Tuberculosis 107 (2017) 119–125

Contents lists available at ScienceDirect

Tuberculosis

journal homepage: http://intl.elsevierhealth.com/journals/tube

Identification of mycobacterial bacterioferritin B for immune screening of tuberculosis and latent tuberculosis infection

Xinyu Yang [a, 1], Jia-bao Wu [a, c, 1], Ying Liu [a], Yanqing Xiong [b], Ping Ji [a], Shu-jun Wang [a], Yingying Chen [a], Guo-ping Zhao [c, d], Shui-hua Lu [b, **], Ying Wang [a, d, *]

[a] Shanghai Jiao Tong University School of Medicine, Shanghai Institute of Immunology, Shanghai, 200025, China
[b] Shanghai Public Health Clinical Center, Key Laboratory of Medical Molecular Virology of MOE/MOH, Fudan University, 2901 Caolang Rd., Shanghai, 201508, China
[c] Department of Microbiology, School of Life Sciences, Fudan University, Shanghai, 200438, China
[d] Shanghai-MOST Key Laboratory of Health and Disease Genomics, Chinese National Human Genome Center at Shanghai, Shanghai, 201200, China

ARTICLE INFO

Article history:
Received 5 January 2017
Received in revised form
14 August 2017
Accepted 20 August 2017

Keywords:
Tuberculosis
Latent tuberculosis infection
BfrB
Cellular response
Immunodiagnosis

ABSTRACT

Objectives: It remains necessary and urgent to search for novel mycobacterial antigens to increase the sensitivity and specificity for tuberculosis (TB) diagnosis and latent TB infection (LTBI) screening. Antigens capable of inducing strong immune responses during *Mycobacterium tuberculosis (M.tb)* infection would be good candidates.

Methods: Cellular responses specific to *M.tb* derived bacterioferritin B (BfrB) were assessed by IFN-γ ELISPOT in three human cohorts, including healthy controls (HCs), LTBI population and pulmonary TB (PTB) patients. Its significance in TB diagnosis and LTBI identification was further analyzed.

Results: BfrB-specific IFN-γ responses in PTB and LTBI groups were significantly higher than that in HCs. However, BfrB-specific IFN-γ release was not as strong as that to ESAT-6 or CFP-10 in PTB patients whereas comparable in LTBI cohort with possible complementary properties to ESAT-6 or CFP-10. More interestingly, there were a considerable number of HCs with high BfrB-specific cellular responses. When HCs with high BfrB-specific cellular responses were subgrouped into ESAT-6/CFP-10^{hi} (SFUs = 3, 4, 5) and ESAT-6/CFP-10^{lo} (SFUs < 3) groups, those who belonged to ESAT-6/CFP-10^{hi} group exhibited higher PPD responsiveness than ESAT-6/CFP-10^{lo} group.

Conclusions: PTB and LTBI groups exhibit higher BfrB-specific IFN-γ responses than HCs. Although BfrB is not as immunodominant as ESAT-6/CFP-10 during acute *M.tb* infection, comparable BfrB-specific cellular immune responses are observed in LTBI population with the potential to increase the sensitivity for LTBI screening. Moreover, strong BfrB-specific IFN-γ release in the healthy cohort is probably cautionary in identifying leaky LTBI from HCs. BfrB might thus be considered as an additional biomarker antigen for LTBI identification.

1. Introduction

Tuberculosis (TB) remains one of the most contagious diseases worldwide. Based on World Health Organization (WHO) 2016 annual report, an estimated 10.4 million people developed TB and 1.5 million died from the disease [1]. TB begins with asymptomatic infection by a low number of *Mycobacterium tuberculosis* (*M.tb*). Most of the pathogens can be eliminated by host immune system. Some become dormant in alveolar macrophages leading to a status called latent TB infection (LTBI). With the alteration of metabolic and replication activity of *M.tb* under certain circumstances, LTBI transforms to active TB with clinical symptoms and spreading capacity [2]. Approximately one third of world population are infected with *M.tb*, 5%–15% of which suffer from the reactivation of *M.tb* during their lifetime [1]. Therefore, TB diagnosis and LTBI screening directly influence the global control of TB.

