Sutherland, Jayne S; Garba, Danlani; Fombah, Augustin E; Mendy-Gomez, Awa; Mendy, Francis S; Antonio, Martin; Townend, John; Ideh, Readon C; Corrah, Tumani; Ota, Martin OC (2012) Highly Accurate Diagnosis of Pleural Tuberculosis by Immunological Analysis of the Pleural Effusion. PLOS ONE, 7 (1). ISSN 1932-6203 DOI: https://doi.org/10.1371/journal.pone.0030324

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DOI: 10.1371/journal.pone.0030324

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Highly Accurate Diagnosis of Pleural Tuberculosis by Immunological Analysis of the Pleural Effusion

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Abstract

Pleural TB is notoriously difficult to diagnose due to its paucibacillary nature yet it is the most common cause of pleural effusions in TB endemic countries such as The Gambia. We identified both cellular and soluble biomarkers in the pleural fluid that allowed highly accurate diagnosis of pleural TB compared to peripheral blood markers. Multi-plex cytokine analysis on unstimulated pleural fluid showed that IP-10 resulted in a positive likelihood ratio (LR) of 9.6 versus 2.8 for IFN-γ; a combination of IP-10, IL-6 and IL-10 resulted in an AUC of 0.96 and positive LR of 10. A striking finding was the significantly higher proportion of PPD-specific IFN-γ+TNF-α+ cell population (PPD-IGTA) in the pleural fluid compared to peripheral blood of TB subjects. Presence of this pleural PPD-IGTA population resulted in 95% correct classification of pleural TB disease with a sensitivity of 95% and specificity of 100%. These data suggest that analysis of the site of infection provides superior diagnostic accuracy compared to peripheral blood for pleural TB, likely due to the sequestration of effector cells at this acute stage of disease.

Introduction

Tuberculosis (TB) still remains one of the top three deadly diseases in developing countries with 1.7 million deaths recorded in 2009 [1]. While effective treatment is available, this relies on early and accurate diagnosis. To date, sputum smear (which is unreliable, particularly in HIV-positive and extrapulmonary TB) and sputum culture (which is time-consuming and expensive) remain the ‘gold-standard’ techniques for diagnosis of TB. Development of point-of-care diagnostics will have vast public health benefits by decreasing morbidity and mortality and reducing transmission rates of TB but this requires determination of biomarkers with high specificity and sensitivity for active TB disease.

Tuberculosis is the most common cause of pleural effusion in developing countries but it is notoriously difficult to diagnose due to its paucibacillary nature: positive cultures are seen in less than 25% of HIV-negative cases but can rise to 75% with HIV-infection [2]. Host immune factors provide greater diagnostic accuracy, including levels of IFN-γ and Adenosine Deaminase (ADA) [3], both of which have >95% specificity and sensitivity, appear to be unaffected by HIV co-infection and do not require any sample preparation [3–7]. However these markers are less definitive in TB-endemic countries and have not been evaluated in a West African cohort.

Tuberculosis infection occurs by inhalation of Mycobacterium tuberculosis (MTb) bacilli. These reside within lung macrophages and can be contained (but rarely eliminated) at early stages of infection: thus close to 2 billion people world-wide are infected with MTb and 10% of these will progress to active disease in their lifetime [1]. Progression to active disease occurs due to changes in bacterial virulence and/or the host immune system: the prime example shown with con-current HIV infection, which is the most potent known risk factor for progression to active tuberculosis [8]. The fact that the localised immune response is occurring in the lung suggests that analysis of the peripheral blood may not be representative of the host immune response, particularly at the acute stage of disease. Indeed, there is extensive literature showing sequestration of immune cells to the lung during TB [9–13], although their role appears to depend on the type and stage of disease [13].

In this study we analysed paired blood and pleural fluid samples from patients presenting with pleural effusion who were subsequently classified as having TB or not (malignancy, pneumonia, liver disease). The cellular and soluble profiles of the pleural fluid easily discriminated between subjects with TB and those without and should be further validated as tools for new and improved TB diagnostics.

Methods

Ethics statement

This study was conducted according to the principles expressed in the Declaration of Helsinki. Ethical approval was obtained from the Gambia Government/Medical Research Council (MRC) Joint Ethics Committee. All patients provided written informed consent for the collection of samples and subsequent analysis.

