Circulating B-Lymphocytes as Potential Biomarkers of Tuberculosis Infection Activity

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Abstract
Accurate biomarkers of Mycobacterium tuberculosis infection activity would significantly improve early diagnosis, treatment and management of M. tuberculosis infection. We hypothesised that circulating B-lymphocytes may be useful biomarkers of tuberculosis (TB) infection status in highly TB-endemic settings. Ex-vivo and in-vitro mycobacteria-specific B-cell ELISPOT assays were used to examine the plasmablast (PB) and memory B-cell (MBC) responses in the peripheral blood of adult, healthy, community controls (n = 151) and of active TB patients (n = 48) living in Uganda. Frequencies of mycobacteria-specific PBs were markedly higher in active TB patients compared to healthy controls, and, conversely, MBCs were markedly higher in the healthy controls compared to active TB patients. In addition, the community controls with evidence of latent TB infection had higher peripheral blood PB and MBC responses than those without evidence of TB infection. These data demonstrate that peripheral blood B-cell responses are differentially modulated during latent and active M. tuberculosis infection, and suggest that the PB to MBC ratio may be a useful biomarker of TB infection activity.

Introduction
Until recently, tuberculosis (TB) was defined as a two-stage infection - active or latent. In contrast, current thinking suggests that TB may induce a broad spectrum of immune responses, dependent on the Mycobacterium tuberculosis load and/or replication activity within infected individuals, so that the interplay of mycobacterial replication and host immune response results in disease, recovery from active infection, or a sub-clinical latent state [1–4]. Understanding the interplay between M. tuberculosis and host immune responses within this clinical spectrum remains a critical research goal [5,6]. In addition, identification of biomarkers that can accurately predict the clinical status of M. tuberculosis infected individuals, and identify those who are at the highest risk of progressing from sub-clinical latent infections to clinically manifested active disease, also remains a fundamental challenge [5–10]. Novel transcriptomic approaches have improved our knowledge of the immune response to, and pathogenesis of, TB, providing new insight into potential biological markers that might eventually stratify M. tuberculosis infected individuals [7,11,12]. One major break-through from such approaches indicated a relationship between a type I-interferon inducible gene signature and disease activity as assessed by chest x-ray [13]. Interestingly, in this paper the healthy latent TB infected individuals had gene signatures that clustered with both active TB and healthy control gene signatures. The authors suggested that the healthy latent TB infected individuals with gene signatures clustering with those of active TB, might be the ones about to develop active TB, although they did not prove this hypothesis in this paper. Subsequently, other studies have reported changes in gene expression profiles of other signatures within whole blood of TB patients before and after treatment, as well as in latently infected individuals and healthy controls [11,14,15]. Although these approaches have generated exciting results, the cost and expertise needed to produce accurate results limit their practicability and efficiency, particularly in highly endemic and resource poor settings. Cheap, simplified, rapid and accurate alternatives to these approaches are therefore still needed.

Given the critical role of T-cells in M. tuberculosis immunity, approaches aiming to identify new TB biomarkers have previously been biased towards T-cells and T-cell associated molecules. However, recent reports suggesting various modulatory roles for B-cells in M. tuberculosis-specific immune responses [16–21] have revitalised interest in B-cells as possible targets for new biomarkers [14,15]. In a previous study, we described higher frequencies of MBCs within the peripheral blood of healthy UK-donors who had previously visited or lived in highly TB-endemic areas compared to their counterparts who had neither visited nor stayed in such areas [22]. More recently, active TB patients who were
successfully cured following treatment were found to present contrasting B-cell associated gene expression patterns within their blood, before and after treatment [14].

