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Biomarkers for discrimination between latent tuberculosis infection and active tuberculosis disease

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Summary *Objective:* We aimed to determine whether combinations of multiplex cytokine responses could differentiate *Mycobacterium tuberculosis* (*Mtb*) infection states.
Methods: *Mtb*-specific antigen-induced and unstimulated cytokines were measured by Luminex assay in supernatants of QuantiFERON® Gold In-Tube assay (QFT) in 48 active pulmonary TB patients (TB), 15 latent TB infection subjects (LTBI), and 13 healthy controls (HCs).
Results: Among the 29 cytokines, eight *Mtb* antigen-specific biomarkers (GM-CSF, IFN-γ, IL-1RA, IL-2, IL-3, IL-13, IP-10, and MIP-1β) in the *Mtb*-infected group were significantly different from those of the HCs. Five *Mtb*-specific biomarkers (EGF, GM-CSF, IL-5, IL-10, and VEGF), two unstimulated biomarkers (TNF-$\alpha_{[Nil]}$ and VEGF$_{[Nil]}$), and one *Mtb*-specific biomarker ratio (IL-2/IFN-γ) showed significant differences between active TB and LTBI. Three unstimulated biomarkers (IL-8$_{[Nil]}$, IL-13$_{[Nil]}$, and VEGF$_{[Nil]}$) and 5 *Mtb*-specific biomarkers (IFN-γ, IL-2, IL-3, IP-10, and VEGF) were significantly different between active TB and non-active TB groups. Combinations of three cytokine biomarkers resulted in the accurate prediction of 92.1

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–93.7% of *Mtb*-infected cases and 92.3–100% of HCs, respectively. Moreover, combinations of five biomarkers accurately predicted 90.9–100% of active TB cases and 80–100% of LTBI subjects, respectively. In discriminating between active TB and non-active TB regardless of QFT results, combinations of six biomarkers predicted 79.2–95.8% of active TB cases and 67.9–89.3% of non-active TB subjects.
Conclusions: Taken together, our data suggest that combinations of whole blood *Mtb* antigen-dependent cytokines could serve as biomarkers to determine TB disease states. Especially, VEGF is highlighted as a key biomarker for reflecting active TB, irrespective of stimulation.
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Introduction

Tuberculosis (TB) is a leading global public health problem with high morbidity and mortality in humans.[1] Although most individuals infected with *Mycobacterium tuberculosis* (*Mtb*) remain healthy, a state referred to as latent TB infection (LTBI), approximately 10% of these individuals will eventually develop active TB.[2] The development of a rapid diagnostic test that can distinguish between active TB and LTBI or active TB and non-active TB, as well as detect *Mtb* infection, is very important for TB control. There is no gold standard for diagnosis of *Mtb* infection, and, until recently, the tuberculin skin test (TST) was the only tool available for detecting LTBI. Interferon (IFN)-gamma release assays (IGRAs), which depend on the detection of a single cytokine, IFN-γ, in response to *Mtb*-specific antigens, have been introduced as an alternative to TST for the diagnosis of *Mtb* infection. However, both TST and IGRA are intrinsically unable to discriminate between active TB and LTBI.[3] In addition, even with active TB patients in a high-burden setting, these assays show low sensitivity because of advanced disease, malnutrition, and immunosuppression in patients, as well as low specificity due to a high background prevalence of LTBI.[4]

To address these limitations, new techniques, including transcript microarrays, flow cytometry of intracellular cytokines, and multiplex microbead-based immunoassay (Luminex assay) of cytokines, have recently been introduced.[5–9] In these studies, the simultaneous detection of multiple analytes rather than a single analyte has been found to be a promising approach to discriminate between active TB and LTBI. More specifically, the combination of soluble CD40 ligand (sCD40L), epidermal growth factor (EGF), IFN-α, interleukin (IL)-1α, IL-1 receptor antagonist (IL-1RA), IL-4, IL-12 (p40), IL-15, IL-17, macrophage inflammatory protein (MIP)-1β, monocyte chemotactic protein (MCP)-1, transforming growth factor (TGF)-α, tumor necrosis factor (TNF)-α, or vascular endothelial growth factor (VEGF) responses after stimulation with *Mtb*-specific antigens, as measured by Luminex assay, has been reported for the rapid differential diagnosis of active TB versus LTBI. However, these studies have selected different cytokines to detect active TB, and far less is known about the reliability of selected cytokines for the diagnosis of active TB. Therefore, the clinical relevance of cytokine responses remains to be clarified.

The aims of the present study were to assess 29 background-corrected *Mtb*-specific antigen-stimulated cytokine/chemokine biomarker levels in supernatants from the whole blood IGRA obtained from 4 major diagnostic groups, including *Mtb*-uninfected healthy controls (HCs), LTBI, IGRA-negative active TB, and IGRA-positive active TB, to examine whether these biomarkers can increase the sensitivity of detecting *Mtb*-infected individuals, especially patients with IGRA-negative active TB, and to determine whether such biomarkers can discriminate between active TB and LTBI or between active TB and non-active TB.

Materials and methods

Subjects

A total of 48 culture-confirmed active TB patients, 15 LTBI subjects, and 13 HCs were recruited at Chonnam National University Hospital, Gwangju, South Korea. All enrolled subjects were negative for anti-human immunodeficiency (HIV) antibody. All active TB were pulmonary TB and was diagnosed based on the guidelines of the American Thoracic Society and the U.S. Centers for Disease Control and Prevention.[10] Active TB patients were divided into two experimental groups according to IGRA results: Active TB patients with negative QuantiFERON® Gold In-Tube (QFT, Qiagen, Carnegie, VA, Australia) results and active TB patients with positive QFT results. All LTBI subjects were household contacts of active TB patients and met the following criteria: (i) no clinical or radiographic evidence of an active TB infection, and (ii) positive QFT at the time of sample collection.[10] All HCs met the following criteria: (i) no known exposure to any individual with active TB, (ii) no symptoms of TB infection, (iii) negative QFT at the time of sample collection, and (iv) no history of an autoimmune disease, an infectious disease, a malignancy, or immunosuppressive therapy.[10] Subjects were excluded if they were known to have received >2 weeks of chemotherapy for tuberculosis or LTBI. Non-active TB defined as subjects who had no active-TB disease therefore this group included LTBI and HCs. The study protocol was approved by the Institutional Review Board of Chonnam National University Hospital. All adult volunteers provided written informed consent. A parent or legal guardian of any child included in the study signed a consent form on their behalf.

Measurement of IFN-γ by ELISA

QFT assay was performed according to the manufacturer's instructions. Briefly, 1 ml of blood was collected into 3 QFT

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