Subjects

Subjects were included in this study if they were above 15 years of age and were seen at the TB clinic, general outpatient’s clinic or admitted to the ward at MRC, Fajara with evidence of pleural...
effusion demonstrated by x-ray. Following informed consent, 50 mL of pleural fluid (PF) and 5 mL of peripheral blood (PB) was taken for immunological analysis. Pleural fluid and sputum (where possible) were sent to the microbiology lab for routine smear and Bactec culture. A sample of pleural fluid was also sent to the biochemistry lab for assessment of glucose and protein levels. HIV testing was performed on all consenting patients. We also performed Mantoux skin test reactivity (2 IU PPD, SSI, Denmark). 200 µL of PB was used to obtain differential cell counts using a Medonic haematology analyser (Merck).

Classification of subjects presenting with pleural effusion

Subjects were classified as having TB if they had an exudative effusion (for this study, exudative effusion was classified as pleural protein >30 g/L and glucose <4 mM) and were bacteriologically culture positive (definite TB) or had a good response to TB treatment and had ADA levels >35 IU/mL and a lymphocytic infiltrate (>75% of PF cells) (probable TB; Table 1). Out of 41 subjects with evaluable data, 7 were confirmed TB by bacterial culture; 23 were classified as having probable TB and 11 were classified as ‘not TB’. Of these, 3 had liver disease (cirrhosis), 5 had pneumonia (Streptococcus pneumoniae), 2 had a malignancy and 1 was unclassified. Two subjects (19 and 21) had extremely high ADA levels; previously been shown to be indicative of empyema [7]. Subject 19 was however culture positive and thus classified as having TB disease while subject 21 was diagnosed radiologically and clinically with pneumonia. Subject 26 had extremely low ADA levels and had a final classification of Cirrhosis. While most of the effusions were lymphocytic, subject 12 had the lowest percentage (15%) and was diagnosed with pneumonia. This patient also had a high peripheral blood WBC count (10.8E9/L) and clinically with pneumonia. Subject 26 had extremely low ADA levels; previously been shown to be indicative of pneumonia rather than TB [14]. 9 subjects were HIV-positive of which 7 were subjects with a classification of TB (4 were culture positive = definite TB and 3 were classified according to ADA levels and response to treatment = probable TB) and 2 with a classification of pneumonia. Subject 6 was Mtb culture positive and thus classified as ‘not TB’. Of these, 3 had liver disease (cirrhosis), 5 had pneumonia (Streptococcus pneumoniae), 2 had a malignancy and 1 was unclassified. Two subjects (19 and 21) had extremely high ADA levels; previously been shown to be indicative of empyema [7].

Intracellular cytokine analysis

Overnight antigenic stimulation. One million PF cells in 200 µL or 200 µL of PB was used for each test of an intracellular cytokine assay. Cells were stimulated with ESAT-6/CFP-10 fusion protein (10 µg/mL), PPD (10 µg/mL), or anti-CD3 (10 µg/mL). The negative control tube was incubated without antigen. Co-stimulation with anti-CD28 and anti-CD49d (Becton-Dickinson, USA) was added to all tubes (final concentration 2 µg/mL each). Tubes were vortexed and incubated for 2 hours. Brefeldin A was then added (final concentration 10 µg/mL), tubes vortexed, covered and incubated overnight (16 h) at 37°C, 5% CO2.

Antibody staining

20 µL of antibody cocktail (CD45RO-PC5, CD27-FTTC, CD8-PB (all from Becton-Dickinson, USA) and CD4-APCA750 (ebioscience, UK) was added to each tube and incubated for 30 min at RT, in the dark. Red blood cells were lysed by addition of 2 mL FACS lysis buffer for 9 min followed by centrifugation at 600gmax for 5 min. Supernatant was poured off and cells resuspended in 500 µL of 1X FACS Perm 2 solution (Becton-Dickinson, USA). Tubes were vortexed and incubated for a further 20 min, at RT, in the dark. Tubes were then centrifuged at 620gmax, supernatant removed and 20 µL of cytokine cocktail (TNF-α-PC7 at 1:80 (ebioscience, UK), IFN-γ-APC at 1:40 and IL-2-PE at 1:80 (both from Becton-Dickinson, USA) added. Cells were incubated for 30 min, RT, in the dark, washed by addition of 1 mL FACS buffer and centrifuged at 620gmax for 5 min. Finally, cells were resuspended in 300 µL FACS buffer for acquisition.

