IL-4 subverts mycobacterial containment in *M. tuberculosis*-infected human macrophages

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ABSTRACT (198/200)

Protective immunity against *Mycobacterium tuberculosis* is poorly understood. The role of interleukin-4 (IL-4), the archetypal T-helper-2 (Th2) cytokine, in the immunopathogenesis of human tuberculosis remains unclear.

Blood and/or broncho-alveolar lavage fluid (BAL) were obtained from participants with pulmonary TB (TB; n=23) and presumed latent TB infection (LTBI; n=22). Messenger RNA expression levels of interferon-gamma (IFN-γ), IL-4, and its splice variant IL-4δ2 were determined by real-time PCR. The effect of human recombinant IL-4 (hrIL-4) on mycobacterial survival/ containment [colony-forming-units (CFU/ml] was evaluated in *M. tuberculosis*-infected macrophages co-cultured with mycobacterial antigen-primed effector T-cells. Regulatory T-cell (Treg) and Th1 cytokine levels were evaluated using flow cytometry.

In blood, but not BAL, IL-4 mRNA levels (p=0.02) and the IL-4/IFN-γ ratio (p=0.01) was higher in TB versus LTBI. hrIL-4 reduced mycobacterial containment in infected macrophages (p<0.008) in a dose-dependent manner and was associated with an increase in Tregs (p<0.001) but decreased CD4$^+$Th1 cytokine levels (CD4$^+$IFN-γ^+: p<0.001; CD4$^+$TNFα^+: p=0.01). Blocking IL-4 significantly neutralized mycobacterial containment (p=0.03), CD4$^+$IFNγ^+ levels (p=0.03) and Treg expression (p=0.03).

IL-4 can subvert mycobacterial containment in human macrophages, likely via perturbations in Treg and Th1-linked pathways. These data may have implications in the design of effective TB vaccines and host-directed therapies.

Keywords: tuberculosis, immunology, interleukin-4, T-helper 2 cells

Take home message (248/256 characters)

TB patients express high levels of IL-4 and exhibit a compartment-specific skewed Th2/Th1 response. *In vitro*, IL-4 subverts mycobacterial containment in *M.tb*-infected human macrophages indicating its potential utility as an immunotherapeutic target.

INTRODUCTION

Tuberculosis (TB) has killed over 1 billion people over the last two centuries and remains a major global public health threat today. Vaccination is the best hope for worldwide elimination of TB but most vaccine candidates are partially effective or ineffective [1]. More recently, there has been a profound interest in host-directed therapies as an adjunct to TB chemotherapy, either for shortening conventional treatment regimens, or to manage cases of programmatically incurable or difficult-to-treat drug-resistant TB [2]. However, novel strategies for the design of new vaccine candidates or immunotherapies will require a deeper understanding of the immune mechanisms underpinning susceptibility and disease progression, as these are currently not well understood.

It has become central dogma that a surrogate of protective immunity against *Mycobacterium tuberculosis* (*M.tb*) is a robust Th1 response. Although, IFN-γ-related gene defects lead to increased susceptibility to TB [3, 4], patients with active TB have robust IFN-γ responses at the site of disease [5-7]. Furthermore, current vaccine candidates, including Bacille-Calmette-Guérin and MVA85A, prioritized based on their Th1-inducing ability, offer limited protection against TB in adults [8, 9].

If a Th1 response alone cannot provide sufficient protection then what other factors, either alone or in tandem, underpin *M.tb*-specific protective host immunity? Several innate and adaptive immune mechanisms have been postulated (reviewed in [10]). One possibility, supported by circumstantial evidence, is that *M.tb* induces a small but significant subversive Th2 response within a dominant Th1 environment, leading to bacterial proliferation and disease progression [11]. Indeed, murine [12] and cellular human data support this hypothesis; TB patients exhibit elevated IL-4 levels [6, 13, 14] correlating with immunopathology [6, 15], and IL-4 predicts progression to active disease in exposed healthcare workers [16] and household contacts [17]. Furthermore, the IL-4-receptor (IL4R) has been implicated in the development of TB-associated tissue pathology in both murine [18] and human TB [19]. Despite these findings, the role of IL-4 in TB remains controversial due to discordant data and the technical challenges of measuring human IL-4 that is active at sub-ELISA concentrations (reviewed in [20]). The existence of IL-4δ2, an alternatively spliced variant and natural antagonist of IL-4 which is associated with protection in TB, further complicates the picture [6, 21, 22], and may explain why some studies failed to detect differences in IL-4 between TB patients and controls [23, 24]. Finally, whether IL-4 can subvert mycobacterial stasis/ killing in human cells has, hitherto, not been investigated.

Thus, it is still unknown whether a Th2-like response is causally related to attenuated immunity, or, merely a consequence of excessive inflammation. To address these questions we interrogated Th2 responses in the peripheral blood and human lung, and further evaluated the effect of human recombinant IL-4 (hrIL-4) on mycobacterial survival in *M.tb*-infected human macrophages.

METHODS

Participant recruitment

Newly diagnosed (<2 weeks of anti-TB therapy), drug-sensitive pulmonary TB (TB) patients were recruited from 4 primary care clinics in Cape Town. TB diagnosis was microbiologically confirmed by MGIT liquid culture. Presumed latently infected (LTBI) controls were asymptomatic with no clinical or radiological evidence of previous or current disease and were exposed persons (close contacts of TB index cases or healthcare workers) with a positive tuberculin skin test *and* interferon-gamma-release-assay (Quantiferon Gold-in-tube) result. Those with HIV co-infection or other chronic immunosuppressive diseases, and any known Th2-associated conditions were excluded. Ethical approval was obtained from the University of Cape Town Research Ethics Committee.

Peripheral blood and broncho-alveolar lavage sample processing

After informed consent, 45-50ml of peripheral blood was collected by venipuncture into PAXgene RNA tubes (2.5ml; Qiagen) for RNA preservation and sodium heparin tubes (~42.5-47.5ml) for peripheral blood mononuclear cell (PBMC) isolation. PBMCs were isolated by density centrifugation for use in downstream functional immunoassays. Broncho-alveolar lavage (BAL) was performed, as previously described [25]. Isolated BAL cells were stored in RNA stabilization buffer to fix the RNA profile.

RNA extraction, reverse transcription and quantitative real-time PCR (qPCR)

RNA was extracted from whole blood and BAL cells using the PAXgene blood RNA kit (Preanalytix) and RNeasy Plus kit (Qiagen), respectively. Following RNA quality assessment

and reverse transcription, transcribed cDNA was amplified by qPCR using primers and probes specific to IFN-γ, IL-4 and IL-4δ2 (Table E1) from published literature [6]. Values were normalized to a validated reference gene, human acidic ribosomal protein (HuPO) [6]. Full methodological details are provided in the online data supplement.

Expression, purification and bioactivity assessment of hrIL-4

Human recombinant IL-4 (hrIL-4) protein was produced in a baculovirus-expression system and subsequently used in the mycobacterial containment assay. Full methods are provided in the online data supplement.

Mycobacterial containment assay

A mycobacterial (*M.tb*) containment assay was used to determine the effect of hrIL-4 on the ability of effector cells and macrophages to control the intracellular containment of *M.tb* within autologous monocyte-derived-macrophages (MDMs) using peripheral blood from active TB patients. MDMs were generated from PBMCs for 5 days, as previously described [26] followed by infection with H37*Rv* for 18 hours at an MOI of 3 [27]. Non-ingested bacteria were removed by washing. Frozen aliquots of H37*Rv* were randomly cultured to confirm the infecting bacterial dose. MDM viability was determined by trypan blue exclusion staining.

Concurrent to MDM generation, PBMCs were also stimulated for 6 days with purified protein derivative (PPD;12μg/ml) with or without hrIL-4 to generate pre-primed effector T-cells (T$_{eff}$ cells). After 6 days, H37*Rv*-infected MDMs and T$_{eff}$ cells were co-cultured for 48 hours. Appropriate controls performed in duplicate included a reference control containing H37*Rv*-infected MDMs only and a positive *M.tb* containment control containing H37*Rv*-infected MDMs

co-cultured with PPD pre-primed effector T-cells (PPD T_{eff}). The effect of IL-4 was assessed by adding various concentrations (5,20,100ng/ml) of hrIL-4 (day 1) together with PPD to PBMCs for 6 days to generate PPD+IL-4 T_{eff} cells prior to co-culture with infected MDMs. After co-culturing for 48 hours, intracellular H37Rv was released by lysis of infected MDMs and plated on Middlebrook 7H10 agar. Colonies were counted and expressed as colony forming units per ml (CFU/ml). In order to normalize the data to account for inter-patient variability, the

$$100 - \left[\frac{\text{Experimental condition (CFU/ml)}}{\text{Reference control (CFU/ml)}} \times 100 \right] = \textbf{\% } \textbf{\textit{M.tb}}\textbf{ containment}$$

percentage (%) *M.tb* containment was also reported, defined as the change in *M.tb* survival compared to the reference control (H37Rv-infected MDMs only; see Table E3 for experimental details):

Cellular mechanisms associated with IL-4-mediated effect on *M.tb* containment

Adherent (MDMs) and non-adherent (T_{eff} cells) cellular fractions in the mycobacterial containment assay were analyzed by flow cytometry to determine the mechanisms contributing to the IL-4-modulation of *M.tb* containment. Adherent cells were lifted by treatment with cold 0.5% EDTA and gentle scraping. Cells were stained using fluorescently-labeled antibodies against CD3, CD4, CD8, CD14, CD16, CD25, dendritic-cell-specific-intercellular-adhesion-molecule-3-grabbing-non-integrin (DC-SIGN; a marker of alternative macrophage activation), Foxp3, IFN-γ, TNFα and IL-10 (BD Biosciences, Biolegend and eBiosciences). Cells were acquired on an LSRII flow cytometer and analyzed using FACSDiva software (BD Biosciences).

Neutralization of hrIL-4

The effect of neutralizing IL-4 was assessed by adding anti-IL-4 antibody (20μg/ml; Abcam) to hrIL-4-containing interventions (day 1) in the mycobacterial containment assay. The effects on *M.tb* containment and associated cellular mechanisms were determined by colony counting (CFU/ml) and flow cytometry, respectively (Table E3 and E4). We also performed similar preliminary experiments to determine the effect of blocking the IL-4 receptor (IL-4R) using anti-IL-4 receptor antibodies (10μg/ml, Abcam).

Statistical analysis

The Mann-Whitney U test was used to assess differences between participant groups and biological compartments. Wilcoxon-matched pairs signed rank test was used to assess differences pre- and post-interventions. A p-value of <0.05 was considered significant. Statistical analyses were performed using GraphPad Prism version 6.0 (Graphpad).

RESULTS

Th1 and Th2 expression levels in the lungs and blood of TB patients and presumed LTBI controls

mRNA levels were assessed using a validated qPCR assay (see online data supplement) in whole blood (WB) and BAL cells of TB patients (n=23 and 8, respectively) and presumed LTBI controls (n=22 and 7, respectively). IFN-γ expression levels (median [interquartile range, IQR]) per 10^6 copies of HuPO) in BAL was ~6-fold higher compared to WB in both TB (6783 [2452-24918] vs. 1721 [692-3161] copies, respectively; p=0.005) and LTBI (6281 [2570-10438] vs.

1871 [1131-3521] copies, respectively; p=0.02, Figure 1a). IL-4 mRNA levels were higher in TB patients versus LTBI controls in WB (126 [45-232] vs. 42 [16-98] copies, respectively; p=0.02) but not in BAL cells (Figure 1b). Expression levels of IL-4δ2 were generally low and expression in some samples, particularly from BAL, were below the detection limit of the assay (Figure 1c). The IL-4/IFN-γ expression ratio, thought to represent the Th2/Th1 balance, was also higher in WB of TB patients compared to LTBI controls (0.046 [0.021-0.155] vs. 0.019 [0.010-0.036]; p=0.01) but not in BAL cells. The IL-4/IFN-γ ratio was much lower in BAL compared to WB in both groups (p<0.0001; Figure 1d). There were no inter-group or inter-compartment differences in the IL-4\IL-4δ2 ratio (Figure 1e). A similar compartment-specific pattern of IFN-γ and IL-4 expression was observed in matched BAL and blood samples in TB (n=5) and LTBI (n=4; Figure E4 in the online supplement) Additionally, no differences in IL-4 mRNA levels or the IL-4/IFN-γ ratio were observed when stratified by smear grade, used as a proxy of disease extent, in the TB group (Figure E5).

Soluble IL-4 protein, as measured by ELISA in TB antigen-driven cell culture supernatants levels were mostly below the detection limit of the assay in both BAL and blood (Figure E6). IL-13 protein levels were detectable but also low, and there were no differences in expression levels between TB patients and LTBI controls (Figure E7).

IL-4-primed effector cells can subvert mycobacterial containment
In TB patients (n=8), the addition of PPD T_{eff} cells to infected MDMs reduced the median *M.tb* CFU/ml from $30.7x10^3$ to $15.7x10^3$ compared to infected MDMs only (p=0.008; Figure 2a), equivalent to a 48% increase in *M.tb* containment (p=0.008; Figure 2b).

In the PPD+IL-4 T_{eff} interventions, there was a significant increase in median CFU/ml at 5-100ng/ml hrIL-4 ($29.1x10^3$ to $43.8x10^3$; p=0.008; Figure 2a) compared to the PPD T_{eff} control in TB patients (n=8). This equated to a decrease in % *M.tb* containment at each of the hrIL-4 concentrations (1%, -73% and -33%, vs. 48% respectively; p=0.008; Figure 2b). The % *M.tb* containment was significantly lower at 100ng/ml compared to 5ng/ml hrIL-4 (p=0.008) indicating that the observed hrIL-4 effect was concentration-dependent.

A similar trend was observed in LTBI participants (n=5) where the median CFU/ml increased in the PPD+IL-4 T_{eff} interventions at 5-100ng/ml hrIL-4 ($45.6x10^3$ to $108.3x10^3$) compared to the PPD T_{eff} control ($28.0x10^3$; Figure E10A). This resulted in decreased % *M.tb* containment at each hrIL-4 concentrations compared to the control (64%, 18% and -6%, vs. 72% respectively; Figure E10B). However, the differences between the PPD+IL-4 T_{eff} interventions and PPD T_{eff} control were not statistically significant (p=0.06). Furthermore, there were no significant differences when equivalent wells were compared between the TB and LTBI groups (Table E5).

IL-4 modulates the expression of regulatory T-cells (Tregs), Th1 cytokines and pattern recognition receptors (DC-SIGN)

In order to determine the effect of hrIL-4 on cellular biomarker expression, cells (MDMs and T_{eff} cells) were harvested from the mycobacterial containment assay and analyzed by flow cytometry.

Regulatory T-cells (Tregs)

The gating strategy for identification of Tregs (CD3$^+$CD4$^+$CD25$^+$FoxP3$^+$) is shown in figure 3a. The median % Treg expression [IQR] was significantly increased in the PPD+IL-4 T$_{eff}$ intervention (100ng/ml hrIL-4; n=16) compared to the PPD T$_{eff}$ control (4.2% [1.2-10.5] vs. 2.1% [1.1-4.2], p=0.0006; Figure 3b).

Th1 cytokine and DC-SIGN expression

The gating strategy for Th1 cytokine (IFN-γ and TNFα) and DC-SIGN expression in lymphocytes (CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$) and macrophages (CD14$^+$CD16$^+$) is shown in Figure 4a. Median CD4$^+$IFN-γ$^+$ expression at both 20 (n=8; 1.8% [0.4-4.1]) and 100ng/ml hrIL-4 (n=16; 1.2% [0.7-3.8]), compared to the PPD T$_{eff}$ control (4.0% [1.2-7.4]), was significantly reduced (p=0.01 and p=0.0005, respectively; Figure 4b). Although IFN-γ expression was higher in the CD8$^+$ lymphocyte population, no significant differences were observed between the control and interventions.

