Modulation of the immune response to *Mycobacterium tuberculosis* during Malaria/*M. tuberculosis* co-infection

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Tuberculosis (TB) causes significant morbidity and mortality on a global scale. The African region has 24% of the world TB cases. TB overlaps with other infectious diseases such as malaria and HIV which are also highly prevalent in the African region. TB is a leading cause of death among HIV-positive patients and co-infection with HIV and TB has been described as a syndemic. In view of the overlapping epidemiology of these diseases, it is important to understand the dynamics of the immune response to TB in the context of co-infection. We investigated the cytokine response to purified protein derivative (PPD) in peripheral blood mononuclear cells from TB patients co-infected with HIV or malaria and compared it to that of malaria- and HIV-free TB patients. A total of 231 subjects were recruited for this study and classified into 6 groups: Untreated TB positive, TB positive subjects on TB drugs, TB and HIV positive, TB and malaria positive, latent TB, and apparently healthy control subjects. Our results demonstrate maintenance of IFN-γ production in HIV and malaria co-infected TB patients in spite of lower CD4 counts in the HIV-infected cohort. Malaria co-infection caused an increase in the production of the Th2-associated cytokine IL-4 and the anti-inflammatory cytokine IL-10 in PPD-stimulated cultures. These results suggest that malaria co-infection diverts immunity response against M. tuberculosis towards a Th-2/anti-inflammatory response which might have important consequences for disease progression.

**Keywords:** tuberculosis, HIV, malaria, co-infection, cytokines
Introduction

Tuberculosis (TB) is an infectious disease that causes significant morbidity and mortality on a global scale [1]. Pulmonary tuberculosis caused by infection with *Mycobacterium tuberculosis* is the leading cause of death due to a bacterial pathogen and is responsible for 1.4 million deaths annually [2]. In 2011, there were an estimated 8.7 million new cases of TB and 1.4 million people died from TB, including almost one million deaths among HIV-negative individuals and 430,000 among people who were HIV positive [3]. TB is second only to HIV/AIDS as the biggest killer worldwide due to a single infectious agent [4].

Host immune responses to infection are primarily characterized by a cell mediated response dominated by T-helper (Th) 1 cells playing a critical role in the secretion of IFN-γ; IFN-γ directs the effector response against mycobacteria through activation of macrophages, allowing them to exert a microbicidal role [5]. The crucial role of IFN-γ in immunity to mycobacteria has been demonstrated in studies of individuals with deficiencies in IFN-γ signalling due to mutations within the *ifngr* gene resulting in increased disease severity, poor granuloma formation and multibacillary lesions [6, 7]. TNF-α also plays an important role in the latent phase of infection as TNF-α blockade in clinical and experimental models results in reactivation of latent tuberculosis [8].

Geographically, the burden of TB disease is highest in Asia and Africa. The African region accounts for 24% of the world’s cases and the highest rates of cases and deaths per capita [4]. TB is fuelled by HIV as well as by social and economic factors [9]. Co-infection with HIV and tuberculosis has been described as a syndemic [10, 11]. In Africa, HIV is the single most important factor contributing to the increase in the incidence of TB [12]. TB is a leading cause of death among people who are HIV-positive; a quarter of all deaths in HIV-infected patients are caused by TB [10]. Malaria is endemic in 109 countries and continues to cause between 189 and 327 million clinical episodes each year, with at least 881,000 associated deaths [13]. TB overlaps with malaria which is prevalent in the African region [14]. Malaria is endemic in Nigeria, particularly the *P. falciparum* species [15, 16]. HIV/AIDS, tuberculosis, malaria and neglected tropical diseases cause 32% of the burden of ill health in Africa, and seriously impact on health outcomes in every region of the world [3].

In view of the overlapping epidemiology of these diseases, it is important to understand the dynamics of the immune response to TB in the context of co-infection. Towards this aim we investigated the cytokine response to TB antigens in peripheral blood mononuclear cells (PBMCs) from TB patients co-infected with HIV or malaria and compared it to that of malaria- and HIV-free TB patients. Our results demonstrate maintenance of IFN-γ production in HIV and malaria co-infected TB patients in spite of lower CD4 counts in the HIV-infected cohort. In contrast, changes were observed in IL-4 and IL-10 production among TB-infected groups. In particular, malaria co-infection resulted in increased IL-4 and IL-10 secretion compared to malaria-HIV-free and HIV-co-infected TB patients. Moreover, on the basis of TB-specific cytokine secretion, we were able to cluster our patient cohorts into groupings that align with their pathogen/treatment grouping which highlights the clinical relevance of our findings. These results suggest that malaria co-infection promotes an anti-inflammatory response against *M. tuberculosis* which might have important consequences for disease progression.
Materials and Methods

Ethics Statement
Ethical approval for the study design was obtained from the Nnamdi Azikiwe University Teaching Hospital ethical committee (ethical approval reference number NAUTH/CS/66/3/21). Written informed consent was obtained from all participants enrolled in the study.