Flow cytometry acquisition and analysis

Cells were acquired using a CyAn ADP™ 9-colour flow cytometer (Beckman Coulter, USA). Lymphocytes were gated according to 90° FSC and SSC and compensation performed. For ex vivo staining, 100,000 lymphocytes were acquired and 200–500,000 were acquired for intracellular cytokine staining. Analysis was performed using FlowJo software version 9.2 (Treestar, USA). Analysis and presentation of distributions was performed using SPICE version 5.1, downloaded from http://exon.niaid.nih.gov/spice [15]. Comparison of distributions was performed using Student’s T test and a partial permutation test as described [15].

Adenosine Deaminase (ADA) assay

To assess ADA levels in the pleural fluid we adapted the standard Galanti and Giusti protocol [7] according to the manufacturer’s instructions (Diazyme, USA). Briefly, the high control provided with the kit was serially diluted to form a standard curve ranging from 14.2 to 24.7 U/L. 5 µL of samples, standards, low control (31.4±2.3 U/L) and calibrator (50 IU/L) were added to 180 µL of R1 (PNP) in duplicate in a flat-
Samples were incubated at 37°C for 5 min then 90 µL of R2 (XOD) was added to each well. Samples were incubated at RT and read on an ELISA plate reader (Multiscan, Labsystems, Finland) at 550 nm after 3 min. Data were analysed using Softmax Pro software (Molecular Devices, USA). A standard curve was constructed using the diluted high standard, the calibrator concentration was determined and results were adjusted accordingly. All samples were read in duplicate and mean values (IU/L) determined. This assay is specific for ADA, has no detectable reaction with other nucleosides and is not affected by serum bilirubin up to 20 mg/dL, hemoglobin up to 200 mg/dL, triglycerides up to 750 mg/dL.

### Table 1. Subject information and classification.

| Subject | PF ADA | PF %L | Culture | Exudate | TST | HIV | Tx | Response | Classification |
|---------|--------|-------|---------|---------|-----|-----|----|----------|----------------|
| 1       | n/d    | 88    | neg     | yes     | pos | neg | pos| improved | Probable TB    |
| 2       | 55     | 87    | neg     | yes     | pos | neg | pos| improved | Probable TB    |
| 3       | 39     | 95    | neg     | yes     | n/d | neg | pos| improved | Probable TB    |
| 4       | 51     | 81    | neg     | yes     | pos | neg | pos| improved | Probable TB    |
| 5       | 110    | 45    | neg     | yes     | neg | pos | pos| not given| Pneumonia      |
| 6       | 41     | 91    | pos     | yes     | n/d | pos | pos| improved | Definite TB    |
| 7       | 47     | 86    | neg     | yes     | pos | neg | pos| improved | Probable TB    |
| 8       | 45     | 73    | neg     | yes     | pos | neg | pos| improved | Probable TB    |
| 9       | 24     | 89    | neg     | yes     | n/d | neg | pos| not given| Cirrhosis      |
| 10      | 59     | 36    | pos     | yes     | neg | pos | pos| died     | Definite TB    |
| 11      | 29     | 15    | neg     | yes     | n/d | neg | pos| died     | Pneumonia      |
| 12      | 45     | 94    | neg     | yes     | pos | neg | pos| improved | Probable TB    |
| 13      | 58     | n/d   | neg     | yes     | neg | pos | pos| improved | Probable TB    |
| 14      | 33     | 86    | pos     | yes     | pos | pos | pos| improved | Definite TB    |
| 15      | 45     | 93    | n/d     | yes     | pos | neg | pos| improved | Probable TB    |
| 16      | 29     | 94    | neg     | yes     | neg | neg | pos| died     | Probable TB    |
| 17      | 16     | 77    | n/d     | yes     | neg | neg | neg| improved | Probable TB    |
| 18      | 58     | 62    | pos     | yes     | n/d | neg | pos| improved | Definite TB    |
| 19      | 689    | 65    | pos     | yes     | pos | neg | pos| improved | Probable TB    |
| 20      | 52     | 34    | pos     | yes     | pos | neg | pos| improved | Definite TB    |
| 21      | 1463   | 40    | neg     | yes     | neg | neg | neg| not given| Pneumonia      |
| 22      | 38     | 46    | neg     | yes     | pos | neg | pos| improved | Probable TB    |
| 23      | n/d    | 39    | neg     | yes     | neg | n/d | not given| Malignancy |
| 24      | 39     | 84    | neg     | yes     | neg | pos | pos| improved | Probable TB    |
| 25      | 26     | 91    | neg     | yes     | pos | neg | pos| improved | Probable TB    |
| 26      | 0.82   | 84    | neg     | no      | neg | neg | pos| not given| Cirrhosis      |
| 27      | 45     | 83    | neg     | yes     | pos | neg | pos| improved | Probable TB    |
| 28      | 37     | 93    | neg     | yes     | pos | neg | neg| not given| Pneumonia      |
| 29      | 15     | 28    | neg     | yes     | neg | neg | neg| died     | Probable TB    |
| 30      | 47     | 85    | neg     | yes     | pos | pos | pos| not given| Pneumonia      |
| 31      | 9      | 91    | n/d     | yes     | neg | neg | not given| Malignancy |
| 32      | 57     | 71    | neg     | yes     | n/d | neg | pos| improved | Probable TB    |
| 33      | 30     | 85    | neg     | yes     | n/d | neg | neg| not given| Unknown        |
| 34      | 6      | 88    | neg     | yes     | neg | neg | pos| improved | Probable TB    |
| 35      | 71     | 82    | pos     | yes     | neg | pos | pos| improved | Definite TB    |
| 36      | 39     | n/d   | neg     | yes     | pos | neg | pos| improved | Probable TB    |
| 37      | 54     | 96    | neg     | yes     | pos | neg | pos| improved | Probable TB    |
| 38      | 59     | 34    | neg     | yes     | neg | pos | pos| Improved | Probable TB    |
| 39      | 0      | n/d   | neg     | no      | neg | n/d | not given| Cirrhosis |
| 40      | 24     | 74    | neg     | yes     | pos | neg | pos| improved | Probable TB    |
| 41      | 68     | 85    | n/d     | yes     | neg | neg | Improved| Probable TB    |