The effect of IL-4 on TNFα expression was less pronounced than CD4$^+$IFN-γ expression. Lower median CD4$^+$TNFα$^+$ expression was observed in the PPD+IL-4 T$_{eff}$ intervention at 100ng/ml hrIL-4 compared to the PPD T$_{eff}$ control (1.4% [0.5-3.1] vs. 1.9% [0.9-6.7], respectively; p=0.02; Figure 4c). These differences were not observed in the CD8$^+$ population.

Macrophage (CD14$^+$CD16$^+$) DC-SIGN expression, a marker of alternative macrophage activation, was significantly reduced in the PPD T$_{eff}$ control compared to the infected MDMs only well (12.3% [4.6-21.2] vs. 23.8% [5.8-45.5] respectively, p=0.02). However, DC-SIGN

expression increased in the PPD+IL-4 T_{eff} intervention at 100ng/ml hrIL-4 (16.8% [5.8-21.5], p=0.02; Figure 4d).

Neutralization of IL-4

The effect of neutralizing rIL-4 was determined in TB patients (n=6). Addition of anti-IL-4 antibody (20μg/ml) to the PPD+IL-4 T_{eff} intervention at 20ng/ml reduced the CFU/ml to levels similar to the PPD T_{eff} control ($76.5x10^3$ to $32.4x10^3$ CFU/ml, p=0.03; Figure 5a). Consequently, the % *M.tb* containment increased following the addition of anti-IL-4 antibodies (-20.0% to 60.0%; p=0.03; Figure 5b).

In terms of biomarker expression, anti-IL-4 (n=6; 20μg/ml) antibody was tested in the PPD+IL-4 T_{eff} intervention at 100ng/ml hrIL-4 only (limitations in sample amount prevented testing of 20 ng/ml hrIL4). Following the addition of antibody, Treg frequency (0.8% [0.8-1.8] to 0.2% [0.2-0.8], p=0.03; Figure 5c) and CD4$^+$IFN-γ$^+$ expression (2.0% [1.4-2.5] to 2.9% [2.1-5.2], p=0.03; Figure 5d) reverted to levels similar to that of the PPD T_{eff} control. No significant effect on TNFα and DC-SIGN expression were observed (data not shown).

The addition of anti-IL-4R antibody produced a similar pattern on mycobacterial containment (n=2) Treg frequency (n=4) and CD4$^+$IFN-γ$^+$ expression (n=4) but no significant differences were observed when compared to wells with no anti-IL-4R antibody added (Figure E12 in the online supplement).

DISCUSSION

The key findings of this study were that: (i) in blood, but not BAL, TB patients exhibited higher IL-4 mRNA expression and a higher IL4/IFN-γ ratio compared to LTBI controls; (ii) overall, IL-4δ2 expression levels were very low; (iii) responses were compartmentalized (higher IFN-γ, lower IL-4, and a lower IL4/IFN-γ ratio in the lungs versus blood) in both TB patients and LTBI controls; (iv) hrIL-4 can subvert *M.tb* containment in human macrophages, and these effects were concentration-dependent; (v) IL-4-driven mycobacterial containment was associated with an increased Treg frequency, reduced CD4$^+$Th1 cytokine expression, and increased macrophage DC-SIGN expression, and (vi) these effects were reversed upon neutralization of IL-4 using anti-IL-4 antibody.

This is the first study to demonstrate that IL-4 can directly impact mycobacterial containment in *M.tb*-infected human macrophages. What could be driving a Th2 response leading to disease progression? Evidence suggests that both the pathogen and environmental factors may be involved [28]. For example, certain bacterial components, such as ManLAM, can stimulate IL-4 production [29] and exposure to environmental mycobacteria or helminths can also drive/facilitate a mixed Th2/Th1 response [11, 30]. The latter, which is common in developing countries, is thought to contribute to the failure of BCG in these areas [11].

What are the implications of these findings? Our data suggest that vaccines, whether prophylactic or therapeutic, should be designed to include antigens that not only induce Th1 immunity but also downregulate Th2 or immunoregulatory responses (for example, *M. vaccae* [31] and *hps65* DNA [32] both induce strong Th1 and cytotoxic T-cell responses, but simultaneously downregulate a Th2 response, and have shown some therapeutic efficacy in

preclinical studies [33] and are now being evaluated in a phase 3 trial [34]). Host-directed therapies also exhibit tremendous potential, either to shorten treatment regimens or expand the limited treatment options available for those with highly drug-resistant TB [2, 35]. Interestingly, dupilimab, a monoclonal antibody against the IL-4-receptor-α chain, which has been approved for use in atopic dermatitis [36], is currently being evaluated for the treatment of persistent asthma [37]. It is intriguing to speculate that such an agent might have utility as an immunotherapeutic agent in TB; further *in vitro* studies are warranted.

Our findings of increased IL-4 in the peripheral blood of TB patients are consistent with other reports [6, 14, 22, 38]. Conversely, some studies failed to show differences in IL-4 levels [23, 24]. Measuring IL-4 can be challenging (reviewed in [20]) and many studies failed to distinguish between IL-4 and IL-4δ2, which can have significant effects on study conclusions [22]). This is the first study to investigate IL-4δ2 levels in both the lung and peripheral blood compartments in a TB-endemic setting. Observations on the protective effect of IL-4δ2, in relation to IL-4, in TB have been discussed elsewhere [11]. Previous studies focused on peripheral blood [14, 22, 38] and only one UK-based study measured compartmental differences in IL-4δ2 expression [6]. These studies found either increased levels [6] or no difference in expression between patients and controls [14, 38]. Our results are consistent with the latter. Low IL4δ2 expression observed in our and other studies is not surprising given the decreased stability of IL-4δ2 mRNA [39] and that splice variant expression can be as little as 15% of the parent cytokine [40]. Evidence suggests that IL-4δ2 protein is antagonistic to IL-4 and acts like a Th1 cytokine in *in vitro* culture [41] but further mechanistic studies are required to elucidate its exact role during TB infection.

Measurement of TB antigen-specific Th2 protein (IL-4 and IL-13) levels in cell culture supernatants revealed no differences in expression between the different compartments or study groups (Figures E6 and E7 in the online supplement). IL-4 protein levels were mostly below the detection limit of the assay which highlights the difficulties in measuring this low expressing cytokine. Like IL-4, IL-13 has been implicated in the development of TB-associated lung pathology in both mice and humans [18, 19]. Although IL-13 and IL-4 share common receptors and signaling pathways, they can have distinct expression profiles and functions in Th2-driven conditions [42], but whether this is also true in TB requires further investigation. This lack of concordance between IL-4 mRNA and IL-13 protein levels observed in our study could be due to these intrinsic differences in expression [42], differences in the abundance and profile of mRNA transcript expression compared to protein secretion [43], and/or recall responses to TB-antigen stimulation compared to direct ex vivo measurement. "

The use of IL-4 and/or IL4δ2 as a biomarker of TB progression or treatment response may have some diagnostic utility but will require large validation studies. However, several technical challenges related to the biological properties of IL-4 and IL4δ2 are likely to hinder the development of an antibody-based diagnostic assay. These include sub-ELISA expression levels ([44, 45] and Figure E6), rapid degradation [46] and reduced bioavailability [47, 48] of IL-4 protein, and lack of commercial antibodies that can distinguish between the two isoforms. Furthermore, detection of the IL4δ2 protein has only been described in asthma [49] but its mechanism of action and precise function remains unclear.

The differential expression of IFN-γ and IL-4 in the lungs compared to peripheral blood suggests a compartment-specific pattern of cytokine expression and cellular trafficking, which may reflect the active recruitment and clonal expansion of IFN-γ-producing T-cells at the site of disease [50] subsequently allowing for a greater expansion of IL-4-producing Th2 cells in the peripheral blood.

In TB patients, a ~50% reduction in *M.tb* survival in infected MDMs by PPD pre-primed effectors (PPD T$_{eff}$ control) is similar to the magnitude of containment previously described in the context of Treg effects [25]. Addition of IL-4 lead to a reduction in *M.tb* containment by 50-120%. The addition of IL-4 to un-primed T-cells before commitment to a specific T-cell lineage, creates a Th2-polarizing microenvironment where IFN-γ expression and Th1 differentiation pathways are inhibited [51]. The IL-4-concentration-dependent effect observed in this intervention suggests that there is some level of competition between PPD-driven Th1 and IL-4-driven Th2 polarization.

A similar reduction in mycobacterial containment was also observed in LTBI participants but this failed to reach statistical significance (p=0.06) likely because there were fewer sample numbers in this group. Furthermore, no differences were observed when TB and LTBI groups were directly compared (Table E5). This suggests that the effect of IL-4 in our model was not restricted to any specific clinical phenotype and similar cellular mechanisms were induced by IL-4 in both groups leading to the observed reduction in containment.

Our data indicate simultaneous expansion of the Treg population and subsequent downregulation of a Th1 response. While IL-4 has been implicated in the development [52] and maintenance of Tregs [53], the data are conflicting and this relationship has never been demonstrated in the context of human TB. There is substantial evidence on the detrimental role of Tregs in drug-sensitive TB [25, 54-56], drug-resistant TB [26, 57], and TB/HIV co-infection [58], including attenuation of mycobacterial containment in vitro [25, 26], and downregulating Th1 responses [25, 56]. There is substantial evidence on the detrimental role of Tregs in TB [25, 26, 56] including attenuation of mycobacterial containment *in vitro* [25, 26], and downregulating Th1 responses [25, 56]. The Treg-mediated effect on Th1 responses in our model seem to be restricted to the CD4 T-cell population (the most important IFN-γ producing cells involved in controlling *M.tb* replication [59]). Although producing very little IL-10 (Table E6), these Tregs may exert their regulatory effects by other cellular mechanisms (reviewed in [60]). Further investigation is required to elucidate these mechanisms.

DC-SIGN expression on macrophages was also increased in the PPD+IL-4 T_{eff} intervention. DC-SIGN facilitates entry of *M.tb* into phagocytic cells and is a hallmark of alternatively-activated M2 macrophages. A number of *M.tb* components that act via DC-SIGN can drive host immunoregulatory mechanisms [61]. In our model, M2 polarization is likely occurring as both IL-4 [11] and FoxP3$^+$ Tregs [62] can drive alternative macrophage activation. M2 macrophages also have anti-mycobactercidal properties [11] and may be contributing to the observed reduction in *M.tb* containment.

Preliminary experiments indicate that blocking of the IL-4 receptor resulted in a similar effect to neutralization of IL-4 on mycobacterial containment, Treg frequency and IFN-γ expression although no significant differences were observed likely due to the limited sample numbers. Previous evidence indicates that IL4Rα-mediated signalling is associated with tissue pathology in TB-infected mice [18] and the development of necrotizing granulomas in human TB [19]. However, further investigations are needed to determine the precise downstream mechanisms that may be driving this effect.

There are a number of limitations to our study: (i) The hrIL-4 concentrations used in the *M.tb* containment assays may, in some cases, have been higher than that encountered physiologically in human tissue (as low as picogram levels). However, there are a number of factors overestimating the concentration of active recombinant IL-4 protein in experimental conditions (for example, (a) recombinant proteins tend to be less stable and active compared to their naturally produced counterparts [63], (b) mechanisms such as chaperone proteins [64] and soluble IL-4 receptors [65] can prolong stability and increase IL-4 bioactivity but are normally absent in an *in vitro* system, (c) proteins expressed in insect cell systems may undergo inappropriate N-glycosylation patterns which can affect protein function and stability [64], and (d) quantification methods, including the Bradford assay, fail to distinguish between functional and non-functional recombinant protein). Moreover, the rIL-4 concentrations used in our *M.tb* containment assays were derived from those used in the ^{3}H-thymidine T-cell proliferation assay (Figure E9C) that produced a significant effect, and other *in vitro* stimulation experiments have used similar IL-4 concentrations [66]. (ii) We did not use alveolar macrophages in the stasis/ containment experiments. It was difficult to obtain sufficient BAL cell numbers to perform all the required experiments given the safety and logistical limitations of the bronchoscopy

procedure (~200 ml of instilled saline), and similar outcomes were obtained either using MDMs or alveolar macrophages in another *M.tb* containment assay [25] (iii) We only performed preliminary functional experiments to determine the effect of IL-4R on *M.tb* containment and selected cellular mechanisms and did not specifically target mechanisms within the signalling pathway. The aim of the study was to determine the ultimate effect of IL-4 on mechanisms known to be associated with TB, including Tregs, the Th1 response and alternative macrophage activation (discussed in [11]). Future studies are required to probe the effects of blocking specific molecules within the IL-4 signalling pathway such as STAT-6, IRS-1 and GATA-3 to determine their effects on *M.tb* containment. (iv) We were only able to measure mRNA expression levels in a limited number of matched BAL and blood samples from TB patients (n=5) and LTBI controls (n=4) either because blood was not collected prior to the bronchoscopy procedure, or samples were excluded due to poor RNA quality or low yield. However, the matched samples (Figure E4 in the online supplement) also showed a compartment-specific effect similar to the complete data set outlined in Figure 1 (characterized by elevated IFN-γ levels were in the lungs whereas IL-4 levels were increased in the peripheral blood).

In conclusion, IL-4 subverted anti-mycobactericidal mechanisms and undermined *M.tb* containment in infected monocyte-derived-macrophages. These data inform the development of vaccines and immunotherapeutic interventions against *M.tb*.

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FIGURES LEGENDS

Figure 1. mRNA expression levels of a) IFN-γ b) IL-4 and c) IL-4δ2 and cytokine expression ratios of d) IL-4\IFN-γ and e) IL-4\IL-4δ2 in cells of broncho-alveolar lavage (BAL) and peripheral whole blood (Blood) from patients with pulmonary tuberculosis (TB; BAL n=8; blood n=23) and presumed latently infected controls (LTBI; BAL=7; blood n=22) measured using a validated quantitative real-time PCR assay. Data is shown on a $\log_{10}$ scale and copy numbers are expressed per million copies of HuPO (validated reference gene). Statistical analyses between groups were performed using the Mann-Whitney test and $p<0.05$ was deemed significant.

Figure 2. The effect of human recombinant IL-4 on mycobacterial containment in infected monocyte-derived macrophages (MDMs) from pulmonary tuberculosis patients (TB; n=8)). A mycobacterial containment assay was performed where H37Rv-infected MDMs were cultured by themselves or co-cultured with peripheral blood mononuclear cells pre-primed with purified protein derivation alone (PPD T_{eff}), or PPD and hrIL-4 (PPD+IL-4 T_{eff})at concentrations of 5, 20 and 100ng/ml hrIL-4. Results are expressed as **a)** median colony forming units (CFU/ml) in TB patients and **b)** The % mycobacterial (*M.tb*) containment, defined as the change in *M.tb* survival compared to the "H37Rv-infected MDMs only" control, were also determined. Increased % *M.tb* containment indicates a reduction in *M.tb* survival whereas decreased % *M.tb* containment indicates an increase in *M.tb* survival. Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test and $p<0.05$ was deemed significant.

Figure 3. The effect of human recombinant IL-4 on regulatory T-cell (Treg) expression in a mycobacterial containment co-culture assay as measured by flow cytometry. a) The flow cytometry gating strategy for identification of Tregs (CD3^{+}CD4^{+}CD25^{+}FoxP3^{+}) within the lymphocyte population and **b)** the frequency (%) of Tregs in the co-cultured lymphocyte population of the mycobacterial

containment assay in pulmonary TB patients. H37Rv-infected MDMs were co-cultured with peripheral blood mononuclear cells pre-primed with PPD alone (PPD T_{eff}), or PPD and hrIL-4 (PPD+IL-4 T_{eff}) at concentrations of 20ng/ml (n=10) and 100ng/ml (n=16) hrIL-4. Tregs were expressed as a % of CD4$^+$ lymphocytes. Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test and p<0.05 was deemed significant.