At present identification of *M.tb* infection, including active TB and LTBI, is not satisfactory enough due to the lack of reliable

* Corresponding author. Shanghai Jiao Tong University School of Medicine, Shanghai Institute of Immunology, Shanghai, 200025, China.
** Corresponding author.
E-mail addresses: lushuihua66@126.com (S.-h. Lu), ywang@sibs.ac.cn (Y. Wang).
[1] Authors contributed equally.

http://dx.doi.org/10.1016/j.tube.2017.08.005
1472-9792/

methods with rapidity and accuracy. Three conventional methods for TB diagnosis are available in clinic, including acid-fast bacilli (AFB) smear, *M.tb* sputum culture and X-ray chest radiograph [3]. As a standard method for TB diagnosis, AFB smear with Ziehl-Neelsen (ZN) staining is positive only when there exist more than 5000 bacilli per mL in the sputum [3]. Bacilli culture positivity is a golden criterion for TB diagnosis. But it takes nearly one month with low success even in smear-positive sputum samples. Immunodiagnosis becomes valuable based on *M.tb*-specific immune responses. Tuberculin purified protein derivative (PPD) based tissue skin test (TST) assay has been used for screening *M.tb* infection with low cost and quickness for a long time. However, the cross-reaction between PPD derived from mycobacterial bacilli and the Bacillus Calmette–Guérin (BCG) vaccine leads to low specificity of TST assay in TB diagnosis, which in turn limits its application as well [4]. Recently, immune effector molecules or cells induced by *M.tb* infection are considered to be reliable biomarkers for TB diagnosis and LTBI screening [5–8]. Interferon-γ release triggered by mycobacterial antigens, including 6 kDa early secretary antigenic target (ESAT-6), 10 kDa culture filtrate protein (CFP-10) and TB10.4, are adapted in commercialized diagnostic kits, such as QFT-GIT and T-SPOT.TB, to detect TB and LTBI [9]. However, a recent meta-analysis including nineteen studies claimed pooled sensitivity of 75%–90% as well as specificity of 71%–77% [10], which is not competent enough for diagnosis. In view of low sensitivity of the available methods, it is suggested to add extra antigens to increase the sensitivity and specificity for distinguishing cases of *M.tb* infection [11].

Bacterioferritin B, known as Rv3841, is a ferritin-like protein identified by whole genome sequencing of *M.tb* H37Rv strain [12]. It functions to uptake iron and regulate the release of the stored iron [13]. A recent study indicates that after knockdown of *lsr-2*, which encodes a global transcriptional regulator of *M.tb* for the adaptation to hypoxia environment [14], there is 5.96 times upregulation of r*v3841* expression compared to wildtype *M.tb* (data unpublished). However, the antigenicity of BfrB after *M.tb* infection is not well studied. Here we assessed BfrB specific cellular responses in human cohorts, including healthy controls (HCs), LTBI and pulmonary TB (PTB) patients to evaluate its potential as a novel biomarker antigen in TB-related diagnosis.

2. Materials and methods

2.1. Study subjects

All PTB patients and healthy volunteers were adults who vaccinated with the BCG Shanghai strain (Shanghai Institute of Biological Products Co. Ltd., Shanghai, China) during childhood. PTB patients (n = 29) were in-patient patients from Shanghai Public Health Clinical Center, including 17 new onset patients and 12 patients undergoing standard treatment, 10 females and 19 males with the age median of 51 (21–83) (Table 1). PTB diagnosis was based on medical history, chest radiograph (X-ray and CT), acid-fast bacilli (AFB) smear and *M.tb* sputum culture. A standard anti-TB therapy, including isoniazid (INH), rifampicin (RFP), ethambutol (EMB), and pyrazinamide (PZA) was performed on 12 PTB patients, among which 6 patients were treated for 1–6 months and the other 6 for 6–24 months. All the patients have signed voluntary informed consents. The healthy volunteers (n = 266) have been defined as no medical history, a normal physical examination (including blood test, serum chemistry, chest X-rays) with no disease symptoms. LTBI was defined as positive in T-SPOT.TB assay among HC groups accordingly [11]. This study was approved by the Ethical Committee of Shanghai Jiao Tong University School of Medicine.