ADA = Adenosine Deaminase; n/d = not done; TST = Tuberculin skin test; PF = pleural fluid; %L = % lymphocytes; Exudate = PF protein >30 g/L and glucose >3.3 mmol/L; pos = positive; neg = negative; HIV = Human Immunodeficiency Virus; Culture = bacteriological culture of sputum or pleural fluid; Tx = treatment.

doi:10.1371/journal.pone.0030324.t001
or ascorbic acid up to 4 mg/dL. Levels greater than 35 IU/L are indicative of TB in lymphocyte-predominant pleural fluid [7].

27-plex cytokine analysis of unstimulated pleural fluid

A 27-plex cytokine-bead kit was used (Bio-Rad, USA) and the assay performed according to the manufacturer’s instructions. Cytokines, chemokines and growth factors assessed were: IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12(p70), IL-13, IL-15, IL-17, Eotaxin, FGF, G-CSF, GM-CSF, IFN-γ, IP-10, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α and VEGF. Following pre-wetting of the filter plate, 50 μl of bead suspension was added to each well and washed twice. 50 μl of pre-diluted stimulation buffer were added to each well and washed twice. 50 μl of samples and standards were then added, the plate was sealed and incubated for 30 min at 300 rpm. The plate was washed 3 times then 25 μl of pre-diluted detection antibody was added. Following shaking, the plate was incubated for 30 min at 300 rpm in the dark. After washing, 50 μl of streptavidin-PE was added to each well and incubated for 10 min. The plate was again washed and resuspended in 125 μl of assay buffer, sealed, mixed and immediately read on the Bioplex analyser using Bioplex manager software (version 4.0; Bio-Rad, USA) and a low PMT setting. All standards were run in duplicate.

Statistical analyses

Data were analysed with Wilcoxon matched pairs test, Kruskal-Wallis test with Dunn’s post-test comparison, Mann-Whitney U-test or multiple logistic regression analysis. Immunological analyses were performed on all subjects (HIGH-positive and negative) and also on HIV-negative alone and we found no statistical difference. As such, all subjects are grouped together unless otherwise indicated. Furthermore, following validation with the TB-definite subjects, all subjects with probable or definite TB were grouped together for statistical purposes.

