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Identification of promising plasma immune biomarkers to differentiate active pulmonary tuberculosis

Fabiana A. Zambuzi [a], Priscilla M. Cardoso-Silva [a], Milena S. Espindola [a], Luana S. Soares [a], Leonardo J. Galvão-Lima [a], Verônica S. Brauer [a], Matheus S. Gomes [b], Laurence R. Amaral [b], Matthew Schaller [c], Valdes R. Bollela [d], Fabiani G. Frantz [a,*]

[a] Faculdade de Ciencias Farmaceuticas de Ribeirao Preto, Universidade de Sao Paulo, Ribeirao Preto, SP, Brazil
[b] Laboratorio de Bioinformatica e Analises Moleculares – INGEB/FACOM, Universidade Federal de Uberlandia, Patos de Minas, MG, Brazil
[c] Department of Pathology, University of Michigan Medical School, Ann Arbor, MI, USA
[d] Faculdade de Medicina de Ribeirao Preto, Universidade de Sao Paulo, Ribeirao Preto, SP, Brazil

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ABSTRACT

Although much research has been done related to biomarker discovery for tuberculosis infection, a set of biomarkers that can discriminate between active and latent TB diseases remains elusive. In the current study we correlate clinical aspects of TB disease with changes in the immune response as determined by biomarkers detected in plasma. Our study measured 18 molecules in human plasma in 17 patients with active disease (APTB), 14 individuals with latent tuberculosis infection (LTBI) and 16 uninfected controls (CTRL). We found that active tuberculosis patients have increased plasma levels of IL-6, IP-10, TNF-α, sCD163 and sCD14. Statistical analysis of these biomarkers indicated that simultaneous measurement of sCD14 and IL-6 was able to diagnose active tuberculosis infection with 83% accuracy. We also demonstrated that TNF-α and sCD163 were correlated with tuberculosis severity. We showed that the simultaneous detection of both plasma sCD14 and IL-6 is a promising diagnostic approach to identify APTB, and further, measurement of TNF-α and sCD163 can identify the most severe cases of tuberculosis.

1. Introduction

Tuberculosis (TB) represents an important public health problem worldwide. In 2014, it was estimated that 9.6 million people developed TB and 1.5 million died from *M. tuberculosis* (Mtb) infection [1]. Accordingly to the World Health Organization, in its Global Tuberculosis report, the male to female ratio of prevalence is around 1.5–2. This prevalence is true whether for Africa countries and for those considered industrialized [1]. Factors contributing to the spread of TB include the ineffectiveness of the BCG vaccine [2,3], the absence of rapid and sensitive techniques for diagnosis [4] and a lengthy and complex treatment regimen to reduce bacterial burden in the host [5,6]. The incidence and the course of TB are related with alcohol abuse [7] tobacco, diabetes and low body mass index, that are risk factors and worsen the scenario, when are combined could triple or quadruple the risk of active TB development [8].

Improved diagnosis of Mtb infection may lead to increases in effective treatment of infected individuals and decreased spread of the disease. To this end there has been a focus on the discovery of biomarkers that can distinguish between latent and active tuberculosis, with the goal of a rapid and simple diagnosis of active infection [9]. A biomarker is a molecule that can be objectively measured and evaluated as a disease indicator and thus used as a diagnostic tool. Ideally, TB biomarkers would be used to determine the stages of TB infection and also as tools for monitoring the success of clinical intervention [10]. Although a single biomarker has not proved sufficient for the identification of TB infection, it is possible that a combination of independent biomarkers (disease biosignature [11]) will provide information needed to accurately diagnose this disease.

Biomarkers and biosignatures are described in many infectious diseases. Sepsis is an example of this, where measuring levels of polymorphonuclear (PMN) CD64, procalcitonin (PCT) and soluble

* Corresponding author at: Faculdade de Ciencias Farmaceuticas de Ribeirao Preto - FCFRP, Universidade de Sao Paulo, ZIP Code: 14041-903, Brazil.
E-mail addresses: fabianazambuzi@usp.br (F.A. Zambuzi), priscilla.mariane@hotmail.com (P.M. Cardoso-Silva), msespindola@usp.br (M.S. Espindola), luanasi-soares@usp.br (L.S. Soares), galvaolima@usp.br (L.J. Galvão-Lima), veronica.brauer@hotmail.com (V.S. Brauer), matheus@ingeb.ufu.br (M.S. Gomes), laurence@ufu.br (L.R. Amaral), mschalle@umich.edu (M. Schaller), bollela@gmail.com (V.R. Bollela), frantz@usp.br (F.G. Frantz).

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triggering receptor expressed on myeloid cells-1 (TREM-1) in serum currently function as a diagnostic tool in critically ill sepsis patients [12]. For TB diagnosis, the accepted approach for biomarker discovery has been the use of gene expression profiles, as demonstrated by Berry et al. [13], in which peripheral blood leukocytes from TB patients were found to have elevated expression of upstream and downstream elements of the type I and II interferon signaling pathway. Another study has demonstrated that expression of CD64, LTF, and Rab33A constitute a biosignature, which allows discrimination between TB patients and healthy donors [14]. Furthermore, serum levels of IL-6 may be a marker to determine the effectiveness of antibiotic treatment in patients with active TB infection [15]. Other investigators have identified immune mediators [reviewed by [16]], metabolic markers [17] and a proteomics fingerprint [18] that form possible biosignatures indicative of TB. If widely used these biosignatures may aid in diagnosis and monitoring of treatment effectiveness in pulmonary TB patients.