Study populations
Two hundred and thirty-one (231) subjects comprising one hundred and thirty-five (135) males and ninety-six (96) females with a mean age of 37 years, median age of 36 years (range 12-68 years) were enrolled in the study at the Nnamdi Azikiwe University Teaching Hospital, Nnewi (Anambra State) and the Mile 4 hospital, Abakaliki (Ebonyi State). The subjects were classified into six groups according to their TB, anti-TB treatment, HIV and malaria parasitemia status:

- **Group A**: Untreated TB positive (n = 41); these subjects are TB positive only (HIV and malaria negative) but had not commenced anti-TB drugs,
- **Group B**: TB positive on drugs (n = 65); TB positive only and have commenced on anti-TB drugs,
- **Group C**: TB and HIV positive (n = 37); newly diagnosed to be TB and HIV positive but malaria-negative subjects who had not commenced anti-TB drugs or anti-retroviral therapy,
- **Group D**: TB and malaria positive (n = 23); TB positive subjects co-infected with malaria but had not commenced on anti-TB therapy or anti-malaria drugs,
- **Group E**: latent TB positive subjects (n = 30); apparently healthy subjects who were found to be latently infected with TB after mantoux screening,
- **Group F**: healthy control subjects (n = 35), these had no reaction after mantoux testing.

The TB disease group were enrolled after clinical assessment and were referred for laboratory testing using Ziehl-Neelsen and Auramine-phenol fluorochrome technique for Acid fast bacilli (AFB), and by Polymerase chain reaction (PCR) using the GeneXpert MTB/RIF assay (Cepheid, California). After samples had been collected from apparently healthy controls, subjects were administered with 0.1ml/5TU purified protein derivative (mantoux testing) to classify them into latently TB infected and control groups. As a precaution, children less than 12 years and adults who had received BCG vaccine in adulthood were excluded to rule out false positives. All the TB patients were classified according to chest X-ray, PCR and sputum smear bacterial density into mild, moderate and severe TB. For uniformity, equal numbers of each category was targeted for each of the four (4) TB disease groups. No significant evidence of increased lung compromise was observed in any TB disease group compared to the other groups. In the TB positive group on drugs, no matter the drug duration, the recruited patients were sputum smear positive, had persistent clinical features of disease, abnormal chest X-ray findings and a positive PCR test. Some patients in this category had multi-drug resistant TB. Based on BCG scars and questionnaires, not all subjects were BCG vaccinated. Approximately 60% from each group was vaccinated. A large number of individuals below 30 years had BCG scars as they were vaccinated as part of routine childhood vaccination.

Subjects were tested for HIV infection using Determine (Inverness Medical, Japan) and STAT-Pak (Chembio, USA) and in the event of discordant results, Uni-Gold (Trinity Biotech, Ireland) was used as the tie breaker according to the national guidelines for HIV counselling and testing [43]. Malaria testing was performed using thin and thick films as well as by using rapid MP test kits (SD...
Bioline, Standard Diagnostics Inc, Korea). Patients and other subjects were excluded if they were pregnant, had any other clinical problems or had commenced anti-retroviral therapy.

**CD4 T cell and differential counts**

Blood samples were collected in EDTA vacutainer blood collection tubes (BD, USA) and CD4 T cell counts were measured within 5 hours of sample collection, using a Cyflow SL3 cytometer for automated CD4+ counts as per manufacturer’s instructions (Partec, Germany). For full differential white blood cell (WBC) counts, thin blood films were prepared, stained by Leishman’s technique and counted manually using x100 magnification following standard procedure.

**Isolation and stimulation of PBMCs**

Fresh EDTA anticoagulated blood was diluted 1:1 in Hanks’ balanced salt solution (HBSS, Sigma-Aldrich). The mixture was then layered over Histopaque-1077 (Sigma-Aldrich) and centrifuged at 800 x g for 30 minutes. PBMCs were collected and washed thrice in HBSS. Cells were re-suspended in RPMI-1640 complete medium (all components from Sigma-Aldrich) containing 10% human AB serum, 2mM L-glutamine, 100U/ml penicillin, 100 µg/ml streptomycin and 10 mM HEPES. Cells were then cultured in sterile 96 well plates (Corning NY) in quadruplicate. Each well contained 200 µl of cell suspension (2x10⁵ cells/well) and PPD (2 µg/ml, BB-NCIP Ltd, Sofia, Bulgaria). Cultures were incubated for 72 hours at 37°C with 5% CO₂ in a humidified incubator. Supernatants were collected; centrifuged at 800 x g for 15 minutes at room temperature and stored in aliquots at -20°C until cytokine measurement.