Figure 4. The effect of human recombinant IL-4 on Th1 cytokine expression in lymphocytes and DC-SIGN expression in macrophages in a mycobacterial containment assay as measured by flow cytometry. a) The flow cytometry gating strategy for identifying IFN-γ and TNFα within the lymphocyte (CD4$^+$ and CD8$^+$) population and DC-SIGN expression within the macrophage (CD14$^+$CD16$^+$) population. The % of CD4$^+$ and CD8$^+$ lymphocytes expressing **b)** IFN-γ, **c)** TNFα and CD14$^+$CD16$^+$ macrophages expressing **d)** DC-SIGN in cells harvested from a mycobacterial containment assay from pulmonary TB patients. H37Rv-infected MDMs were co-cultured with peripheral blood mononuclear cells pre-primed with PPD alone (PPD T_{eff}), or PPD and hrIL-4 (PPD+IL-4 T_{eff}) at concentrations of 20ng/ml (n=10) and 100ng/ml (n=16) hrIL-4. Statistical analyses were performed using the Wilcoxon matched-pairs signed rank test between interventions and the Mann Whitney U test between CD4+ and CD8+ lymphocytes. A p-value<0.05 was deemed significant.

Figure 5. The effect of neutralizing human recombinant IL-4 on mycobacterial containment and on cytokine and regulatory T-cell expression in a mycobacterial containment assay. H37Rv-infected MDMs were co-cultured with peripheral blood mononuclear cells pre-primed with PPD alone (PPD T_{eff}) or PPD and hrIL-4 (PPD+IL-4 T_{eff}) at 20 and 100ng/ml hrIL-4. To the IL-4 interventions, anti-IL4 antibody (20μg/ml) was added at the same time as hrIL-4 (day 1). In TB patients (n=6), mycobacterial containment was measured in terms of **a)** colony forming unit/ml (CFU/ml) and **b)** % mycobacterial (*M.tb*) containment, defined as the change in *M.tb* survival compared to the "H37Rv-infected MDMs only" control. Increased % *M.tb* containment indicates a reduction *M.tb* survival whereas decreased %

M.tb containment indicates an increase in *M.tb* survival. The % of CD4$^+$ lymphocytes that were **c)** Treg (CD25$^+$FoxP3$^+$) and expressing **d)** IFN-γ were also determined within the system (cells co-cultured within the *M.tb* containment assay) by flow cytometry. In this case, IL-4 neutralisation was assessed using anti-IL-4 (n=6). Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test and p<0.05 was deemed significant.

a)

IFNγ copies (per 10^6 HuPO copies)

**p=0.005
*p=0.02

BAL — TB, LTBI
Blood — TB, LTBI

b)

IL-4 copies (per 10^6 HuPO copies)

**p=0.005
*p=0.02
**p=0.003

BAL — TB, LTBI
Blood — TB, LTBI

c)

IL-452 copies (per 10^6 HuPO copies)

*p=0.04

BAL — TB, LTBI
Blood — TB, LTBI

d)

IL-4:IFNγ ratio

****p<0.0001
****p<0.0001
*p=0.01

BAL — TB, LTBI
Blood — TB, LTBI

e)

IL-4:IL-452 ratio

BAL — TB, LTBI
Blood — TB, LTBI

FIGURE 1

FIGURE 2

a)

**p=0.008
**p=0.008
**p=0.008
**p=0.008
**p=0.008

x 10³ colony foring units/ml

H37*Rv* infected MDMs	+	+	+	+	+
PPD T_eff	-	+	-	-	-
PPD+IL-4 T_eff (ng/ml of hrIL4)	-	-	5	20	100

b)

**p=0.008
*p=0.015
**p=0.008
**p=0.008

% Mycobacterial containment
(D CFU/ml relative to MDM only control)

Increased *M. tb* containment

MDM only control baseline

Reduced *M. tb* containment

H37Rv infected MDMs	+	+	+	+
PPD T_eff	+	-	-	-
PPD+IL-4 T_eff (ng/ml of hrIL4)	-	5	20	100

FIGURE 3

a)

b)

H37Rv infected MDMs	+	+	+
PPD T$_{eff}$	+	-	-
PPD+IL-4 T$_{eff}$ (ng/ml of hrIL4)	-	20	100

FIGURE 4

FIGURE 5

a)

x10² colony forming units/ml

*p=0.03
*p=0.03

H37Rv infected MDMs	+	+	+	+	+	+
PPD T_eff	-	+	-	-	-	-
PPD+IL-4 T_eff (ng/ml of hrIL4)	-	-	20	20	100	100
Anti-IL-4 antibody (μg/ml)	-	-	-	20	-	20

b)

% Mycobacterial containment
(D CFU/ml relative to MDM only control)

Increased *M. tb* containment
MDM only control baseline
Reduced *M. tb* containment

*p=0.03
*p=0.03

H37Rv infected MDMs	+	+	+	+	+	+
PPD T_eff	+	-	-	-	-	-
PPD+IL-4 T_eff (ng/ml of hrIL4)	-	20	-	20	100	-
Anti-IL-4 antibody (μg/ml)	-	-	20	20	-	20

c)

% CD4 that are Tregs (CD25+FoxP3+)

*p=0.03
*p=0.03

PPD T_eff	+	-	-
PPD+IL-4 T_eff (ng/ml of hrIL4)	-	100	100
Anti-IL-4 antibody (μg/ml)	-	-	20

d)

% CD4 cells expressing IFNγ

*p=0.03
*p=0.03

PPD T_eff	+	-	-
PPD+IL-4 T_eff (ng/ml of hrIL4)	-	100	100
Anti-IL-4 antibody (μg/ml)	-	-	20

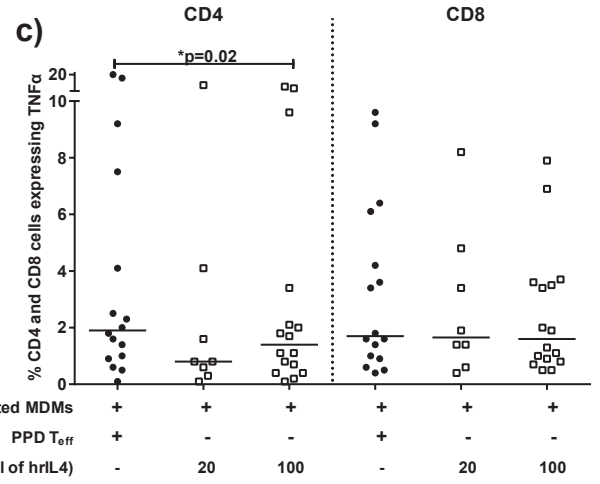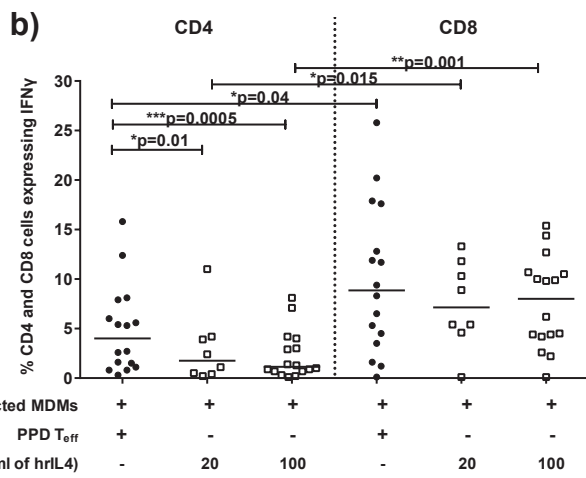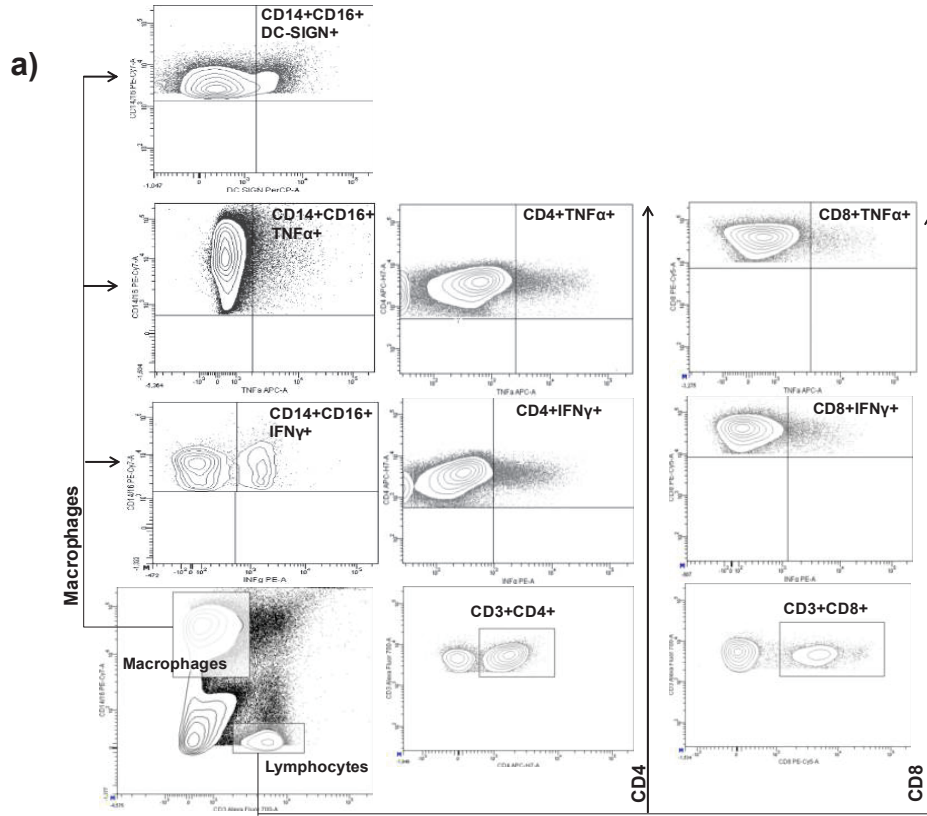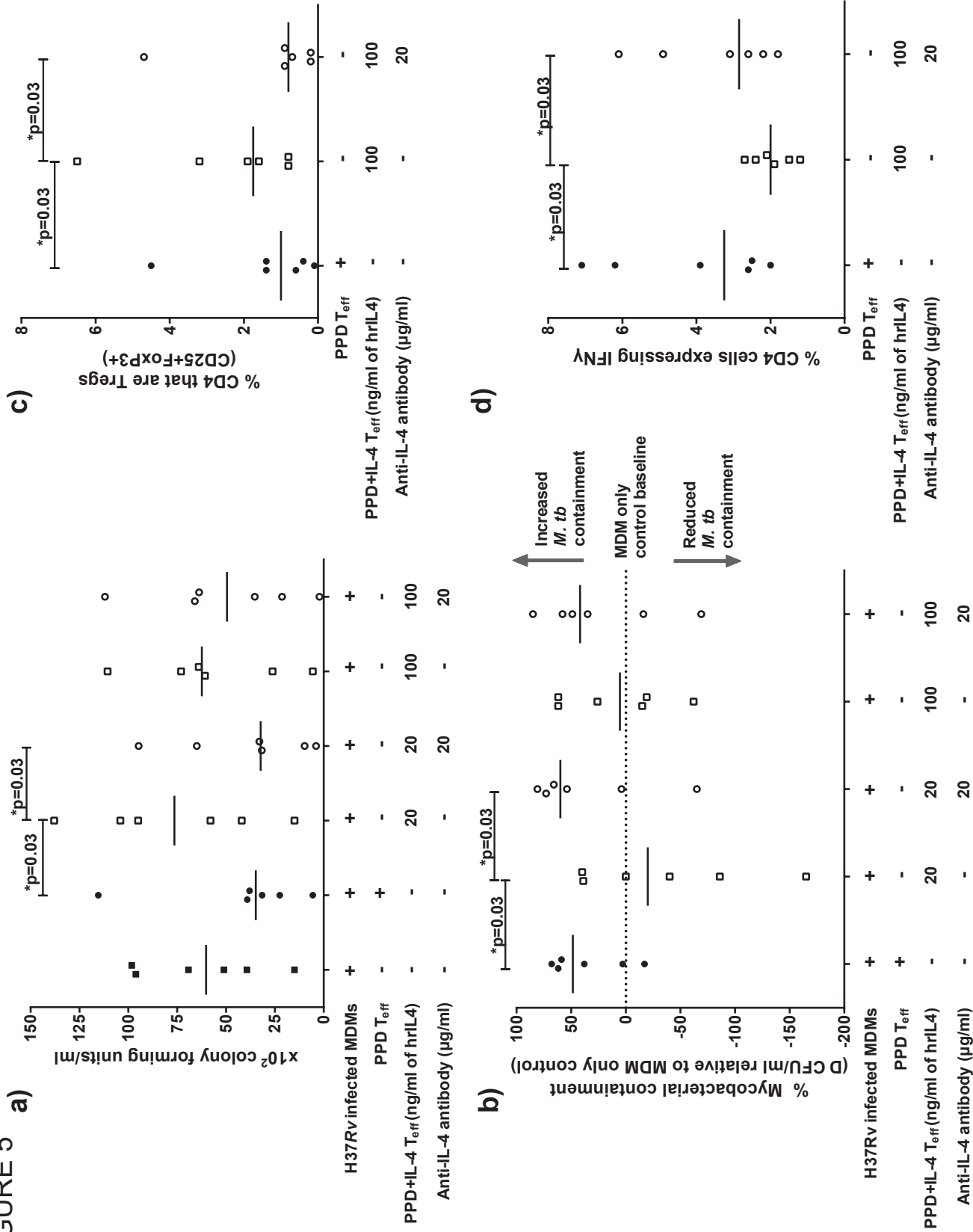**IL-4 subverts mycobacterial containment in *M. tuberculosis*-infected human macrophages**

ONLINE DATA SUPPLEMENT

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METHODS

Quantitative real-time polymerase chain reaction (qPCR)

Choice of primers, hydrolysis probes and amplification targets used for quantitative PCR

The primer and hydrolysis probes sequences were taken from published sequences in the literature (1,2) and are shown in Table E1. The primers were designed to span exon-exon boundaries so as not to amplify genomic DNA and thus increase assay specificity. Furthermore, the design of intro-spanning primers was an absolute requirement to differentiate between the IL-4 and IL-4δ2 transcripts. Primers and probes were synthesized by IDT (Integrated DNA Technologies Ltd.) on a scale of 25nM and 100nM, respectively. Primers were purified by standard desalting whereas probes underwent HPLC purification. A fluorophore (FAM) and two fluorescent quencher (FQ) molecules (an internal ZEN FQ and IOWA BLACK FQ at 3'-end) were incorporated into the hydrolysis probes. Primer sequence specificity was confirmed by melt curve analysis on the Rotor-Gene Q (Qiagen) using the Rotor-Gene SYBER Green PCR kit (Qiagen) assay, according to the manufacturer's instructions.

RNA extraction and assessment of RNA quality

RNA was extracted from whole blood and BAL cells using the PreAnalytiX PAXgene Blood RNA kit (Qiagen) and RNeasy Plus Mini Kit (Qiagen), respectively. Genomic DNA was removed in each case by DNase I treatment and use of a gDNA eliminator column, respectively. All standard protocols for cleaning, handling and storage during RNA isolation procedures were strictly followed. Quality and quantity assessment of isolated RNA was performed on an Agilent Bioanalyzer 2100 (Agilent) using the Agilent RNA 6000 Nano kit. Total RNA obtained ranged

from 0.5-5μg. Samples were only used in reverse transcription reactions if an RNA Integrity Number (RIN) of ≥7.5 was obtained.