Results

The immune cell profile of the peripheral blood is significantly different to the site of infection in pleural TB

We analysed the cellular content of the PF and compared this to the PB for each subject. Analysis of the lymphocyte and CD4+ T cell proportions in the PF indicated a likelihood of TB (Fig. 1a). Subject 11 was classified with Streptococcus Pneumoniae infection and had a granulocytic effusion (90%) whereas Subject 6, classified with TB (culture-confirmed) had a lymphocytic infiltrate. Furthermore, only 4.6% of the lymphocytes were CD4+ in Subject 11 compared to 58.7% in Subject 6 (Fig. 1a). Flow cytometry also allowed us to determine other underlying causes of the effusion, particularly malignancy and showed distinct differences between the blood and PF profiles. For example, assessment of the lymphocyte population for subject 33 showed normal blood levels of CD3+ T cells but elevated B cell levels (22% median %) and in the pleural fluid they had only 0.8% CD3+ T cells but 97% B cells (Fig. 1b). Flow cytometry of an ascites sample was also performed although this was not included in the statistical analysis. The ascites profile again was very distinct to that seen in the peripheral blood. The blood had a large Vz24+CD4+ proportion which was not present in the ascites (Fig. 1c). This patient was subsequently confirmed to have both TB and lymphoma.

Comparison between the PB and the PF for each patient was performed using a Wilcoxon matched-pairs test (Table 2). There was a significantly higher proportion of CD3+ T cells in the PF compared to the PB (median IQR = 72[43–80] for PF and 44[33–51] for PB; p = 0.0045) which was due to an increase in CD4+ T cells but not CD8+ T cells (p = 0.9001 for CD4+ T cells; Table 2), resulting in an increased ratio of CD4+:CD8+ T cells (p = 0.0001). Further classification of the CD4+ T cells based on CD27 and CD45RO expression showed a significant decrease in the proportion of naïve cells (CD27+CD45RO−) but increase in central memory (CM) cells (CD27+CD45RO+) (p = 0.0004 and p = 0.0166 respectively; Table 2) in the PB compared to PB. Although the overall CD4+ T cell proportion was similar between the PF and PB, there was a significantly higher proportion of naïve CD8+ cells and lower proportion of CD8+ terminal effector (TE; CD27−CD45RO−) cells (p = 0.0175 and p = 0.0005 respectively; Table 2) in the PF. There was also an increased expression of CD127 (IL7Ra) on both the CD4+ and CD8+ cells (p = 0.0175 and p = 0.0005 respectively) in the PF compared to the PB. Increased expression of the activation marker, CD38, was also seen on the CD4+ T cells in the PF compared to the PB (median IQR = 6.3[4.0–15.1] for PF compared to 2.2[1.4–9.4] for PB; p = 0.0143; Table 2).

The proportion of CD56+ NK cells was significantly lower in the PF compared to the PB (median IQR = 2.5[1.6–4.6] and 8.6[4.6–13] respectively; p = 0.0000) but there was no difference in the proportion of B cells, γδ T cells, NK T cells or CD14+ monocytes in the PF compared to PB. The proportion of CD4+CD25+ and CD4+CD25+CD127lo regulatory (Treg) cells were both significantly higher in the PF compared to the PB (p = 0.0016 and p = 0.0002 respectively). However, the ratio of Treg/effector cells did not change (median 4.6 for PB and 4.9 for PF; Table 2).

We next compared the PB subsets of TB and non-TB patients in order to see if PB markers could be used as a surrogate for pleural TB (Table 2). Interestingly, the proportion of CD4+ T cells was similar in TB and non-TB subjects in the blood (median IQR = 26[21–32] for TB and 22[15–27] for non-TB) but there was a significantly lower CD4:CD8 ratio in TB patients (median = 1.6 for TB and 2.7 for non-TB; p = 0.0192) due to a higher (but not significant) proportion of CD8+ T cells (median IQR = 16[11–24] for TB and 8.4[4.8–17] for non-TB; Table 2). We also saw a significantly higher level of CD56+ NK cells in the PB of TB patients (median IQR = 8.6[4.6–13] compared to 5.3[3.5–9.0] for non-TB; p = 0.0296; Table 2). We did not see any differences in the total WBC levels, granulocyte, lymphocyte proportions (or ratio), MCV, hematocrit, hemoglobin or platelet levels (data not shown) between TB and non-TB subjects.