**Cytokine Assay**

The cytokine levels in supernatants of cultured PBMCs were measured using commercial ELISA kits (Abcam, Cambridge, UK) according to manufacturer’s instructions. Samples were assayed in duplicate for IFN-γ, TNF-α, IL-2, IL-4, and IL-10. The lower limit for assay sensitivity were <5 pg/ml for IFNγ, <10 pg/ml for TNF-α, <10 pg/ml for IL-2, <0.5 pg/ml for IL-4, <5 pg/ml for IL-10 as stated by the manufacturer. Cytokine release by unstimulated and PPD-stimulated cultures were assessed in two samples per patient group as controls. In all instances, unstimulated cultures produced less cytokines than PPD-stimulated cultures which indicates that the cells were responsive to stimulation under our assay conditions.

**Statistical analysis**

Data were analysed using Kruskall-Wallis test with Dunn’s multiple comparison correction (Graph pad prism version 5.03) and the level of significance set at P<0.05. For clustering analysis data were analyzed using SPSS (IBM Version 21). Briefly, samples were transformed to Z-scores to enable agglomerative hierarchical clustering. Z-scores were used due to the variable nature of the scale of cytokine production both within individual cytokine variables and across the cytokine variables. A pre-defined number of clusters were set at 12 to allow for potential variation, i.e. high cytokine responder or non-responder, within each of the six nominal groups of patients recruited to the study. Clustering was performed twice using two measures of distance to define the distance between clusters, complete and average linkage. The distance measures were first sorted to determine cytokine similarity and further sorted to allow alignment of patients.
Results

Demographics and CD4+ T-cell counts of patient groups
The demographics of the study population and each patient group are displayed in Table 1. The % of male subjects in the patient groups was higher than the % of female patients, ranging from 56.7% to 60.9%. The median age ranged from 36±10.1 to 38.2±7.8 and patients in the lower socio-economic status were overrepresented in all TB subjects except the TB-malaria co-infected group.

CD4+ T-cell counts (cells/μl) (Figure 1) were significantly lower in all the active TB infected groups (untreated TB subjects [737 ± 266], TB positive subjects on TB drugs [807 ± 252], TB and HIV positive subjects [258 ± 203] and TB and MP positive subjects [611 ± 271], but not in the latent TB positive subjects (1046 ± 305) when compared with the control (1083 ± 281) group (P < 0.01). The total lymphocyte counts (x10⁹) were 1.81 ± 0.99 in the untreated TB positive subjects, 1.79 ± 0.67 in TB positive subjects on drugs, 1.62 ± 0.94 in TB and HIV positive subjects, 2.28 ± 1.00 in TB and MP positive subjects, 2.13 ± 0.79 in Latent TB positive subjects and 2.07 ± 0.76 in apparently healthy controls. There were no significant differences between the test and control groups (P>0.05). Other WBC counts such as eosinophils, monocytes and basophils showed no divergence from the expected normal ranges (see supplementary Table 1). Monocyte/lymphocyte ratios were also calculated and no differences among groups were observed (data not shown). The lack of eosinophilia and basophilia would suggest no underlying helminth infection. Hypoalbuminemia is a non-specific indicator of helminth infection; serum albumin levels of all patients were examined. In agreement with the eosinophil data, no changes were found in the albumin levels among groups with respect to normal ranges of 35-50g/L (data not shown).

Cytokine production by patients’ PBMC cultures
PBMCs from subjects belonging to each patient group were stimulated with PPD for 72 hours as described in materials and methods and supernatants were collected and tested for the presence of the effectors cytokines TNF-α and IL-2 (Figure 2), the Th1/Th2 signature cytokines IFNγ and IL-4 (Figure 3) and the regulatory cytokine IL-10 (Figure 4).

The median values obtained for TNF-α (pg/ml) were 138 (range 1.9 – 574) for the untreated TB positive subjects, 130 (range 30 – 2,267) for the TB positive subjects on drugs, 139 (range 24 – 421) for TB and HIV positive subjects, 213 (105 – 526) for TB and malaria positive subjects, 215 (range 78 – 613) for the latent TB positive subjects and 0.0 (range 0 – 103) for the apparently healthy controls. TNF-α levels were significantly increased in all TB patients compared to controls with differences among the TB infected only reaching significance when comparing the latent TB group and active TB group on anti-TB drugs (p=0.0156).