Reverse transcription and qPCR amplification

The iScript cDNA synthesis kit (BioRad) was used to convert RNA samples to cDNA. The kit uses a modified MMLV-derived RNase H+ transcriptase. RNA quantity limited the amount of input RNA to 500ng per reaction. A 20μl reaction was set up containing 500ng of sample RNA, reverse transcriptase, nuclease free water and iScript mastermix (containing a proprietary mixture of oligo (dT), random hexamers and RNase inhibitor). The reaction was performed in a G-Storm thermal cycler (G-Storm) using following conditions: 5 minutes at 25ºC; 30 minutes at 42ºC; 5 minutes at 85ºC. Appropriate control reactions containing no reverse transcriptase enzyme were also included.

Amplification of cDNA was performed on a Rotor-Gene Q real-time PCR machine (Qiagen). Reactions were set up manually using the Rotor-Gene probe PCR kit. The 2x Rotor-Gene probe PCR Master Mix contained HotStar Taq *Plus* DNA Polymerase, dNTP mix and Rotor-Gene Probe PCR buffer (containing a proprietary mixture of Tris-Cl, KCl, NH_4Cl, $MgCl_2$ and Q-Bond). Primers were used at pre-validated concentrations of 500nM for HuPO and IFN-γ and 250nM for IL-4 and IL-4δ2. Probes were used at concentrations of 250nM for all analytes, as previously described (1). 2μl of sample cDNA and 5μl of 10-fold serial dilutions of linearized plasmid standards (described below) were added to the appropriate sample and standard tubes, respectively. The final PCR reaction volume was 20μl. The amplification conditions were as follows: initial step of 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds and

60°C for 30 seconds. Standards were run in triplicate and samples were run in duplicate. Appropriate no template controls (NTC) and no reverse transcriptase controls were includes in each qPCR run.

Method of mRNA quantification

Absolute quantification, where the mRNA copy number in a sample is expressed in relation to a standard curve containing known numbers of nucleic acid copies, was chosen as the method of mRNA quantification. It allows for greater control of inter-assay variation in PCR efficiency and experimental reproducibility. This is beneficial especially when large sample numbers are assayed over a period of time from different study sites (3). As such, an external standard curve using recombinant DNA inserted into a plasmid was chosen because of its long term stability, high reproducibility and its ability to more appropriately mimic the length of native mRNA. Furthermore, it shows higher sensitivity compared to recombinant RNA standards (4).

Generation of cDNA targets of interest

Peripheral blood mononuclear cells (PBMCs) from a healthy control were isolated and $1x10^6$ cells were stimulated for 16 hours with 10μg/ml of phytohaemmaglutinin (PHA) in a 96-well round bottom plate. Total RNA was extracted and reverse transcribed using 1μg of RNA. cDNA was amplified using the Kappa Taq PCR kit (Kappa Biosystems) and cloning primers (Table E2) according to the manufacturer's instructions. The cloning primers were designed to amplify a region of the cDNA containing the target qPCR sequence for IFN-γ and HuPO. The PCR reaction conditions were as follows: initial step of 95°C for 2 minutes, followed by 30 cycles of 95°C for 1 minute 30 seconds, 50°C for 1 minute, 72°C for 1 minute and a final extension step of

72°C for 10 minutes. The amplified PCR product was run on a 1% Agarose gel at 200V for 1 hour. The bands of interest were excised and purified using Zymoclean Gel DNA Recovery Kit (Zymo Research). pGEM-T Easy (Promega) plasmids containing IL-4 and IL-4δ2 cDNA were generated during cloning procedures to express recombinant IL-4 protein (described below).

Sub-cloning of cDNA into pGEM-T Easy plasmid

Purified cDNA was ligated into the pGEM-T Easy plasmid (Promega) at a vector:insert ratio of 3:1, transformed in JM109 chemically competent *E. coli* cells (Promega) and purified using the GeneJet Plasmid Miniprep kit (Thermo Scientific), according to the manufacturer's instructions. Plasmids were sequenced by Inqaba Biotech Ltd using an ABI 3500XL DNA sequencer to confirm the successful insertion of the target DNA into the plasmid.

Linearization and serial dilutions of the plasmid standards

The HuPO, IFN-γ, IL-4 and IL-4δ2 plasmids were linearized using *SacI* FastDigest enzyme (Thermo Scientific), according to the manufacturer's instructions. The plasmids were linearized because circular plasmid standards tend to overestimate copy numbers and reduce PCR efficiency (5). Each plasmid was identified to have a single *SacI* restriction site outside the target cDNA sequence. Linearized plasmid concentrations were re-measured and the exact copy number per tube was determined using the following formula:

$$\left(\frac{\text{Amount of plasmid (ng) x Avogaro's constant}}{\text{length of plasmid x } 1*10^9 \text{ x } 655} \right) = \textbf{copy number}$$

10-fold serial dilutions containing down to 10^1 copies were made in nuclease-free H_2O and added to appropriate PCR tubes (Qiagen) for qPCR amplification reactions.

Data Analysis and Normalization strategy

Data analysis was performed using the Rotor-Gene Q series software 2.0 (Qiagen). The cycle threshold (Ct) was automatically determined by the software for each qPCR run. Samples that were not reproducible (technical replicates with variations in Ct values >0.5 were considered outliers) or that were below the detection limit of the assay were given an arbitrary value of 1 copy in the final analysis, as previously described (1). Copy numbers were normalized using human ribosomal protein (HuPO) as a reference gene. The suitability of HuPO as a reference gene in pulmonary tuberculosis gene expression studies has been pre-validated (6) and used in similar study settings(2,7). Gene of interest copy numbers are expressed per million copies of HuPO. Coefficient of variance was used to assess inter- and intra-assay variation.

Soluble protein levels of IL-4 and IL-13 in TB antigen driven cell culture supernatants

BAL cells and PBMCs were seeded in 96-well round bottom plates at a concentration of $1x10^6$ /ml and stimulated with the *M.tb*-specific antigens ESAT-6 and CFP-10 (Oxford Immunotec) using manufacturer recommended concentrations (which are proprietary and cannot be disclosed) for 5 days. Supernatants were harvested and measured by ELISA (IL-4; eBiosciences) or Luminex multiplex assay (IL-13; Millipore) according to the manufacturer's instructions.

Expression and purification of human recombinant IL-4 (hrIL-4)

Human recombinant IL-4 (hrIL-4) was expressed in a baculovirus insect cell system, using a method described in (8). Full length human IL-4 cDNA (Accession number: NM_000589.2) was purchased from Origene (Origene Technologies) and subsequently cloned into an in-house baculovirus transfer vector (8) designated pAP01 (sequence provided at the end of the online supplement) using *BamH1* and *AvrII* restriction enzymes (Thermo Scientific). A decahistidine (His) tag was inserted downstream of the IL-4 sequence using specific primers to facilitate protein purification. The IL-4-His cDNA from the newly designated pAP01-IL4-His vector construct was transferred to linearized bacmid viral DNA (pBAC10:KO$_{1629}$) by co-transfection into *Spodoptera frugiperda* (*Sf*21) cells using lipofectin (Sigma-Aldrich). Virus-containing cell supernatants were used in several rounds of viral amplification to generate high-titre viral stocks (10^7-10^8 plaque forming units/ml), as measured by quantitative real-time PCR, for large-volume protein expression. Initial small-scale expression studies were performed to determine the optimal multiplicity of infection (MOI) and the time-to-harvest post-infection (PI). Cell morphology and viability were periodically assessed by trypan blue exclusion staining and examination under a microscope to confirm infection of cells. Large-scale IL-4 expression was performed at 28^oC in a 500ml culture of *Sf*21 cells (1.5×10^6/ml in Insect Xpress media (Lonza) supplemented with 2% fetal bovine serum (Lonza)) infected at an MOI of 10 and harvested 72 hours PI. hrIL-4 was purified from the virus-infected cell culture supernatants, supplemented with 1% Benzonase (Novagen) and 1x EDTA-free protease inhibitor cocktail (Roche), by Ni^{2+}-NTA affinity purification using the His spin miniprep kit (Zymo Research). Protein identity was determined by sodium-dodecyl-sulfate-polyacrylamide-gel-electrophoresis (SDS-PAGE) and Western blot using IL-4-specific HRP-conjugated antibody (Abcam). Protein concentration was determined by Bradford assay (Sigma-Aldrich).

Assessment of hrIL-4 bioactivity

T-cell proliferation

The effect of hrIL-4 on T-cell proliferation was assessed by ^{3}H thymidine incorporation assay. 5×10^4 PBMCs were stimulated with anti-CD3 antibody (eBiosciences) for 3 days at 37°C and 5% CO_2, followed by the addition of hrIL-4 (5, 20 and 100ng/ml) alone or together with anti-IL4 antibody (0.5, 5 and 20μg/ml; Abcam). Cells were incubated for a further 3 days followed by the addition of 1μCi of ^{3}H-Thymidine (AEC Amersham) to each well for 18 hours. The cells were harvested and counted by liquid scintillation spectrophotometry. The mean counts per minute (CPM) for each condition was used to calculate the % proliferation, as shown below:

$$\left[\frac{\text{Mean CPM of hrIL4 intervention - Mean CPM of negative control}}{\text{Mean CPM of negative control}} \times 100 \right] = \textbf{\% Proliferation}$$

CD23 expression in B cells

Flow cytometry was used to determine the effect of hrIL-4 on CD23 expression in B cells. PBMCs at 1.5×10^6 per well in a 24-well plate were stimulated with various concentrations of hrIL-4 (0.5-50ng/ml) for 3 days at 37°C and 5% CO_2. Cells were harvested and stained with fluorescently labeled antibodies against CD3, CD19 and CD23 (eBiosciences). Cells were acquired on an LSRII flow cytometer and analysed using FACSDiva software (BD Biosciences).

Mycobacterial containment assay

Preparation of H37Rv stocks

H37*Rv* (donated by State Laboratory) was grown in Middlebrook 7H9 broth (Difco) supplemented with 10 % OADC (oleate-albumin-dextrose-catalase) enrichment media (BD Biosciences) and 0.02% glycerol (Merck). Cultures were grown in a tissue culture flask (Corning) at 37°C and 5% CO_2 in a humidified incubator and shaken daily. Growth was assessed periodically until cultures reached mid-log phase. 1ml aliquots of culture were then snap frozen in 10% glycerol using liquid nitrogen and stored at -80°C.

Colony forming units (CFU)/ml, were determined using three randomly selected frozen H37Rv tubes. The bacterial suspension was homogenised by aspiration using a 1ml insulin syringe. 10-fold serial dilutions were made from the stock and the three lowest dilutions (10^2, 10^1, 10^0 CFUs) were plated in replicates of 6 onto Middlebrook 7H10 gridded agar plates (BD Biosciences) supplemented with 10% OADC. Plates were incubated in a 37°C oven for 10-16 days or until visible colonies were formed. Colonies were counted using an inverted microscope and the CFU/ml for each tube was calculated using the flowing formula:

$$\left(\begin{array}{ccc} \text{Number of colonies} \\ \text{(Avg of 6 replicates)} \end{array} \times 100 \times \begin{array}{c} \text{Dilution} \\ \text{factor} \end{array} \right) = \text{CFU/ml}$$

Immunofluorescence staining

Cells were harvested into 15x75mm 5ml polystyrene tubes (BD Biosciences), washed in 2ml FACS buffer (0.01% sodium azide, 10% human AB serum, 1xPBS) and centrifuged at 1200rpm

for 5 minutes. The supernatants were removed and the cell pellet was resuspended in 100ul of

FACS buffer. Appropriate fluorescently conjugated antibodies for cell surface markers were

added at previously titrated volumes to the cells and incubated in the dark for 15 minutes at room

temperature. Cells were then washed to remove unbound antibodies and fixed in 100µl of BD

Cell Fix diluted 1:10 in H_2O (BD Biosciences) for 15 minutes in the dark. After another wash

step, cells were permeabilized in 500ul PERM II solution diluted 1:10 (BD Biosciences) for 10

minutes in the dark. Fixation and permeabilization was performed using Human FoxP3 buffer set

(BD Biosciences) for Treg staining. Appropriate fluorescently conjugated antibodies for

intracellular markers was added to each tube at previously titrated volumes and incubated for 30

minutes in the dark. The cells were then washed to remove unbound antibodies and 100ul of 4%

paraformaldehyde solution was added to each tube for at least 30 minutes.

RESULTS

Validation of qPCR assay

Primer specificity was confirmed by melt curve analysis (Figure E1). qPCR assay validation was

performed on plasmid standards prior to analysis of samples. The limit of detection of the assay

was determined to be 10 copies as the replicate Ct values below 10 copies were very variable

(difference in Ct replicates >0.5). In most experiments, PCR efficiency approached 100% and R^2

value was >0.99. No transcripts were detectable in the 'no template' controls (NTC) or in the 'no

reverse transcriptase' controls (no RT controls) indicating a lack of genomic DNA

contamination. The coefficient of variance between technical replicates within a single assay

(intra-assay variation) was <~20% at each concentration. Figure E2 shows an example of IL-4

copy number determination by absolute quantification in a validated qPCR assay. PCR

efficiency, copy numbers and Ct values were measured in 5 separate experiments and compared in order to assess inter-assay variation. The mean PCR efficiency was 100% (95% CI; 96.1-103.9) and the coefficient of variance at each dilution was <10% (Figure E3A). Inter-assay variations in Ct values are shown in figure E3B.

Expression, identify confirmation and bioactivity of hrIL-4

The full nucleotide sequence of the transfer vector pAP01-IL4-His containing IL-4 used for co-transfection with bacmid DNA is shown in figure E4. The presence of the IL-4-His cDNA construct following transfection was determined by PCR amplification of P_0 viral DNA and visualization of the amplified inserts by agarose gel electrophoresis. In the large-volume protein expression harvested at 3 days PI, cells were elongated and enlarged at ~50-60% viability indicating viral infection of cells. hrIL-4 protein yield from a 500ml culture ranged between 60-120μg. Analysis of the hrIL-4 protein purification fractions by SDS-PAGE indicated the presence of 2 bands at ~19kDa and ~17kDa in the purified and concentrated fraction (Figure E5A). The expected size of His-tagged rIL-4 is ~17-18kDa and these two bands could represent different glycosylated forms of IL-4, which has also been shown to occur in baculovirus expressed swine rIL-4 (9). The purity in this fraction was ~85-90% as determined by band densitometry using Image J software (lanes 5 of Figure E5A). Expression and purification using empty pAP01 vector (with no IL-4 cDNA inserted) did not produce equivalent bands of this size. Western blot analysis using anti-IL-4 antibodies produced a similarly sized band confirming that this protein was indeed IL-4 (Figure E5B). The expected protein size and reactivity to anti-IL-4 antibody provides sufficient evidence to confirm the identity of hrIL-4.

Bioactivity of hrIL-4 was assessed by its effect on T-cell proliferation and CD23 expression on B-cells. Addition of hrIL-4 induced T-cell proliferation in a dose-dependent manner (179% at 5ng/ml rIL-4 (p=0.03), 398% at 20ng/ml rIL-4 (p=0.002) and 522% at 100ng/ml rIL-4 (p=0.001) compared to the negative control; Figure E5C) while the subsequent addition of neutralizing IL-4 antibody (at 0.5, 5 and 20μg/ml) suppressed this observed hrIL-4-induced proliferation (suppression at 20ng/ml hrIL-4 was 54%, 71% (p=0.04) and 76% (p=0.04); suppression at 100ng/ml was 22%, 62% (p=0.02), 69% (p=0.02), respectively). Similarly, there was a dose-dependent induction of CD23 (a low affinity IgE receptor) expression on B-cells (CD3$^-$CD19$^+$) when hrIL-4 was added at different concentrations. The median CD23 frequency [IQR], expressed as a % of B-cells, was 25.6% [10.7-41.1] at 0.5ng/ml (p=0.005), 50.2% [30.5-64.2] at 5ng/ml (p=0.002) and 64.6 [56.8-69.7] at 50ng/ml of hrIL-4 (p=0.001; Figure E5E).