We next compared the PB subsets between TB and non-TB patients and found an increase in CD8+ T cells (median IQR = 17[12–23] for TB and 10[9.9–16] for non-TB (p = 0.0099) but no difference in the proportion of CD4+ T cells (Table 2). However, within the CD4+ subset, there was a shift to a central memory phenotype with a median of 49% in TB compared to 23% in non-TB subjects (p = 0.0048). Despite the increase in proportion of CD8+ T cells, no difference in the proportions of naïve and memory subsets within the CD8+ T cell population was observed (Table 2). There was a 4-fold increase in the proportion of Treg cells in the pleural fluid of TB patients (p = 0.0291) but again no difference in the ratio of Treg to effector cells (Table 2). We also saw a significantly increased expression of CD38 on both the CD4+ (median % = 6.3 for TB and 2.4 for non-TB) and CD56+ cells (median % = 17 for TB and 4.1 for non-TB) (p = 0.0065 and p = 0.0031 respectively; Table 2). However, we saw no difference in the proportion of Ki-67 or perforin positive cells within the CD4+, CD8+ or CD56+ subsets (data not shown).
Figure 1. Flow cytometry comparison between peripheral blood and pleural fluid. (a) Ex vivo assessment of the cell phenotypes within the pleural fluid. Shown are representative flow cytometry profiles from a patient with pneumonia and one who was diagnosed with TB. Profiles are first gated on the lymphocyte population as determined by FSC and SSC. Following singlet and CD3 gating, analysis of CD4+ and CD8+ T cell populations is performed. (b) Analysis of a subject with pleural effusion caused by malignancy. In this case CD3 and CD19 are plotted together to illustrate T and B cell proportions in the blood (left) and pleural fluid (right) of a subject with a malignancy (subject 33). (c) Flow cytometry profile from blood and ascites where the blood had a distinct Vv24+CD4+ T cell population; absent from the ascites. (d) Functional analysis of PF cells. Overnight stimulation of PF cells with PPD was followed by intracellular cytokine detection. Following gating on the CD4+ T cells, TNF-α and IFN-γ positive cells were
Functional responses to TB-specific and non-specific stimuli are significantly higher in the PF compared to PB.

The function of pleural and blood lymphocytes was evaluated by intracellular cytokine detection following overnight stimulation with TB-specific antigens (PPD and ESAT-6/CFP-10 (EC)) or positive control (αCD3/CD28 stimulation). Subjects classified with TB (probable or definite) had a distinct population of IFN-γ+TNF-α+ (IGTA) double-positive cells in the PF following stimulation with PPD and EC at much higher levels than the peripheral blood (Fig. 1d red box; Fig. 2a). Analysis of the PPD-IGTA population in the pleural fluid identified pleural TB with a sensitivity of 95% (95%CI = 75–100) and specificity of 100% (95%CI = 63–100) and was never seen in patients who did not have TB (negative predictive value of 100%). The memory phenotype of the IGTA population was also assessed based on CD27 and CD45RO expression (Figs. 1e and 1f). As expected, the majority of the responding cells were of an effector memory phenotype (CD27+CD45RO+; Figs. 1e and 1f) compared to naïve (CD27+CD45RO−) or central memory (CD27+CD45RO+) cells (p < 0.001). Unexpectedly, the response in the PB was magnitudes lower than in the PF, even for the positive control stimulation (Fig. 2a). Furthermore, the quality of the response was also significantly different. Analysis of total IFN-γ, TNF-α or IL-2 producing cells showed a highly significant increase in the proportion of CD4+IFN-γ+ and CD4+TNF-α+ cells in TB patients compared to non-TB (p = 0.0124 and p = 0.0060 respectively; Fig. 2b). Analysis of the combinatorial responses showed a significant increase in the proportion of triple positive (IFN-γ+IL-2+TNF-α+) and double positive (TNF-α+IFN-γ+) cells in TB compared to non-TB patients following both PPD and EC stimulation (p = 0.0011 and p = 0.0001 respectively; Figs. 2c and 2d). This was only seen in the PF and not the PB where the majority of responses were single-positive cytokines (Figs. 2c and 2d).