The median values obtained for IL-2 in pg/ml were 141.0 (range 36 – 503) for the untreated TB positive subjects, 116.0 (range 30 – 480) for the TB positive subjects on drugs, 115 (range 39 – 215) for TB positive HIV positive subjects, 136 (66 – 315) for TB and malaria positive subjects, 156 (range 86 – 321) for the Latent TB positive subjects, 175 (range 86 – 410) for the apparently healthy controls. IL-2, a surrogate marker of T cell activation, was readily detected in all cultures. The findings reveal that IL-2 levels were significantly lower in the TB positive group on drugs and...
the HIV co-infected TB group when compared to the latent TB positive subjects and healthy control subjects. However, there were no significant differences when comparing the MP co-infected and the untreated TB group to the latent and control group (Fig 2).

The median values for IFN-γ in pg/ml were 554.0 (154 – 1399) for the untreated TB positive subjects, 596.0 (170 -1710) for the TB positive subjects on drugs, 543.0 (136 – 1048) for the TB and HIV positive subjects, 536.0 (108 – 1360) for the TB and malaria positive subjects, 817.5 (360 – 1430) for the Latent TB positive subjects and 215.0 (106 – 430) for the control subjects. IFN-γ, an indicator of Th1 polarisation was detected in all instances with significantly higher amounts being measured in the TB-infected groups compared to control group (Fig 3). As expected, the latent TB group produced significantly higher amounts of IFN-γ than the control subjects upon PPD stimulation. Latent TB subjects produced significantly more IFN-γ than the TB-infected group on drugs (p = 0.0341), TB-HIV co-infected group (p = 0.0097) and the TB-malaria co-infected group (p = 0.0084). No differences in IFN-γ production were observed among the active TB-infected groups.

IL-4, an indicator of Th2 polarisation was detected in low amounts in all instances as expected for PPD-stimulated cultures. The median value obtained for IL–4 in pg/ml were 7.6 (0.0 – 229.0) for untreated TB positive subjects, 5.7 (0.0 – 25.6) for TB positive subjects on drugs, 2.3 (0.0 – 47.0) for TB and HIV positive subjects, 33.0 (3.8 – 72.0) for TB and MP positive subjects, 1.6 (0.0 – 36.0) for the Latent TB positive subjects, 38.0 (0.0 – 182) for the apparently healthy controls. The control group produced significantly higher amounts of IL-4 compared to all TB-infected groups with the exception of the TB patients co-infected with malaria (Fig 3). It is interesting to note from our findings that the TB-malaria co-infected patient subset produced significantly higher levels of IL-4 than all other TB infected groups with production levels similar to that found in the control group. This level of production in TB-malaria indicates an increase in anti-inflammatory response to \textit{M. tuberculosis}.

Finally, IL-10 production differentiated patient groups into low producers (control and Latent TB), mid producers (treated TB, untreated TB and TB patients co-infected with HIV) and high producers (TB patients co-infected with malaria). The IL – 10 median values in pg/ml were 110.0 (range 5.0 – 241.0) for untreated TB positive subjects, 114.0 (56 – 312) for TB positive subjects on drugs, 119.0 (49.0 – 216) for TB and HIV positive subjects, 221.0 (118.0 – 563.0) for TB and malaria positive subjects, 4.6 (0.0 – 49.0) for the Latent TB positive subjects, 8.0 (0.0 – 52) for the apparently healthy controls. Graph shows median and 5-95 percentiles. No differences between control and Latent TB were observed. Differences between control and Latent TB groups and all active TB groups were highly significant for IL-10 production. The TB-malaria co-infected group produced significantly higher levels of IL-10 compared to untreated TB patients (p = 0.0006), TB patients on drugs (p = 0.001) and TB-HIV co-infected patients (p = 0.0036). The high IL-10 production in the TB-malaria co-infected group indicates the prevalence of more anti-inflammatory conditions in this particular group of patients.
Cluster analysis of patient groups
To determine if the cytokine responses we determined corresponded to valid patient clusters/groupings we undertook agglomerative hierarchical cluster analysis. The resulting dendrogram (Figure 5A) illustrates the resulting clusters. Clustering was undertaken twice using two differing definitions of distance – complete and linkage. On both occasions the resulting clusters indicated the same relationships – there are two major clusters one corresponding to control, latent TB and TB-HIV co-infected patients and the second corresponding to TB patients on drugs, TB patients not on drugs and TB-malaria co-infected patients. In cluster one latent TB and TB-HIV co-infected patients were more closely associated and in cluster two TB patients on drugs and TB patients not on drugs were most closely related. These relationships were then represented using a heatmap (Figure 5B). Cytokines production was calculated as % increase over the control group average for each individual within the remaining patient groups. Thereafter, patient group responses were averaged to produce the heatmap which demonstrates that the greatest gradients in responses across groups were seen in IL-10 and TNF-α.
Discussion

