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DIAGNOSTICS

Use of lateral flow assays to determine IP-10 and CCL4 levels in pleural effusions and whole blood for TB diagnosis

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SUMMARY

One of the key problems in combating TB is the lack of fast and accurate diagnostic tests that are affordable and easy to use in resource-limited settings. We have used a field-friendly up-converting phosphor (UCP) reporter technology in a lateral flow (LF) based test for the diagnosis of respiratory infections. In this study we analysed samples obtained from patients presenting with symptoms suggestive of TB but prior to confirmation by microbiology in The Gambia. Following clinical and microbiological evaluation they were classified as either having TB or other respiratory disorder (ORD). Analysis of blood was performed for those with pulmonary TB and pleural fluid for those with pleural TB. UCP-LF test for detection and quantitation of IP-10 and CCL4 were used being the two chemokine markers that have been shown to increase in active TB disease. UCP-LF test accurately determined concentrations of both markers as compared to ELISA and multiplex cytokine array. However, only IP-10 could discriminate between TB and ORD, and this was significantly enhanced by analysing the site of infection (pleural fluid), which showed 92% correct classification. Future work will assess the use of multiple markers to increase diagnostic accuracy.

1. Introduction

Tuberculosis (TB) is caused by infection with *Mycobacterium tuberculosis* (Mtb), which is transmitted via aerosolized droplets from index cases with active TB disease [1]. In 2013, 8.6 million people were diagnosed with TB and 1.5 million people died [2]. One of the major challenges in combating active TB is the lack of fast and accurate diagnostic tests. Current tests such as Acid Fast Bacilli (AFB) smear microscopy, Mtb culture and molecular based diagnostic tests (i.e. GeneXpert MTB-RIF) all have limitations, most notably the requirement for infrastructure, thus limiting roll-out to peripheral clinics, where the majority of patients are seen [3]. In addition, analysis of the pathogen in TB is challenging in paucibacillary patients including children [4]. Current blood-based tests utilising host-derived production of interferon gamma (IFN-γ) following stimulation with Mtb antigens (IFN-γ release assays (IGRA)) also require infrastructure and cannot differentiate active and latent forms of TB [5]. However, recent work has shown increased diagnostic accuracy when a combination of analytes other than IFN-γ [6,7] or samples from the site of infection [8,9] were analysed. Progression to the development of user-friendly rapid tests for TB, based on host-derived markers would be of major global public health importance.

Diagnostics employing the rapid lateral flow (LF) assay format require little infrastructure and are routinely used for home testing of pregnancy, monitoring of diabetes and screening for infectious diseases such as HIV and malaria [10–12]. Previous studies have described the application of sensitive fluorescent up-converting phosphor (UCP) reporter technology combined with the LF assay

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format to diagnose bacterial and viral infections [13]. User-friendly dry reagent formats for storage at ambient temperature combined with lightweight portable strip readers have enabled implementation as a field friendly device in different sites in Africa [14–16]. Similar types of UCP-LF assays have also been developed for the quantitative analysis of blood samples to determine levels of IFN-γ [17], IL-10 [18] and chemokine IP-10 [16,17] and CCL4 [Corstjens et al., in press].

While there are many analytes of interest in regards to TB diagnostics, two candidates that hold great promise are IP-10 and CCL4 (MIP-1β). IP-10 is a chemokine produced by monocytes and other immune cells. It is highly elevated in both pleural effusions and whole blood supernatants of TB patients and has been suggested as a potential biomarker for treatment monitoring and disease progression [19,20]. Moreover, we have shown that levels in pleural effusions are not affected by the HIV status of the patient [8,21]. CCL4 is a chemoattractant for natural killer cells, monocytes and other immune cells to the site of infection. It induces the synthesis and release of other pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α from fibroblasts and macrophages [22,23]. In addition a human CD8+ regulatory T cell subset has been identified that mediates suppression of Th1 cell immunity to mycobacteria through CCL4 [24]. CCL4 has previously been shown to discriminate between TB and ORD in saliva and serum [25] as well as between tuberculoid and borderline tuberculoid (TT/BT) leprosy and exposed healthy individuals [26].

The study described here is the first to apply UCP-LF assays using pleural fluid as a potential tool for the diagnosis of pleural TB. We analysed IP-10 and CCL4 levels in pleural effusions or Mtb antigen-stimulated whole blood supernatants from subjects with pleural or pulmonary TB respectively, and compared these to subjects with other respiratory disorders (ORDs). Our findings hold promise for future development of an IP-10 based rapid diagnostic test for TB in resource-poor settings.