In the present study, we investigated whether the ratio of plasmablasts (PBs) to memory B-cells (MBCs) may accurately predict the clinical status of *M. tuberculosis* infected individuals living in a highly TB-endemic area. Plasmablasts are short-lived antibody secreting cells (ASCs) and are readily detected in peripheral blood during an early or on-going active infection, but they are rarely present in the circulation in the absence of antigen and, or, of viable, replicating pathogens [23]. In contrast, MBCs are long-lived and may persist throughout the lifetime of the host [23–27]. MBCs rapidly and specifically respond to antigenic re-stimulation, differentiating into new ASC populations [27–30]. Given their prolonged lifespan and rapid response, MBCs may contribute to the rapid clearance of pathogens following re-exposure throughout the host’s lifetime [24,25], although this has not been demonstrated for infection with *M. tuberculosis*. Nevertheless, the B cell response to infection with *M. tuberculosis* may be promising in predicting the immunological (and consequently clinical) status of *M. tuberculosis* infected individuals. We reasoned that the presence of antigen-specific PBs in the absence of MBCs within the peripheral circulation may reflect a current or on-going active *M. tuberculosis* infection (clinically manifested disease); that the presence of PBs in addition to MBCs may reflect a resolving active or a subclinical latent infection; and that the presence of MBCs in the absence of PBs may reflect sterile immunity following infection and thus a healthy state.

**Results**

**PB and MBC responses to *M. tuberculosis* antigens**

Little is known about the peripheral blood B-cell responses induced by *M. tuberculosis* infection. We therefore examined these responses by employing short-term *ex-vivo* and 6-day *in-vitro* ELISPOT assays to determine Mycobacteria-specific PB and MBC responses, respectively, in the peripheral blood of adult, healthy, community controls (n = 151) and active TB patients (n = 48). PB and MBC responses were present and readily detected within the peripheral blood of these individuals (Figure 1). Of the 116 healthy controls tested for MBC responses, 74 had positive MBC responses to PPD, 84 to ESAT-6, 76 to CFP-10 and 74 to Ag85A (Figure 1A, open circles). Of another 35 healthy controls tested for PB response, three participants had positive PB responses to PPD, five to ESAT-6, four to CFP-10 and four to Ag85A (Figure 1A, closed circles). Conversely, of the 18 active TB patients tested for MBC responses, five had positive responses to PPD, 10 to ESAT-6, six to CFP-10 and five to Ag85A (Figure 1B). Of another 30 active TB cases, tested for PB responses, 24 had positive PB responses to PPD, 27 to ESAT-6, 25 to CFP-10 and 24 to Ag85A (Figure 1B). Healthy community controls had significantly higher frequencies of mycobacteria-specific MBCs than of PBs (Figure 1A and Figure S1A), and active TB patients had significantly higher frequencies of mycobacteria-specific PBs than of MBCs (Figure 1B and Figure S1B). We observed strong correlations amongst the detected antigen-specific PB and MBC responses, (Table 1), showing that individuals who responded to any one antigen were likely to respond to all antigens tested.

**Differential modulation of Mycobacteria-specific PB and MBC responses during *M. tuberculosis* infection**

Healthy community controls were not assessed for latent TB infection using conventional assays such as the tuberculin skin test (TST) or interferon-gamma release assays (IGRAs). Thus, participants in this group may have been latently infected or truly TB-naive. To explore this possibility, we stratified our community controls into those who had responses to ESAT-6 or CFP-10 (n = 89) and those who lacked responses to both these *M. tuberculosis* – specific antigens (n = 26) in either of the two B-cell assays. We then compared the PB and MBC responses detected in these individuals to those detected in the active TB patients. Consistent with our hypothesis, and as described above, high PB numbers were characteristic of active TB patients, although MBCs were also detected within their peripheral blood (Figure 2, filled circles). On the other hand, high MBC numbers to PPD and Ag85A were characteristic of healthy individuals with responses to ESAT-6 or CFP-10, although a few PBs were detected in some individuals (Figure 2, half filled triangles). All healthy individuals lacking responses to both ESAT-6 and CFP-10 also lacked PB responses to PPD and Ag85A, and only low numbers of MBCs were present within their peripheral blood (Figure 2, open circles). This data is consistent with the generally held definition that healthy individuals with responses to ESAT-6 and CFP-10 are latently infected, and those lacking responses to both of these antigens are uninfected [31]. For the purpose of our further analyses, by analogy with T-cell, IGRAs [31], we defined individuals who had a response to either of the two *M. tuberculosis* – specific antigens in either B-cell assay as having probable latent TB infection. Together, these data demonstrate that peripheral blood B-cell responses are differentially modulated during latent and active *M. tuberculosis* infection, and that the frequencies of *M. tuberculosis*-specific PBs and MBCs in peripheral blood may be able to accurately predict the clinical status of *M. tuberculosis* infected individuals living in highly TB-endemic settings.