The main findings of this study are the changes that were observed in the TB-malaria co-infected group which displayed increased IL-4 and IL-10 secretion compared to other TB-infected groups. In addition, the patient cohorts could be clustered into groupings that align with their pathogen/treatment characteristics which highlight the relevance of our results. The observations regarding socio-economic status in this study were in agreement with the link between TB infection and socioeconomic status previously described with prevalent TB significantly associated with poverty and lower household socio-economic positions [17, 18].

In this study, immunological markers in TB were investigated by assessing CD4+ counts and quantifying cytokine production by PPD-stimulated PBMCs in controls and TB-infected groups. CD4+ T cell counts (Figure 1) and total lymphocyte counts (see supplementary Table 1) in the Latent TB group were comparable to what was observed in the control group and higher than values observed in active TB samples showing that in these individuals CMI is optimal. TNF-α, IL-10 and IFN-γ were preferentially detected in TB-infected samples although substantial IFN-γ could also be detected in control samples. These results indicate that production of these cytokines was largely due to the presence of memory cells. IL-2 and IL-4 were detected in both control and TB-infected supernatants. IL-2 levels were significantly reduced in TB patients on drugs and TB-HIV co-infected samples compared to control and Latent TB. Levels of IL-4 were low in general and controls and TB-malaria co-infected samples contained significantly more IL-4 than the rest of the groups, particularly when compared to the Latent TB group that produced the lowest amounts of IL-4.

Differences between Latent TB vs. active TB groups

In general PPD-induced responses in Latent TB was characterized by similar production of TNF-α, IL-2, IFN-γ and lowest production of IL-4 and IL-10 suggesting a robust Th1-dominated response in line with the fact that TB is controlled in these patients. These results agree with that of Handzel et al (2007) [19] who reported that PPD-stimulated cells of latently infected subjects secreted more IFN-γ and IL-2 and may contribute to protection from overt disease. Functional capacity to produce certain cytokines is associated with mycobacterial load [20]. Day et al (2011) [20] reported that patients with latent TB infection produced higher levels of cytokines than those with clinical TB. They suggested that there is progressive impairment of mycobacterium-specific T cell responses with increasing mycobacterial load. However, in contrast to our findings some researchers [21] reported higher IFN-γ level in active TB cases compared to latently infected individuals.

IL-2, IFN-γ and other Th1 cytokines play a role in the control of intracellular infections while type 2 cytokines (IL-4, IL-5, IL-10) are either irrelevant or exert a negative influence [22]. IFN-γ is a key cytokine in control of TB infection [23, 24, 25]. TNF-α induces inflammatory responses and is critical to host resistance to TB [26, 27, 28]. IL-4 is the most powerful trigger for Th2 development and is antagonistic to Th1 cytokines [29]. Diminished production of IL-4 has been reported in TB infected individuals compared with healthy controls [30]. IL-10 has emerged as a key immunoregulator during infection that can inhibit both Th1 and Th2 responses [31]. It has been
reported that experimental ablation of IL-10 or inhibition of IL-10 signalling restores pathogen control and reduces the severity of disease [31].