Table 2. Comparison of immune subsets in peripheral blood and pleural fluid of TB and non-TB patients.

| Subset (%) | TB (n = 30) | non-TB (n = 11) | TB PF vs PB | PF TB vs no TB |
|-----------|-------------|----------------|-------------|---------------|
|           | PB          | PF             | p-value     | p-value       |
| CD3       | 44[33–51]   | 72[43–80]      | 0.0045      | 0.7168        |
| CD4       | 26[21–33]   | 45[33–61]      | 0.0001      | 0.2147        |
| CD8       | 16[11–24]   | 17[12–23]      | 0.0009      | 0.0099        |
| A/B ratio | 1.5[0.9–2.1]| 3.4[1.5–5.0]   | 0.0001      | 0.7145        |
| CD19      | 8.2[5.5–12.4]| 3.9[1.1–10]    | 0.0001      | 0.0813        |
| CD56      | 8.6[4.6–13] | 2.5[1.6–4.6]   | 0.0001      | 0.0813        |
| γεTCR     | 3.0[0.8–3.7]| 1.5[1.0–2.8]   | 0.0002      | 0.0291        |
| CD14      | 7.9[4.5–12]| 2.2[0.8–7.3]   | 0.0002      | 0.0291        |
| CD11b/CD11c | 2.5[0.8–7.5]| 1.6[0.5–6.1]   | 0.0016      | 0.0579        |
| CD4+CD25* | 1.4[0.8–2.4]| 2.8[1.8–4.2]   | 0.0004      | 0.0004        |
| Treg/Teff | 1.0[0.4–1.5]| 2.3[1.4–3.1]   | 0.0004      | 0.0486        |
| CD4 N     | 36[25–53]   | 21[12–37]      | 0.0016      | 0.0048        |
| CD4 CM    | 27[15–42]   | 49[30–63]      | 0.0016      | 0.0048        |
| CD4 EM    | 15[6.1–24]| 13[4.5–20]     | 0.0173      | 0.2722        |
| CD4 TE    | 7.8[5.3–11]| 3.9[2.3–5.4]   | 0.0173      | 0.2722        |
| CD8 N     | 34[21–54]   | 52[22–74]      | 0.0173      | 0.2722        |
| CD8 CM    | 5.9[4.5–17]| 14[2.8–27]     | 0.0173      | 0.2722        |
| CD8 EM    | 7.0[2.9–14]| 3.0[1.5–14]    | 0.0173      | 0.2722        |
| CD8 TE    | 38[34–56]   | 12[4.6–26]     | 0.0173      | 0.2722        |
| CD4+CD127+| 61[39–83]| 73[53–84]      | 0.0173      | 0.2722        |
| CD6+CD127+| 46[36–57]| 67[56–83]      | 0.0173      | 0.2722        |
| CD4+CD38+ | 2.2[1.4–9.4]| 6.3[4.0–15]    | 0.0173      | 0.2722        |
| CD56+CD38+| 34[10–50]| 17[7.2–50]     | 0.0173      | 0.2722        |

Values expressed as median[interquartile range] of 30 subjects with pleural TB and 11 with pleural effusions caused by other diseases (non-TB); PB = peripheral blood; PF = pleural fluid; Treg = regulatory T cells (CD4+CD25+CD127+); Teff = effector T cells (CD4+); N = naïve; CM = central memory; EM = effector memory; TE = terminal effector.

doi:10.1371/journal.pone.0030324.t002

Diagnosis of Pleural TB

assessed. There was no response from the patient with a bacterial infection but a striking response from the patients with definite and probable TB. In particular, note the presence of a distinct IFN-γ+TNF-α+ double-positive cell population (IGTA; red box). (e) The CD4+ IGTA population was gated and the naïve/memory phenotype determined by CD27 and CD45RO expression (red-dot overlay). (f) Analysis of 12 subjects with evaluable data was performed using a Kruskal-Wallis test followed by Dunn’s post-test comparison. We found the majority of the IGTA+ cells were of an effector memory phenotype (EM; CD27−CD45RO+), bottom right quadrant of (d). N = naïve (CD27+CD45RO−); CM = central memory (CD27+CD45RO+); EM = effector memory (CD27−CD45RO+) and TE = terminal effectors (CD27−CD45RO−).

doi:10.1371/journal.pone.0030324.g001
Soluble biomarkers for pleural TB classification