In the current study we correlated clinical aspects of specific stages of TB infection with changes in the immune response, as determined by measurement of cytokines detected in plasma. We demonstrated that the plasma levels of cytokines in patients with active pulmonary TB were elevated when compared to latently infected individuals or uninfected controls. Specifically we found increased levels of TNF-α, IL-6, IP-10 and monocyte activation markers, sCD163 and sCD14. Of these, we found that IL-6 and sCD14 were sufficient to discriminate active TB disease from both latently infected and uninfected individuals. Analyzed together, levels of these molecules may discriminate between latent and active infection and thus provide an effective TB biosignature. Additionally we demonstrate that sCD163 and TNF-α may be used to indicate the severity of Mtb infection.

2. Materials and methods

2.1. Ethical aspects

This research has approval of the Ethics Committee at the Hospital das Clínicas de Ribeirão Preto and FMRP-USP (Protocol #6481/2013). All patients and controls provided a written informed consent form for participation in the study.

2.2. Study group

Active TB patients with treatment-time less than one month (n = 17), latent infected individuals (n = 14) and non-infected controls (n = 16) from both genders, aged between 18 and 65 years and without HIV coinfection, were recruited from Hospital das Clínicas de Ribeirão Preto e Centro de Saúde Escola, FMRP-USP. Active disease was diagnosed through clinical or radiological signs and confirmed by microbiological results. Healthy individuals were classified based on the Tuberculin Skin Test (TST) status into latent (TST reactor) or non-infected (TST-) groups [19]. Active TB patients were also classified regarding the severity of disease according to pulmonary radiographic images using a double blind test and classified as minimal, moderate and advanced disease, as described by Abakay et al. [20]. Thus, the lesions were considered minimal (stage 1), when there was no evidence of cavitation, the tissue density was classified as mild or moderate, and location was above the second chondrosternal junction. Moreover, these stage 1 lesions involved only a single segment of one or both lungs and the extent of combined lesions did not exceed the volume of a single lung. In moderate disease (stage 2), the lesions were densely confluent, but the area occupied by these lesions could not occupy more than a third of the volume of a lung. In addition, the overall diameter of cavitation did not exceed 4 cm in patients classified at this stage. Patients with advanced disease (stage 3), had lesions that were of greater extent than defined in moderate disease. Table 1 presents the demographic and clinical characteristics of active pulmonary tuberculosis patients (APTB), latent infected subjects (LTBI) and non-infected controls (CTRL).

2.3. Immune biomarkers quantification

Blood was collected using Heparin blood collection tubes (BD Biosciences, San Diego, CA), and plasma separation was performed to quantify cytokine, chemokine and monocyte/macrophage biomarkers. To evaluate IL-1β, IL-4, IL-5, IL-6, IL-10, IL-12p70, IFN-α2, TNF-α, IFN-γ, IP-10, RANTES, MCP-1, GM-CSF, IL-17, MIP-1α and MIP-1β, we used a customized multiplex kit (16-plex, EMD Millipore Corporation, Billerica, Massachusetts, USA) and followed the manufacturer's instructions. Following incubation with fluorescent magnetic microspheres coated with capture antibodies specific for the above listed cytokines, samples were evaluated in a fluorescent bead-based instrument (Luminex® MAGPIX® System; Luminex Corporation, Austin, Texas, USA). Luminex bead-based data were analyzed using Milliplex Analyst software v3.5 (Millipore; VigeneTech Inc., Boston, Massachusetts, USA) and a three-parameter logistic curve fit. The concentration of soluble monocytes/macrophages plasma biomarkers CD14 and CD163 were measured using a DuoSet ELISA kit (R&D Systems, Minneapolis, Minn), according to the manufacturer's instructions.

2.4. Statistical analysis

Conventional statistical analyses were performed using GraphPad Prism 5.0 software (Graph-Pad Software, San Diego, CA, USA). Differences between groups were first evaluated to test their normality. Considering the nonparametric nature of all data sets, statistical analyses between the groups were performed by the Mann-Whitney test. The correlation between immune biomarkers and stages of pulmonary lesions visualized by radiography were calculated using Spearman rank correlation. Values of $P < 0.05$ were considered significant.

Table 1
Baseline characteristics of the cohort.

Demographic and clinical characteristics	CTRL	LTBI	APTB
Age (yr)	27±	31,4±	39,6±
Men:Women	3:13	3:11	14:3
Smoking (%)	0	0	29,4
Alcoholism (%)	0	0	23,5
Drug addiction (%)	0	0	23,5
Sign and symptoms			
Productive or unproductive cough (n)	–	–	15
Weight loss (n)	–	–	14
Fever (n)	–	–	11
Smear (Number of individual)			
+	NA	NA	9
++	NA	NA	2
+++	NA	NA	3
Negative	NA	NA	3
Status of chest radiograph (Number of individual)			
Minimal	NA	NA	8
Moderate	NA	NA	5
Advanced	NA	NA	4

General characteristics of the groups involved in the study showing the number of subjects per group (n), the mean age (yr: years old), gender, addictions, sign/symptoms; n: number; and status of chest radiograph. CTRL: Non-infected Control; LTBI: Latent Tuberculosis Infection; APTB: Active Pulmonary Tuberculosis, NA: not applied.