Co-infection with HIV and malaria modulate anti-TB responses

Human populations are rarely exposed to one pathogen alone, particularly in high incidence regions [32]. In comparison to mono-infection, co-infection presents a more complex challenge to the host immune system [33]. It has been reported that TB co-infection with parasitic infections in animals present enhanced inflammatory immune responses as reflected by exacerbated leukocyte infiltrates, tissue pathology and hypercytokinemia accompanied by altered T cell responses [32]. It is estimated that 14 million people are co-infected with HIV and TB, with tuberculosis being the most common opportunistic infection in HIV and accounting for over a quarter of AIDS-related deaths [34]. Given the complexity of the immune response, there are multiple ways that HIV can alter the immune response to M. tuberculosis [35] including CD4+ T cell depletion. CD4+ T cell depletion was associated with decline in memory (CD27CD45RO') CD4+ T cells that recognize M. tuberculosis antigens [36]. Multivariate analysis placed TB-HIV co-infected samples closest to controls and Latent TB samples indicating a distinct effect of HIV infection on TB-specific cytokine responses during active TB. The effect of HIV infection does not appear to be caused by general immunosuppression. Immune cells from TB-HIV co-infected patients produced levels of cytokines similar to those observed in other patient groups in spite of the significantly lower numbers of CD4+ T cells detected in these donors at the time of sampling. For IL-4 TB-HIV co-infected patients tended to align with the Latent TB group (lowest IL-4 production), while for IL-10 the TB-HIV co-infected donors produced levels similar to those produced by the untreated and treated TB groups (intermediate IL-10 production). In the case of TNF-α, this cohort could not be differentiated from the Latent TB and other TB-infected cohorts. For IL-2 and IFN-γ this group could be differentiated from controls and Latent TB but no other TB-infected groups. These observations suggest a Th1 bias with increased regulatory potential (intermediate IL-10) in these TB-HIV patients compared to the TB only groups (both treated and untreated). This would be in agreement with the suppression of cellular immune responses by regulatory T cells induced by HIV infection in TB previously described [37].

In this study, among the individuals who were co-infected with malaria parasite and TB, none had severe anaemia, acute respiratory distress syndrome, neurological disorders and other manifestations characteristic of severe malaria. None of the patients complained of malaria symptoms during questioning in spite of the parasite counts (median 29,520 parasites/µl, 5% 17,595 parasites/µl and 95% 67,520 parasites/µl) which, although modest should cause symptomatic malaria. There could be reasons for this. First, clinically, malaria infection causes a range of symptoms from asymptomatic or mild flu-like illness (uncomplicated malaria), particularly in individuals immune to malaria in endemic areas, to the uncommon complications of severe disease [38]. The study area is a malaria endemic region where Plasmodium falciparum causes most malarial disease. Adults with some level of immunity to malaria may be infected with malaria, have few clinical symptoms and rarely experience severe complications (with the exception of pregnant women) [39]. Additionally, co-infection with TB could mask the malaria symptoms since the symptoms of both diseases overlap. Moreover, it has been reported that TB-induced potentiation of type 1 immune responses is associated with protection against lethal murine malaria and this protective process is believed to relate to IFN-γ induction [40]. Riley et al [41] reported that this induction of cellular immune responses is related to the ATP-binding
protein (ATPBP) of plasmodium species. Also according to Zheng et al [42], heat shock protein 70 (HSP 70) from \textit{M. tuberculosis} was associated with the induction of a strong humoral and cellular response directed against \textit{Plasmodium falciparum} which further support an interplay between TB and malaria immune responses.

For TNF-α, IL-2 and IFN-γ production the TB-malaria co-infected cohort could not be differentiated from the other TB-infected cohorts. This pro-inflammatory response could be protective against severe malaria.

It is interesting to note that the TB-malaria co-infected group produced highest levels of IL-4 and IL-10, which are unique characteristics of this group. This observation was validated by the multivariate analysis. These observations indicate there is reduced Th1 bias and increased regulatory potential in the TB-malaria co-infected group and support a major effect of malaria infection in anti-TB immunity. No differences were observed in between TB patients undergoing chemotherapy and those untreated. Since there were significant differences in all the TB infected groups (with the exception of the TB-malaria co-infected group) compared to the control group, this then indicates that \textit{M. tuberculosis} suppresses the production of IL-4. On the other hand, there was no significant difference in IL-4 production between the control group and the TB-malaria co-infected subset. This suggests that co-infection with malaria increases production of IL-4 and favours an anti-inflammatory response to \textit{M. tuberculosis}. It can be argued that perhaps the TB-malaria co-infected group have more severe disease and this could be responsible for increased production of IL-4. This is however not the case as this group had no evidence of increased lung compromise when compared to other TB infected groups. The control group produced relatively high levels of IL-4 and helmint infection could be suspected. Though stool analysis was not performed to assess for helmint infection, the low eosinophil numbers in all samples (Supplementary Table 1) indicate that helmint infection would be unlikely not just in the control group but in all groups studied. Thus, the probability of helmint infection would be the same for all study groups.