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TABLES

Table E1: Primer and probe sequences to determine cytokine mRNA levels by quantitative real-time PCR.

Gene of Interest (NCBI Accession numbers)	Sequence ○ Forward primer 5' - 3' ● Reverse primer 5'- 3' ◊ Probe 5'- 6-FAM/ZEN/IOWA BLACK FQ - 3'	Tm (°C)	Product size (bp)
HuPO (NM_001002.3)	○ GCTTCCTGGAGGGTGTCC ● GGACTCGTTTGTACCCGTTG ◊ TGCCAGTGT/ZEN/CTGTCTGCAGATTGG	58.0 55.9 61.3	106
IFN-γ (NM_000619.2)	○ TTCAGCTCTGCATCGTTTTG ● TCCGCTACATCTGAATGACCT ◊ TGGCTGTTA/ZEN/CTGCCAGGACCCA	53.7 55.6 63.0	112
IL-4 (NM_000589.3)	○ GCTGCCTCCAAGAACACAAC ● TGTAGAACTGCCGGAGCAC ◊ AAACCTTCT/ZEN/GCAGGGCTGCGAC	56.8 57.3 62.5	71
IL-4δ2 (NM_172348.2)	○ CCTCACAGAGCAGAAGAACAC ● TGTAGAACTGCCGGAGCAC ◊ AAACCTTCT/ZEN/GCAGGGCTGCGAC	55.4 57.3 62.5	74

Table E2. Cloning primers used to amplify cDNA regions containing the qPCR targets of interest for IFN-γ and HuPO. The amplified regions were used to generate the plasmid standards.

Gene of Interest	Sequence ○ Forward cloning primer 5' - 3' ● Reverse cloning primer 5 '- 3'	Tm (°C)
HuPO (NM_001002.3)	○ TTCGACAATGGCAGCATCTACAACC ● AAGGTGTAATCCGTCTCCACAGAC	59.5 58.3
IFN-γ (NM_000619.2)	○ TTGGCTTAATTCTCTCGGAAACGATG ● AGAGTTCCATTATCCGCTACATCTG	57.3 56.2

Table E3. Details of experimental setup including cell numbers and stimulant concentrations in the mycobacterial containment assay to determine the effect of IL-4 on mycobacterial containment.

		DAY 1						DAY 6
		MDMs	PBMCs	Stimulant				Cells co-cultured with infected MDMs
Condition		cell number	cell number	PPD (μg/ml)	hrIL-4 (ng/ml)	anti-IL-4 Abs (μg/ml)	anti-IL-4R Abs (μg/ml)	
controls	H37Rv infected MDMs only	2×10^5	-	-	-	-	-	-
controls	PPD T_{eff}	2×10^5	2×10^5	12	-	-	-	PPD T_{eff}
interventions	PPD+IL-4 T_{eff}	2×10^5	2×10^5	12	5, 20 & 100	-	-	PPD+IL4 T_{eff}
interventions	IL-4 neutralization	2×10^5	2×10^5	12	20 & 100	20	-	PPD+IL4 T_{eff}
interventions	IL-4 receptor neutralization	2×10^5	2×10^5	12	20 & 100	-	10	PPD+IL4 T_{eff}

Table E4. Details of experimental setup including cell numbers and stimulant concentrations in the mycobacterial containment assay to determine the mechanisms involved in the IL-4 associated modulation of mycobacterial containment by flow cytometry.

	Condition	DAY 1						DAY 6
		MDMs	PBMCs					Cells co-cultured with infected MDMs
				Stimulant				
		cell number	cell number	PPD (μg/ml)	hrIL-4 (ng/ml)	anti-IL-4 Abs (μg/ml)	anti-IL-4R Abs (μg/ml)	
controls	H37Rv infected MDMs only	6×10^5	-	-	-	-	-	-
controls	PPD T$_{eff}$	6×10^5	6×10^5	12	-	-	-	PPD T$_{eff}$
interventions	PPD+IL-4 T$_{eff}$	6×10^5	6×10^5	12	20 & 100	-	-	PPD+IL4 T$_{eff}$
interventions	IL-4 neutralization	6×10^5	6×10^5	12	100	20	-	PPD+IL4 T$_{eff}$
interventions	IL-4 receptor neutralization	6×10^5	6×10^5	12	100	-	10	PPD+IL4 T$_{eff}$

Table E5: Comparison of colony forming units (CFU/ml) and % mycobacterial containment in the control and IL-4 interventions wells in the *M.tb* containment assay. Statistical analysis between the TB and LTBI groups were performed using Mann-Whitney test.

Group	Outcome measure	Controls		Interventions		
		H37*Rv* infected MDMs	PPD T_{eff}	PPD+IL4 T_{eff} (5ng/ml IL-4)	PPD+IL4 T_{eff} (5ng/ml IL-4)	PPD+IL4 T_{eff} (100ng/ml IL-4)
TB	CFU/ml; median [IQR]	30.7×10^3 [6.5; 88.8]	15.7×10^3 [3.8; 60.4]	29.1×10^3 [6.8; 91.6]	42.6×10^3 [7.7; 92.7]	43.8×10^3 [8.5; 127.9]
	% *M.tb* containment [IQR]	-	46% [12; 60]	1% [-49; 34]	-73% [-167; 1]	-33% [-96; -17]
LTBI	CFU/ml; median [IQR]	101.7×10^3 [41.3 - 217.0]	28.0×10^3 [16.6; 79.7]	45.6×10^3 [29.2; 190.0]	70.0×10^3 [49.6; 180.0]	108.3×10^3 [62.2; 232.0]
	% *M.tb* containment [IQR]	-	72% [28; 77]	64% [-144; 71]	18% [-88; 41]	-6% [-229; 42]
	*p-value	-	0.22	0.61	0.27	0.43

*p-value for comparison between % Mtb containment between LTBI and TB group using Mann Whitney test

Table E6. Median frequency of regulatory T-cell (Tregs) expressing IL-10 in cells of the mycobacterial containment assay. Values are expressed as the median % of CD4+CD25+FoxP3+ Tregs and interquartile range in parentheses (IQR).

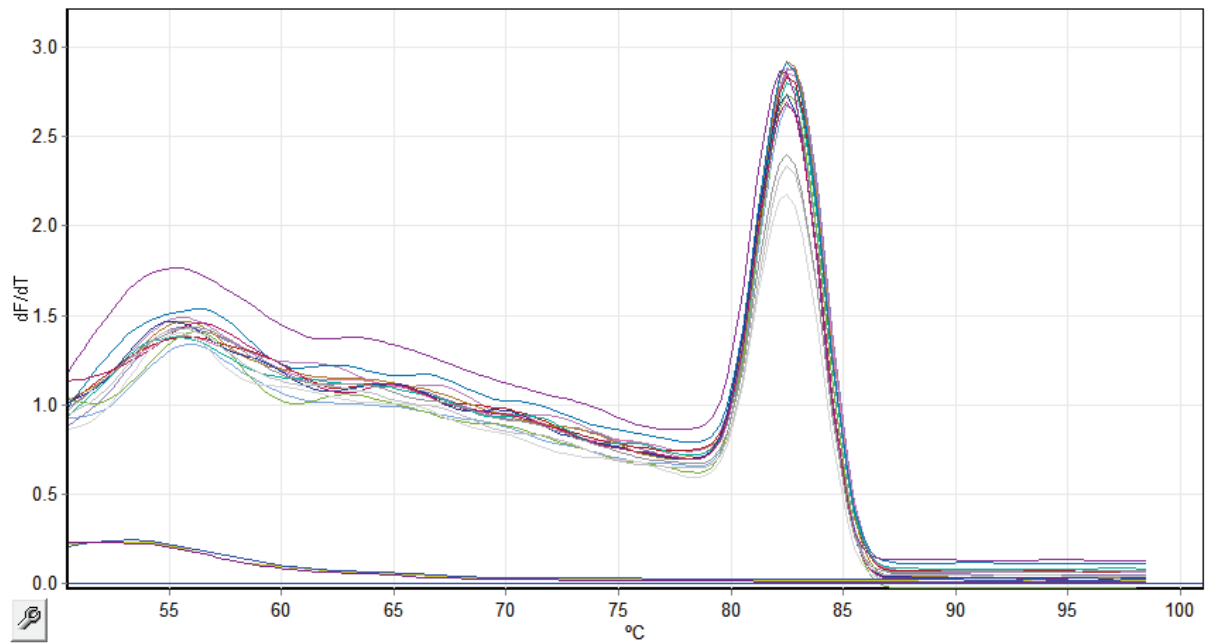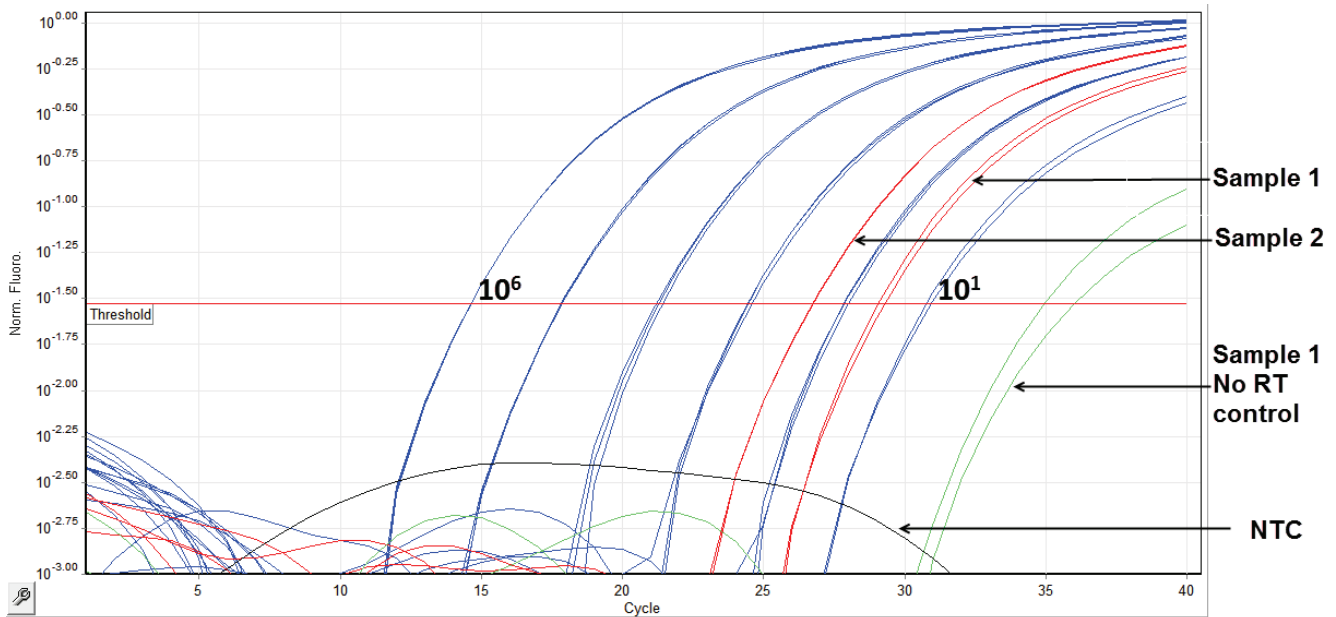| Analyte	Infected MDMs +		
	PPD T_{eff}	PPD+IL-4 T_{eff} (20ng/ml)	PPD+IL-4 T_{eff} (100ng/ml)
% Tregs expressing IL-10 Median % (IQR)	3.3 (0.5-24.5)	4.6 (1.6-13.6)	1.1 (0.6-11.4)

FIGURES

Figure E1. Melt curve analysis to determine IL-4 quantitative PCR primer sequence specificity using Rotor Gene Q software.

Name	Type	Ct	Given Conc	Calc Conc	% Var
10^6	Standard	14.6	1,000,000	1,020,782	2.10%
10^6	Standard	14.62	1,000,000	1,010,603	1.10%
10^6	Standard	14.61	1,000,000	1,011,025	1.10%
10^5	Standard	17.83	100,000	105,806	5.80%
10^5	Standard	17.85	100,000	104,097	4.10%
10^5	Standard	17.84	100,000	105,145	5.10%
10^4	Standard	21.18	10,000	10,097	1.00%
10^4	Standard	21.26	10,000	9,527	4.70%
10^4	Standard	21.4	10,000	8,669	13.30%
10^3	Standard	24.42	1,000	1,037	3.70%
10^3	Standard	24.57	1,000	934	6.60%
10^3	Standard	24.41	1,000	1,048	4.80%
10^2	Standard	27.83	100	95	5.00%
10^2	Standard	28	100	85	15.50%
10^2	Standard	27.84	100	94	6.10%
10^1	Standard	30.94	10	11	7.20%
10^1	Standard	30.76	10	12	21.00%
Sample 1	Unknown	29.28		34	
Sample 1	Unknown	29.05		40	
Sample 2	Unknown	26.73		205	
Sample 2	Unknown	26.75		203	
sample1 noRT	Unknown	35.98		0	
sample1 noRT	Unknown	34.93		1	
NTC	NTC				

Cycling A.Green (Page 1):
R=0.99975
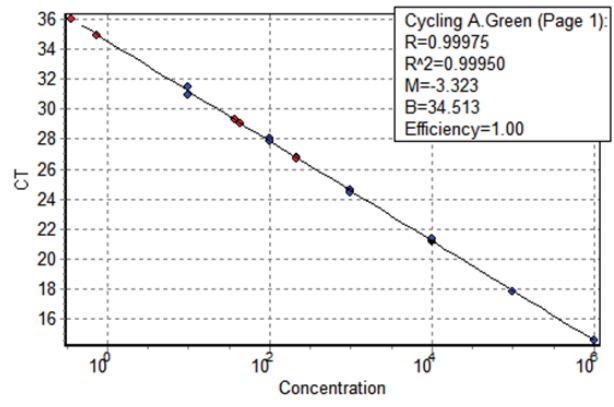R^2=0.99950
M=-3.323
B=34.513
Efficiency=1.00

