimental error, a too-short interval between M. tuberculosis contact and the evaluation of the specific immune responses against M. tuberculosis antigens, spontaneous clearance of the bacteria, or an incorrect administration and/or reading of the TST. These results underline the importance of using PPD as a signal of previous mycobacterial infection when performing the nHBHA-IGRA. Indeed, when excluding the PPD-negative results, the 24-h nHBHA-IGRA offers a sensitivity and specificity of 89% and 92%, respectively.

The specificity of the nHBHA-based IGRA is further increased, as it provides a better discrimination between LTBI and TB than TST and QFT-GIT. Although the overlap between the nHBHA-IGRA results in LTBI and TB patients persists, it is certainly a solid basis for ongoing and future improvement. Currently, work is ongoing to enhance the LTBI-TB discriminant power of the nHBHA-IGRA by combining the results with other M. tuberculosis antigen-specific IGRA’s (such as the nHBHA/recombinant early secretory antigen target 6 [rESAT-6] ratio) and/or other cytokine secretion measurements. BCG vaccination independency, offered by the commercialized IGRA’s but not the TST, is also an essential feature for an LTBI screening test. nHBHA is produced off the commercialized IGRAs but not the TST, is also an essential feature for an LTBI screening test.
In addition to these numerous advantages, the 24-h nHBHA-IGRA, like the 96-h nHBHA-IGRA, detected both recent and remote *M. tuberculosis* infections. Classically, remote *M. tuberculosis* infections are believed to be identified through Tcm responses detected with long-incubation IGRA (3,4). However, the 24-h nHBHA-IGRA detected remote *M. tuberculosis* infections through CD4+ H11001 Tem responses. The detection of remote infections therefore appears to be determined by the specificity of the nHBHA for latency.

In this study, implication of both CD4+ and CD8+ T-cell lymphocytes in the nHBHA-induced IFN-γ synthesis is confirmed (10,20). More specifically, we demonstrate that the CD4+ Tem responses play a key role in the 24-h nHBHA-IGRA but also in the 96-h nHBHA-IGRA, albeit with more heterogeneous results. In viral infections, such as hepatitis C, Tem responses have been associated with ongoing viral replication and Tcm to viral clearance (21). Similarly, in recent studies on intracellular organisms, the persistence of functional memory CD4+ T cells has been shown to depend on antigen persistence (22,23). Nelson et al. (22) demonstrated in a mouse model of *Salmonella enterica* subsp. *enterica* serovar Typhimurium infection that CD4+ T-cell maintenance is dependent upon persistent peptide-major histocompatibility complex (MHC) class II presentation. This indicates that T-cell receptor (TCR) stimulation by the persistent infection of antigen-presenting cells is the key signal needed for the maintenance of CD4+ T cells. Such circulating memory CD4+ T lymphocytes were shown in a mouse model of chronic malaria to be predominantly Tem, able to produce cytokines on short-term restimulation, whereas antigen-independent Tcm mostly resided in secondary lymphoid organs (23). The circulating CD4+ Tem in this mouse model seemed to survive acute infection and to be continuously stimulated to control chronic infection. Whether these characteristics can be transposable to a *M. tuberculosis* infection is unknown, but this has been suggested in a study by Goletti et al. that underlined the protective role of Tem in TB (24). By analogy, we suggest that the detection of IFN-γ-producing CD4+ Tem in response to the nHBHA-IGRA is not simply a measure of the immunological response but reflects the persistence of *M. tuberculosis* antigens and therefore a real state of latency. As the 24-h nHBHA-IGRA shows a more homogenous Tem response than the 96-h nHBHA-IGRA, this novel assay appears to be more robust from an immunological point of view. Although the exact role of IL-7 in these differences was not investigated, it seems unlikely that these observations could be solely explained by the presence of this cytokine, which is known to maintain and optimize preexisting T-cell functions.
Overall, the 24-h nHBHA-IGRA is a good candidate for LTBI screening programs. The test shows a good correlation with the previously validated 96-h assay, maintaining its principal features, namely its high specificities for LTBI, BCG independency, and the detection of both recent and remote M. tuberculosis infections. No specific drawbacks were identified for the 24-h nHBHA-IGRA compared to the original assay. Moreover, this novel test shows specific drawbacks were identified for the 24-h nHBHA-IGRA detection of both recent and remote screening programs. The test shows a good correlation with the pre-

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