2. Materials and methods

2.1. Study participants and samples

This study was approved by the Medical Research Council (MRC) and Gambia government joint ethics committee. Adult human immune-deficiency virus (HIV) uninfected participants were recruited following written informed consent. Participants presented at the MRC outpatients department with symptoms suggestive of TB (i.e. cough > 2 weeks plus one other system such as weight loss, night sweats) but prior to microbiological confirmation. Patients were classified as having TB or ORD based on bacteriological, clinical observations and chest X-ray (CXR) as described previously [9]. Patients with evidence of pleural effusion by CXR had pleural fluid drawn and biochemical evaluation performed as described previously [8]. Pleural TB was diagnosed based on clinical and/or microbiological confirmation. Venous blood was collected in heparinised tubes for whole blood antigen stimulations and pleural fluid in a 50 ml falcon tube for pleural effusion analysis. Aliquots of pleural fluid were frozen at −20 °C after centrifugation.

2.2. Whole blood antigenic stimulations

Within 2 h of sample collection, 480 μl of undiluted blood was added to each well of a 48-well plate and incubated at 37 °C, 5% CO_2 with ESAT-6/CFP-10 fusion protein, Rv0081, Rv2034 (10 μg/ml final, LUMC), PPD (10 μg/ml final; SSI, Denmark), and PHA (5 μg/ml final; Sigma, USA). After 24 h incubation, approximately 200 μl of supernatants were collected from each well, and stored at −20 °C prior to analysis.

2.3. UCP-LF materials and UCP-LF test protocol for IP-10 and CCL4

The IP-10 and CCL4 UCP-LF assay materials were shipped from the Netherlands to Gambia as dry reagents (100 ng) in 0.65 ml tubes stored in aluminium foil pouches with silica dry pack [15]. The shipment included IP-10 and CCL4 LF strips and lyophilized High Salt Lateral Flow (HSLF; 100 mM Hepes pH 7.4, 270 mM NaCl, 1% w/v BSA, 0.5% w/v Tween-20) assay buffer. The IP-10 and CCL4 lyophilised standard controls (948 pg) were reconstituted in 150 μl HSLF and serial diluted to final concentrations of 316, 100, 32, 10, 3.2, 1, 0.32, 0.1, 0.01 and 0 pg per 50 μl. Pleural samples or whole blood assay supernatants were diluted 50-fold in HSLF buffer. The UCP reporter conjugates were rehydrated with 50 μl of HSLF buffer for 5 min. They were then incubated with 50 μl of the chemokine standard series or 50 μl of the 50-fold diluted pleural fluid and incubated on a thermoshaker at 37 °C for 1 h at 900 rpm. At the end of the incubation period immunochromatography was initiated by inserting the LF strips into UCP reporter tubes and left at room temperature (RT) until they were completely dry. Strips were analysed using the lightweight portable UCP-Quant reader ([27], Qiagen Lake Constance GmbH, Stockach, Germany). UCP-LF strips used to generate the standard curves were analysed with the UCP-Quant reader and the area under the curves for both the 'test' (T) and 'flow control' (FC), was measured. The ratio (R) of both curves, T/FC, was calculated to generate a standard curve of the R value versus the IP-10 or CCL4 concentration, which was used to translate R obtained with the pleural fluid samples to concentration. Standard series were incorporated in each run in order to control for day to day variability.

2.4. IP-10 and CCL4 ELISA

ELISA kits for IP-10 (Diaclone, France) and CCL4 (R&D, USA) were obtained from Gen-Probe (Diaclone, France). 96-well plates were coated with a 100 μl/well of coating antibody and incubated overnight at 4 °C. The next day, plates were washed with PBS/0.05% Tween 20 (PBS-T), 250 μl/well of blocking solution (PBS/BSA) added and incubated for 2 h at RT. Another wash with PBS-T was performed and the plate left to dry for 1 h at RT. Standards and samples were diluted in 1% (w/v) BSA in PBS, 50 μl/well added in duplicate and incubated for 2 h at RT. After washing, 50 μl/well of biotinylated antibody was added and incubated for 1 h at RT, followed by addition of HRP-streptavidin (100 μl/well) for 20 min at RT. A final wash was performed and substrate added. Plates were incubated for 15 min in the dark until a blue colour developed. Stop solution (H_2SO_4) was then added (100 μl/well) and plates read at 450 nm using Softmax Pro software v6.2.2 (Molecular Devices, USA).

2.5. Multiplex cytokine- and chemokine analysis

The analysis of the concentrations of IP-10 and CCL4 was included as part of our previous multiplex cytokine analysis (MCA) of pleural fluid and whole blood assay (WBA) supernatants [8,9]. Both analytes were measured using the Bio-Plex suspension array system (Bio-Rad Laboratories, Belgium) as part of a pre-mixed 27-plex kit. After pre-wetting the filter plates with assay-solution, beads were added and the plate washed twice. Samples, controls and standards were added and the plate incubated for 1 h at RT. After washing, detection antibody was added and incubated at RT for 30 min. After washing, 1× strepavidin-PE was added and incubated for 10 min at RT. After a further wash step, assay buffer was added to each well and analysed using Bio-Plex Manager™ software 6.0 (Bio-Rad laboratories). Sample concentrations were interpolated from standard curves.

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