The ratio of mycobacteria-specific PBs to MBCs is likely to be associated with *M. tuberculosis* clinical status

Having observed contrasting peripheral blood PB and MBC responses in the community controls and active TB patients, we next considered that the ratio of PBs to MBCs (PB:MBC) might be a more accurate approach for predicting the clinical status of *M. tuberculosis* infected individuals. Considering that we conducted most of our assays in different individuals, we could not directly assess this approach. Nevertheless, we reasoned that comparing median PB and MBC responses in active TB patients to those in the latently infected or uninfected community controls might reveal a pattern that would reflect likely PB:MBC ratios. Indeed, we observed that the median antigen-specific PB response was higher in the TB patients than in the uninfected and latently infected controls, but also higher in the latently infected than in the uninfected controls (Table 2). In contrast, the median antigen-specific MBC response was higher in the latently infected controls than in the active TB patients and uninfected controls (Table 2). This data further suggests that PBs may be associated with an active TB phenotype and MBCs with a latent state. To explore this further, we analysed data from the group of 35 healthy community controls for whom we had data on both PB and MBC responses (Figure 3). The majority of these healthy participants had more peripheral blood MBCs than PBs, presenting a low PB:MBC ratio (Figure 3). Of interest, one participant, who had not been BCG-vaccinated, presented with a ratio consistent with that of the active TB patients (Figure 3, indicated with an open square). We postulate that this individual may be more at risk of developing active TB infection than the other healthy volunteers, or may be progressing from latency towards active disease. Together, these data suggest that the ratio of *M. tuberculosis*-specific PBs to MBCs in peripheral blood may predict the clinical
status of *M. tuberculosis* infected individuals, and thus could be a useful biomarker of *M. tuberculosis* infection status, particularly in individuals living in highly TB-endemic settings.

**Discussion**

We have shown that peripheral blood mycobacteria-specific B-cell responses are differentially modulated during active and latent *M. tuberculosis* infection - PB responses are significantly elevated whereas MBC responses are depleted from the peripheral circulation during active TB disease. These observations may be explained by the increase in mycobacterial loads due to the presence of viable replicating mycobacteria within these TB patients. These elevated mycobacterial loads may activate circulating mycobacteria-specific MBCs, which subsequently differentiate into new PB populations, and thus contribute to the elevation of PBs detected within the peripheral blood of active TB patients. In addition, the increase in mycobacterial loads may accelerate the activation and differentiation of naive B-cells, thereby contributing to the PB pool. Plasmablasts contribute to the increase in serum levels of protective antibodies during active infection, regardless of the pathogen. Increased serum antibodies are readily detected during TB infection [32,33], although why such antibodies are not protective remains unknown.

It should be noted that we used frozen PBMCs from active TB patients and fresh PBMCs from healthy volunteers in our experiments. There is some suggestion that the freeze/thaw cycle may affect cell viability and/or a selective loss of cell populations, notably activated cells [34,35]. Given this potential issue, we might expect the freeze/thaw process to selectively reduce numbers of PBs, which are activated B cells; however, we could clearly identify PB populations in our thawed PBMCs from active TB patients. Thus, if the freeze/thaw process did negatively affect PB numbers, we are likely to have underestimated the high PB:MBC ratio in these patients. In addition, a recent study showed that the freeze/thaw process did not affect influenza-specific PB numbers [36], and in a more recent report, MBC responses were also largely unaffected by this process [37]. It is therefore likely that the freeze/thaw process did not significantly alter the numbers of PBs or MBCs that we report here.