Figure E2. Generation of IL-4 standard curve to determine unknown values by absolute quantification method using Rotor-Gene Q software. Serial 10-fold dilutions of IL-4 plasmids

standard (blue) and 2 unknown samples (red) were amplified in a quantitative PCR assay. No reverse transcriptase (no RT; green) controls and no template controls (NTC; black) and were also included. The fluorescence plot (top) was used to generate the standard curve (bottom right). PCR efficiency was 100% and R^2 value was 0.9995 for this assay. The calculated copy numbers (Calc conc) for the samples (red), no RT control (Green) and NTC (black) derived from the standard curve are shown in the adjacent table. The variance (%Var) for individual standards was <22% and coefficient of variance (intra-assay variation) for each dilution was <20%. Both controls did not have any detectable copies.
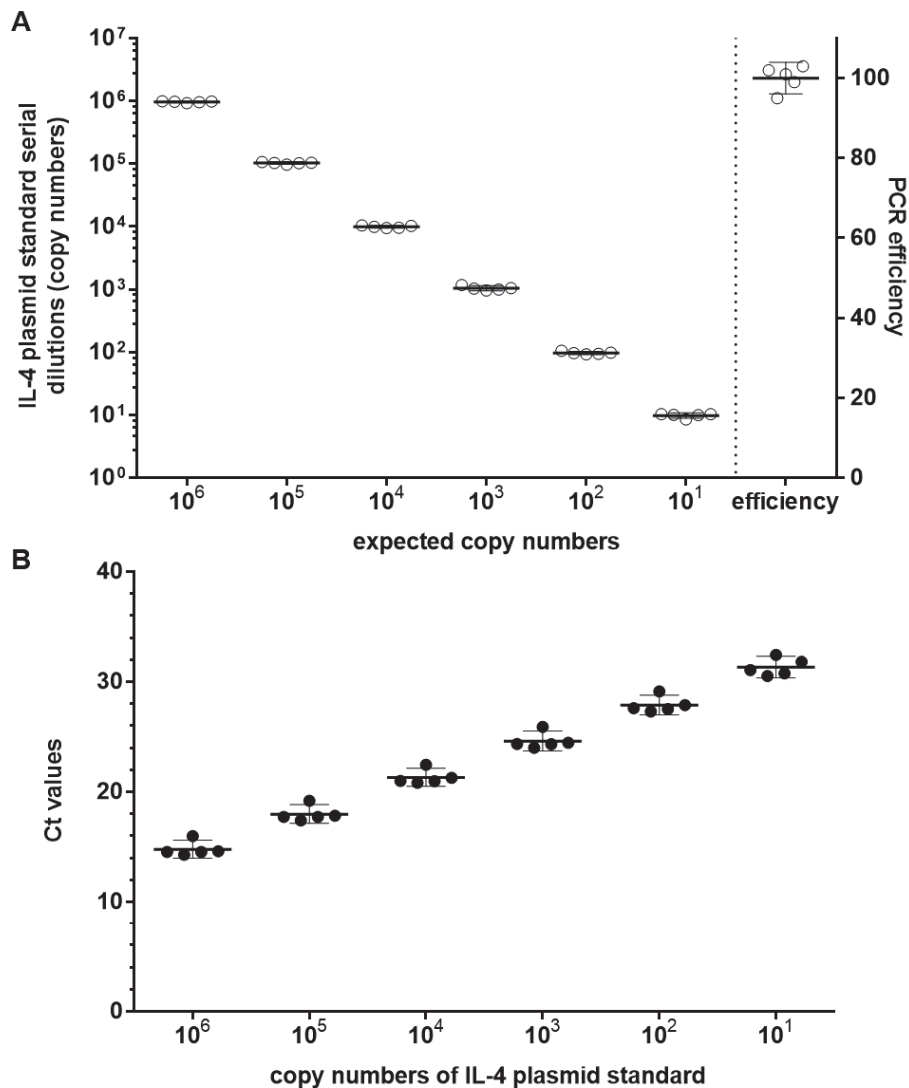
Figure E3. Inter-assay variability in (A) copy number and (B) Ct value of a quantitative PCR (qPCR) assay to determine IL-4 plasmid standard copy number. qPCR amplification was performed on serial 10-fold dilution (10^6-10^1) of linearized IL-4 plasmid standard in five separate runs. Each circle represents a single experiment. In (A) the x-axis show the expected copy numbers and the left y-axis show the expected copy numbers while the right y-axis shows the % PCR efficiency. In (B), the x-axis show the expected copy numbers and the y-axis show CT values. Lines and error bars represent mean values and 95% confidence intervals, respectively. In (A), the coefficient of variance at each dilution and for PCR efficiency (mean 100.0; 95% CI 96.1-103.9) was <10%.
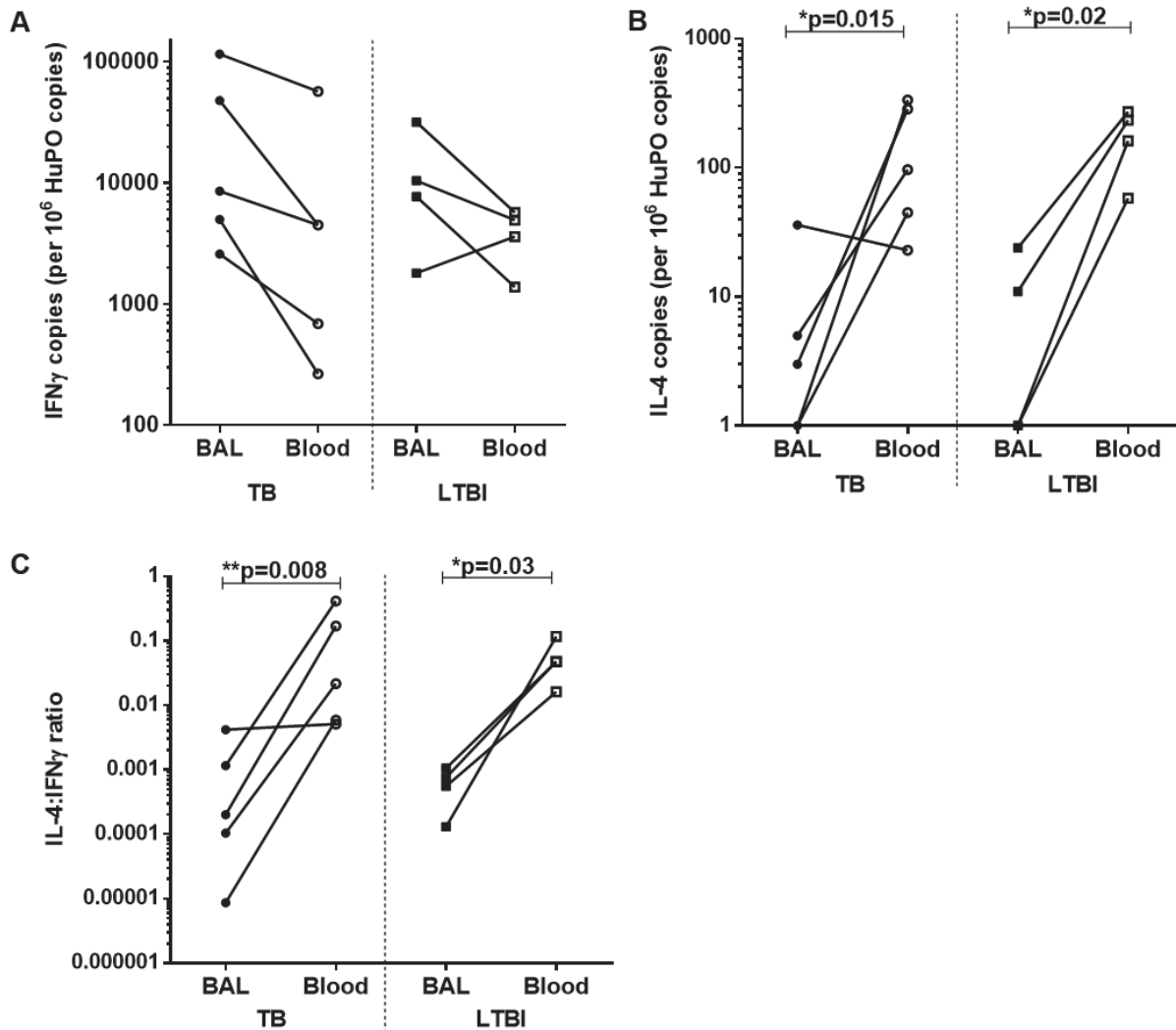
Figure E4. mRNA expression levels of A) IFN-γ B) IL-4 and the cytokine expression ratio of C) IL-4\IFN-γ in cells of broncho-alveolar lavage (BAL) and peripheral whole blood (Blood) from matched patients with pulmonary tuberculosis (TB; n=5) and presumed latently infected controls (LTBI; n=4) measured using a validated quantitative real-time PCR assay. Data is shown on a $\log_{10}$ scale and copy numbers are expressed per million copies of HuPO (validated reference gene). Statistical analyses between groups were performed using the Mann-Whitney test and p<0.05 was deemed significant.
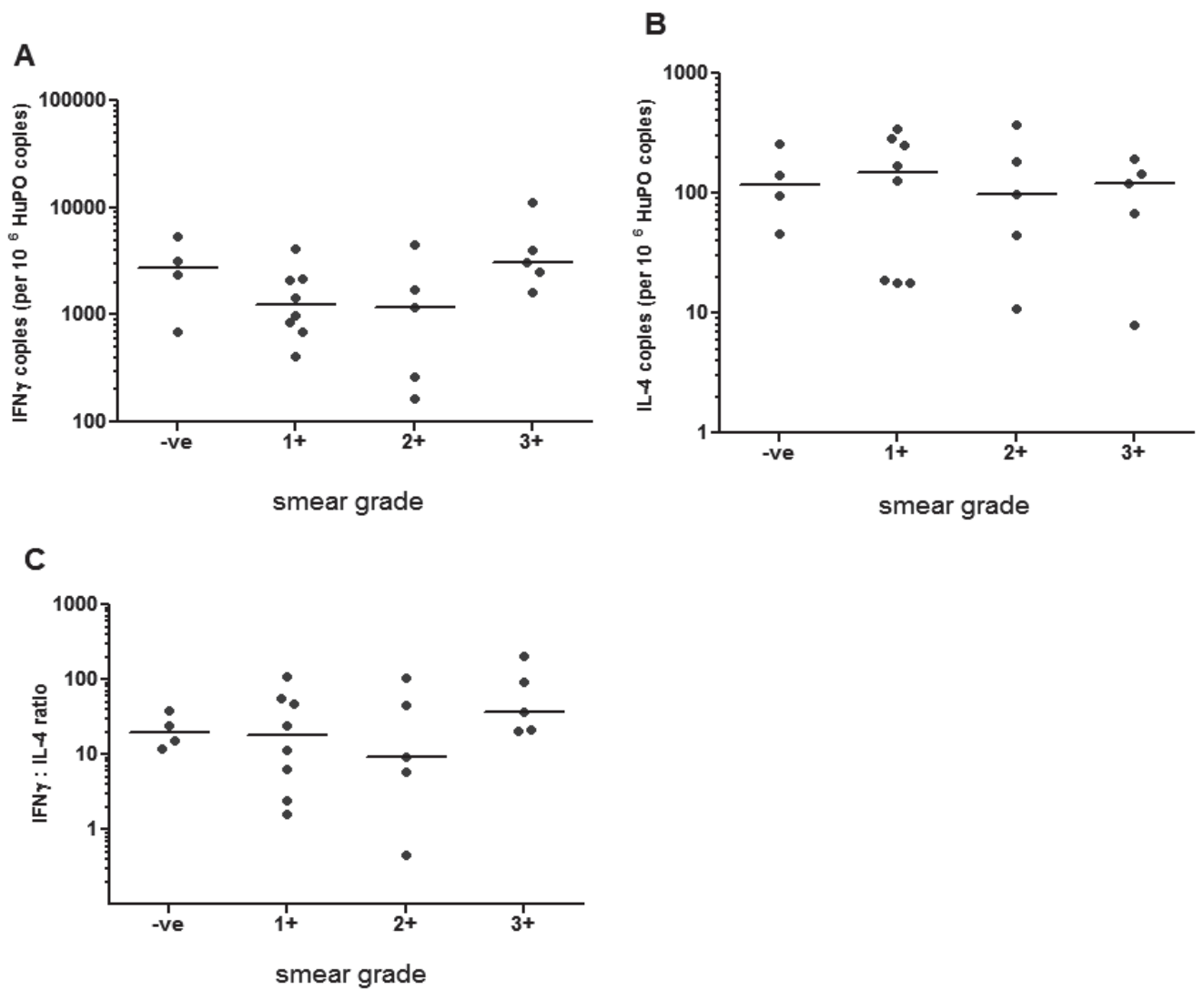
Figure E5. mRNA expression levels of (A) IFN-γ (B) IL-4 and (C) IFN-γ:IL-4 ratio stratified by sputum smear status in peripheral whole blood from patients with pulmonary tuberculosis (TB; Blood n=23) as measured by quantitative real-time PCR. Data is shown on a $\log_{10}$ scale and copy numbers are expressed per million copies of HuPO. Statistical analyses were performed using the Mann-Whitney test and $p<0.05$ was deemed significant
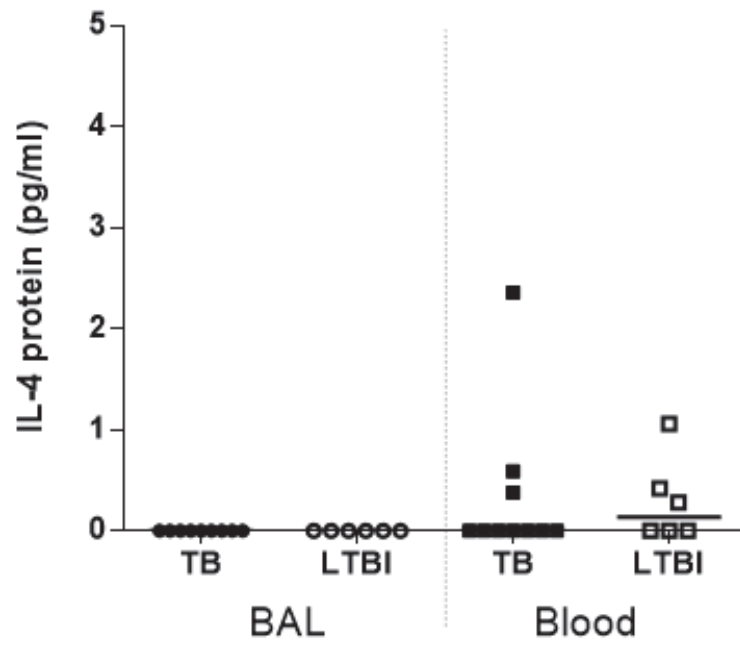
Figure E6: Measurement of IL-4 protein in broncho-alveolar lavage and peripheral blood mononuclear cell culture supernatants from TB patients and LTBI controls.
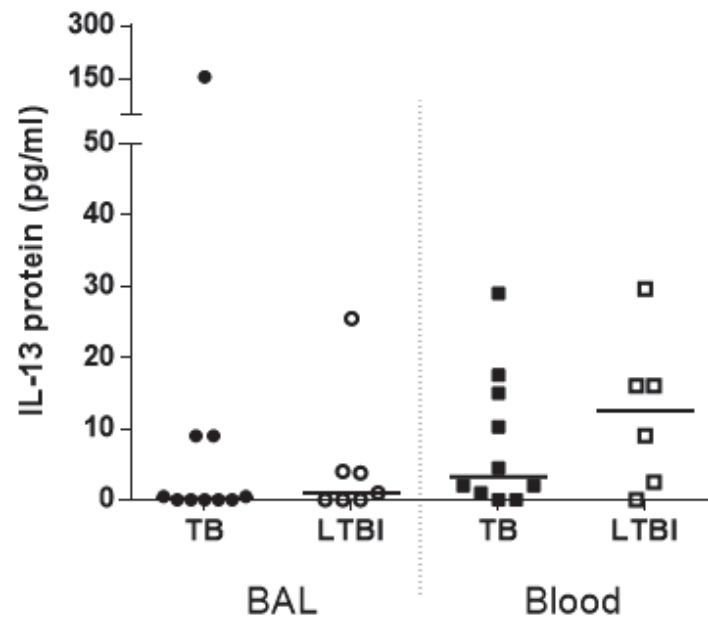
Figure E7: Measurement of IL-13 protein in broncho-alveolar lavage and peripheral blood mononuclear

TB-antigen stimulated cell culture supernatants from TB patients and LTBI controls.

