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IMMUNOLOGICAL ASPECTS

# High granulocyte/lymphocyte ratio and paucity of NKT cells defines TB disease in a TB-endemic setting

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SUMMARY

Most people infected with *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB) actually maintain a strong immune response and are able to control bacterial growth (deemed latently infected (LTBI)), while approximately 10% progress to disease resulting in almost 2 million deaths per year. Determining the immune 'footprint' at specific stages of infection and disease will allow for better diagnostics, treatments and ultimately development of new vaccine candidates. In this study we performed multi-factorial flow cytometry on fresh blood from 56 TB cases, 46 Tuberculin Skin Test (TST) positive (LTBI) and 39 TST negative household contacts. We found a highly significant increase in granulocytes and decrease in B cells and invariant (Vα24 + Vβ11+) NKT cells in TB cases compared to TST+ contacts ($p < 0.0001$, $p = 0.007$ and $p = 0.01$ respectively) which were restored to LTBI levels following 6 months of TB treatment. Using support vector analysis, we found a combination of granulocyte and lymphocyte and/or NKT cell proportions allowed almost 90% correct classification into *M. tuberculosis* infection or disease. This work has important public health benefits in regards to diagnosis and treatment of TB in sub-Saharan Africa and in furthering our understanding of the requirements for protective immunity to TB.



## 1. Introduction

Tuberculosis (TB) is caused mainly by the intracellular pathogen, *Mycobacterium tuberculosis* (Mtb) and is one of the major health problems in developing countries.[1] One unique feature about TB is that although one third of the world population is infected with Mtb, only about 5–10% of them progress to active disease, with higher rates among those co-infected with HIV.[1] The majority remain healthy but latently infected (LTBI). Comparison of the immune system between individuals with active TB disease and those with LTBI will help to determine what is required for a protective immune response and will therefore enable specific immune responses to be targeted for future vaccine developments.[2,3] Determination of biomarkers for diagnosis of active TB disease, other than acid-fast bacilli (AFB) in sputum, is also essential in TB-endemic settings where less than 50% of individuals are positive[2,3] and in diagnostically difficult groups such as children, HIV co-infected subjects or those with extra-pulmonary TB where the current diagnostic tests on the market are unreliable.

A number of changes in the innate and adaptive arms of the immune system have been described with active TB disease. Neutrophils provide an innate immune resistance to TB most likely via chemokine secretion[4] with risk of Mtb infection being inversely associated with neutrophil counts in contacts of pulmonary TB (pTB) patients.[5] CD4+ T cells are specifically required for generating an optimal immune response to TB[6,7] mainly through production of IFN-γ which forms the basis of TB diagnostics to date.[2,8] Interestingly, with increasing disease severity, CD4 responses decrease[7] and CD8+ T cells become more important although their contribution to human disease pathology is complicated and appears to reflect different stages of disease and bacterial burden.[9,10] A recent study demonstrated that a combination of moderate levels of both memory CD4+ T cells and CD8+ T cells were most effective in achieving protection; illuminating the requirement for vaccination strategies to target both subsets of cells.[11] NKT cells provide a link between the innate and adaptive immune systems and have been shown to be decreased in TB disease[12,13] with higher levels seen in fast treatment responders.[14] Other cell types such as γδ T cells,[15] regulatory T cells,[16] B cells[17] and NK cells[18] all appear to interact and control the eventual output response to TB.

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In this study we performed multi-factorial analysis of the immune system using fresh, un-fractionated blood from subjects with active pTB disease (pre- and post-treatment) and compared this to Mtb-infected (TST+) and non-infected (TST−) household contacts. We found that increased granulocytes and decreased NKT cells could distinguish between TB disease and infection with almost 90% correct classification. These findings may have important public health implications in the development of new, simplistic diagnostic tools.

## 2. Materials and methods

### 2.1. Participants

Fifty-six sputum smear-positive pTB cases and 85 of their *Mycobacterium*-exposed healthy household contacts were consecutively recruited as part of ongoing tuberculosis case contact studies at the MRC (UK) unit in The Gambia and the Gates Grand Challenge 6, Biomarkers for TB.[3] Subjects were considered for inclusion if they were >18 years of age, were HIV-1 sero-negative. All cases and contacts underwent a clinical assessment, including a screen for malaria and inter-current illness. Of the confirmed pTB cases, 2.5% had Grade I (+), 27.5% Grade 2 (++) and 70% Grade 3 (+++) smears (taken as highest grade out of 2 consecutively positive smears). Ethical approval was obtained from the Gambia Government/Medical Research Council joint ethics committee. Tuberculin skin tests (TST; 2 tuberculin units [TU], PPD RT23, SSI, Denmark) were performed in order to further classify the household contacts into TST+ and TST−. Due to the influence of prior BCG vaccination and environmental mycobacteria in this TB-endemic setting, subjects with skin test induration of ≥10 mm diameter were categorised as TST positive (likely Mtb infected; 46/85; 57%). A chest X-ray was conducted on all cases and TST+ contacts. TB cases were further classified according to the degree of pulmonary involvement seen by chest X-ray into subjects with minimal disease (21/56; 38%), moderate disease (24%) and advanced disease (38%). Other clinical data such as night sweats, cough duration and weight loss were obtained from the TB cases where possible. TB treatment consisted of 2-months intensive phase with rifampycin, pyrazinamid, isoniazid and ethambutol followed by a 4-month continuation phase with isoniazid and ethambutol only. As a control for the general effects of infection, we also retrospectively analysed 9 adult bacterial pneumonia patients with evaluable hematological data.