Table 1
Population information.

	PTB	LTBI	HC
N	29	59	207
Gender (Female/Male)	10/19	26/33	105/102
Median age, years (range)	51 (21–83)	46 (18–76)	38 (18–73)

N: sample size.

2.2. Preparation of M.tb antigens

The recombinant plasmids were obtained from NIH Biodefense and Emerging Infection Research Resources Repository (NIAID, NIH, USA), including pMRLB.5 containing *rv3841* (Protein BfrB) (NR-13278), pMRLB.7 containing *rv3875* (Protein ESAT-6) (NR-36431) and pMRLB.46 containing *rv3874* (Protein CFP-10) (NR-13297) from *M.tb*. Target genes were subcloned into pET28a expressing vectors by PCR. The recombinant plasmids were transformed to *E.coli* BL21 (DE3) strain and the proteins were induced for 4 h with 0.5 mM Isopropyl-β-D-thiogalactoside (IPTG) (Beyotime, Jiangsu, China) at 30 °C. Bacteria were lysed and proteins were purified by affinity chromatography with Ni-NTA His-Bind Resin (Qiagen, NRW, Germany) accordingly. Endotoxins were removed from purified antigens using Triton X-114 two-phase separation as previously described [15]. Briefly, Triton X-114 was added to the proteins to a final concentration of 1% and incubated for 30 min at 4 °C with constant agitation, followed by 10 min incubation at 37 °C and 16,000×*g* centrifugation at 25 °C for 10 min. Six cycles of Triton X-114 phase separation were performed for sufficient endotoxin depletion. Triton X-114 was removed by dialysis against phosphate buffer saline (PBS). The remaining endotoxin in proteins was detected by Tachyleus Amebocyte Lysate Kits (Gulangyu, Xiamen, China) according to the manufacturer's protocol. Protein concentration was detected by BCA Protein Assay Kit (Pierce, Waltham, MA, USA).

2.3. Peptide synthesis

A total of 19 peptides were synthesized by Sangon Biotech (Shanghai, China). These peptides consisted of 20 amino acid residues in length and covered the entire sequence of ESAT-6 and CFP-10 with 10 amino acids overlapping.

2.4. Peripheral blood mononuclear cell isolation

10 mL peripheral blood was collected in tubes containing EDTA. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-hypaque density gradient centrifugation with Lymphoprep™ solution (AXIS-SHIELD Poc AS, Oslo, Norway) at 860×*g* for 20 min at room temperature (RT). The supernatant (plasma) was collected and stored at −80 °C. The mononuclear cell layer was carefully transferred to a new 15 mL conical tube and washed twice with RPMI 1640 (GIBCO, Grand Island, USA) by centrifuging at 480×*g* for 10 min at RT. PBMCs were resuspended at a concentration of 2.5×10^6/mL in RPMI 1640 culture medium containing 10% fetal bovine serum (FBS) (Merck Millipore, Darmstadt, Germany), 100 units/mL penicillin (GIBCO) and 100 μg/mL streptomycin (GIBCO).

2.5. Interferon-gamma (IFN-γ) ELISPOT assay

Antigen-specific IFN-γ response was detected by an enzyme-linked immunospot (ELISPOT) assay according to the manufacturer's instructions (U-CyTech, Utrecht, Netherlands). Briefly, 96-well Polyvinylidene fluoride (PVDF) plates (Millipore) were coated with 50 μl anti-human IFN-γ coating antibody overnight at 4 °C. The wells were blocked with 200 μl blocking buffer for 1 h at