Boxed – start and stop codons
<mark style="background:yellow">Exon 1 of IL-4</mark>
<mark style="background:magenta">Exon2 of IL-4</mark>
<mark style="background:green">Exon 3 of IL-4</mark>
<mark style="background:cyan">Exon 4 of IL-4</mark>
<mark style="background:silver">Decahistidine tag</mark>
Restriction enzyme sites

```
1       GGAACGGCTC CGCCCACTAT TAATGAAATT AAAAATTCCA ATTTTAAAAA ACGCAGCAAG
61      AGAAACATTT GTATGAAAGA ATGCGTAGAA GGAAAGAAAA ATGTCGTCGA CATGCTGAAC
121     AACAAGATTA ATATGCCTCC GTGTATAAAA AAAATATTGA ACGATTTGAA AGAAACAAT
181     GTACCGCGCG GCGGTATGTA CAGGAAGAGG TTTATACTAA ACTGTTACAT TGCAAACGTG
241     GTTTCGTGTG CCAAGTGTGA AAACCGATGT TTAATCAAGG CTCTGACGCA TTTCTACAAC
301     CACGACTCCA AGTGTGTGGG TGAAGTCATG CATCTTTTAA TCAAATCCCA AGATGTGTAT
361     AAACCACCAA ACTGCCAAAA AATGAAAACT GTCGACAAGC TCTGTCCGTT TGCTGGCAAC
421     TGCAAGGGTC TCAATCCTAT TTGTAATTAT TGAATAATAA AACAATTATA AATGCTAAAT
481     TTGTTTTTTA TTAACGATAC AAACCAAACG CAACAAGAAC ATTTGTAGTA TTATCTATAA
541     TTGAAAACGC GTAGTTATAA TCGCTGAGGT AATATTTAAA ATCATTTTCA AATGATTCAC
601     AGTTAATTTG CGACAATATA ATTTTATTTT CACATAAACT AGACGCCTTG TCGTCTTCTT
661     CTTCGTATTC CTTCTCTTTT TCATTTTTCT CTTCATAAAA ATTAACATAG TTATTATCGT
721     ATCCATATAT GTATCTATCG TATAGAGTAA ATTTTTTGTT GTCATAAATA TATATGTCTT
781     TTTTAATGGG GTGTATAGTA CCGCTGCGCA TAGTTTTTCT GTAATTTACA ACAGTGCTAT
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901     TGGGATCGTC GGTTTTGTAC AATATGTTGC CGGCATAGTA CGCAGCTTCT TCTAGTTCAA
961     TTACACCATT TTTTAGCAGC ACCGGATTAA CATAACTTTC CAAAATGTTG TACGAACCGT
1021    TAAACAAAAA CAGTTCACCT CCCTTTTCTA TACTATTGTC TGCGAGCAGT TGTTTGTTGT
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1801    TGAGCGCATG CAAGCTGATC CGGGTTATTA GTACATTTAT TAAGCGCTAG ATTCTGTGCG
1861    TTGTTGATTT ACAGACAATT GTTGTACGTA TTTTAATAAT TCATTAAATT TATAATCTTT
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1981    TATTAAATCC TCAATAGATT TGTAAAATAG GTTTCGATTA GTTTCAAACA AGGGTTGTTT
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2101    ATCTTGTAGC AGCAATCTAG CTTTGTCGAT ATTCGTTTGT GTTTTGTTTT GTAATAAAGG
2161    TTCGACGTCG TTCAAAATAT TATGCGCTTT TGTATTTCTT TCATCACTGT CGTTAGTGTA
2221    CAATTGACTC GACGTAAACA CGTTAAATAG AGCTTGGACA TATTTAACAT CGGGCGTGTT
2281    AGCTTTATTA GGCCGATTAT CGTCGTCGTC CCAACCCTCG TCGTTAGAAG TTGCTTCCGA
2341    AGACGATTTT GCCATAGCCA CACGACGCCT ATTAATTGTG TCGGCTAACA CGTCCGCGAT
2401    CAAATTTGTA GTTGAGCTTT TTGGAATTAT TTCTGATTGC GGGCGTTTTT GGGCGGGTTT
2461    CAATCTAACT GTGCCCGATT TTAATTCAGA CAACACGTTA GAAAGCGATG GTGCAGGCGG
2521    TGGTAACATT TCAGACGGCA AATCTACTAA TGGCGGCGGT GGTGGAGCTG ATGATAAATC
2581    TACCATCGGT GGAGGCGCAG GCGGGGCTGG CGGCGGAGGC GGAGGCGGAG GTGGTGGCGG
2641    TGATGCAGAC GGCGGTTTAG GCTCAAATGT CTCTTTAGGC AACACAGTCG GCACCTCAAC
2701    TATTGTACTG GTTTCGGGCG CCGTTTTTGG TTTGACCGGT CTGACGACGAG TGCGATTTTT
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2821    TTCCGTCGGC ATTGGTGGAG CGGGCGGCAA TTCAGCATC GATGGTGGTG GTGGTGGTGG
2881    AGGCGCTGGA ATGTTAGGCA CGGGAGAAGG TGGTGGCGGC GGTGCCGCCG GTATAATTTG
2941    TTCTGGTTTA GTTTGTTCGC GCACGATTGT GGGCACCGGC GCAGGCGCCG CTGGCTGCAC
3001    AACGGAAGGT CGTCTGCTTC GAGGCAGCGC TTGGGGTGGT GGCAATTCAA TATTATAATT
3061    GGAATACAAA TCGTAAAAAT CTGCTATAAG CATTGTAATT CGCTATCGT TTACCGTGCC
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3241    ACACTTCGCT GTCGTCGAGG TTTAAACGCT TCCTCGCTCA CTGACTCGCT GCGCTCGGTC
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3361    TCAGGGGATA ACGCAGGAAA GAACATGTGA GCAAAAGGCC AGCAAAAGGC CAGGAACCGT
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3781    TCGCCACTGG CAGCAGCCAC TGGTAACAGG ATTAGCAGAG CGAGGTATGT AGGCGGTGCT
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4201    AGTTACCAAT GCTTAATCAG TGAGGCACCT ATCTCAGCGA TCTGTCTATT TCGTTCATCC
4261    ATAGTTGCCT GACTCCCCGT CGTGTAGATA ACTACGATAC GGGAGGGCTT ACCATCTGGC
4321    CCCAGTGCTG CAATGATACC GCGAGACCCA CGCTCACCGG CTCCAGATTT ATCAGCAATA
4381    AACCAGCCAG CCGGAAGGGC CGAGCGCAGA AGTGGTCCTG CAACTTTATC CGCCTCCATC
4441    CAGTCTATTA ATTGTTGCCG GGAAGCTAGA GTAAGTAGTT CGCCAGTTAA TAGTTTGCGC
4501    AACGTTGTTG CCATTGCTAC AGGCATCGTG GTGTCACGCT CGTCGTTTGG TATGGCTTCA
4561    TTCAGCTCCG GTTCCCAACG ATCAAGGCGA GTTACATGAT CCCCCATGTT GTGCAAAAAA
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4681    CTCATGGTTA TGGCAGCACT GCATAATTCT CTTACTGTCA TGCCATCCGT AAGATGCTTT
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4981    AGCGTTTCTG GGTGAGCAAA AACAGGAAGG CAAAATGCCG CAAAAAAGGG AATAAGGGCG
5041    ACACGGAAAT GTTGAATACT CATACTCTTC CTTTTTCAAT ATTATTGAAG CATTTATCAG
5101    GGTTATTGTC TCATGAGCGG ATACATATTT GAATGTATTT AGAAAAATAA ACAAATAGGG
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5221    GCGGCGGGTG TGGTGGTTAC GCGCAGCGTG ACCGCTACAC TTGCCAGCGC CCTAGCGCCC
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5341    CTAAATCGGG GGCTCCCTTT AGGGTTCCGA TTTAGTGCTT TACGGCACCT CGACCCCAAA
5401    AAACTTGATT AGGGTGATGG TTCACGTAGT GGGCCATCGC CCTGATAGAC GGTTTTTCGC
5461    CCTTTGACGT TGGAGTCCAC GTTCTTTAAT AGTGGACTCT TGTTCCAAAC TGGAACAACA
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5581    TGGTTAAAAA ATGAGCTGAT TTAACAAAAA TTTAACGCGA ATTTTAACAA AATATTAACG
5641    TTTACAATTT CCCATTCGCC ATTCAGGCTG CGCAACTGTT GGGAAGGGCG ATCGGTGCGG
5701    GCCTCTTCGC TATTACGCCA
```

Figure E8. The full nucleotide sequence of the bacmid transfer vector containing IL-4, pAP01-IL4 - His (5720bp), used to express human recombinant IL-4. The start and stop codons, the IL-4 sequence, decahistidine tag and restriction sites used for insertion of the IL-4 cDNA sequence are also shown are all annotated
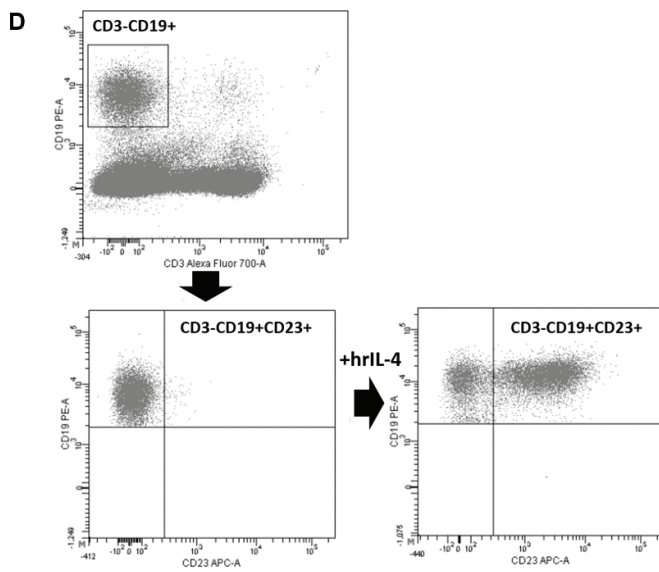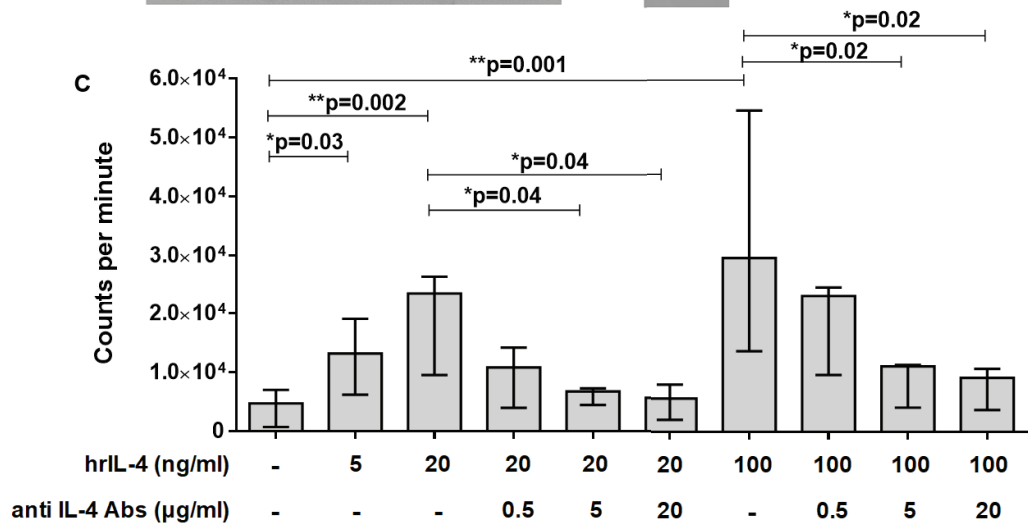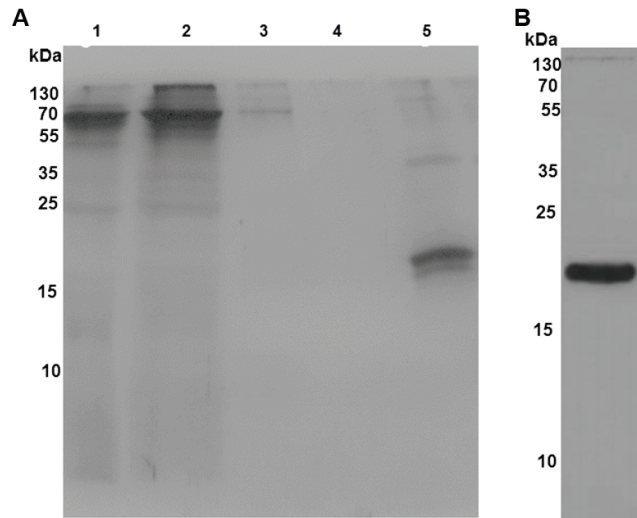
A

	1	2	3	4	5
kDa					
130					
70					
55					
35					
25					
15					
10					

B

kDa	
130	
70	
55	
35	
25	
15	
10	

C

Counts per minute

**p=0.001

**p=0.002

*p=0.03

*p=0.04

*p=0.04

*p=0.02

*p=0.02

| hrIL-4 (ng/ml) | - | 5 | 20 | 20 | 20 | 20 | 100 | 100 | 100 | 100 |
| anti IL-4 Abs (µg/ml) | - | - | - | 0.5 | 5 | 20 | - | 0.5 | 5 | 20 |

D

CD3-CD19+

CD19 PE-A

CD3 Alexa Fluor 700-A

CD3-CD19+CD23+

CD19 PE-A

CD23 APC-A

+hrIL-4

CD3-CD19+CD23+

CD19 PE-A

CD23 APC-A

E

% CD19 cells positive for CD23

**p=0.005

**p=0.002

**p=0.001

hrIL-4 concentrations (ng/ml)

Figure E9. Expression, purification and bioactivity of human recombinant IL-4 (hrIL-4). hrIL-4 was expressed using a baculovirus expression system and purified using Ni^{2+} affinity columns. (A) a 15% sodium-dodecyl-sulfate-polyacrylamide-gel-electrophoresis (SDS-PAGE) gel of hrIL-4 purification fractions showing (1) post-infection *Sf21* insect cell supernatant (2) flow-through after column binding (3) 1st wash with 50mM imidazole (4) 2nd wash with 50mM imidazole (5) elution of rIL-4 at 500mM imidazole. The eluted protein had a size of ~17kDa (lane 5). (B) Western blot analysis of the purified hrIL-4 fraction probed with anti-IL4 antibody at 1:2500. Molecular weight markers are shown on the left in kiloDaltons (kDa) and 10-15μl of each fraction was loaded onto the gel. (C) hrIL-4 induces proliferation of T-cells pre-activated with anti-CD3 in peripheral blood mononuclear cells of healthy participants (n=6) in a concentration dependent manner. The addition of anti-IL-4 neutralization antibodies reduces the IL-4-mediated T-cell proliferation (n=3) also in a concentration dependent manner. Proliferation was measured in a ^{3}H-Thymidine proliferation assay. hrIL4 induces expression of CD23 on B-cells (CD3+CD19+) in peripheral blood mononuclear cells of healthy participants (n=6) in a concentration dependent manner as measured by flow cytometry. The gating strategy for identification of CD3-CD19+CD23+ cells is shown in (D). The median frequency (%) and interquartile range of CD3-CD19+CD23+ cells at different concentrations of rIL-4 are shown in (E). Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test and $p<0.05$ was deemed significant.
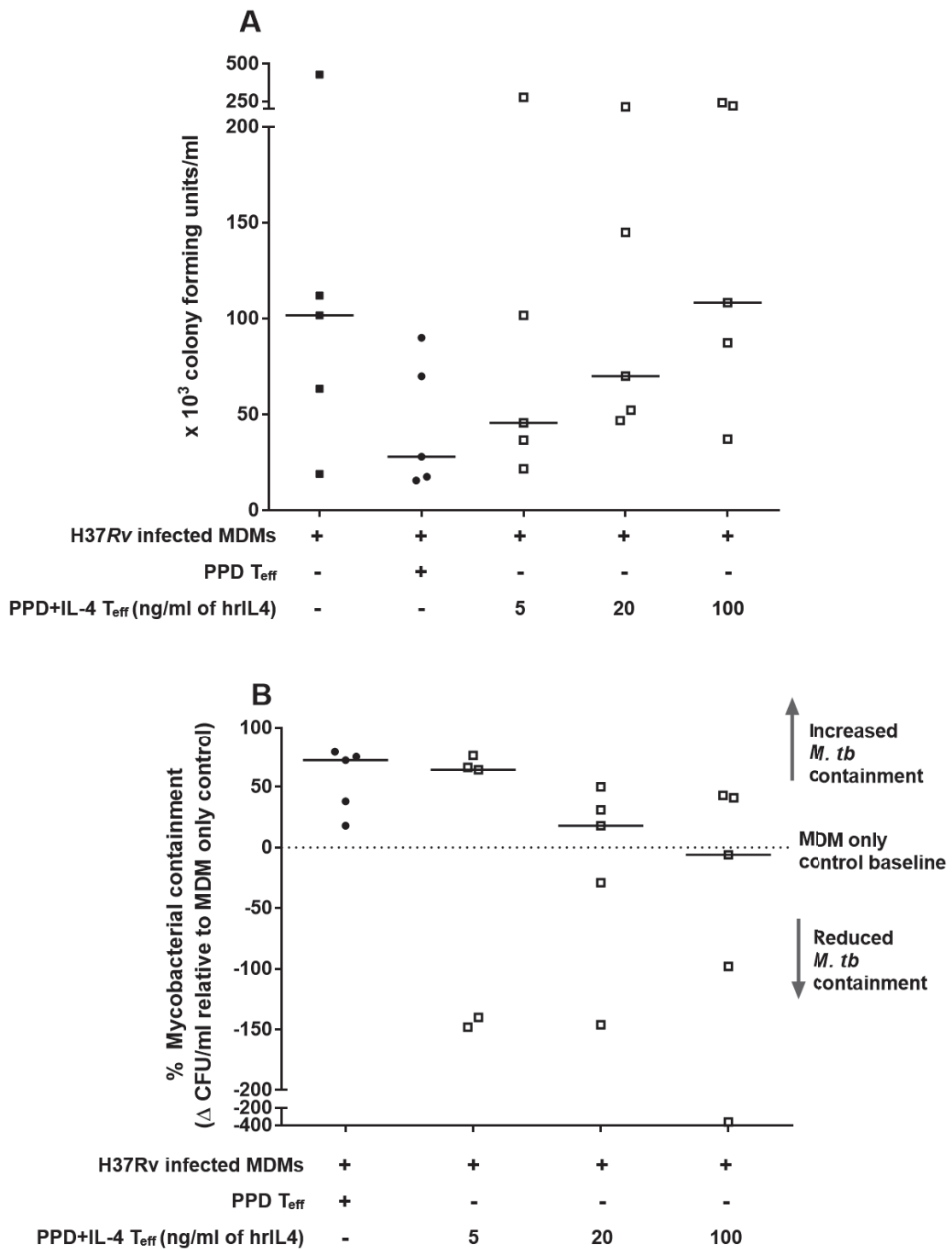
Figure E10. The effect of human recombinant IL-4 on mycobacterial containment in infected monocyte-derived macrophages (MDMs) from healthy participants with latent TB infection (LTBI;