### 2.2. Cell counts and phenotypic analysis

Blood samples were taken from TB cases prior to treatment and from 26 of them at 6-months post-treatment. Blood was taken from household contacts at recruitment prior to skin testing. Blood was collected into heparin vacutainers (BD Biosciences) and white blood cell (WBC), granulocyte and lymphocyte numbers determined using a Medonic™ hematology cell analyzer (Boule Medical, Sweden). Cell phenotypes were analysed using a whole blood assay: 20 μl of the appropriate antibody cocktail was added to 100 μl whole blood and incubated in the dark at room temperature (RT) for 30 min. Red blood cells were removed by adding 1 mL of FACS lysing buffer (Becton–Dickinson) and incubating in the dark for 10 min at RT. Thereafter samples were centrifuged at $600_{gmax}$, supernatant decanted, cells washed twice in PBS/FCS/Azide, and finally resuspended in 1% paraformaldehyde (PFA) prior to acquisition. Antibodies used were CD4-PerCP, CD8-Pacific Blue, CD27-APC, CD45RO-PE, CD56-PE, CD56-PECy7 (all from BDPharmingen, USA); CD3-PE-Cy7, CD19-APCAlexa750 (all from eBioscience, UK) and Vα24-FITC and Vβ11-PE (Beckman Coulter, USA).

### 2.3. Ki-67 and perforin detection

Surface staining for CD4-PerCP, CD8-Pacific Blue, and CD56-APC was performed as described above. Following RBC lysis, samples were resuspended in 500 μl of FACS permeabilising solution 2 (Becton–Dickinson, USA) and incubated for 20 min at RT in the dark. Washed samples were incubated with anti-Ki67-FITC and anti-perforin-PE (or the appropriate isotype control) (Pharmingen, USA) for 30 min at RT, in the dark. Samples were then washed and resuspended in 1% PFA prior to acquisition.

### 2.4. Flow cytometry acquisition and analysis

All samples were acquired with a 7-colour (9-parameter) CyAn ADP™ flow cytometer (Beckman Coulter, USA). Prior to acquisition, calibration and compensation were performed and lymphocytes gated according to 90° forward and side scatter plots. FACS plots were analysed using FlowJo software (Treestar, OR), version 6.1.1.

### 2.5. Statistics

Linear regression was used to assess the differences between confirmed TB cases and TST+ and TST– contacts, adjusting for possible confounders. All data were $Log_{10}$ transformed to satisfy the constant variance and normality assumptions of regression. To allow for the multiplicity of tests resulting from multiple responses and multiple comparisons within response, a false discovery rate (FDR) of 10% was assumed.[19] Due to the large number of tests family wise error rate correction methods, were too conservative. Multivariate discrimination was tested using a support vector machine,[20] with testing and training sets selected using holdout cross-validation with half the data allocated to each training set. Correlation with pulmonary involvement was performed using Spearman rank analysis. All analyses were performed using STATA version 9.1 (Stata Corporation, USA) and Matlab version 7.6 (Mathworks, Natwick, 2008).

## 3. Results

### 3.1. Study population

The median age of the TB cases was significantly higher than the contacts (28 compared to 23 years; $p = 0.004$). The majority (77%) of TB cases were male while 54% of contacts were female ($p < 0.0001$) and all were HIV negative. To allow for these possible confounding effects all analyses were adjusted for age and gender. Univariate results are presented in Table 1 with significant test results between TB cases and TST+ contacts shown based on a FDR of 10%. All subsets were significantly different between TB cases and TST− contacts. Figures are presented as a comparison between TB cases and TST+ contacts unless otherwise stated.

### 3.2. WBC counts are highly increased in TB but return to non-disease levels following treatment

Whole blood from TB cases and their contacts was analysed using a Medonic cell analyser for total WBC, lymphocyte and granulocyte numbers (Figure 1A and Table 1). WBC levels were significantly higher in TB cases compared to TST+ contacts ($p < 0.0001$), predominantly due to a significant increase in granulocyte numbers ($p < 0.0001$; Figure 1A and Table 1). Both the percentage and absolute number of granulocytes were increased in TB cases while lymphocyte numbers showed a decrease in percentage but not in absolute number indicating this was a specific effect on the granulocyte compartment (Table 1). Following chemotherapy for TB, WBC and granulocyte