Our study lacked conventional tests such as the TST or an IGRA that could independently confirm latent TB infection (LTBI) in our healthy controls. Nevertheless, our data is consistent with what we might expect from such assays: the proposed LTBI...
**Figure 2. Mycobacteria-specific PB and MBC responses in the active TB patients and community controls.** Frequencies of antigen-specific PBs and MBCs were determined by 6-hour ex-vivo and 6-day in-vitro ELISPOTs respectively. Frequencies of antigen-specific PBs and MBCs are presented as antibody secreting cells per million PBMCs. (A) Frequencies of mycobacteria-specific PBs in the active TB patients (filled circles), community controls responding to ESAT-6/CFP-10 (half filled triangles) and healthy community controls lacking ESAT-6 and CFP-10 responses (open circles) (B) Frequencies of mycobacteria-specific MBCs in the active TB patients (filled circles), community controls responding to ESAT-6/CFP-10 (half filled triangles) those without ESAT-6 and CFP-10 responses (open circles). **** P<0.0001, ***, P<0.001; **, P<0.01.
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**Figure 3. Paired mycobacteria-specific PB and MBC responses in healthy community controls.** Matched frequencies of antigen-specific PBs and MBCs were determined by 6-hour ex-vivo and 6-day in-vitro ELISPOTs respectively. Matched frequencies of PPD- (A), ESAT-6- (B), CFP-10- (C), Ag85A- (D) and Ag85B- (E) specific PBs and MBCs in healthy community controls are shown. Open square across all panels represents an interesting donor who presented with a higher PB:MBC ratio on various antigens tested. Frequencies of antigen-specific PBs and MBCs are presented as antibody secreting cells per million PBMCs.
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Concluding Remarks

This study investigated whether differences in peripheral blood B-cell responses accurately reflect the clinical status of M. tuberculosis infected individuals. We demonstrate a differential modulation in peripheral blood PB and MBC responses during active and latent M. tuberculosis infection, reflected in a significant increase in the peripheral blood PB:MBC ratio during active M. tuberculosis infection. We conclude that the ratio of M. tuberculosis-specific PBs to MBCs within the peripheral blood may be a useful biomarker of TB infection activity and thus warrants further investigation. Prospective studies aiming to test the kinetics of this ratio among untreated, M. tuberculosis latently infected individuals who might progress towards active infection, or in newly identified active TB patients undergoing treatment, may provide better insight and validation of this concept. This may thus serve as a significant improvement to currently available methods for detecting M. tuberculosis infection, which cannot accurately distinguish between active and latent infections.

Materials and Methods

Ethics statement

The ethics committees at Makerere University, Kampala, Uganda, the Uganda Virus Research Institute (UVRI) and the Uganda National Council of Science and Technology (UNCST) approved these studies. Informed written consent was obtained from all study participants. Participants provided up to 20 ml of heparinised venous blood that was used in subsequent experiments.

Study participants

Forty-eight (48) newly diagnosed, adult, BCG-vaccinated, HIV negative, untreated, sputum positive TB patients, 18–50 years of age, were included from a larger prospective study, at two health clinics in Kampala, Uganda. In addition, 151 adult, HIV negative, clinically healthy, community controls, 18–55 years old, were recruited through an HIV counselling and testing programme at the UVRI clinic in Entebbe, Uganda, as part of a cross-sectional study. Of the 151 healthy community controls recruited, only four had not been previously vaccinated with BCG.

Antigens and mitogens

Mycobacterium tuberculosis ESAT-6 (NR-14868), CFP-10 (NR-14869), Ag85A (NR-14871) and Ag85B (NR-14870) were obtained through BEI Resources (NIAID, USA) and purified protein derivative for in-vitro use (PPD) from Statens Serum Institut (SSI, Denmark). Non-adsorbed tetanus toxoid (TT) was obtained from the National Institute for Biological Standards and Control (NIBSC, UK). Phytolacca americana (PWM) and Staphylococcus aureus (SAC) were obtained from Sigma-Aldrich, UK. CpG-oligonucleotide (ODN-2006, 5′-tcgtcgttttgtcgttttgtcgtt-3′) was obtained through Eurofins MWG/Operon, Germany.

Study design

In-vitro and ex-vivo B-cell ELISPOT assays were used to examine the mycobacteria-specific memory B cell (MBC) and plasmablast (PB) responses, respectively, in both active TB patients and the healthy community controls. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll Paque Plus (Amersham Pharmacia Biotech, UK) density-gradient centrifugation, washed twice in Hank’s balanced salt solution (HBSS, Invitrogen, UK) and resuspended in RPMI-1640 culture media (Invitrogen, UK).