We performed 27-plex cytokine analysis on unstimulated pleural fluid to determine which marker(s) could best predict pleural TB. A high level of IFN-\(\gamma\) has previously been seen in unmanipulated pleural fluid samples from TB patients in South Africa [16]. In the present study IFN-\(\gamma\) levels >1171 pg/mL resulted in a high degree of sensitivity and specificity and 88% correct classification of pleural effusions caused by TB or not (\(p = 0.0003\); Fig. 3 and Table 3). We also found a number of other markers that accurately discriminated between TB and non-TB in unstimulated pleural fluid. These included significantly higher levels of Eotaxin (\(p = 0.0243\)), IL-10 (\(p = 0.0034\)), IL-13 (\(p = 0.0025\)), IL-6 (\(p = 0.0005\)) and IP-10 (\(p = 0.0004\)) in subjects with TB compared to those without (Fig. 3). Logistic regression analysis showed that levels of IP-10 >36,695 pg/mL could discriminate between subjects with TB and those without with a specificity of 82% and sensitivity of 85% (AUC of 0.84) (Fig. 3 and Table 3). This was slightly lower than IFN-\(\gamma\) but IP-10 resulted in the best likelihood ratio (positive and negative = 9.6 and 0.1 respectively compared to positive LR of 2.8 for IFN-\(\gamma\); data not shown). Levels of IL-6 >23,254 pg/mL resulted in a sensitivity of 93% but a comparatively low specificity (64%); Table 3). IL-10 was 100% sensitive but again, low in specificity (64%) at levels >17 pg/mL. Multivariate analysis showed that a combination of IL-6, IL-10 and IP-10 increased the AUC to 0.96 (Table 3), with the positive likelihood ratio increasing to 10.
However, few studies have directly compared the blood and shown to be significantly higher in pulmonary and extrapulmonary TB [10], whilst PPD-specific responses in the BAL have been stimulation, a recent paper showed high levels of polyfunctional T cells; most likely non-specific. TB patients were an increase in CD8 Teff cells. The role in protecting against excessive inflammation that is associated with the increase in effector memory population to the site of infection. Indeed, analysis of the PPD-responsive cells in the pleural fluid resulted in 95% correct classification of TB disease or not. Cellular analysis of the pleural effusions also allowed us to diagnose other underlying causes, particularly malignancies, and was also applicable for analysis of ascites fluid. Our results indicate that use of blood-based diagnostics may be inaccurate at this acute stage of disease and possibly accounts for the reduced sensitivity for active TB disease with the current IFN-γ release assays (IGRAs) [17]. We believe flow cytometry should be included in a diagnostic algorithm for extrapulmonary TB where feasible; however its use as a rapid diagnostic test is limited in resource-poor settings.

Therefore, the final aim of this study was to determine soluble biomarkers for pleural TB, which presents the most viable option for development of a field-friendly, rapid diagnostic test. Previous studies have found high levels of IFN-γ in extrapleural fluid [16] with a recent meta-analysis showing close to 100% specificity and sensitivity for pleural TB [18]. However, we found much lower sensitivity using IFN-γ alone in our study despite the relatively high levels in unstimulated fluid, presumably reflecting genetic differences in the study sites. Alongside IFN-γ, we found high levels of IP-10 and IL-6 with a combination of IP-10, IL-6 and IL-10 resulting in 96% correct classification of pleural TB. IP-10 is primarily (but not solely) induced by IFN-γ (with enhanced production seen with dual TNF-α and IFN-γ stimulation) [19] and is a potent chemo-attractant for activated T cells [19]. It has previously been shown to be elevated in tuberculous pleurisy [19,20], although the discriminatory power compared to IFN-γ for both pulmonary and pleural TB appears to be variable [19–23]. IL-6 has also been shown to be increased in pleural TB [11], is important in the pro-inflammatory response and has recently been shown to be one of the most important biomarkers in TB, alongside IP-10 and IL-10 [24].

In conclusion, this study defines both cellular and soluble biomarkers that provide high levels of sensitivity and specificity for pleural TB in a West African cohort. Whilst cellular biomarkers are currently not applicable for generation of a rapid diagnostic test, the use of flow cytometry as part of the diagnostic algorithm for extrapulmonary TB is important where feasible. However, a combination of soluble biomarkers (IP-10, IL-10 and IL-6), resulted in high specificity and sensitivity for pleural TB, were not affected by HIV status and, once validated, hold great promise for development of a rapid diagnostic test for pleural TB.

Acknowledgments

We thank all the staff at the TB clinic, outpatient’s clinic and ward at the MRC Unit in The Gambia for subject enrolment and consent. We also thank Professor Gerhard Walzl for critical review of the manuscript. Finally, we thank all study subjects and their families.

Author Contributions

Conceived and designed the experiments: JSS MOCO TC. Performed the experiments: JSS FSM AM-G. Analyzed the data: JSS JT. Contributed reagents/materials/analysis tools: MA. Wrote the paper: JSS MOCO. Subject recruitment and clinical assessment: RCI DG AEF.

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