n=5). A mycobacterial containment assay was performed where H37*Rv*-infected MDMs were cultured by themselves or co-cultured with peripheral blood mononuclear cells pre-primed with purified protein derivation alone (PPD T$_{eff}$), or PPD and hrIL-4 (PPD+IL-4 T$_{eff}$) at concentrations of 5, 20 and 100ng/ml hrIL-4. Results are expressed as (**A**) median colony forming units (CFU/ml) in TB patients and (**B**) The % mycobacterial (*M.tb*) containment, defined as the change in *M.tb* survival compared to the "H37Rv-infected MDMs only" control, were also determined. Increased % *M.tb* containment indicates a reduction in *M.tb* survival whereas decreased % *M.tb* containment indicates an increase in *M.tb* survival. Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test and $p < 0.05$ was deemed significant.
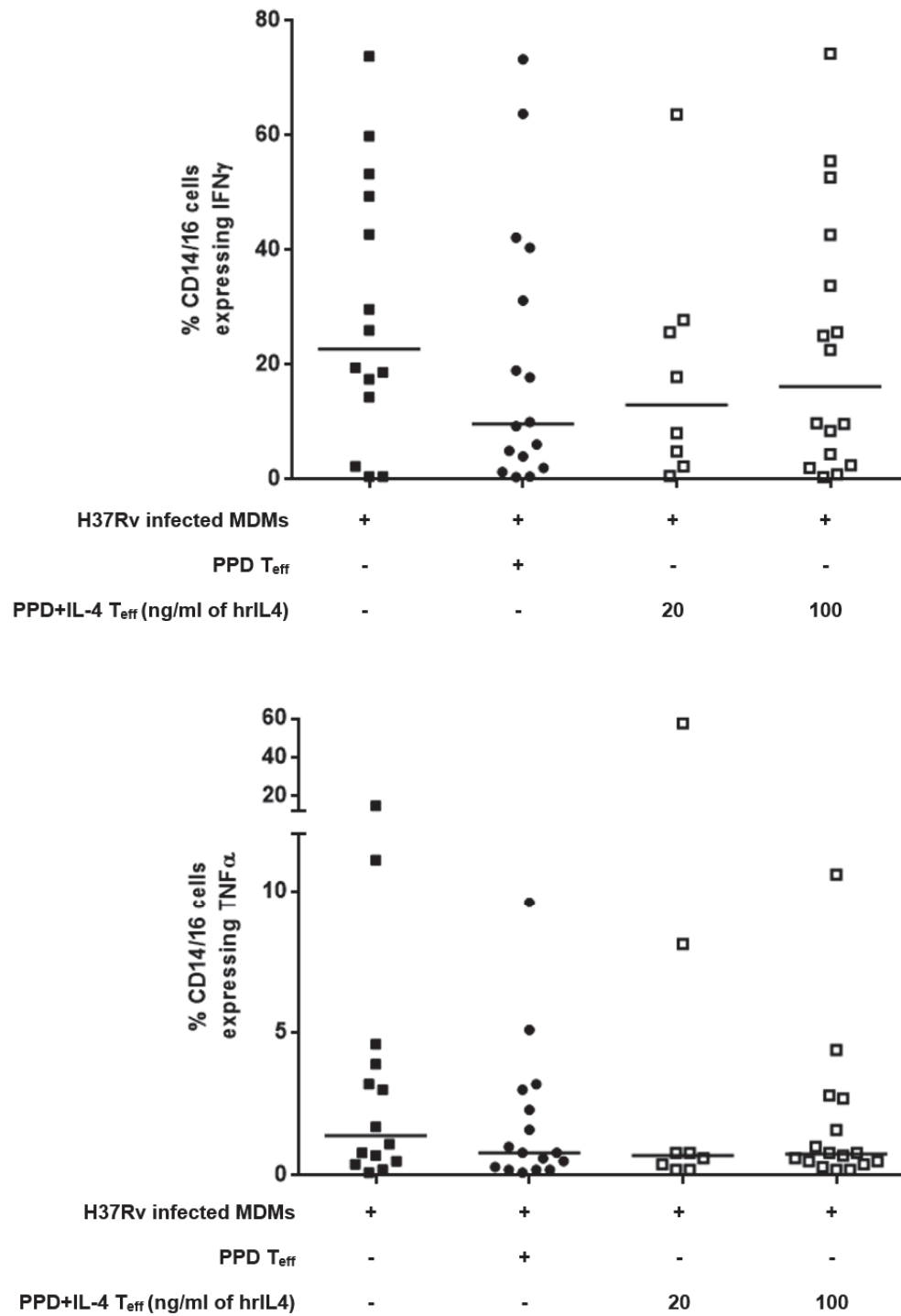
Figure E11. The effect of human recombinant IL-4 on Th1 cytokine expression in in macrophages in a mycobacterial containment assay as measured by flow cytometry. The % of CD14$^+$CD16$^+$

macrophages expressing (A) IFN-γ and (B) TNFα in cells harvested from a mycobacterial containment assay from pulmonary TB patients. H37Rv-infected MDMs were co-cultured with peripheral blood mononuclear cells pre-primed with purified protein derivation (PPD) alone (PPD T_{eff}) or PPD and hrIL-4 (PPD+IL-4 T_{eff}) at concentrations of 20ng/ml (n=10) and 100ng/ml (n=16). Statistical analyses were performed using the Wilcoxon matched-pairs signed rank test between interventions and the Mann Whitney U test between CD4+ and CD8+ lymphocytes. A p-value<0.05 was deemed significant.

A

x10^2 colony forming units/ml

150
125
100
75
50
25
0

H37*Rv* infected MDMs	+	+	+	+	+	+
PPD T$_{eff}$	-	+	-	-	-	-
PPD+IL-4 T$_{eff}$ (ng/ml of hrIL4)	-	-	20	20	100	100
Anti-IL-4R antibody (µg/ml)	-	-	-	10	-	10

B

% Mycobacterial containment
(Δ CFU/ml relative to MDM only control)

100
50
0
-50
-100

Increased *M. tb* containment

MDM only control baseline

Reduced *M. tb* containment

H37Rv infected MDMs	+	+	+	+	+
PPD T$_{eff}$	+	-	-	-	-
PPD+IL-4 T$_{eff}$ (ng/ml of hrIL4)	-	20	20	100	100
Anti-IL-4R antibody (µg/ml)	-	-	10	-	10

C

% CD4 that are Tregs (CD25+FoxP3+)

4
3
2
1
0

PPD T$_{eff}$	+	-	-
PPD+IL-4 T$_{eff}$ (ng/ml of hrIL4)	-	100	100
Anti-IL-4R antibody (µg/ml)	-	-	10

D

% CD4 cells expressing IFNγ

8
6
4
2
0

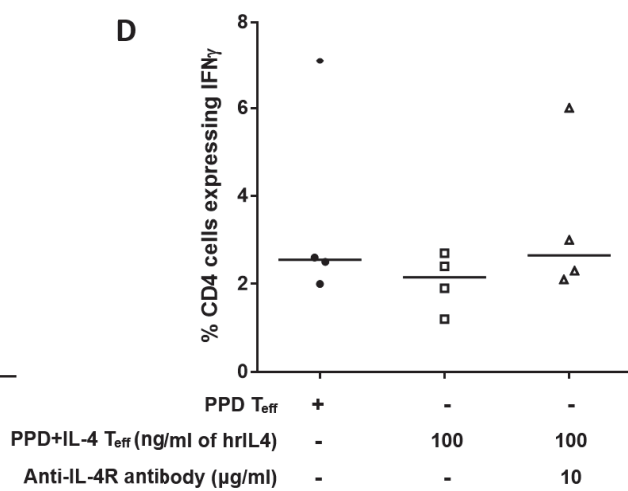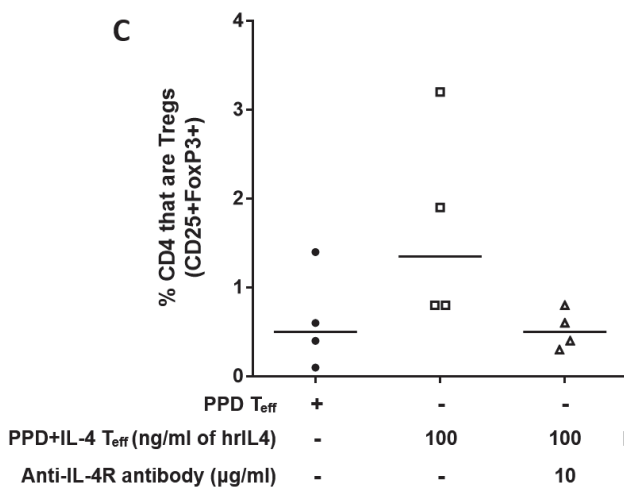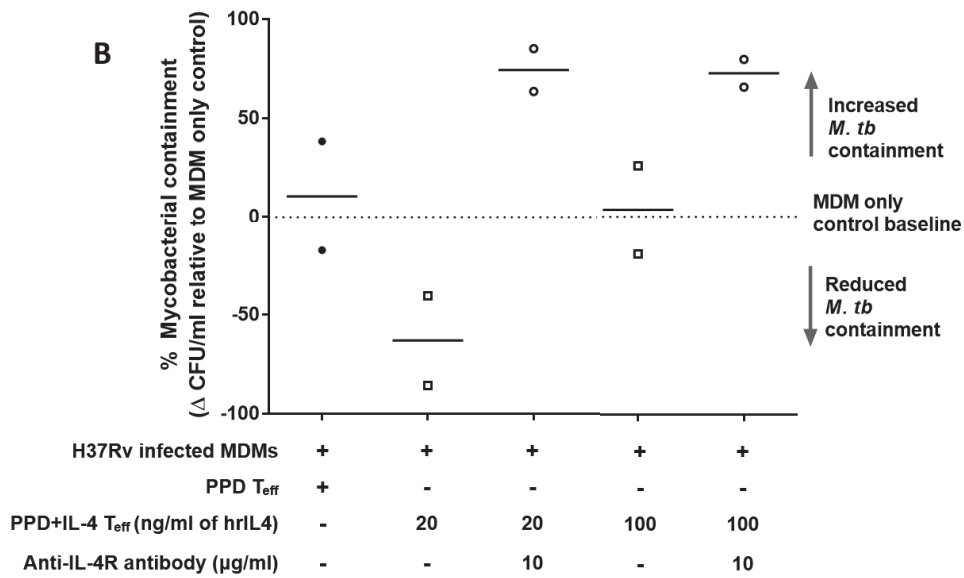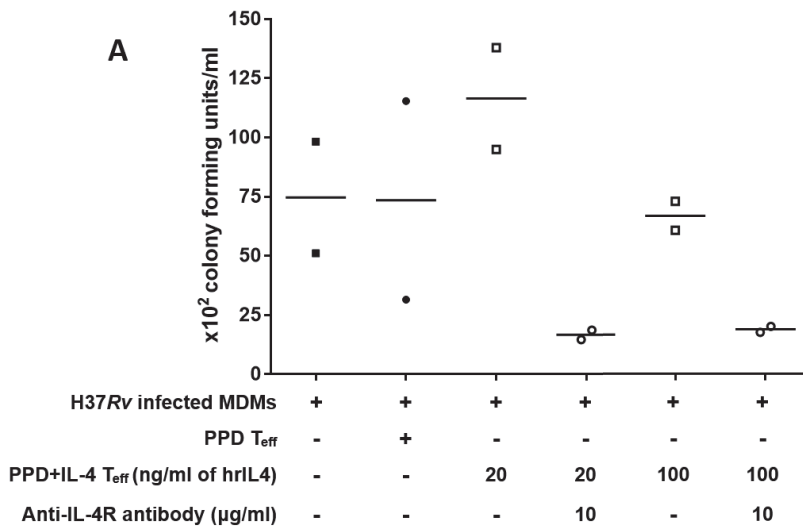| PPD T$_{eff}$	+	-	-
PPD+IL-4 T$_{eff}$ (ng/ml of hrIL4)	-	100	100
Anti-IL-4R antibody (µg/ml)	-	-	10

Figure E12. The effect of blocking IL-4 receptor on mycobacterial containment and on cytokine and regulatory T-cell expression in a mycobacterial containment assay. H37Rv-infected MDMs were co-cultured with peripheral blood mononuclear cells pre-primed with PPD alone (PPD T$_{eff}$) or PPD and hrIL-4 (PPD+IL-4 T$_{eff}$) at 20 and 100ng/ml hrIL-4. To the IL-4 interventions, anti-IL4-receptor (IL-4R) antibody (10µg/ml) was added at the same time as hrIL-4 (day 1). In TB patients (n=2), mycobacterial containment was measured in terms of **a)** colony forming unit/ml (CFU/ml) and **b)** % mycobacterial (*M.tb*) containment, defined as the change in *M.tb* survival compared to the "H37Rv-infected MDMs only" control. Increased % *M.tb* containment indicates a reduction *M.tb* survival whereas decreased % *M.tb* containment indicates an increase in *M.tb* survival. The % of CD4$^+$ lymphocytes that were **c)** Treg (CD25$^+$FoxP3$^+$) and expressing **d)** IFN-γ were also determined within the system (cells co-cultured within the *M.tb* containment assay) by flow cytometry. In this case, IL-4R blocking was assessed using anti-IL-4R antibodies (n=4; at 100ng/ml of IL-4). Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test and p<0.05 was deemed significant.