Table 2. Median PB and MBC responses in the community controls and active TB patients.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Healthy</th>
<th>LTBI</th>
<th>Active</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag85A</td>
<td>0/0</td>
<td>11/14</td>
<td>20/1</td>
</tr>
<tr>
<td>CFP-10</td>
<td>0/0</td>
<td>0/13</td>
<td>14/0</td>
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<tr>
<td>ESAT-6</td>
<td>0/0</td>
<td>13/23</td>
<td>27/5</td>
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<tr>
<td>PPD</td>
<td>0/0</td>
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containing 10% Fetal Bovine Serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine (all from Sigma-Aldrich, UK). Purified PBMCs from the blood of the active TB patients were stored in liquid nitrogen until use; those from the healthy community controls were used fresh in the ELISPOT assays. In-vitro ELISPOT assays were used to determine MBC responses in 116 healthy community controls. Separately, paired ex-vivo and in-vitro ELISPOT assays were used to determine matched PB and MBC responses in PBMCs from another 35 clinically healthy participants. For the active TB patients, PBMCs from 30 participants were analyzed using the ex-vivo ELISPOT to determine their PB responses. PBMCs from another 18 active TB cases were analyzed using the in-vitro ELISPOT assay to determine their MBC response. Due to limited blood volumes and PBMC numbers obtained from the active TB patients, we were unable to conduct both assays on samples from the same patient.

In-vitro ELISPOT assay

Frequencies of MBCs in peripheral blood were determined using an in-house in-vitro B-cell enzyme-linked immunosorbenent spot (ELISPOT), previously described [22]. Briefly, purified PBMCs (1x10^6/ml) were stimulated with a cocktail of 6 μg/ml CpG, 0.5 μg/ml PWM, 1.2 μg/ml SAC, 25 ng/ml recombinant human IL-10 (R&D systems, UK) and 50 μM β-mercaptoethanol for six days at 37°C in a humidified 5% CO2 incubator. Polyclonal stimulated cells (4x10^5 cells/ml) were then transferred directly into wells of a 96-well filter plate (Millipore, MAHAS4510) that was coated with either 100 μl of PBS containing Mycobacterium-specific recombinant proteins (6 μg/ml PPD, 10 μg/ml ESAT-6, Ag85A, Ag85B, and CFP-10), 2 μg/ml Tetanus toxoid (TT, positive control) or with PBS alone (negative control). Plates were developed with a biotin-SP-conjugated AffiniPure fragment donkey anti-human IgG (Jackson ImmunoResearch), strepavidin-AKP (BD biosciences) and its substrate (AP-conjugate substrate kit, Biorad, USA). Developed spots were air-dried and the number of resultant spots was enumerated using an AID ELISPOT reader. Frequencies of antigen-specific PBs and MBCs were calculated and later presented as spot forming units per million PBMCs.

Statistical analysis

Collected data were entered into Excel and GraphPad Prism software. Statistical analysis was performed using Stata 10.1. Frequencies of PBs and MBCs were not normally distributed and therefore non-parametric statistical tests were used. Comparisons of antigen-specific PB and MBC responses between healthy community controls and TB patients were made using an unpaired Wilcoxon sign rank test. Matched PB and MBC responses in a selected set of healthy community controls were compared using a paired Wilcoxon sign rank test. Spearman’s rank correlation coefficient was used to test for correlations amongst various antigen-specific PB and MBC responses. Only statistically significant differences are shown.

Supporting Information

Figure S1 Mycobacteria-specific PB and MBC responses in the healthy community controls and active TB cases. Frequencies of antigen-specific PBs and MBCs were determined by 6-hour ex-vivo and 6-day in-vitro ELISPOTs respectively. Frequencies of antigen-specific PBs and MBCs are presented as antibody secreting cells per million PBMCs. Frequencies of mycobacteria-specific PBs in healthy community controls (open circles) and active TB patients (closed circles) (A) and MBCs in healthy community controls (open circles) and active TB patients (closed circles) (B) are shown. ***P<0.001, **P<0.01, *P<0.05. (TIF)

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Author Contributions

Conceived and designed the experiments: IS HMD AME SC. Performed the experiments: IS. Analyzed the data: IS HMD AME SC. Contributed reagents/materials/analysis tools: IS IAB. Contributed to the writing of the manuscript: IS IAB HMD AME SC.
B-Cells as TB Biomarkers