In conclusion, findings from this study suggests that the higher pro-inflammatory cytokines seen in Latent TB is an evidence of protection while higher anti-inflammatory and regulatory cytokines in other TB test subjects may indicate failure of protection. Thus, a balance of these two types of cytokines (inflammatory and anti-inflammatory) groups may be critical to non-progression to TB disease. Our results demonstrate maintenance of IFN-γ production in HIV and malaria co-infected TB patients in spite of lower CD4 counts. This study also suggests that co-infection with TB may modulate the immune responses to confer immunologic protection against severe malaria while weakening responses to TB. Malaria co-infection caused a marked increase in the production of the anti-inflammatory cytokines IL-10 and IL-4 in response to TB antigen. These results suggest that malaria co-infection promotes an anti-inflammatory response against \textit{M. tuberculosis} which might have important consequences for disease progression. In view of these findings, future work will focus on comparison of TB disease progression in TB versus TB and malaria co-infected patients.
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Disclosure Statement

The authors declare no conflict of interest.
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Footnote

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Key = Untreated TB positive subjects (A), TB positive subjects on drugs (B), TB and HIV positive subjects (C), TB and malaria positive subjects (D) Latent TB subjects (E), Apparently healthy control group (F)
Figure legends

Figure 1.
CD4+ T-cell counts from patient groups.
Patients were grouped according to disease diagnosis plus or minus TB chemotherapy into the following groups; controls (Cont), Latent TB (Lat-TB), active TB without chemotherapy (TB+Drug-) or with chemotherapy (TB+Drug+), TB-HIV co-infection (TB+HIV+) and TB-malaria (TB+MP+) co-infection. CD4 counts were determined using blood collected in EDTA analyzed with an automated Cyflow SL3 cell cytometer. Counts are expressed as cells/μl. Graph shows median and 5-95 percentiles. Statistical significance between groups was determined using a Kruskal-Wallis test with Dunn’s multiple comparison correction; *p<0.05, **p<0.01, ***P<0.001, ****p<0.0001. Significant differences among Controls and Latent-TB and active TB groups are shown in grey. Significant differences among active TB groups are shown in black.

Figure 2.
Production of TNF-α and IL-2 by TB patient groups.
Patients were grouped according to disease diagnosis plus or minus TB chemotherapy into the following groups; controls (Cont), Latent TB (Lat-TB), active TB without chemotherapy (TB+Drug-) or with chemotherapy (TB+Drug+), TB-HIV co-infection (TB+HIV+) and TB-malaria co-infection (TB+MP+). Concentrations of TNF-α and IL-2 in supernatants were quantified using ELISA as described in materials and methods. Graph shows median and 5-95 percentiles. Statistical significance between groups was determined using a Kruskal-Wallis test with Dunn’s multiple comparison correction; *p<0.05, **p<0.01, ***P<0.001, ****p<0.0001. Significant differences among Controls and Latent-TB and active TB groups are shown in grey. Significant differences among active TB groups are shown in black.

Figure 3.
Production of IFN-γ and IL-4 by TB patient groups.
Patients were grouped according to disease diagnosis plus or minus TB chemotherapy into the following groups; controls (Cont), Latent TB (Lat-TB), active TB without chemotherapy (TB+Drug-) or with chemotherapy (TB+Drug+), TB-HIV co-infection (TB+HIV+) and TB-malaria co-infection (TB+MP+). Concentrations of IFN-γ and IL-4 in supernatants were quantified using ELISA as described in materials and methods. Graph shows median and 5-95 percentiles. Statistical significance between groups was determined using a Kruskal-Wallis test with Dunn’s multiple comparison correction; *p<0.05, **p<0.01, ***P<0.001, ****p<0.0001. Significant differences among Controls and Latent-TB and active TB groups are shown in grey. Significant differences among active TB groups are shown in black.
Figure 4.
Production of IL-10 by TB patient groups.
Patients were grouped according to disease diagnosis plus or minus TB chemotherapy into the following groups; controls (Cont), Latent TB (Lat-TB), active TB without chemotherapy (TB+Drug-) or with chemotherapy (TB+Drug+), TB- HIV co-infection (TB+HIV+) and TB-malaria co-infection (TB+MP+). Concentrations of IL-10 in supernatants were quantified using ELISA as described in materials and methods. Statistical significance between groups was determined using a Kruskal-Wallis test with Dunn’s multiple comparison correction; \*p<0.05, **p<0.01, ***P<0.001, ****p<0.0001. Significant differences among Controls and Latent-TB and active TB groups are shown in grey. Significant differences among active TB groups are shown in black.

Figure 5.
Cluster analysis of patient cytokine production.
Patients were grouped according to disease diagnosis plus or minus TB chemotherapy into the following groups; controls (Cont), Latent TB (Lat-TB), active TB with chemotherapy (TB+drug+) or without chemotherapy (TB+drug-), TB-HIV co-infection (TB+HIV+) and TB-malaria co-infection (TB+MP+).(A) Cluster analysis was undertaken using SPSS (Version 21 – IBM). Cytokine values were transformed to Z-scores prior to undertaking clustering. Two distance measures were used, complete and linkage distance. A finite number of clusters, 12, were pre-determined.(B) A heatmap was generated corresponding to each cluster for average cytokine production. Control values were averaged and individual values from remaining patient groups were calculated as a % change over control values.
Supplementary Table 1:

Mean (+ SD) of CD4 + T cell counts, WBC and differential counts in tuberculosis infected subjects and the apparently healthy control subjects.
<table>
<thead>
<tr>
<th>Groups</th>
<th>CD4 (cells/µl)</th>
<th>TWBC (10^9/l)</th>
<th>ABSOLUTE LYMPH (x10^9)</th>
<th>ABSOLUTE NEUT (x10^9)</th>
<th>LYMPH (%)</th>
<th>NEUT (%)</th>
<th>MONO (%)</th>
<th>EOSIN (%)</th>
<th>BASO (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated TB pos. subjects (n=41) (A)</td>
<td>737±266</td>
<td>3.95±2.36</td>
<td>1.81±.99</td>
<td>2.05±.15</td>
<td>46.8±11.1</td>
<td>49.9±11.5</td>
<td>2.2±0.9</td>
<td>0.8±1.0</td>
<td>0.3±0.7</td>
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<tr>
<td>TB positive subjects on drugs (n=65) (B)</td>
<td>807±252</td>
<td>3.79±1.51</td>
<td>1.79±.67</td>
<td>1.88±.99</td>
<td>48.3±10.0</td>
<td>48.9±10.0</td>
<td>2.1±0.9</td>
<td>0.8±0.8</td>
<td>0.5±0.8</td>
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<tr>
<td>TB and HIV positive subjects (n=37) (C)</td>
<td>258±203</td>
<td>3.98±2.15</td>
<td>1.62±.94</td>
<td>2.27±.15</td>
<td>41.2±10.9</td>
<td>55.9±11.3</td>
<td>2.00±0.9</td>
<td>0.7±0.8</td>
<td>0.4±0.7</td>
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<tr>
<td>TB and MP positive subjects (n=23) (D)</td>
<td>611±271</td>
<td>4.57±2.42</td>
<td>2.28±1.0</td>
<td>2.20±1.80</td>
<td>53.3±17.3</td>
<td>44.0±17.6</td>
<td>2.5±0.8</td>
<td>0.7±0.9</td>
<td>0.08±0.2</td>
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<tr>
<td>Latent TB positive subjects (n=30) (E)</td>
<td>1046±305</td>
<td>4.17±1.67</td>
<td>2.13±.79</td>
<td>1.9±1.09</td>
<td>52.1±10.5</td>
<td>45.2±10.4</td>
<td>1.9±0.9</td>
<td>0.8±1.1</td>
<td>0.73±0.8</td>
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<tr>
<td>Apparently healthy controls (n=35) (F)</td>
<td>1083±281</td>
<td>3.82±1.23</td>
<td>2.07±.76</td>
<td>1.6±.65</td>
<td>54.9±10.0</td>
<td>41.3±9.6</td>
<td>2.4±0.9</td>
<td>1.00±0.8</td>
<td>0.43±0.6</td>
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<tr>
<td>F Value</td>
<td>46.86</td>
<td>0.69</td>
<td>2.78</td>
<td>1.43</td>
<td>6.83</td>
<td>7.18</td>
<td>1.87</td>
<td>0.43</td>
<td>2.22</td>
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<tr>
<td>P Value</td>
<td>0.00</td>
<td>0.63(ns)</td>
<td>0.01</td>
<td>0.21(ns)</td>
<td>0.00</td>
<td>0.00</td>
<td>0.10(ns)</td>
<td>0.31(ns)</td>
<td>0.05(ns)</td>
</tr>
</tbody>
</table>
Figure 1

CD4 counts (cells/µl)

Cont  Lat-TB  Drug -  Drug†  HLV†  MP†
TB+

190x275mm (300 x 300 DPI)
Figure 2

TNFα (pg/ml)

IL-2 (pg/ml)

190x275mm (300 x 300 DPI)
Figure 3

190x275mm (300 x 300 DPI)
Figure 4

IL-10 (pg/ml)

Cont  Lat-TB  Drug -  Drug +  HIV +  MP +  TB +

190x275mm (300 x 300 DPI)
Figure 5

190x275mm (300 x 300 DPI)